Long-Lasting Memory Impairments after Subchronic Immune Challenge

by

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TABLE OF CONTENTS

LIST OF TABLES

TABLE

LIST OF FIGURES

FIGURE

ABSTRACT

Long-lasting dysfunction of memory, emotion, and cognition is observed in individuals who have experienced a systemic inflammatory event, including a critical illness or major surgery. I have developed an animal model in which we can study short-term and long-term changes in memory and affective processes after a systemic inflammatory event in males and in females. To examine the short-term and long-lasting changes in memory after a systemic inflammatory insult, I used systemic, subchronic immune challenge (5 intraperitoneal injections, each spaced 3 days apart) to trigger transient systemic immune activation and tested animals for changes in memory either one or eight weeks after last injection (Chapter 2). I found sex-specific patterns of hippocampal-dependent deficits after subchronic immune challenge, with females showing deficits in object recognition memory one and eight weeks after last injection while males showed impairments in object recognition and fear conditioning only eight weeks after last injection. Subchronic immune challenge impacts specific memory processes as deficits in memory formation, but not retrieval nor extinction were observed months after injection. To determine whether subchronic immune challenge dysregulates both memory and affective processes, I assessed changes in anxiety-like or depression-like behaviors weeks after immune challenge (Chapter 3). We tested different aspects of depression-like behaviors, including despair-like behavior with forced swim test and anhedonia-like behavior using sucrose preference test. I found no long-lasting increases in anxiety-like nor depression-like behaviors after subchronic immune challenge. Collectively, the findings from chapter 2 and 3 suggest that a

mild systemic inflammatory insult results in sex-specific cognitive impairments, with persistent changes in processes important for memory but not affective behaviors.

We next determined molecular substrates that may mediate long-lasting changes in memory and hippocampal dysfunction in males and in females (Chapter 4). Changes in hippocampal gene expression were examined three months after subchronic LPS challenge using a large scale and unbiased approach, RNA-sequencing (RNA-seq). I found sex-specific, enduring changes in hippocampal gene expression after subchronic immune challenge. Males showed greater dysregulation of gene expression, primarily with changes in immune-related and neuroplasticity-associated targets, while females showed changes mainly in targets related to monoaminergic signaling. Interestingly, a subsequent systemic inflammatory insult months after subchronic immune challenge also resulted in sex-specific patterns of hippocampal gene expression, with greater dysregulation in females compared with males.

As subchronic immune challenge resulted in persistent dysregulation of immune- and neuroplasticity-related genes, I have explored the involvement of sustained changes in neuroimmune mechanisms, including blood-brain barrier permeability and microglial activation, and neuroplasticity-associated processes, such as changes in activity-dependent induction of c-Fos, that may contribute to the long-lasting memory deficits (Chapter 5). I found no sustained blood-brain barrier permeability nor microglial activation months after subchronic immune challenge. However, subchronic immune challenge resulted in persistent changes in activity-dependent c-Fos induction after fear conditioning in a brain region important for memory, the dorsolateral entorhinal cortex, suggesting that persistent alterations in neuroplasticity-related mechanisms may underlie the long-lasting memory deficits. Together, these studies provide evidence of novel mechanisms of memory dysregulation in males and in females after a mild

systemic inflammatory event that will inform development of preventative and therapeutic strategies for memory dysfunction after a systemic inflammatory event in men and in women.

Chapter I

General Introduction

Long-Lasting Consequences Systemic Inflammatory Event on Memory and Emotion

A systemic inflammatory event leads to long-lasting cognitive dysfunction. Patients who experience a systemic inflammation event, including a critical illness or major surgery, develop cognitive deficits that persist for months to years after resolution of inflammation (Gharacholou *et al*, 2011; Sakusic and Rabinstein, 2018; Semmler *et al*, 2013). It is estimated that at least 25% of 1,064 patients develop cognitive dysfunction after surgery, a condition referred to as post-operative cognitive dysfunction (POCD) (Monk *et al*, 2008). These changes in cognition include impairments in executive functions, attention, processing speed, and moderate to severe memory deficits in verbal learning, working memory, and long-term memory (Langa *et al*, 2012; Rengel *et al*, 2019; Semmler *et al*, 2013). A great proportion of individuals who have recovered from a systemic inflammatory event develop changes in emotion, including depression, anxiety, or post-traumatic disorder (Prescott and Angus, 2018), which can often co-occur with changes in emotion (Han *et al*, 2016). Thus, a systemic inflammatory event has debilitating consequences for an individual's mental health.

The consequences of a systemic inflammatory event on memory, emotion, cognition, and well-being differ in men and women. Women are more susceptible to diseases associated with systemic immune dysfunction, such as development and disease progression of autoimmune diseases including multiple sclerosis, systemic lupus, erythematosus, and rheumatoid arthritis (Ortona *et al,* 2016). Yet, it is male rather than female MS patients who show worst performance

on cognitive tasks when matched for age, education, and other neurologic and emotional measures (Aupperle *et al*, 2002). These findings suggest that systemic inflammatory events affect cognitive processes differently in men and women. Understanding the mechanisms by which systemic inflammation leads to cognitive decline in both men and women will provide novel targets for prevention of long-term disabilities in all patients who have experienced a systemic inflammatory event.

Memory deficits observed after a systemic inflammatory event are indicative of various aging and memory-related disorders. For example, both major surgery, such as coronary bypass graft, and systemic infections have been associated with the risk of developing dementia years later and repeated systemic inflammation has been associated with decline in episodic memory (Evered *et al*, 2016; Holmes *et al*, 2009; Tampubolon, 2016). Presence of immune-associated markers in middle age is also a predictor of cognitive decline decades later (Rafnsson *et al*, 2007). This is of great clinical relevance given that women are more susceptible to developing Alzheimer's disease. Therefore, systemic immune activation may not only be a contributor to cognitive decline, but also to severe neuropathological states, including neurodegenerative diseases. As neuroinflammation can precede the development of neurodegenerative diseases, it is important to determine how systemic inflammation leads to this long-term neural dysfunction.

Rodent models have been used to study how a systemic inflammatory event induces long-lasting changes in memory, emotion, and cognition. Such animal models have shown impairments in various types of memory weeks and months after the inflammatory insult. Most studies have focused on changes in memory at least a week (ten days) after surgery and have shown deficits in Morris water maze, inhibitory avoidance (Barichello *et al*, 2007; Tuon *et al*, 2008). The few studies that have examined long-lasting changes in memory after the

inflammatory insult have shown deficits in inhibitory avoidance, novel object recognition, context fear conditioning or extinction, and spatial and working memory tasks one to two months after surgery of immune challenge (Chavan *et al*, 2012; Huerta *et al*, 2016; Singer *et al*, 2016; Weberpals *et al*, 2009; Zhu *et al*, 2017). Some memory deficits can even persist four to ten months after the inflammatory insult (Chavan *et al*, 2012; Ming *et al*, 2015; Semmler *et al*, 2007). Similarly, long-lasting changes in affective regulation are also observed, with anxiety-like and depressive-like behaviors persisting a month after peripheral immune challenge (Anderson *et al*, 2015). Yet, there are some discrepancies in the literature with regards to the persistence of memory deficits, as not all animal studies show memory deficits at least a month after immune challenge (Anderson *et al*, 2015), and some memory deficits recover after the inflammatory insult (Tuon *et al*, 2008). Similarly, not all animal studies show anxiety-like behaviors even weeks after inflammatory insult (Barichello *et al*, 2007). Therefore, further investigation of the factors that contribute to long-lasting changes in memory and affective processes after systemic immune activation is necessary through continued development of appropriate animal models.

Long-lasting changes in cognitive functions, including memory and affective processing, in animal models have been studied only in males, and therefore there is a large gap in the literature on the long-lasting impact of transient systemic immune activation on memory and emotion in females. This is inherently problematic as males and females show differences in peripheral immune responses that may impact neural function in a sex-specific manner. Females have stronger immune responses to a wide range inflammatory stimuli, including bacteria, viruses, parasites, fungi, and vascular trauma (Scotland *et al*, 2011) and vaccines (Klein and Flanagan, 2016), which allows them to also have increased resistance to inflammatory diseases and infection than males (Schwarz and Bilbo, 2011) and protection from injury (Rosen *et al*,

2017). Females also have greater adaptive immune responses and stronger Th2 responses than males which mediates neuroprotection (Huber and Pfaeffle, 1994; Klein and Flanagan, 2016). The implications of these sex differences in peripheral immune activation for memory and cognition remain to be explored.

Neuroimmune Activation After Systemic Inflammatory Event and its Consequences for Behavior

Neuroimmune system

The neuroimmune system consist of cells constructing the blood-brain barrier as well as glial cells, including microglia and astrocytes. The blood brain barrier is a highly- selective semi-permeable barrier found at the boundary between the blood and the brain parenchyma that regulates the entry of blood-borne substances, including toxic molecules, into the brain. Endothelial and perivascular cells as well as tight junctions proteins, such as claudins and occludins, make up an intact blood-brain barrier (Abbott *et al*, 2010; Ballabh *et al*, 2004; Banks, 2015; Daneman and Prat, 2015; Stamatovic *et al*, 2016). Microglia are the primary innate immune cells in the brain that respond to infection and injury (Kreutzberg, 1996). Microglia can be in a resting state (morphologically "ramified") or an activated state (morphologically "amoeboid"). When the brain becomes exposed to pathogens during illness or to neural damage during injury, microglia switch from the ramified into the ameboid state and phagocytose the invading pathogens or damaged cells. Astrocytes are a major type of glia in the brain are crucial for maintaining proper brain homeostasis. Astrocyte participate in several processes necessary for mediating neural functions, including neurotransmission and synaptic plasticity (Eroglu and Barres, 2010; Vasile *et al*, 2017; Zhang and Barres, 2010). Prior to immune activation, and intact

blood-brain barrier protects the brain from entry of toxic substances and maintains brain homeostasis and proper neuronal and glial function.

Neuroimmune activation after systemic inflammatory insult

During illness or injury, peripheral immune cells secrete signaling proteins called cytokines as part of the elimination process for the invading pathogens, such as bacteria or viruses, or injured cells (Barrientos *et al*, 2015; Foex and Shelly, 1996). These cytokines can either enter the blood brain barrier and directly communicate with neuroimmune cells or activate the vagal nerve (McCusker and Kelley, 2013), all of which propagate the immune derived signal in the brain (Dantzer *et al*, 1998). The blood-brain barrier becomes altered in response to systemic immune activation, with changes in permeability or alterations in cells and proteins that keep the blood-brain barrier intact. Increased blood-brain barrier permeability after systemic immune activation results in entry of peripheral immune cells into the brain and an elevated inflammatory profile of the blood-brain barrier cells (Quan and Banks, 2007). Inflammatory insults may also alter blood-brain barrier function through decreased levels of transporters for organic anions & amino acids (Wittmann *et al*, 2015). Thus, systemic immune activation leads to a neuroinflammatory response, which typically protects the brain from the infection or injury (Gadani *et al*, 2015). The neuroinflammatory response includes changes to the neuroimmune system, including activity of glial cells such as microglia and astrocytes. Activated microglia change shape from ramnified to ameboid state and secrete cytokines that impact the function of other cells in the brain (Lynch, 2009). Astrocytes respond to these inflammatory signals secreted by microglia by changing in morphology and function (John *et al*, 2004; Schiweck *et al*, 2018; Tian *et al*, 2012). These activated astrocytes in turn produce cytokines that regulate neuroimmune processes (Farina *et al*, 2007; Norden *et al*, 2015).

Recent studies show that there are sex differences in the activation of microglia (Bodhankar *et al*, 2015; Morrison and Filosa, 2016; Schwarz and Bilbo, 2011) and astrocytes (Acaz-Fonseca *et al*, 2015; Cordeau *et al*, 2008; Santos-Galindo *et al*, 2011) after various inflammatory insults. Males show greater damaging neuroinflammatory responses while females show more protective responses after immune challenge (Santos-Galindo *et al*, 2011). Additionally, greater damaging (M1) microglial activation is observed after ischemic stroke in males (Morrison and Filosa, 2016) while greater protective (M2) microglial activation was observed after stroke in females (Bodhankar *et al*, 2015). Females have also been shown to be protected from other immune-inducing insults, including traumatic brain injury (Kim *et al*, 2019). Together, findings show that males may surmount greater neuroinflammatory responses during inflammatory insults, which may have more detrimental consequences for their memory long after resolution of an inflammatory event.

Inducing systemic immune and neuroimmune activation using LPS and Poly I:C

Animal models used to study the long-lasting consequences of a systemic inflammatory event on cognition have either used surgical procedures, including cecal ligation and puncture, or ligands that induce activation of innate immunity-related receptors sensing pathogen-associated and danger-associated molecular patterns, or toll-like receptors. Toll-like receptors are located on antigen presenting cells in the periphery, including B cells, dendritic cells, monocytes, and macrophages (Kawai and Akira, 2006). Activation of toll-like receptors leads to a systemic inflammatory response that can be used to mimic the inflammatory response observed during a systemic inflammatory event (Lin and Yeh, 2005).

In the brain, toll-like receptors are present on microglia, astrocytes, oligodendrocytes, and neurons, with preferential localization of toll-like receptor 3 on astrocytes and toll-like receptor 4

on microglia (Hanke and Keilian, 2011; Okun *et al,* 2012). Ligands that bind to these toll-like receptors also induce a neuroinflammatory response. One such ligand is the gram-negative bacterial endotoxin lipopolysaccharide (LPS), which can bind to its ligand toll-like receptor 4 (TLR4) on microglia. TLR4 activation results in microglial activation (Norden *et al,* 2016), cytokine production, and subsequent neuroimmune signaling (Okun *et al,* 2012). Neurons and glia also respond to viral DNA or RNA through activation of toll-like receptor 3. Toll-like receptor 3 recognizes double stranded viral RNA and can be activated with synthetic ligands such as polyinosinic:polycytidylic acid**,** or Poly I:C (Jiang *et al,* 2003). TLR3 activation leads to cytokine production and neuroimmune signaling (Park *et al,* 2006). The synthetic dsRNA analog Poly I:C is commonly used to mimic the acute phase of the immune responses to viruses (Traynor *et al,* 2004).

Behavioral changes after LPS and Poly I:C

Peripheral immune signaling causes activation of the neuroimmune system, which regulates acute physiological responses (e.g., fever, increased sleep), behaviors (e.g., sickness behaviors). Immune-inducing agents such as LPS and Poly I:C have prominent impacts on the animal's behavior and physiology, memory, affective processes, and cognition. Peripheral injection of LPS or Poly I:C induces transient sickness behaviors, which is characterized by decreased food consumption, body weight, voluntary wheel running, open field activity, or locomotor responses (Dantzer *et al,* 2008; Teeling *et al,* 2007; Vichaya *et al,* 2019). Both LPS and Poly I:C induce transient changes in fever and other "sickness behaviors" (Hopwood *et al,* 2009) that can be similar in time course and magnitude (Fortier *et al,* 2004). While these sickness behaviors are observed with acute injections of immune-stimulants such as LPS or Poly I:C, repeated injections of LPS or Poly I:C have been shown to induce tolerance, a phenomenon by

which weaker immune responses are observed in an organism during repeated administration of immune-stimulants (Engeland *et al*, 2001; Soszynski *et al*, 1991; Wickens *et al*, 2018). Tolerance can be overcome by using higher doses in the repeated injections (Musaelyan *et al*, 2018; Wickens *et al*, 2018). The sickness behaviors observed in animals after peripheral injections of LPS or Poly I:C mimics the sickness responses animals show after an infection.

Memory: Types of Memory, Circuits, and Molecular Mechanisms

Memory types

Memory is the process by which information about our world is encoded, stored, and retrieved and is critical for using past experiences to adjust to environmental changes and events (Kandel *et al*, 2014). Memory can be categorized into subtypes that differ in their purpose, salience, strength, and in underlying neural circuitry. Object recognition and object location memory tests rely on the rodent's ability to detect a particular object in an environment as well as on a rodent's ability and preference for novelty (object or location). The novel object recognition and object location tests do not require rule learning, necessary for reference memory, or additional motivation, reward, or punishment for required for associative learning (Antunes and Biala, 2012). On the other hand, fear memories depend on the rodent's ability to form associations between a novel context (CS) and an aversive stimulus (US), such as a foot shock that predicts the aversive outcome (shock in context) (Maren *et al*, 2013; Rudy *et al*, 2004). Fear memories are thus associative memories that are not only emotionally salient, given their associations with a negative outcome but also very strong, long-term memories that form only after a single CS-US pairing (Radulovic *et al*, 1998; Stiedl and Spiess, 1997). Similar to context fear conditioning, in auditory-cued fear conditioning, the animal associates the cue (e.g.

a tone) with a shock, also resulting in a strong, emotionally salient long-term memory (Rogan *et al*, 2003).

Circuits for novel object recognition, location, and fear conditioning

The distinct types of memory also use different brain regions and neuronal circuitry. Object recognition memory uses anterior subhippocampal cortex, including entorhinal and perirhinal cortices (Antunes and Biala, 2012). The perirhinal cortex is essential for analyzing visual aspects of object features (Jacklin *et al*, 2016), and familiarity discrimination, cognitive tasks not requiring hippocampus (Barker and Warburton, 2011). Object location is typically more hippocampal-dependent (Assini *et al*, 2009), with connections between the hippocampus and with the fornix, cingulate cortex, and hypothalamic nucleus (Ennaceur *et al*, 1997). The hippocampus is critical for contextual memory formation necessary to detect place of objects (Balderas *et al*, 2008; Winters, 2005). More specifically, the "*what"* information presented during an object recognition session is conveyed through the perirhinal cortex, while the "*where"* information is transmitted through the parahippocampal and entorhinal cortices. The "*what"* and "*where"* information converges in the hippocampus, with familiarity attributed to the perirhinal cortex and recollection to the hippocampus (Hampstead *et al*, 2016). Several brain regions play critical roles in fear memory. The dorsal and ventral hippocampus as well as cortical regions such as entorhinal cortex, piriform cortex, and cingulate cortex are important for fear memory (Anagnostaras *et al*, 2010; Huang *et al*, 2013; Rudy *et al*, 2004). The amygdala allows for successful association of the aversive stimulus (footshock) with the context (Ledoux, 2000; Maren, 2001). During presentation of the auditory-cue (e.g. tone), auditory thalamus-auditory cortex-basal amygdala networks become activated and converge with incoming somatosensory information to form the cue-shock association (Blair, 2001).

Memory processes may differ in males and females as each sex uses different strategies and molecular mechanisms to complete memory tasks. For example, males and females differ in spatial navigation strategies (Bettis and Jacobs, 2009) and processing of emotionally salient memories (Bellace *et al*, 2013). There are also sex differences in molecular mechanisms crucial for memory, including signaling pathways (Gresack *et al*, 2009; Keiser *et al*, 2017; Kudo *et al*, 2004) and related gene expression important for memory formation (Antunes-Martins *et al*, 2005; Mizuno and Giese, 2010). Along with the sex differences in specific memory processes, such as fear memory retrieval, males and females recruit different brain regions (Keiser *et al*, 2017) and likely different neural pathways to complete similar tasks. Therefore, given these sex differences in peripheral and neuroimmune processes as well as differences in learning strategies and mechanisms of memory, it is likely that a systemic inflammatory event will have different consequences for neural function, memory, emotion, and cognition in males and females.

Immune Modulation of Memory and Cognitive Functions

Memory modulation by immune signaling and neuroimmune cells

Memory can be modulated by various environmental experiences (Baldi and Bucherelli, 2007), that lead to stronger or weaker memories of certain experiences (Roozendaal and McGaugh, 2012). Immune signaling has been shown to modulate memory processes and their underlying mechanisms (Donzis and Tronson, 2014; Yirmiya and Goshen, 2011). For example, increased cytokine signaling in the brain can impair memory and dysregulate expression of memory-relevant genes such as *BDNF* (Bilbo *et al*, 2008). However, several cytokines, including but certainly not limited to IL-1β, TNFα, and IL-6, have regulatory functions, such as modulating neuronal plasticity at baseline (Yirmiya and Goshen, 2011). Cytokines exert their

neuromodulatory effects through networks signaling cascades rather than isolated pathways, and their impacts of acute neuroimmune signaling on neural function may depends on the pattern of cytokine signaling rather than simply which cytokines are activated (Donzis and Tronson, 2014). As such, acute cytokine signaling can lead to both enhancement and impairment of memory (Barrientos *et al*, 2002; Brennan *et al*, 2004; Gonzalez *et al*, 2013; Goshen *et al*, 2007; Yirmiya *et al*, 2002). The transient interactions of cytokine signaling cascades with memory-related pathways and associated gene expression may have long-lasting consequences for memory and cognitive processes.

Neuroimmune cells such as microglia can modulate memory and cognitive functions through their interactions with neurons via the chemokine CX3CL1-CX3CLR as well as through cytokine signaling (Justin *et al*, 2012; Sheridan *et al*, 2014) that can alter AMPA/ NMDAr currents and engulfment of synaptic material necessary for plasticity (Paolicelli *et al*, 2014; Riazi *et al*, 2015). Astrocytes are uniquely positioned at the tripartite synapse to provide energy to neurons, regulate neurotransmitter uptake and ionic balance, as well as regulate synaptic transmission and plasticity (Alberini *et al*, 2018; Nortley and Attwell, 2017; Suzuki *et al*, 2011). Microglia-astrocytic interactions are also important for processes that mediate memory, including neural plasticity (Pascual *et al*, 2011). Both of these neuroimmune cells may therefore play an important role in long-term memory formation (Steinman *et al*, 2016). Additionally, both microglia and astrocytes secrete cytokines, which participate in neural plasticity and neurotransmission, thereby mediating memory processes (Donzis and Tronson, 2014; Yirmiya and Goshen, 2011). While a wealth of studies has determined the role of acute neuroimmune signaling in modulation of memory processes, the mechanisms by which systemic immune

activation leads to long-lasting changes in neural function and memory still requires further exploration.

Acute alterations of memory, affective, cognitive processes after systemic immune activation

Systemic immune activation, either resulting from surgical interventions or peripheral of immune-inducing agents show short-term changes in learning and memory. Memory impairments in working or short-term memory, including deficits in the Y-maze and novel object recognition, are observed in sepsis models (Hou *et al*, 2016; Moraes *et al*, 2015). Acute, systemic LPS treatment produces impairments in several hippocampal-dependent memory processes, including passive avoidance learning and memory (Abareshi *et al*, 2016; Noorbakhshnia and Karimi-Zandi, 2017), contextual fear memory consolidation (Pugh *et al*, 1998) and reconsolidation (Kranjac *et al*, 2012), retrieval of context discrimination (Czerniawski and Guzowski, 2014; Kranjac *et al*, 2011) as well as short-term memory in novel object recognition (Carvalho *et al*, 2017; Sayed and El Sayed, 2016). Poly I:C has been shown to disrupt contextual fear memory consolidation (Kranjac *et al*, 2011). Unlike contextual fear conditioning, auditory-cued fear conditioning is not impaired after peripheral treatment of LPS nor Poly I:C (Kranjac *et al*, 2011; Pugh *et al*, 1998).

Studies examining the effects of more subchronic immune challenge have shown deficits across various memory tasks. For example, seven daily injections of Poly I:C for a week impaired performance on an associative memory task, such as contextual fear conditioning (Weintraub *et al*, 2014). Repeated peripheral LPS injections has been shown to impair novel object recognition (Eduviere *et al*, 2016; Zarezadeh *et al*, 2017) as well as other associative memory types, including the anticipatory gustatory response (Cloutier *et al*, 2012). However,

whether repeated peripheral injections of these immune-inducing agents results in long-lasting alterations in memory remains to be explored. Few studies have directly compared the effects of LPS or Poly I:C on memory processes, especially in females, and determining the long-lasting behavioral consequences of LPS or Poly I:C in both sexes will provide insights into the types of inflammatory insults that are most deleterious for males and for females.

Systemic immune challenge can also lead to depressive-like behaviors, which include appetite loss, sleep disturbance, reduced activity, and reduced social interest (Dantzer *et al*, 1998). Acute peripheral administration of LPS has been shown to increase different types of depressive-like behaviors, including anhedonia-like behavior, as measured by sucrose preference test (Salazar *et al*, 2013; Sens *et al*, 2017) and despair-like behavior, as measured by forced swim test and tail suspension test (Dinel *et al*, 2014; Mousavi *et al*, 2018). Repeated and intermittent LPS administration can also result in prolonged anhedonia-like behavior in sucrose preference test and in despair-like behavior in forced swim test (Kubera *et al*, 2013; Wickens *et al*, 2018). It remains to be explored whether subchronic immune challenge induces such long-lasting depressive-like behaviors in both sexes.

Mechanisms of Long-Lasting Memory Dysfunction after Systemic Inflammatory Event

Several mechanisms by which systemic immune activation induces memory and emotional dysregulation have been proposed to date. In patients, long-lasting changes in neuroimmune processes, blood-brain barrier function, and neuronal processes (Annane and Sharshar, 2015). In animal models similar neuroimmune mechanisms have been proposed, including microglial activation, disrupted blood–brain barrier, as well as metabolic changes,

including oxidative stress and changes in glucose metabolism (Semmler *et al*, 2008; Weberpals *et al*, 2009). Therefore prior interventions have targeted the blood–brain barrier, glial activation, and oxidative stress have shown promise in prevention of cognitive dysfunction in various experimental models of sepsis (Cunningham and Hennessy, 2015). Yet, sustained neuroimmune activation is not observed in all animal models long after the inflammatory event. Instead, persistent neuronal changes are observed long after a systemic inflammatory event.

Long-lasting changes in neuronal processes, including synaptic morphology and structure as well as neural substrates necessary for neuronal plasticity, have also been observed after a systemic inflammatory insult. Sepsis induces persistent neuronal loss in hippocampal subregions and prefrontal cortex (Semmler *et al*, 2007). More specifically, progressive decreases in CA1 neurons along with increases in nucleioli size have been observed from 14 to 60 days are sepsis (Guo *et al*, 2017). There can also be persistent loss of innervation of cholinergic in the parietal cortex (Semmler *et al*, 2007). However, these results are inconclusive as gross neuroanatomical changes and neurodegeneration are not always observed after sepsis (Chavan *et al*, 2012; Singer *et al*, 2016). Instead of neurodegeneration, mechanisms important for memory that are dysregulated long after sepsis include neurogenesis and synaptic plasticity. Persistent decreases in neural stem cell progenitor proliferation, newborn neurons and their synaptic contacts, and neurogenic reserve have been observed weeks after sepsis (Anderson *et al*, 2015; Ormerod *et al*, 2013; Valero *et al*, 2014). In some studies, these decreases in neurogenesis correlate with mild spatial memory impairments (Ormerod *et al*, 2013; Valero *et al*, 2014). Sepsis has been shown to result in long-lasting synaptic changes important for memory, such as decreased dendritic spine turnover two months (Kondo *et al*, 2011) and decreases in spine density and dendritic processes of CA1 neurons weeks and months after the inflammatory insult (Chavan *et al*, 2012; Huerta *et*

al, 2016). These changes in dendritic spine dynamics do not occur one or two weeks after the resolution of the immune response (Kondo *et al*, 2011; Volpe *et al*, 2015), suggesting the synaptic changes develop progressively but persist long after resolution of the systemic inflammatory event. Along with the reduction in synaptic spines, alterations in synaptic proteins have been observed months after sepsis, including increased synaptotagmin and decreased synaptophysin in the hippocampus eight weeks after LPS or CLP (Neves *et al*, 2016; Weberpals *et al*, 2009). These findings suggest that alterations in mechanisms important for neuroplasticity may underlie the long-lasting memory deficits observed after a systemic immune activation.

Persistent changes in neural substrates important for memory have also been observed after sepsis, including neurotransmitter systems and neuropeptide signaling that modulates neuroplasticity networks underlying memory. For example, dysregulation of hippocampal insulin signaling, including decreases in protein levels and phosphorylation of glycogen synthase kinase 3β (GSK3β)at serine residue 9 (GSK3βpSer9), Akt phosphorylated at serine residue 473(AktpSer473) persists a month after sepsis and are associated with impaired aversive and recognition memory (Neves *et al*, 2016). Persistent changes in cortical acetylcholine metabolism, including increases in acetylcholinesterase and increases cortical inhibition, which correlate with deficits in novel object recognition (Ming *et al*, 2015). Modulation of glutaminergic transmission, through the NMDA receptor agonist D-cycloserine or the antagonist MK-801, has been shown to reduce long-term cognitive impairment after systemic immune activation (Liraz-Zaltsman *et al*, 2016; Miranda *et al*, 2017). It is surprising that both increasing and decreasing glutaminergic transmission after sepsis can alleviate the memory deficits. Similarly, alterations in transcription factors involved in neuroplasticity, such as decreases in protein levels and phosphorylation CREB at serine133, are observed months after sepsis (Neves *et al*, 2016). As

activity-dependent expression is necessary for regulating neuroplasticity underlying memory formation (Hawk and Abel, 2011), changes in activation of these immediate early genes after memory tests such as contextual fear conditioning may alter how the memory is stored. Thus, long-lasting changes in neuronal processes, including dendritic spine density or plasticity as well as in protein levels of immediate early genes, that persist months after a systemic inflammatory insult (Anderson *et al*, 2015; Huerta *et al*, 2016; Kondo *et al*, 2011; Volpe *et al*, 2015) and are associated with memory deficits (Huerta *et al*, 2016). These long-lasting decreases in neuronal processes may underlie delayed or persistent memory deficits after a systemic inflammatory event.

Together, these studies suggest that the memory deficits that persistent long after systemic immune activation are not caused only by ongoing systemic inflammatory or neuroimmune processes. Rather, the transient interactions between peripheral inflammatory signaling and neuronal networks relevant for memory and affective processes during systemic immune activation must trigger long-lasting changes in neural function that results in the long-lasting changes in memory, cognition, and emotion (Donzis and Tronson, 2014; Tchessalova *et al*, 2018). As previous animal models have solely focused on the mechanisms of long-lasting memory dysfunction in males, it is imperative that these mechanisms be delineated in females. Studies including both sexes will allow to determine whether males and females show similar or differential patterns of memory deficits after a systemic inflammatory event and to identify molecular mechanisms mediating the changes in memory in males and females.

Goal Dissertation Project and Summary Hypotheses

The goal of this dissertation project is to characterize a mouse model in which we can study how a mild, systemic inflammatory event leads to long-lasting changes in memory and affective processes in males and in females. I first determine whether subchronic immune challenge induces long-lasting changes in particular memory or affective processes in males and in females. I then explore the mechanisms by which systemic immune activation may lead to long-lasting cognitive dysfunction. My guiding hypothesis is that a mild systemic inflammatory event will lead to memory deficits along with increases in anxiety-like and depressive-like behaviors weeks and months after the inflammatory insult. Along with the persistent changes in memory, I anticipate long-lasting changes in mechanisms of memory modulation, that may differ between males and females. Alterations in neuronal processes rather than neuroimmune processes are likely involved in memory modulation long after subchronic immune challenge. *Based on this, I postulate that mild systemic immune activation persistently dysregulates memory and affective processes via sex-specific mechanisms.*

Specifically, I postulate:

1. **Persistent, sex-specific deficits in different memory processes after subchronic immune challenge.** In Chapter 2, I determined short-term and long-lasting changes in memory after subchronic immune challenge in males and in females. This chapter explored how different types of memory, including object recognition, object location, and fear-associated memory, as well as how different types of memory processes, such as memory formation and retrieval, are impacted by prior subchronic immune challenge. I hypothesized that subchronic immune challenge (LPS or Poly I:C treatment) would result

in persistent impairments in object recognition, object location, and fear-associated memories, deficits in both memory formation and retrieval, sex-specific memory deficits, with females being more protected against the negative consequences of systemic immune activation than males, as well as some differences in behavioral consequences of LPS or Poly I:C challenge.

2. **Long-lasting affective dysregulation after subchronic immune challenge.** In Chapter 3, I determined whether subchronic immune challenge alters affective processes in males and in females long after injection. We focused on anxiety-like behaviors as well as different types of depressive-like behaviors. I hypothesized that subchronic LPS or Poly I:C treatment will result in persistent increases in anxiety-like behaviors in males and females, increased despair-like behavior in both sexes, and increased anhedonic-like behavior in males and females.

3. **Enduring dysregulation of hippocampal gene expression after subchronic immune challenge**. The goal of Chapter 4 was to discover novel molecular substrates that underlie long-lasting hippocampal dysfunction after subchronic immune challenge in males and in females. We focused on the dysregulation of the hippocampal transcriptome not only after the subchronic immune challenge but also after subchronic immune challenge and a secondary, acute inflammatory insult. Specifically, I hypothesized that subchronic immune challenge will result in persistent upregulation of immune-related substrates, including mediators of immune signaling, downregulation of memory-related genes such as immediate-early genes and growth factors (e.g. IFG1/2, BDNF), and downregulation

of genes important for affective processing, including monoaminergic targets. A secondary, acute immune insult months after subchronic immune challenge will likely result in upregulation of immune-related genes, including cytokines, as well as dysregulation of genes important for neural functions, such as neurotransmission and neuroplasticity.

4. **Neuroimmune and neuronal processes are involved in memory modulation months after subchronic immune challenge.** In Chapter 5, I determined whether overt neuroimmune processes, including blood-brain barrier permeability and microglial activation, continue months after subchronic immune challenge. I then identified sustained changes in plasticity-related neuronal processes, such as activity-dependent induction of immediate early gene c-Fos after a memory test. I anticipated no persistent blood-brain barrier permeability nor microglial activation months after subchronic immune challenge. Yet, I expected that changes in neuroplasticity-related mechanisms, such as dysregulation of the activity-dependent induction of the immediate early gene c-Fos, are associated with the long-lasting memory impairments.

Chapter II

Long-Lasting Changes in Memory after Subchronic Immune Challenge

Abstract

Memory impairments and cognitive decline persist long after recovery from major illness or injury, and correlate with increased risk of later dementia. Here we developed a subchronic peripheral immune challenge model to examine delayed and persistent memory impairments in females and in males. We show that intermittent injections of either lipopolysaccharides or Poly I:C cause memory decline in both sexes that are evident eight weeks after the immune challenge. Importantly, we observed sex-specific patterns of deficits. Females showed impairments in object recognition one week after challenge that persisted for at least eight weeks. In contrast, males had intact memory one week after the immune challenge but exhibited broad impairments in memory tasks including object recognition, and both context and tone fear conditioning several months later. Together, these data suggest that subchronic immune challenge results in differential vulnerabilities of females and males to memory decline after immune challenge. This model will be an important tool for determining the mechanisms in both sexes that contribute to memory impairments that develop over the weeks and months after recovery from illness. Future studies using this model will provide new insights into the role of chronic inflammation in the pathogenesis of long-lasting memory decline and dementias.

Introduction

Long-lasting memory dysfunction is common after a critical illness or major surgery, such as sepsis or cardiac surgery. More than 25% of patients develop memory impairments that persist for months to years after recovery from major illness (Gharacholou *et al*, 2011; Semmler *et al*, 2013). In animal models, there are long-lasting consequences of an overwhelming immune challenge on neural and cognitive function and for neuroimmune signaling. The cecal ligation and puncture (CLP) model of sepsis, results in impairments of memory that emerge soon after surgery (e.g.,Barichello *et al*, 2007) and persist for months (e.g.,Huerta *et al*, 2016). Similarly, a single high-dose injection of lipopolysaccharides (LPS) results in memory deficits at least 5 months post-injection (Ming *et al*, 2015). Nevertheless, since disease processes persist long after the initial immune challenge in these models, it is not clear whether sustained neuroimmune dysregulation or immune-triggered changes in neural processes mediates memory deficits long after a systemic immune challenge.

Lower intensity immune challenges may be more relevant for understanding the impact of systemic immune activation on neural processes as well as for modeling progressive memory impairments as a consequence of chronic, low-grade inflammation (Yaffe *et al*, 2003). Repeated, lower dose immune challenges also cause impairments of memory (Kahn *et al*, 2012; Weintraub *et al*, 2013, 2014) and neural plasticity (Maggio *et al*, 2013) in the first weeks after injection. There is also some question as to whether different types of immune challenge result in similar or different outcomes. Systemic injections of LPS have been extensively used to trigger immune activation and model sepsis and its effect on central nervous system in adult animals (Ming *et al*, 2015; Pugh *et al*, 1998; Weberpals *et al*, 2009). In contrast, the viral mimic Polyinosinic:polycytidylic acid (Poly I:C) is commonly used in models of maternal or early-life

immune challenge (Arsenault *et al,* 2014). In adult animals, systemic immune challenge with either LPS and Poly I:C result in memory impairments (Cloutier *et al,* 2012; Frühauf *et al,* 2015; Kranjac *et al,* 2011, 2012), albeit via different mechanisms (Doyle *et al,* 2003) and with some differences in behavioral effects (Arsenault *et al*, 2014; Hopwood *et al,* 2009).

Given the greater vulnerability of women to memory disorders such as Alzheimer's disease (Snyder *et al,* 2016), we are particularly interested in the differential impact of subchronic immune challenge on males and females. Sex differences in peripheral responses are well described (Ghosh and Klein, 2017; Scotland *et al,* 2011) and there is growing evidence for sex differences in neuroimmune responses and function (Acaz-Fonseca *et al,* 2016; Engler *et al,* 2016; Sorge *et al,* 2016; Speirs and Tronson, 2018). It is likely that males and females are differentially susceptible to memory decline after subchronic immune challenge.

In this chapter, we determined changes in memory weeks to months after a moderate, subchronic immune challenge. To date, all studies of the lasting consequences of immune challenge on memory have been conducted with male animals. Here we identified a causal role for subchronic immune activation on memory deficits in the weeks and months following recovery in both sexes. *I hypothesized that systemic, subchronic immune challenge, both with LPS or Poly I:C, will result in persistent deficits in hippocampal-dependent memory, including in novel object recognition, novel object location, and context fear conditioning, and not in amygdala-dependent processes, such as auditory-cued fear conditioning. I anticipated that females may be more protected than males from memory impairments.* Instead, we demonstrated that males show delayed memory deficits in hippocampal- and amygdala-dependent tasks that emerged several months after immune challenge. Females showed impaired object recognition memory soon after immune challenge and these deficits persisted for at least eight weeks.

Materials and methods

2.1 Animals. 9-11 week old male and female C57BL/6N mice from Envigo (Indianapolis, IN) were used in all experiments. Mice were individually housed with mouse chow and water provided *ad libitum* as previously described (Keiser et al., 2017). Individual housing in mice prevents fighting-induced stress (Meakin et al., 2013) and is ethologically appropriate for males and females (Becker & Koob, 2016). Individual housing is suitable for testing novel object recognition (Vogel-Ciernia & Wood, 2015) and contextual fear conditioning (Keiser et al., 2017) and follows the University of Michigan Institutional Care and use Committee policies on managing fighting in mice. The facility is ventilated with constant air exchange (60 m^3/ h), temperature (22 ±1°C), and humidity (55±10%) with a standard 12 h light-dark cycle. Experiments were performed during the light portion of the cycle. Mice were acclimated to the colony room for at least seven days prior to injections. All experimental methods used in these studies were approved by the University of Michigan Committee on the Use and Care of Animals.

2.2 Immune stimulants. Lipopolysaccharides (LPS, Escherichia coli, serotype 0111:B4; Sigma-Aldrich, St. Louis) was dissolved in saline (12.5μg/mL) and was injected intraperitoneally (i.p.; 250μg/kg; Fruhauf et al., 2015). Polyinosinic:polycytidylic acid (Poly I:C, P9582; Sigma-Aldrich) lyophilized powder was dissolved in distilled deionized water (10mg/ml), heated to 50°C and cooled to allow re-annealing, and injected i.p. (6mg/kg; Cunningham, Campion, Teeling, Felton, & Perry, 2007). These doses of LPS and Poly I:C were chosen based on previous reports of efficacy of these doses on memory-related paradigms (e.g., (Frühauf et al.,

2015; Kranjac et al., 2011), for their similar and transient effects on weight loss (Fig. 1), and for their minimal effect on observed sickness behaviors in our laboratory in either sex.

2.3 Subchronic Immune Challenge. Mice received five intermittent injections of LPS (250μg/kg; n= 8-9), Poly I:C (6 mg/kg; n= 8-9), or saline control (n = 8-9), spaced three days apart. All injections were performed at the same time of day (Roberts, 2000). Mice were weighed daily throughout the injection period and weekly until testing. Changes in weight were assessed using a repeated-measures (Day × ImmuneChallenge) ANOVA.

2.4 Behavioral Testing. Memory tests began one (Figs. 2-3) or eight weeks (Figs. 4-7) after the final injection. All testing was completed within 12 weeks after subchronic immune challenge. In experiments in which animals were tested on multiple tasks (e.g., Figs. 4 & 6; Figs. 5B-D & 7B-E), novel object recognition (3-8 days of testing) was always conducted first, followed by context fear conditioning (3 days of testing), with 3-10 days between tasks. Estrus cycle phase was determined by obtaining wet vaginal smears approximately 1hr prior to behavioral testing and assessing vaginal cytology under the light microscope (Caligioni, 2009).

2.4.1 Novel Object Recognition. The testing arena consisted of two rectangular opaque white chambers, (LWD: 40cm × 32cm × 32.5cm; 45 lux at center). Two novel object recognition protocols were used: (1) a hippocampal-dependent protocol where memory persists for at least 24 hours (Vogel-Ciernia & Wood, 2015); and (2) a more commonly assessed protocol that typically results in short-term novel object recognition when animals are tested 3 hours after training (Ballaz, Akil, & Watson, 2007).

To assess long-term novel object recognition memory, mice were first habituated to

testing chambers (10 mins/day for 6 days). Mice received two 10-minute training trials spaced 3 hours apart in which they explored two identical objects. A single test session occurred 24 hours after the first training session, in which mice were replaced in the arena with one familiar object (from training) and one novel object (Vogel-Ciernia & Wood, 2015). Novel and familiar objects were counterbalanced across animals. The time spent exploring each object (animal's nose within 2 cm of object) was measured automatically (Ethovision XT 9.0 tracking software; Ballaz, Akil, & Watson, 2007) and corroborated by an experimenter blind to experimental conditions. Novel object preference was calculated as the percent time spent at the novel object {100*[(Time exploring novel object)/(time spent exploring both objects)]}.

Short-term novel object recognition was assessed as a single, 10-minute training session followed 3 hours later by a test session as described above (Ballaz et al., 2007).

Data Analysis and statistics. Habituation was analyzed using repeated measures (Day × ImmuneChallenge) ANOVA. Separate repeated measures (ObjExpl × ImmuneChallenge) ANOVA were conducted for each sex (e.g., Darcet et al., 2014). Planned comparisons were used (with LSD) to examine comparisons between exploration of novel and familiar objects for each group.

2.4.2 Novel Object Location (NOL). To test novel object location, visual cues were added to the north and south walls of testing chambers used for novel object recognition. Mice were habituated to testing chambers for three days (10 mins per day). Training was identical to novel object recognition. Twenty-four hours after the first training session, one of objects was placed in the same (familiar) location and one was placed in a novel location (Vogel-Ciernia and Wood, 2015). Automated and hand-scoring criteria for object location exploration were identical to those used in novel object recognition. For all novel object recognition and location experiments,

boxes were cleaned in between animals with 70% ethanol.

Data analysis and Statistics: Separate repeated measures ANOVA were conducted to assess the effect of immune challenge on novel object recognition and location for each sex. Habituation was analyzed using repeated measures ANOVA. Post hoc tests were used to examine specific comparisons.

2.4.3 Context Fear Conditioning. The context fear conditioning apparatus consisted of rectangular chambers (LWD: 9.75″ × 12.75″ × 9.75″) containing grid floor rods connected to a shock generator, an enclosed sound-attenuating system, and a NIR camera (VID-CAM-MONO-2A) and Video Freeze software for automatic scoring of freezing behavior (MedAssociates, VT). During background context fear conditioning, mice were placed in the training context (rectangular box with white walls, lights on, an evenly sized grid floor, 70% ethanol odor) for 3-min, after which a 30-sec tone (10 kHz, 75 dB SPL) was presented co-terminating with a 2-sec 0.8mA footshock (Tronson et al., 2010). Mice were returned to their home cages immediately following training. We assessed automatically recorded locomotor activity during training to determine whether subchronic immune challenge alters movement or exploration prior to the shock (locomotor activity) (Cunningham & Sanderson, 2008) and locomotor response during the 2-sec shock (shock reactivity) to identify whether prior immune challenge resulted in differences in sensitivity to the aversive US (Tronson et al., 2010).

Twenty-four hours later, context fear memory was assessed. Mice were replaced in the training context for 3 minutes and freezing behavior was measured (Keiser et al., 2017). The following day, mice were tested for fear conditioning to the tone in a novel context (black angled walls, house lights off, staggered grid floors, 1% acetic acid odor). After 90 seconds in the novel context, three 30-sec tones separated by 60-sec intertrial intervals were presented. Freezing to the

training context and to the tones were automatically scored using Video Freeze software (MedAssociates) (Anagnostaras et al., 2010; Keiser et al., 2017).

To assess the effect of subchronic immune challenge on late-occurring impairments in memory retrieval, animals were trained on background context fear conditioning one week after immune challenge and re-tested for context- and tone- fear memory seven weeks later. Foreground context fear conditioning was conducted as above, without presentation of the tone or tone fear tests (Keiser et al., 2017). In extinction trials, animals were re-exposed to the training context without shock for 3 mins for nine consecutive days (Guedea *et al*, 2011).

Data Analysis and Statistics. Separate one-way ANOVA were used to assess the effect of prior immune challenge on context fear conditioning for each sex, and repeated measures ANOVA (Tone × ImmuneChallenge) were used to assess freezing to the cue in background fear conditioning. Post hoc tests (with LSD) were used to further assess specific group differences. Because males and females were always tested separately, group differences were compared within sex.

Results

3.1 Systemic effects of Poly I:C and LPS.

To assess acute and long-lasting systemic effects of subchronic Poly I:C and LPS, we measured changes in weight over the eight week post-challenge period (Fig. 1). All mice gained weight across the 15 day treatment period (Injection: Males: $F(4,88) = 43.01$, $p < 0.001$; Females: $F(4,96) = 42.72$, $p < 0.001$). Across all 5 injections, male and female mice treated with LPS or Poly I:C showed decreased weight the day after injection (Males: ImmuneChallenge × Cycle: $F(4,44) = 4.32$, $p < 0.01$; LPS: $p < 0.01$; Poly I:C $p < 0.05$ vs injection day; Females:

ImmuneChallenge × Cycle: $F(4,48) = 4.63$, $p < 0.01$; LPS $p < 0.05$, Poly I:C $p < 0.01$ vs injection day Fig. 1B,C). For male mice treated with LPS, this weight loss persisted on the second day after injection (LPS: $p < 0.05$; Poly I:C $p = 0.40$ vs injection day). Saline-treated males showed no change in weight on average across the 2 days after injection ($p = 0.22$ and $p = 0.52$, respectively vs injection day) and saline treated females showed a small but significant weight gain ($p < 0.01$, $p = 0.01$, respectively vs injection day). Across the eight week time period, mice gained weight at equivalent rates regardless of prior treatment (Males: ImmuneChallenge × Day: $F(2,22) = 1.93$; $p = 0.17$; Females: $F(2,22) = 1.91$, $p = 0.17$).

3.2 Deficits in 24-hour novel object recognition memory months after subchronic immune challenge in both males and females.

To assess enduring changes in memory, we tested novel object recognition memory eight weeks after LPS or Poly I:C using a memory paradigm that requires hippocampal-dependent processes 24 hours after training (Vogel-Ciernia & Wood, 2015; Fig. 2A). During test, we observed a significant detrimental effect of prior immune challenge on exploration of the novel object compared to the familiar object in males (ObjExpl × ImmuneChallenge: $F(2,22) = 3.90$, $p < 0.05$), with saline ($p < 0.01$), but not LPS ($p = 0.76$) nor Poly I:C ($p = 0.93$) treated males showing intact object recognition memory (Fig. 2B). In females, we observed a trend towards decreased novel object recognition after immune challenge (ObjExpl × ImmuneChallenge: $F(2,14) = 2.97$, $p = 0.07$). Importantly, only saline-treated females showed significantly more exploration of the novel object than the familiar object (saline: $p < 0.05$; LPS: $p = 0.93$; Poly I:C $p = 0.46$; Fig. 2C). Immune challenge disrupted novel object recognition in both males and in females.

There were no differences in locomotor activity nor habituation to the testing chamber for either sex (Males: Day: $F(5,110) = 3.95$, $p < 0.01$; Day × Drug: $F(10,110) = 1.29$, $p = 0.27$; Females: Day $F(5, 120) = 21.17$, $p < 0.001$; Day × Drug: $F(10,120) < 1$; Fig. 2D,F). During training, there were no differences in locomotor activity (Males: Drug: $F(2,22) < 1$; Females: $F(2,24) < 1$), and all animals showed similar exploration of objects, regardless of prior treatment (Males: $F(2,24) < 1$; Females $F(2,26) < 1$; Fig. 2E,G). Prior subchronic LPS or Poly I:C treatment did not affect locomotor activity, habituation to a new arena, or object exploration in males or females. Object recognition memory did not differ between females in different stages of the estrous cycle at training or testing, suggesting that estrous cycle phase does not alter the long-lasting impact of subchronic immune challenge on object recognition memory in females (data not shown).

3.3 Disruption of 3-hour novel object recognition months after subchronic Poly I:C or LPS.

To further examine memory deficits eight weeks after subchronic Poly I:C in males, we used a separate cohort of animals and a short-term novel object recognition paradigm that is less dependent on hippocampus (Ennaceur, Neave, & Aggleton, 1997). There were no differences between groups in object exploration ($t(14) = 1.19$, $p = 0.25$; Fig. 3C) nor locomotor activity ($t(14) <1$; Fig. 3D) during training. At test, novel object preference was significantly impaired in the Poly I:C-treated compared with the saline-treated mice (ObjExpl × ImmuneChallenge: $F(1,14) = 21.55$, $p < 0.01$; Fig. 3B), with significantly greater novel object exploration after saline ($p < 0.05$) and significantly lower novel object exploration after Poly I:C ($p < 0.01$).

Similarly, short-term novel object recognition was impaired in both sexes eight weeks after subchronic LPS challenge compared with saline-treated controls (Males: ObjExpl ×

ImmuneChallenge $F(1,14) = 8.03$, $p < 0.05$; Females: (ObjExpl × ImmuneChallenge $F(1,14) = 32.88$, $p < 0.01$; Fig. 3E,H). Only saline-treated animals showed preference for the novel object (Males: $p < 0.05$; Females $p < 0.01$), and LPS-treated animals showed no preference (Males: $p = 0.23$). Prior LPS had no effect on object exploration (Males: $t(14)= 1.04$, $p = 0.31$; Females: $t(14) = 1.47$, $p = 0.17$; Fig. 3F,I) or locomotor activity during training (all $t(14) <1$; Fig. 3G,J). Novel object recognition memory was not affected by estrous cycle (data not shown). Together, the data from both short- (Fig. 3) and long-term (Fig. 2) memory paradigms, demonstrate that novel object recognition memory is robustly impaired several months after either subchronic Poly I:C or LPS challenge.

3.4 Novel object location memory months after subchronic immune challenge in both males and females. To further assess long-lasting changes in hippocampal-dependent location memory, we tested males and females for object location memory 9 weeks after subchronic LPS or Poly I:C challenge. We observed no significant disruption of novel object location memory between experimental groups in both males and females (Males: ObjExpl: $F(2,22) <1$, ObjExpl x ImmuneChallenge: $F(2,22) = 1.75$; $p = 0.20$); Females: $F(2,22) = 1.36$, $p = 0.26$; ObjExpl x ImmuneChallenge: $F(2,22) < 1$; Figure 4B,C). There were no differences in locomotor activity (Males: $F(2,22) = 1.02$, $p = 0.38$; Females: $F(2,24) = 0.92$, $p = 0.41$), habituation to the testing chambers (Males: Day $F(2,44)=12.45$; $p < 0.001$; Day x Drug: $F(4,44) < 1$; Females: Day $F(2,48) < 1$; DayxDrug $F(4,48) = 1.07$, $p = 0.37$; Figure 4D,F), or object exploration during training (Males: $F(2,24) < 1$; Females: $F(2,26) < 1$; Figure 4 E,G). As there were several animals that explored the objects less than 3 seconds, this data was not included in the manuscript for *Neurobiology of Learning and Memory*.

3.5 Impaired context fear conditioning three months after Poly I:C challenge in males but not females.

We next tested whether subchronic immune challenge alters hippocampal-dependent background fear conditioning. Given prior testing in these animals in novel object recognition (see Fig. 3), fear conditioning took place approximately 10 weeks after the subchronic immune challenge (Fig. 5A). In males, prior Poly I:C but not LPS challenge caused deficits in context fear conditioning ($F(2,24) = 3.63$, $p < 0.05$; Poly I:C $p < 0.05$, LPS: $p = 0.97$ cf saline; Fig. 5B). In contrast, females showed no deficits in context fear conditioning after either LPS or Poly I:C ($F(2,24) < 1$; Fig. 5C). Neither locomotor activity during training (Males: $F(2,24) <1$, Females: $F(2,24) < 1$; Fig. 5D,F) nor response to shock (Males: $F(2,22) = 2.08$, $p = 0.15$, Females: $F(2,24) = 1.22$, $p = 0.32$; Fig. 5E,G) differed across groups in either sex, thus deficits in context fear conditioning were not due to a failure to detect the shock US. Freezing levels during test did not differ between females in different stages of the estrous cycle during training or testing, suggesting that estrous cycle stage does not alter fear conditioning in females (data not shown). Together, these findings demonstrate that males, but not females, are sensitive to disruption of fear conditioning long after subchronic Poly I:C challenge.

Due to the surprisingly long-lasting nature of the fear memory deficits after subchronic Poly I:C challenge in males, we next determined whether this finding was robust and replicable. We used a separate cohort of animals that underwent the short-term novel object recognition a day prior to context fear conditioning (Fig. 3). Here, the animals were tested in context fear conditioning 9 weeks after last injection. Once again, in mice previously tested on novel object recognition we observed that Poly I:C induced disruption of background context fear conditioning after subchronic immune challenge in males. During test, mice displayed

significantly lower freezing in the training context compared with saline controls ($t(14) = 2.75$, $p < 0.05$; Fig. 5I). There were no observed changes in locomotor activity ($t(14) < 1$; Fig. 5J) or reactivity to the shock ($t(14) < 1$; Fig. 5K) in Poly I:C-treated mice.

It is particularly striking that the same female animals showed deficits in hippocampal-dependent novel object recognition (Fig. 2C) but not context fear conditioning (Fig. 5C), which also depends on hippocampus. Similarly, after LPS, the same males showed impairments of novel object recognition (Fig. 2B) but not context fear conditioning (Fig. 5B).

3.6 Mild impairment in tone fear conditioning months after Poly I:C challenge in males but not females.

In males, fear conditioning to the tone was also impaired, with lower freezing to the first test tone in Poly I:C-treated males (ImmuneChallenge: $F(2,22) = 4.87$, $p < 0.05$; Tone × ImmuneChallenge: $F(4,44) = 2.77$, $p < 0.05$; Tone: $F(2,44) = 1.50$ $p = 0.24$; $p < 0.05$ *cf* saline*; p* < 0.01 cf LPS; Fig. 6B). There were no differences between groups in freezing to the novel context ($F(2,22) = 2.15$, $p = 0.14$; 6B). In contrast, females showed no alterations of tone fear conditioning after either immune challenge (ImmuneChallenge: $F(2,44) = 1.31$, $p = 0.29$; Tone × ImmuneChallenge $F(4,48) = 1.38$; $p = 0.25$; Tone: $F(2,48) = 19.48$, $p < 0.001$ Fig. 6C), with no differences in freezing to the novel context ($F(2,24) < 1$; Fig. 6C). We replicated these findings in a separate cohort of males only, whom underwent context fear conditioning in section 3.5 (Figure 5). Tone fear conditioning was once again impaired after Poly I:C (ImmuneChallenge $F(1,14) = 6.67$, $p < 0.05$; Tone: $F(2, 28)$, $F < 1$; ImmuneChallenge × Tone $F(2,28) = 1.27$, $p = 0.23$), with no differences in freezing to the novel context ($t(14) = 1.15$, $p = 0.27$; Fig. 6I).

3.7 No deficits in context fear conditioning two months after subchronic immune challenge.

In a separate experiment, we assessed the effect of subchronic LPS on persistent memory deficits in both sexes, but with alterations to the timeline used in experiments in section 3.5 (Figure 5). Because the previous experiment was conducted several weeks after the 8-week timepoint at which we observed novel object recognition deficits (Fig. 5A), we tested the possibility that LPS-induced memory deficits recover more quickly than those induced by Poly I:C by testing animals at eight weeks after subchronic LPS challenge (Fig. 7A). We showed that subchronic LPS caused no disruption in context fear conditioning in either sex eight weeks after challenge (ImmuneChallenge $F(1,28) = 3.56$, $p = 0.07$; Sex $F(1,28) < 1$; ImmuneChallenge × Sex $F(1,28) < 1$; Fig. 7B,C). There were no observed changes in locomotor activity or reactivity to the shock (all F< 1). Together with data from 3.5, these findings demonstrate that long-lasting memory deficits in context fear conditioning are observed months after Poly I:C, but not LPS.

3.8 No deficits in fear extinction 8 weeks after subchronic LPS challenge.

As we observed no differences in memory retrieval 8 weeks after subchronic immune challenge, we determined whether immune challenge resulted in persistent changes in a different type of memory, fear extinction. We observed no differences in freezing to the training context between the experimental groups across six days of extinction in both sexes (ExtinctionDay x Drug: $F(6,162) = 1.85$, $p = 0.12$, ExtinctionDay x Drug x Sex: $F(6,162) = 1.04$, $p = 0.39$). However, there were differences in extinction between males and females, with females but not males showing extinction across the extinction days (ExtinctionDay: $F(6,162) = 23.08$, $p < 0.0$; ExtinctionDay x Sex: $F(6,162) = 2.35$, $p = 0.033$) (Data not shown).

3.9 Females, but not males, show object recognition memory deficits soon after subchronic immune challenge.

Novel object recognition memory was tested one week after Poly I:C using a paradigm resulting in a hippocampal-dependent memory 24 hours after training (Vogel-Ciernia & Wood, 2015; Fig.2A). In males, we observed preference for exploration of the novel compared to the familiar object in both saline- and Poly I:C-treated groups (ObjExpl*:* $F(1,14) = 7.82$, $p < 0.05$) and no differences between treatments (ObjExpl × ImmuneChallenge: $F(1,14) < 1$; Fig. 2B). Therefore, males showed no deficit in object recognition one week after immune challenge. In contrast, subchronic immune challenge caused impairments of novel object recognition in females (ObjExpl × ImmuneChallenge: $F(1,14) = 7.96$, $p < 0.05$; Fig. 2C). Whereas saline-treated mice showed significantly greater exploration of the novel vs the familiar object ($p < 0.01$), Poly I:C-treated mice showed no such preference ($p = 0.46$). Thus females, but not males, are sensitive to impairments in memory in the first weeks after a subchronic immune challenge.

These memory deficits were not due to changes in locomotor activity or exploration. There were no differences in habituation to the testing chambers (Males: Day $F(5,70) = 5.75$; $p < 0.01$; Day × Drug: $F(5,70) < 1$; Females: Day $F(5,70) = 5.75$, $p < 0.01$; Day × Drug $F(4,48) = 1.07$, $p = 0.37$; Fig. 2D,F). All groups also showed similar locomotor activity (Males: $t(14) <1$; Females: $t(14) < 1$) and object exploration during training (all $t < 1$; Fig. 2E,G). No decreases in body weights were observed in Poly I:C-treated mice prior to habituation one week or two weeks after the last injection, prior to novel object recognition training (Males: ImmuneChallenge × Day: $F(2,28) <1$; Females: $F(2,28) < 1$).

3.10 Novel object location memory is intact in males and females 2 weeks after immune challenge.

Novel object location was tested two weeks after last injection. No deficits in novel object location were observed between Saline and Poly I:C treated males (Main effect ObjectExploration: $F(1,14) = 40.18$; $p < 0.01$; ObjExplxImmuneChallenge $F(1,14) < 1$; Fig. 9B) nor females (Main effect ObjectExploration: $F(1,14) = 22.98$; $p < 0.01$; ObjExplxImmuneChallenge: $F(1,14) = 2.35$, $p = 0.15$; Fig. 9C) after Poly I:C injection. Again, subchronic Poly I:C challenge did not alter locomotor activity (Males: $t(1,14) < 1$; Females: $t(1,14) < 1$), habituation to the testing chambers (Males: Day $F(2,14) = 9.20$, $p < 0.01$; Day x Drug: $F(2,14) < 1$; Females: Day $F(2,14) = 2.85$, $p = 0.092$; Day x Drug $F(2,14) = 3.25$, $p = 0.071$; Fig 9D,F), or object exploration during training in novel object location (Males: $t(1,14) < 1$; Females: $t(1,14) < 1$; Fig 9E,G). Thus, males nor females are sensitive to impairments in memory soon after a subchronic immune challenge.

3.11 Context and cued-fear conditioning is intact soon after immune challenge.

In a separate cohort of mice, we examined whether context- dependent fear conditioning was modulated one week after subchronic immune challenge, and if remote memory or retrieval processes were impacted seven weeks later (Fig. 3A). In males, we found no memory deficits after training one week after immune challenge, or during the remote memory test seven weeks later. Contrary to expectations, we observed that males showed an increase in freezing to context one week after Poly I:C (ImmuneChallenge $F(1,13) = 5.17$, $p < 0.05$; Time × Immune Challenge: $F(1,13) = 2.19$, $p = 0.16$; Time: $F(1,13) < 1$; 1week: $p < 0.01$; 8 weeks: $p = 0.67$; Fig. 3B). Subchronic immune challenge did not result in either memory deficits at one week, or in retrieval

deficits eight weeks after the final injection.

In females, there were no effects of prior Poly I:C on context fear conditioning one week after subchronic immune challenge (ImmuneChallenge: $F(1,14) = 2.1$, $p = 0.17$; Time × ImmuneChallenge: $F(1,14) < 1$; Fig. 3C). However females in both groups showed a dramatic decrease in freezing to the context seven weeks after training (Time: $F(1,14) = 27.30$, $p < 0.001$; Fig. 3C), suggesting a decrease in memory retrieval, independent of prior immune challenge, at a remote time point in females. We observed no effect of Poly I:C on cued fear conditioning or retrieval one week or eight weeks after the final injection in either sex (Males: ImmuneChallenge: $F(1,13) < 1$; ImmuneChallenge × Time $F(1,13) < 1$; ImmuneChallenge × Time × Tone $F(2,26) < 1$; Fig. 3D; Females: ImmuneChallenge: $F(1,14) = 3.32$, $p = 0.09$; ImmuneChallenge × Time × Tone $F(2,28) < 1$; Fig. 3E) demonstrating that Poly I:C does not cause short-lasting changes in auditory fear conditioning or long-lasting changes in amygdala-dependent fear memory retrieval. Importantly, there were no differences in locomotor activity (Males: $t(13) = 1.13$, $p = 0.28$; Females: $t(13) < 1$; Fig. 3F,H) or response to the footshock during training (Males: $t(13) = 1.57$, $p = 0.14$; Females: $t(13) < 1$; Fig. 3G,I).

Discussion

These findings are the first to demonstrate sex-specific patterns of memory deficits after a subchronic, systemic immune challenge. Object recognition memory was impaired in both sexes eight weeks after immune challenge, but females also exhibited early deficits on novel object recognition. Furthermore, males but not females showed delayed deficits in context and tone-dependent fear conditioning after Poly I:C. Retrieval of an established memory as well as extinction memory were unaffected, suggesting that prior immune challenge specifically caused

dysregulation of memory formation. Importantly, memory deficits were independent of sustained sickness as indicated by normal weight gain. Thus, my hypothesis that subchronic immune challenge will induce persistent hippocampal-dependent memory deficits in males was not supported as we did not observe memory impairments at one week after immune challenge. Additionally, we observed mild amygdala-dependent deficits in cued fear conditioning in males. Females were more protected from the deleterious impacts of subchronic immune challenge on memory as they did not display deficits in context fear conditioning. Together, these findings demonstrate progressive changes and sex-specific patterns of memory deficits emerging over the weeks and months following a mild, subchronic immune challenge.

In males, both LPS and Poly I:C disrupted object recognition, however only Poly I:C resulted in deficits in context and auditory fear conditioning. This suggests that specific patterns of immune activation lead to different patterns of neural dysfunction. Indeed, distinct types of immune responses are observed in various animal models of inflammation (Heremans *et al*, 1989). Here, LPS and Poly I:C bind to different toll-like receptors (TLR4 and TLR3, respectively), recruit different signaling pathways, and thereby exert differential effects on the brain (Doyle *et al*, 2003). Acutely, both LPS and Poly I:C cause memory impairments (Cloutier *et al*, 2012), but LPS causes greater decreases in wheel running and locomotor activity (Hopwood *et al*, 2009), and Poly I:C causes more deleterious effects in developmental models (Arsenault *et al*, 2014). We observed that prior Poly I:C, but not LPS, disrupted fear conditioning, suggesting that viral mimetics induce a greater long-lasting impact on fear-related circuitry, including amygdala. We chose doses of LPS and Poly I:C based on similar effects on behavior and weight loss over the two-week injection period and similar doses to those previously compared (Arsenault *et al*, 2014). It is possible, however, that differences in the

intensity or duration of immune activation caused the broader impairments in memory after Poly I:C compared with LPS. Indeed, tolerance to repeated injections is commonly observed with LPS but not to Poly I:C (Soszynski *et al*, 1991), suggesting that in our experiments, animals treated with Poly I:C may have experienced more chronic neuroimmune activity than those treated with LPS. Nevertheless, given the differences in patterns of cells (Starkhammar *et al*, 2012), cytokines (Kimura *et al*, 2004), and downstream effectors (Suh *et al*, 2013) activated by LPS and Poly I:C, directly comparing levels of immune activation remains difficult. Identifying how each kind of immune challenge (e.g., polymicrobial sepsis, gram-negative and gram-positive bacteria, viral mimics, injury/surgery/heart attack, etc.) targets different neural structures and functions will be critical for understanding dysregulation of cognitive and affective processes that persist long after a transient immune event.

In contrast to the delayed memory deficits in males, females showed significant impairments of novel object recognition in the first week after the immune challenge, and these deficits persisted at least 8 weeks. Further work is required to directly assess whether the apparent differences at this time point reflect robust and meaningful sex differences in vulnerability to immune challenge soon after immune challenge. A pattern of early and persistent deficits in females in contrast to delayed, broader memory impairments in males is consistent with recent findings from our lab and others showing differential regulation of neuroimmune activation (Acaz-Fonseca *et al*, 2015; Bodhankar *et al*, 2015; Santos-Galindo *et al*, 2011; Speirs and Tronson, 2018), and with sex differences in peripheral immune responses (Furman *et al*, 2014; Klein and Flanagan, 2016). Whether and how males and females differ in vulnerability to other types of memory dysfunction soon after immune challenge remains to be determined.

That males, but not females show deficits in fear conditioning after a subchronic immune

challenge also suggest sex differences in susceptibility to immune modulation across brain regions. This is consistent with previous studies of sex differences in neuroimmune activity. For example, after chronic stress, males but not females showed ongoing microglial activation in basolateral amygdala (Bollinger *et al*, 2017). Alternatively, sex differences in strategy and mechanisms of fear memory formation may mediate differential susceptibility of males to disruption by prior Poly I:C (Keiser *et al*, 2017; Shansky, 2018). Fear conditioning triggers sex-specific patterns of activation across brain regions (Keiser *et al*, 2017; Lebron-Milad *et al*, 2012) and signaling mechanisms (Keiser and Tronson, 2015; Mizuno and Giese, 2010). Male-specific disruption of fear conditioning may be due to sex differences in neuroimmune signaling, and/or to disruption of circuits or mechanisms required for fear conditioning in males but not females.

Interestingly, in our subchronic immune challenge model, it is fear memory formation, but not fear memory retrieval that is impacted. As fear memory retrieval utilizes more cortical networks (Frankland and Bontempi, 2005; Silva *et al*, 2019; Wheeler *et al*, 2013) different transcriptional regulatory mechanisms (Peixoto *et al*, 2015), it is therefore likely that these cortical regions and transcriptional regulatory mechanisms necessary for retrieval may not be as severely impacted as the hippocampus is by subchronic immune challenge. Similarly, we did not observe any long-lasting deficits in fear extinction after immune challenge in either sex. This further supports the idea that subchronic immune challenge alters specific memory processes. Long-lasting impairments in fear extinction have been observed months after systemic immune activation (Singer *et al*, 2016). Given that this was a sepsis model and that a different fear conditioning protocol was used in these studies, it is possible that either the differences in the type of systemic inflammatory insult or training protocol may impact may influence whether fear extinction is impaired. As fear extinction and fear conditioning uses unique circuits (e.g.

engagement of infralimbic medial prefrontal cortex along with hippocampal-amygdala circuit in fear extinction) and molecular mechanisms (e.g. differences in signaling patterns between fear conditioning and fear extinction) (Rudenko *et al*, 2013; Tronson *et al*, 2010), it is possible that both the differences in circuits and molecular mechanisms mediating fear extinction are intact in males and in females months after subchronic immune challenge. Additionally, other previous studies have shown that either acute LPS or early-life LPS-insults can impair auditory-cued memory processes, but related to auditory fear extinction rather than memory consolidation (Quinones *et al*, 2017). While we did not observe differences in fear extinction to the context, it is possible that there are differences in extinction to the tone months after subchronic immune challenge. Further investigation of which circuits and molecular substrates underlying these changes will provide insights into how mild systemic inflammatory insults alters memory in a sex-specific manner.

In the animal model of subchronic immune challenge, we observed long-lasting memory impairments without persistent changes in the animal's well-being, including sickness behaviors, such as sustained decreases in weights as well as other measures of sickness including changes in locomotor activity during behavioral testing. These studies suggest that in the animal model of subchronic immune challenge, unlike in the animal models of sepsis, memory deficits likely persist without sustained physiological changes that may impact the animal's health and cognitive functions. However, the changes in animal weights that we have observed, while statistically significant, are minimal and may not accurately represent animal sickness. The animals, on average, lose less than a gram of their body weight the day after the first injection and this weight loss decreases as they build tolerance to the immune challenge. Yet, many factors in the laboratory setting can induce such small changes in weight loss, including stress

from handling, injections, testing, etc. Therefore, future studies using this model will need to examine changes in other sickness behaviors, including food consumption, rectal temperature, and visual piloerection, and hunched back (observed by researcher blinded to experimental conditions), minutes, hours, and days after each injection and weeks to months after the last injection. These studies will determine the sickness behavior profile after LPS or Poly I:C treatment and provide insights into the impact of each type of immune challenge on the strength and persistence of the sickness response after injection.

Rodent models that use exogenous stimuli including LPS and Poly I:C have limitations in their ability to mimic human illnesses. For example, although high doses of LPS is commonly used in animal models of sepsis, there are concerns about differences in immune response to endotoxin in humans versus rodent models limiting the efficacy of this model (Fink, 2014; Rittirsch *et al*, 2007). In neuroscience research, Poly I:C has been predominantly used to model maternal viral infection and maternal immune activation on the effects of neural development (Reisinger *et al*, 2015). Neither LPS nor Poly I:C accurately model the course of bacterial or viral infection, or the full time-course of an illness. Nevertheless, these are important tools for triggering transient and robust immune – and neuroimmune – activation, and thereby useful models for identifying both short- and long-term changes in cognitive and affective processes, neural function, and their underlying molecular mechanisms in the absence of ongoing disease processes.

Importantly, this model is sensitive to sex differences in vulnerability to and time course of emergent memory deficits, and differential effects of bacterial- and viral-like immune triggers. These findings demonstrate that subchronic systemic inflammation causes sex-specific patterns of memory decline over the following months. Both males and females are susceptible to

impairments in memory after immune challenge, but females are more vulnerable to early-onset memory deficits, whereas males are more susceptible to later development of impairments across multiple memory systems. Additional studies using this model will be required to identify immune and non-immune mechanisms that drive memory deficits that mediate such delayed and persistent memory impairments. As such, this subchronic immune challenge model will be a valuable tool for identifying how systemic inflammation initiates memory decline and memory-related disorders. Understanding the persistent neural changes as a consequence of transient neuroimmune activation will be critical for development of strategies to prevent cognitive decline after major illness or chronic inflammation in women and in men.

Figures

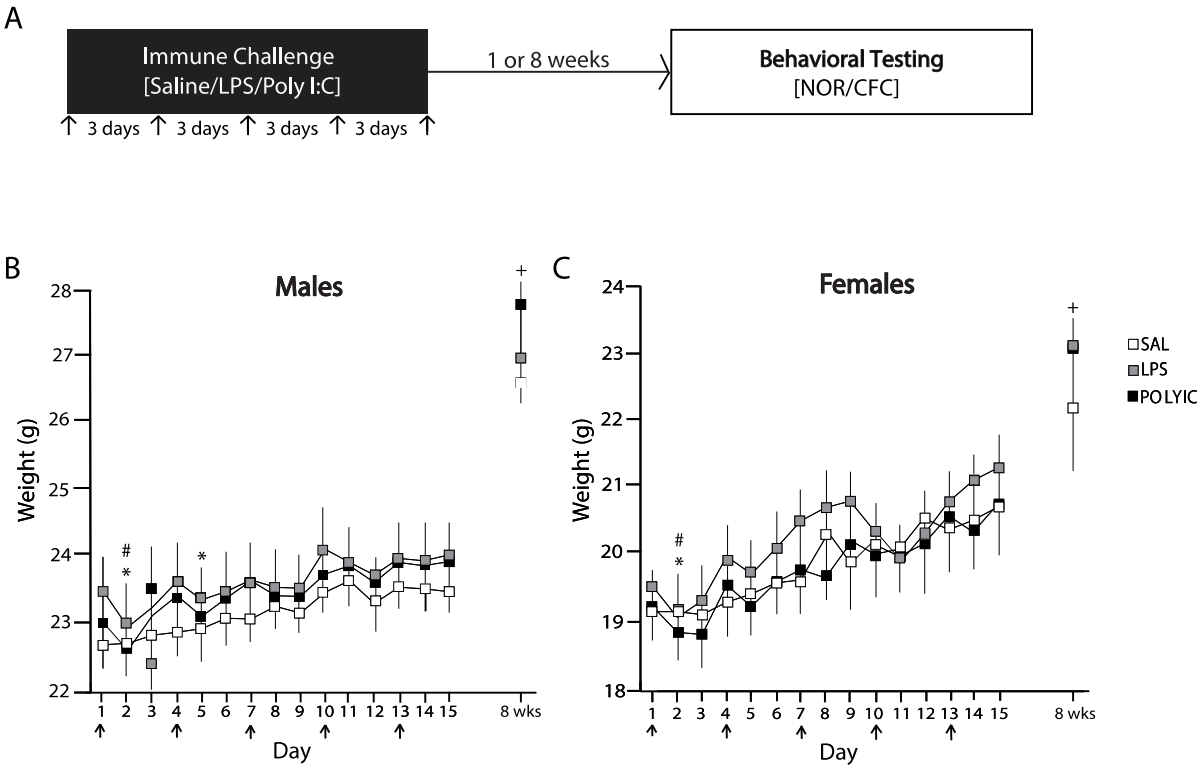


Figure 2.1. Experimental design and mouse weights after immune challenge. (A) Males (n = 8-9 per group) and (B) females (n = 9 per group) showed weight loss after initial LPS and Poly I:C injections, but showed no persistent changes in weight months after immune challenge. Arrows indicate injection days. SAL: saline; LPS: lipopolysaccharide; Poly I:C: Polyinosinic:polycytidylic acid. *$p < 0.05$ compared with injection day LPS group; #$p < 0.05$ compared with injection day Poly I:C group, +$p < 0.05$ Poly I:C vs Saline. Data is presented as group means with error bars representing SEM.

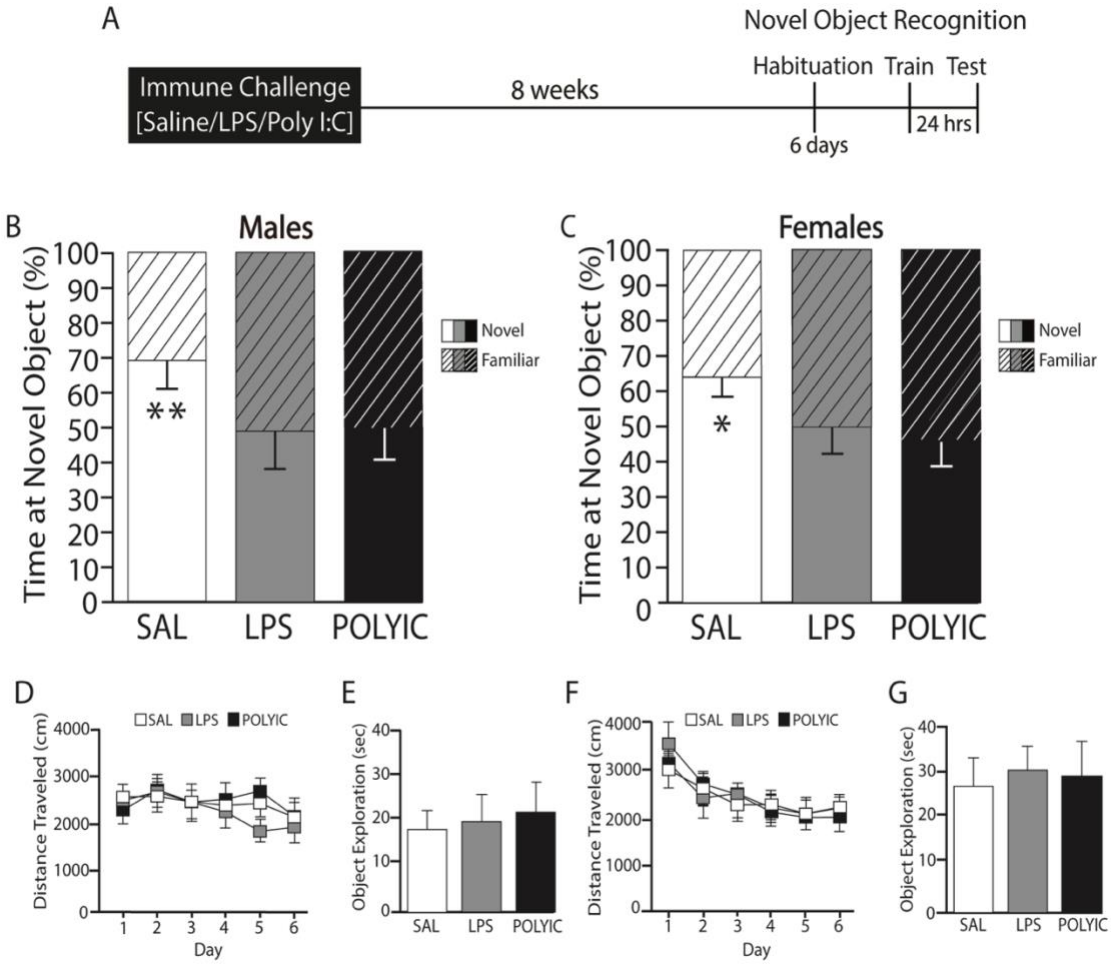


Figure 2.2. Impairments of hippocampal-dependent novel object recognition memory persisted long after subchronic immune challenge. (A) Experimental timeline. Habituation (6 days) in males (n = 8-9 animals/ group) and females (n= 9 animals./group) began 8 weeks after last injection, training the day after habituation, and testing 24 hours after training. (B,C) Saline-treated males and females spent more time exploring the novel object (solid bar) compared with the familiar object (striped bar) 24 hours after training. Long-term memory for novel object recognition was disrupted in LPS- and Poly I:C treated mice. (D,F) Neither locomotor activity nor habitation were affected by prior immune challenge. (E,G) There were no differences in total object exploration during training. *$p < 0.05$, **$p < 0.01$ *cf* Familiar Object. Data is presented as group means with error bars representing SEM.

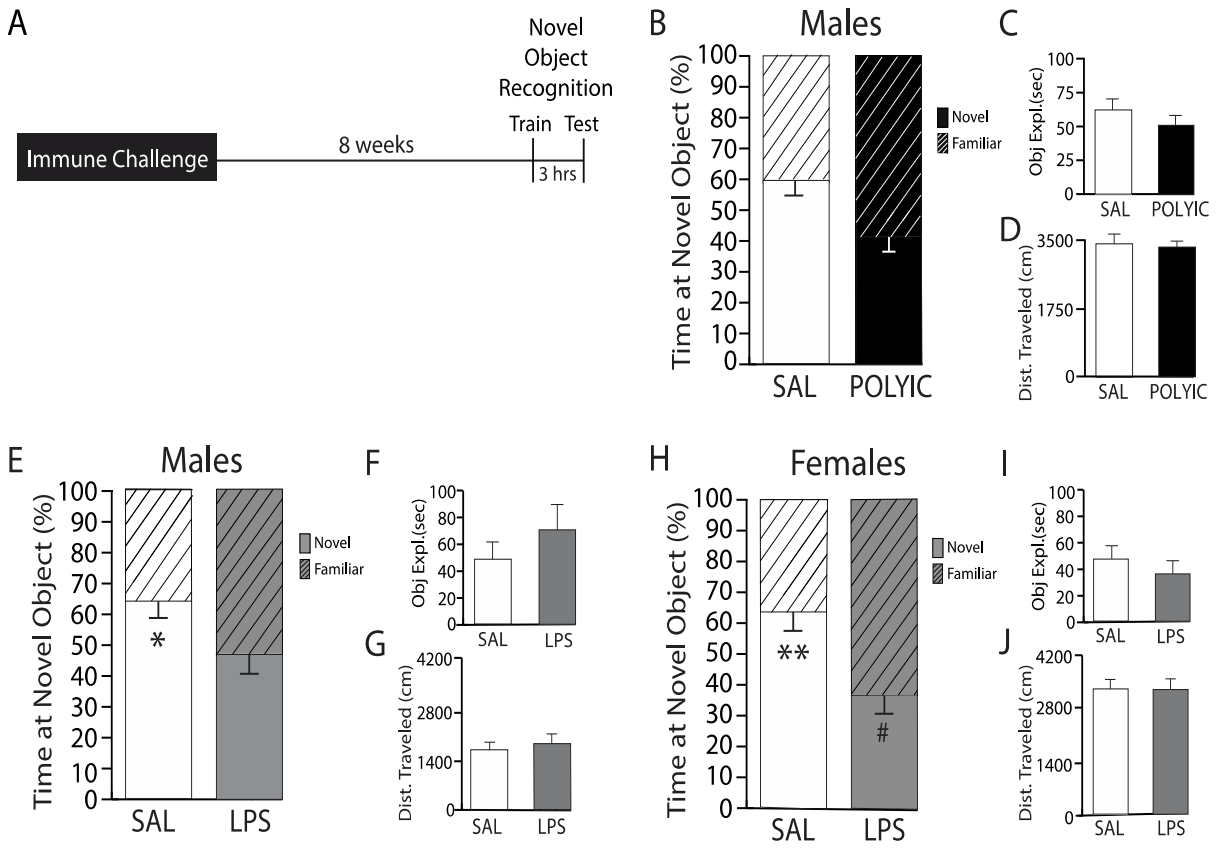


Figure 2.3. Subchronic immune challenge caused impaired 3-hour novel object recognition long after subchronic Poly I:C or LPS challenge. (A) Experimental timeline, with novel object recognition training and testing 8 weeks after last injection. (B) Saline-treated mice showed preference for the novel object when tested 3 hours after training, and this was disrupted months after Poly I:C (n = 8 per group). (C,D) Total object exploration and locomotor activity during novel object training were similar between groups. (E,H) Short-term memory for novel object recognition was impaired in males and females when tested 3 hours after training. (G,I) Total exploration during training and (H,J) locomotor activity were not altered by prior LPS in either sex. Data is presented as group means with error bars representing SEM.

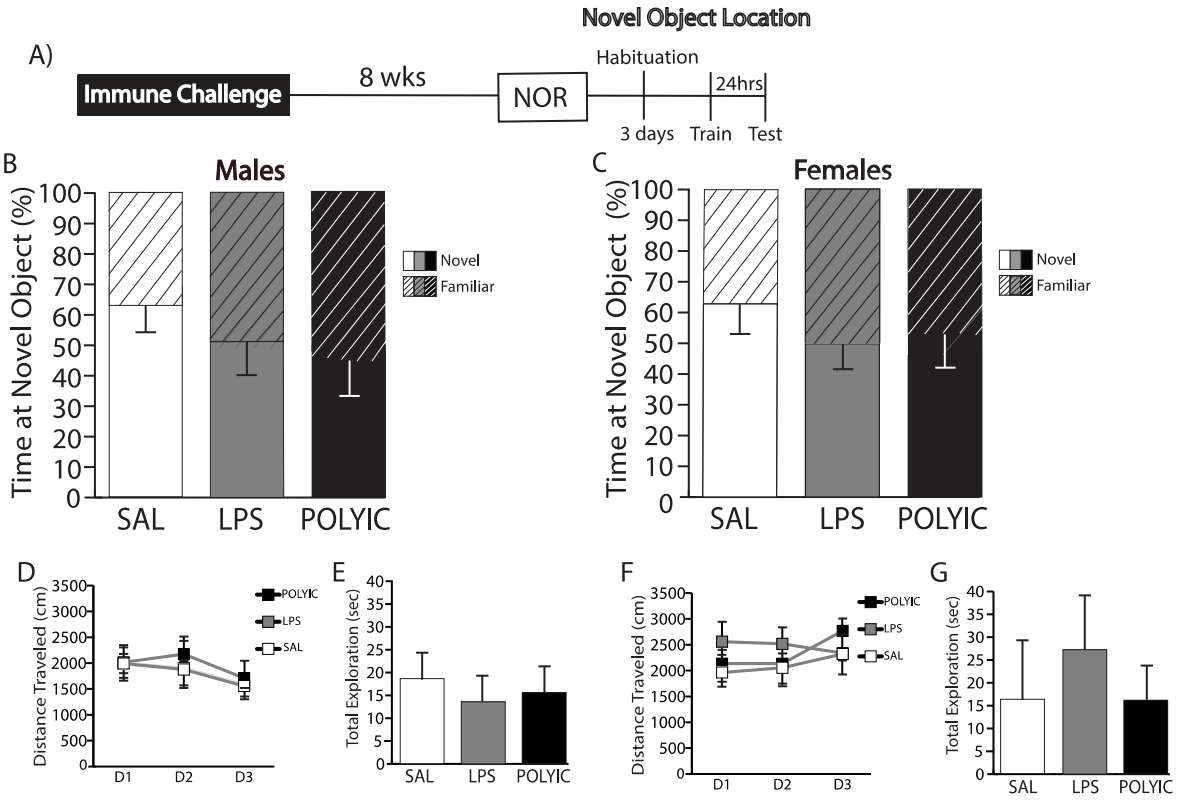


Figure 2.4. No hippocampal-dependent novel object location memory deficits months after subchronic immune challenge. (A) Experimental timeline. Habituation (3 days) in males (n = 8-9 animals/ group) and females (n= 9 animals./group) began weeks after last injection, training the day after habituation, and testing 24 hours after training. (B,C) Time exploring the novel object (solid bar) compared with the familiar object (striped bar) 24 hours after training did not differ between experimental groups. (D,F) Neither locomotor activity nor habitation were affected by prior immune challenge. (E,G) There were no differences in total object exploration during training. **p* < 0.05 *cf* Familiar Object. Data is presented as group means with error bars representing SEM.

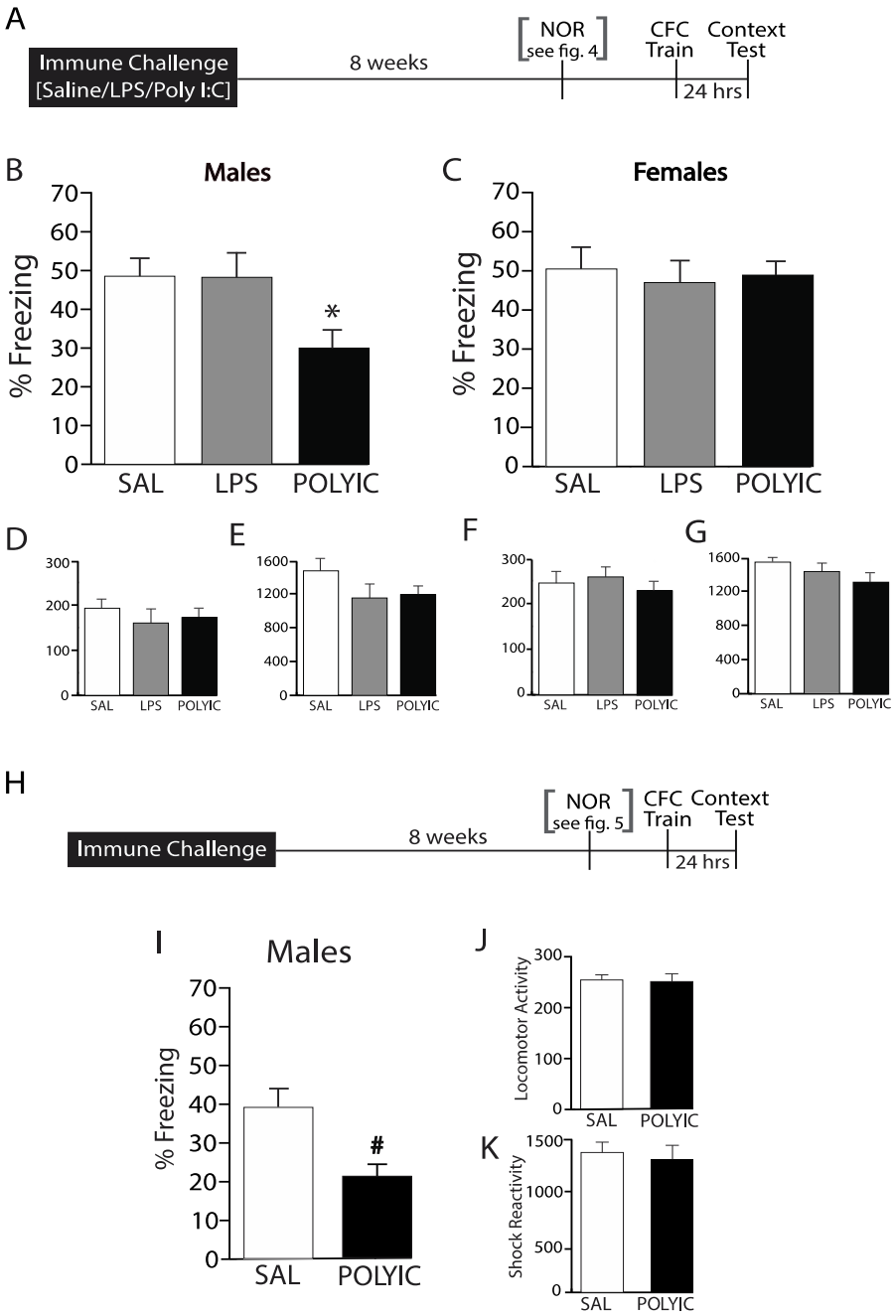


Figure 2.5. Context- fear conditioning was impaired in males long after subchronic Poly I:C. (A) Experimental timeline in one cohort of animals Context fear conditioning was conducted in animals previously tested in novel object recognition (results in Fig. 4). (B) Freezing to the training context in males is decreased after subchronic Poly I:C, but not LPS treatment (n = 8-9 per group). (C) Context fear conditioning was not impaired in females after LPS or Poly I:C (n = 9 per group). (D,F) Locomotor activity and (E,G) shock reactivity during training was not altered after LPS or Poly I:C either sex. (H) Experimental timeline in a separate cohort of males: replicate. Context fear conditioning was conducted in animals previously tested in 3-hour novel object recognition (results in Fig. 3). (I) Context fear conditioning was decreased in males (n = 8 per group) eight weeks after subchronic Poly I:C challenge. (J,K) Locomotor activity and shock reactivity during training was not altered after Poly I:C. *$p < 0.05$ *cf* Saline. # $p < 0.05$ *cf* saline, ##$p < 0.01$ *cf* saline. Data is presented as group means with error bars representing SEM.

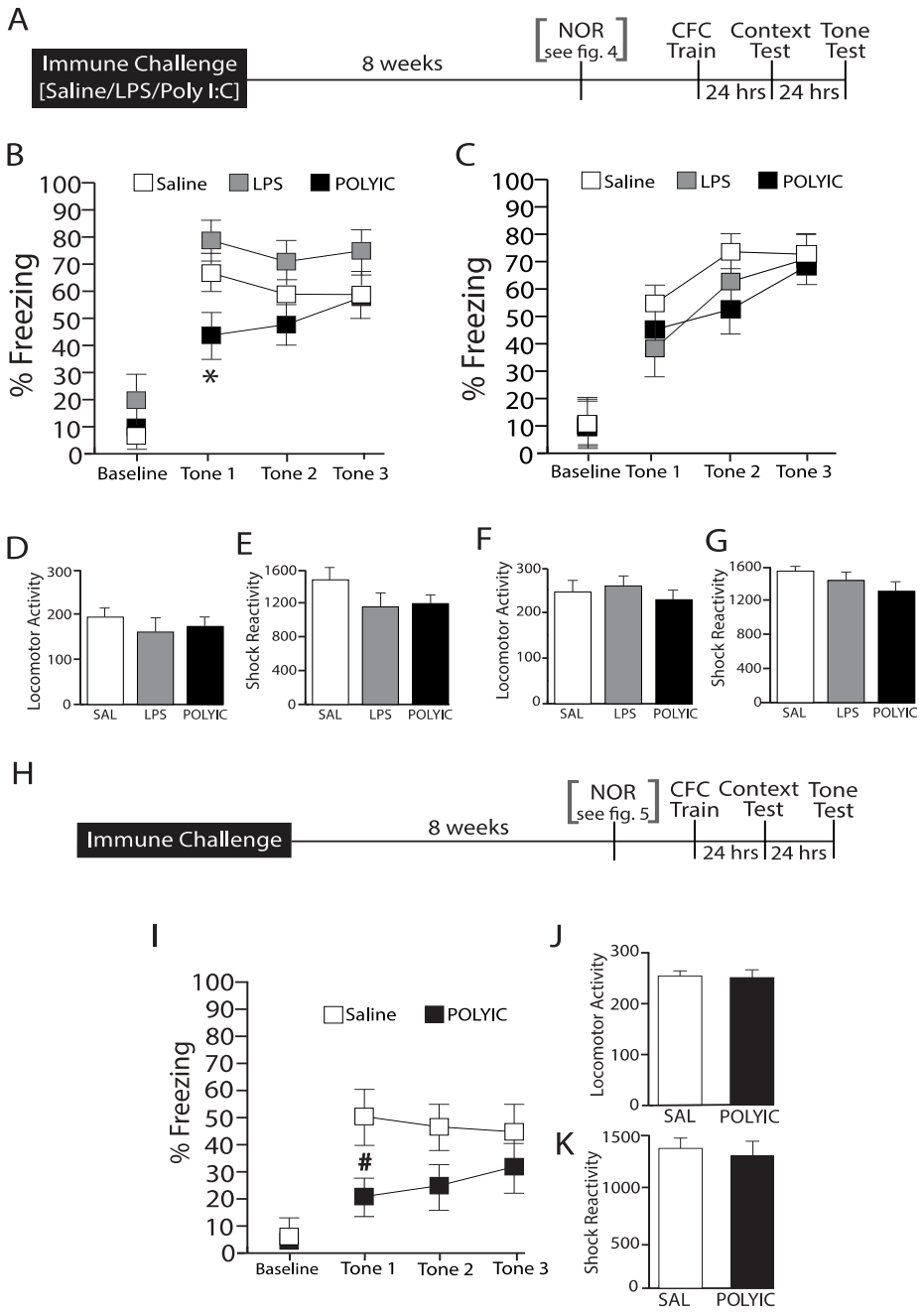


Figure 2.6. Mild impairment in cued fear conditioning in males long after subchronic Poly I:C. (A) Experimental timeline in one cohort of animals. (B) In males, Poly I:C, but not LPS, resulted in a mild impairment in tone fear conditioning. (C) Females showed no disruption of cued-fear conditioning after either immune challenge. (D,F) Locomotor activity and (E,G) shock reactivity during training was not altered after LPS or Poly I:C either sex. (H) Experimental timeline in a separate cohort of males: replicate. Tone fear conditioning was conducted in animals previously tested in 3-hour novel object recognition (results in Fig. 3). (I) Tone fear conditioning was mildly impaired in males (n = 8 per group) eight weeks after subchronic Poly I:C challenge. (J,K) Locomotor activity and shock reactivity during training was not altered after Poly I:C. *$p < 0.05$ *cf* Saline. # $p < 0.05$ *cf* saline, ##$p < 0.01$ *cf* saline. Data is presented as group means with error bars representing SEM.

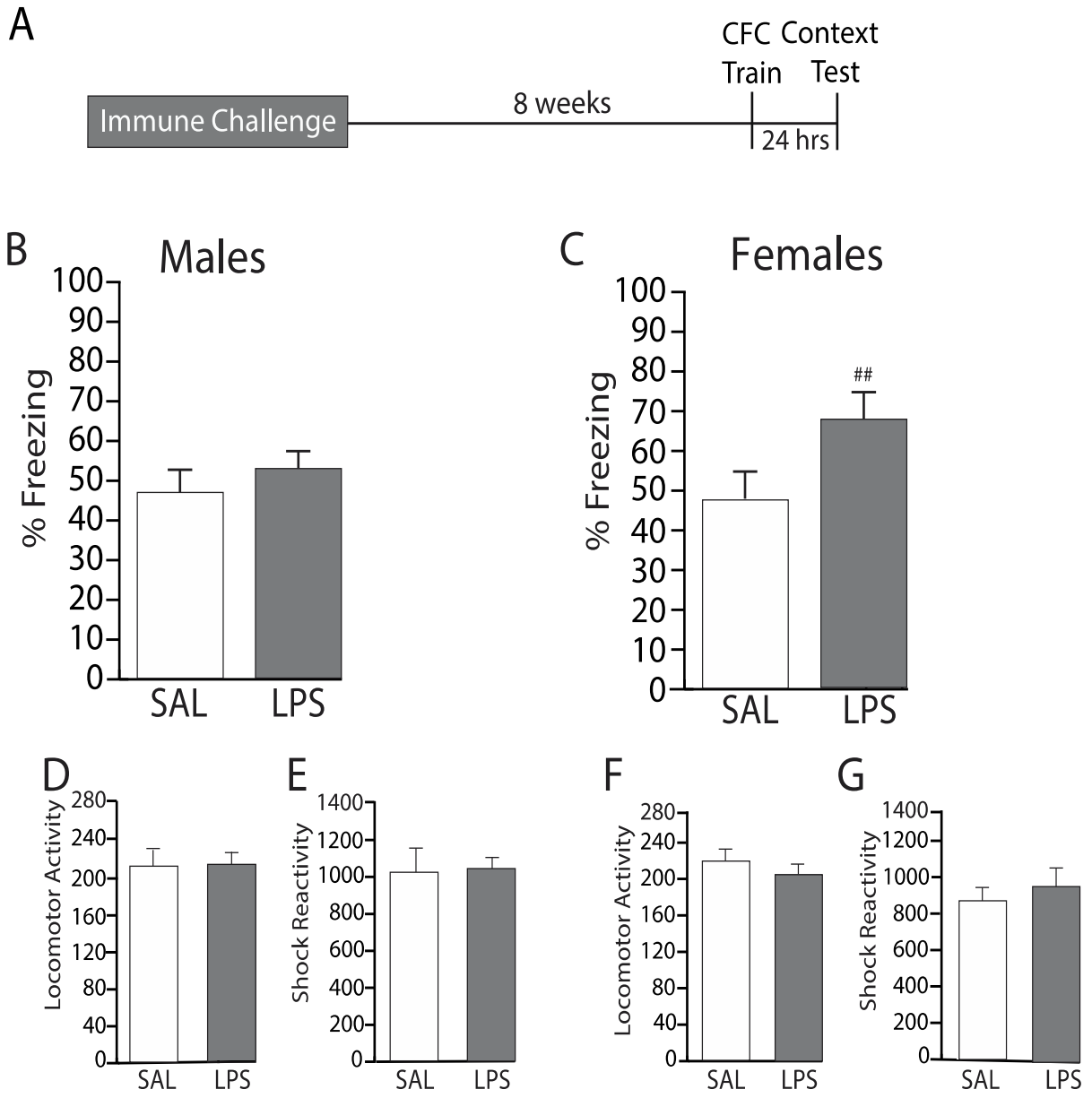


Figure 2.7. No disruption of foreground fear conditioning eight weeks after subchronic immune challenge. (A) Experimental timeline. Context fear conditioning was conducted in behaviorally naïve mice (n = 8 per group per sex) 8 weeks after LPS injections. (B,C) Foreground context fear conditioning was intact in both sexes. (D,F) Locomotor activity and (E,G) shock reactivity were not different between groups. # $p < 0.05$ *cf* saline, ##$p < 0.01$ *cf* saline. Data is presented as group means with error bars representing SEM.

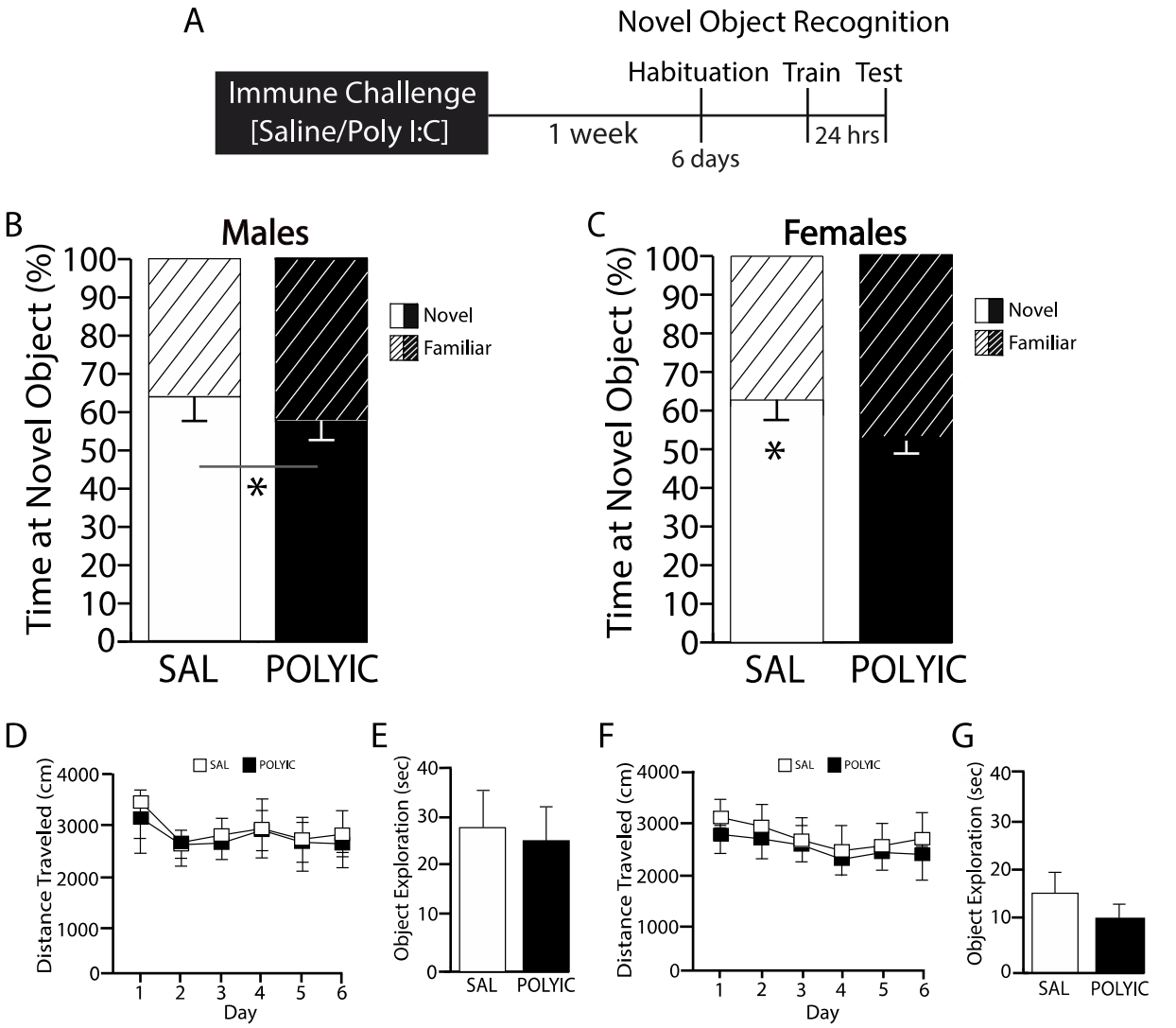


Figure 2.8. Hippocampal-dependent novel object recognition memory was disrupted only in females soon after subchronic Poly I:C. (A) Experimental timeline. Novel object recognition consisted of 6 days of habituation starting 8 weeks after last injection, training the day after habituation. Tests occurred 24 hours after training. (B) Intact novel object recognition in males shortly after Poly I:C (n = 8 per group). (C) Subchronic Poly I:C treatment disrupted long-term novel object recognition memory in females in the weeks soon after the final injection (n = 8). (D, F) Neither habituation nor locomotor activity were altered one week after Poly I:C. (E, G) Total object exploration was similar amongst experimental groups during training in males and in females. * $p < 0.05$ *cf* Familiar Object. Data is presented as group means with error bars representing SEM.

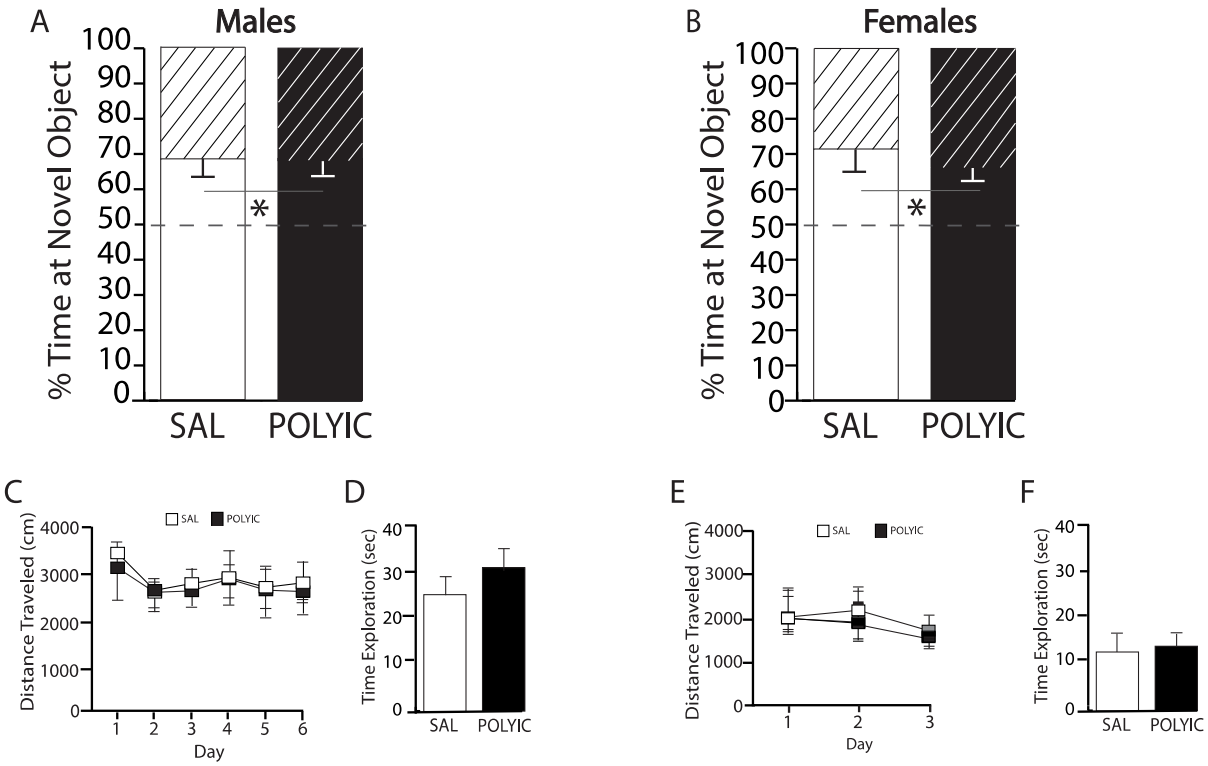


Figure 2.9. Hippocampal-dependent novel object location memory was not disrupted soon after subchronic Poly I:C. (A) Experimental timeline. Novel object recognition consisted of 3-6 days of habituation starting 2 weeks after last injection, training the day after habituation. Tests occurred 24 hours after training. (B) Intact novel object location in males (B) and in females (C) shortly after Poly I:C (n = 8 per group). (D, F) Neither habituation nor locomotor activity were altered one week after Poly I:C. (E, G) Total object exploration was similar amongst experimental groups during training in males and in females. * $p < 0.05$ *cf* Familiar Object. Data is presented as group means with error bars representing SEM.

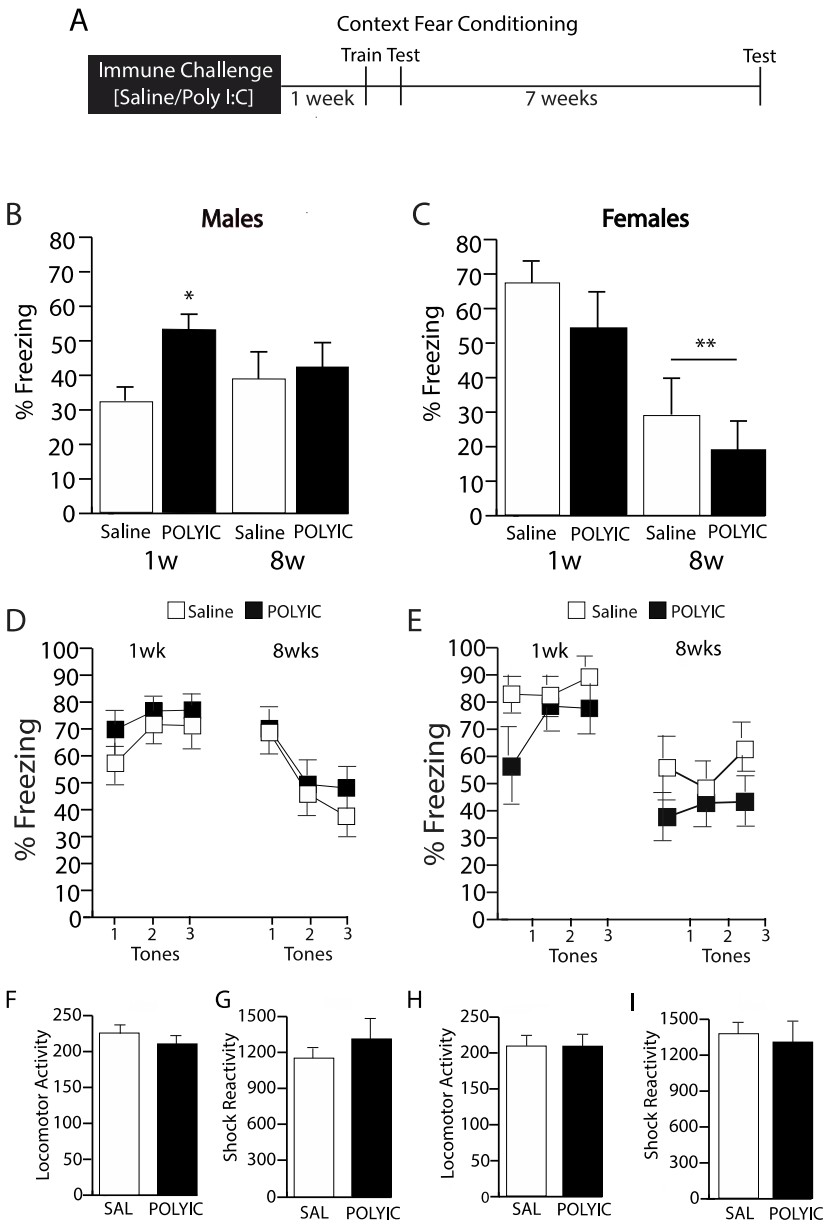


Figure 2.10. Subchronic Poly I:C did not cause early context fear memory impairments or retrieval deficits. (A) Experimental timeline. Animals were trained in context fear conditioning one week after the final injection. Context fear conditioning was assessed 24 hours, and auditory- cued fear test 48 hours after training. Animals were tested for fear memory retrieval seven weeks after training. (B) Prior Poly I:C challenge did not disrupt context fear conditioning soon after immune challenge, or during memory retrieval seven weeks later in males (n = 7-8). (C) In females (n = 8 per group), no disruption of context fear conditioning was observed soon after immune challenge; and, all females showed decreased freezing at the remote test, with no effect of prior immune challenge. (D,E) Tone fear conditioning remained intact soon after subchronic immune challenge in both sexes, and retrieval remained intact seven weeks later. (F,H) Locomotor activity and (G,I) shock reactivity were equivalent across all groups. *p <0.05 *cf* Saline; #p < 0.001 *cf* 1week. Data is presented as group means with error bars representing SEM.

Chapter III

Implications of Subchronic Immune Challenge on Long-Lasting Depressive-like Behaviors and Anxiety-Like Behaviors

Abstract

Long-lasting emotional dysregulation, including depression and anxiety is common amongst men and women who have experienced an illness or major surgery. Animals models of sepsis have also shown increased depression-like behaviors weeks after the systemic inflammatory insult, suggesting that systemic immune activation can impact memory and affective processes in males. However, few animal studies have determined the long-lasting consequences of a systemic inflammatory event on affective processes in females nor have they explored the role of milder systemic immune activation in initiation and progression of depression-like or anxiety-like behaviors. We use subchronic, systemic immune challenge (LPS or Poly I:C) to determine short-term and long-term changes in these affective processes in males and females weeks to months after the inflammatory insult. Subchronic immune challenge did not induce persistent changes in anxiety-like behaviors. While males and females showed no increases in depressive-like behaviors one week or eight weeks after subchronic LPS or Poly I:C treatment, females showed increases in immobility and decreased climbing in the forced swim test at one weeks post immune challenge and males showed these behaviors at least eight weeks post last injection. Sucrose preference was unaltered at least eight weeks after last injection suggesting that no persistent changes in anhedonia-like behavior result from subchronic immune

challenge. These studies demonstrate that subchronic immune challenge does not persistently alter affective processing, including anxiety-like and depressive-like behaviors.

Introduction

In the previous chapter, we determined that subchronic immune challenge results in memory deficits that persist for weeks to mnths after the immune challenge, which has been important for understanding the long-lasting consequences of a systemic inflammatory event on memory and cognition in patients. This research supports clinical studies showing that patients who undergo a systemic inflammatory event develop long-lasting memory deficits. However, clinical studies have also shown that patients who have experience a systemic inflammatory event show changes in emotion months after recovery, including increased anxiety and associated disorders as well as depression. For example, post-traumatic stress disorder is observed months after critical illness (Sukantarat *et al*, 2007; Wintermann *et al*, 2017) or years lung injury (Bienvenu *et al*, 2019). Similarly, individuals who have experienced a critical illness show major depressive disorder months to years after recovery from illness (Wintermann *et al*, 2018). Yet, how anxiety or depression and associated disorders develop after recovery from the systemic inflammatory event remains unknown.

Animals models aimed at understanding the long-lasting consequences of a systemic inflammatory event on changes in affective processing have shown anxiety-like behaviors two to four weeks after systemic immune activation in males (Anderson *et al*, 2015; Denstaedt *et al*, 2018; Frey *et al*, 2014). Similarly, long-lasting depression-like behaviors were also observed weeks to months after systemic immune activation in males (Barichello *et al*, 2007; Anderson *et al*, 2015; Frey *et al*, 2014) and in females (Kubera *et al*, 2013). In rodents, depression-like

behaviors include despair-like behavior, interpreted as a state of hopelessness for the animal, and anhedonic-like behavior, interpreted as a state of reduced pleasure. Despair-like behavior is typically tested using the forced swim test, in which increased immobility and climbing are considered to be effective measures of depressive-like behavior (Bogdanova *et al*, 2013) while changes in anhedonic-like behavior are assessed using the sucrose preference test (Scheggi *et al*, 2018). Systemic immune activation has been shown to induce persistent increases in both despair-like and anhedonic-like behavior (Anderson *et al*, 2015; Kubera *et al*, 2013). Compared with males, few studies have focused on long-lasting changes in affective processing after a systemic inflammatory insult. Given sex differences in prevalence of emotional dysregulation, with women, for example, being twice as likely to develop major depressive disorder (Grigoriadis and Robinson, 2007), it is crucial to determine long-lasting changes in affective processes in both males and females emotion.

The purpose of this chapter is to determine whether subchronic immune challenge induces long-lasting changes in affective processes in males and in females. Mice were tested at least eight weeks post last injection for anxiety-like behaviors using the elevated plus maze and open field test. We determined whether subchronic immune challenge induced alterations in two types of depressive-like behaviors at least eight weeks post last injection, including changes in despair-like behavior using the forced swim test, and anhedonic-like behavior using the sucrose preference test. Additionally, we assessed short-term changes in despair-like behavior after subchronic immune challenge. In these studies, animals were run on multiple behavioral tests to determine whether they exhibited long-lasting changes in memory alone or both in memory and affective processes. *I hypothesized that subchronic immune challenge will induce both short-term (1 week) and long-term (8 weeks) increases in anxiety-like and depressive-like behaviors in*

males and in females. I anticipate that prior immune challenge will cause persistent increases in despair-like behavior and increases in anhedonic-like behavior in both sexes. Surprisingly, subchronic immune challenge did not persistently increase anxiety-like or depression-like behaviors in males nor females. Findings from this work will help to understand the role that mild, systemic immune activation plays in affective processing.

Materials and methods

2.1 Animals: 9-11 week old male and female C57BL/6N mice from Envigo (Indianapolis, IN) were used in all experiments. Mice were individually housed with mouse chow and water provided *ad libitum* as previously described (Keiser et al., 2017). Individual housing in mice prevents fighting-induced stress (Meakin et al., 2013) and is ethologically appropriate for males and females (Becker & Koob, 2016). Individual housing is suitable for testing novel object recognition (Vogel-Ciernia & Wood, 2015) and contextual fear conditioning (Keiser et al., 2017) and follows the University of Michigan Institutional Care and use Committee policies on managing fighting in mice. The facility is ventilated with constant air exchange (60 m^3/ h), temperature (22 ±1°C), and humidity (55±10%) with a standard 12 h light-dark cycle. Experiments were performed during the light portion of the cycle. Mice were acclimated to the colony room for at least seven days prior to injections. All experimental methods used in these studies were approved by the University of Michigan Committee on the Use and Care of Animals. Males and Females used for memory tests were also assessed for anxiety-like and depressive-like behavior.

2.3 Subchronic Immune Challenge. Mice received five intermittent injections of LPS

(250μg/kg; n= 8-9), Poly I:C (6 mg/kg; n= 8-9), or saline control (n = 8-9), spaced three days apart. All injections were performed at the same time of day (Roberts, 2000). Mice were weighed daily throughout the injection period and weekly until testing and weights data is available in Chapter 2, Figure 1.

2.4 Behavioral Testing. All behavioral testing was completed within 12 weeks after subchronic immune challenge. Estrous cycle stage was assessed prior to behavioral testing as described in section 2.4 of Chapter 2.

2.4.1 Elevated plus maze (EPM): The maze consisted of four arms, including two open arms (35 x 7.5 cm) and two closed arms (35 l x 7.5 w x 32 h cm). The apparatus consisted of black closed arms and white open arms with small black edges and was elevated 68 cm from the ground. Animals were tested individually in a 10 in. test, during which each was placed in center facing a closed arm. Measures used to assess anxiety-like behaviors in the elevated plus maze include time spent in the open arms and number of entries to the open arms (Anderson *et al,* 2015). Arm entry was counted when all four of an animals' paws were inside the arm.

2.4.2 Open field test: The open field consisted of a gray square box 76cm long/wide and 31.5cm high. Each session consisted of 10 minutes, during which animals were individually placed into the center of the open field. Ethovision XT 9.0 computer software (Noldus information Technologies, Leesburg, VA; (Kikusui *et al*, 2004)) was used to separate the arena into 16 equally-sized squares, with 4 center squares and 12 peripheral squares. Anxiety-like behaviors were assessed by calculating time spent in the four center quadrants and number of crossings into the center. Locomotor activity can also be assessed using the open test and were measured by calculating total distance traveled and velocity (cm/s) (Anderson *et al*, 2015).

2.4.3 Forced swim test (FST): For the FST, a 4 liter clear plexiglass beaker (17.5 cm diameter 25 cm height) was filled to up 15.5cm with water (23-25 °C). The apparatus was set up so that neither the mouse's tails nor paws could not touch the bottom of the beaker and the mice could not climb out of the beaker. Each test lasted six minutes, with the first minute serving as a habituation period to the water and the next five minutes as test. During the test, despair-like behavior was assessed by calculating the time mice spent immobile (no movement of paws or movement of only one paw to stay afloat). Escape-directed behaviors were measured by number of climbs in each trial. Fecal material was cleaned out of the beaker between every 2 animals using a mesh net to prevent associated smells confounding despair-like behavior.

2.4.5 Sucrose Preference Test: Sucrose preference was carried out using two protocols with a computerized lickometer. Females in Figure 6 are tested in sucrose preference using the first protocol while females in Figures 7-9 were tested using the second protocol.

Protocol 1: Sucrose preference testing consisted of two days of habituation. Prior to each habituation day, mice were water deprived overnight. During the first habituation day, small plastic bottles (150 ml) were filled ¾ of the way with sucrose solution dissolved in tap water, with one bottle placed in the front and one in the back of the Med-Associates testing. Mice were individually placed into the chambers and the MedPC-IV software and the Lickometer 60MIN 2-Bottle Choice protocol (Ford et al. 2018) was used to count the number of licks per bottle during the 60 minute session. Each animal's preference for the front or back side was determined. During the second habituation day, the same lickometer protocol was used, except that one bottle contained sucrose solution and one bottle contained water placed on the non-preferred side. Testing consisted of one day, during which mice were presented with one bottle containing sucrose solution and the other water. The sucrose solution was placed on the non-preferred

licking side (front vs. back), as determined from the habituation trials. The Lickometer 60MIN 2-Bottle Choice protocol was used to measure number of sucrose and water licks during the 60 min. session. After testing, the water bottles were placed back into the animals' home cages. Sucrose preference with only 1% sucrose was performed using this protocol.

Protocol 2: Sucrose preference was carried out as described above, except with the following alterations. During habituation, animals were presented only with sucrose bottles. Testing consisted of two days, during which mice were presented with one bottle containing sucrose solution and the other water. After the first testing day, mice were given water in their home cages after the test. They were water deprived again overnight prior to the second testing day. After testing was complete, water placed back into their home cages. Mice were tested for sucrose preference using multiple sucrose solutions (1%, 1.5%, and 2%) through this protocol and were left in the home cage 2-3 days prior to retesting. This protocol was more advantageous as using a higher percentage of sucrose abolished the differences in the number of total licks in animals between different experimental conditions (see Figures 7-9c).

Sucrose preference was averaged between the two experimental days and was determined by the following two methods:

A) Comparing # water licks vs # sucrose licks between vehicle and LPS-treated animals
B) Assessing % Sucrose Preference: $\frac{(\text{\# sucrose licks})}{(\text{\# sucrose licks} + \text{\# water licks})} \times 100$

Data analysis and statistics: The effect of subchronic immune challenge on the number of sucrose *vs* water licks (method A) and number of total licks on habituation days 1 and 2 were analyzed using repeated measures. Post hoc tests (with LSD) were used to further assess specific

group differences. Percent sucrose preference (method B) and total number of licks were analyzed using unpaired t-tests.

Results

3.1. No anxiety-like behaviors months after subchronic LPS.

To assess long-lasting changes in anxiety-like behaviors after subchronic immune challenge, we tested animals in elevated plus maze and open field test at least 8 weeks after last injection. Both males treated with subchronic LPS challenge did not show differences in time spent in the open arms (ImmuneChallenge: $F(1,28) = 0.001$, $p = 0.97$; ImmuneChallenge x Sex: $F(1,28) = 0.80$, $p = 0.38$; Figure 1A) nor time spent in closed arms (ImmuneChallenge: $F(1,28) = 0.02$, $p = 0.89$; ImmuneChallenge x Sex: $F(1,28) = 0.23$, $p = 0.64$; Figure 1B). Similarly, no differences were observed between the experimental groups in number of crossings into the open arms (ImmuneChallenge: $F(1,28) = 0.64$, $p = 0.43$; ImmuneChallenge x Sex: $F(1,28) = 3.15$, $p = 0.087$; Figure 1C) nor in number of entries into the closed arms (ImmuneChallenge: $F(1,28) = 0.52$, $p = 0.48$; ImmuneChallenge x Sex: $F(1,28) = 0.00$, $p = 1.00$; Figure 1D) in both sexes. There was a trend towards decreased closed arm entries in LPS-treated females ($p = 0.079$). Males and females did not differ in any of the measures used to assess anxiety-like behaviors, including time in open arms (Sex: $F(1,28) = 0.00$, $p = 0.98$; Figure 1A) nor in number of entries into closed arms (Sex: $F(1,28) = 0.059$, $p = 0.81$; Figure 1D). There was a trend towards higher number of open arm entries in females (Sex: $F(1,28) = 3.69$, $p = 0.065$).

We observed no differences in time spent in center at least than 8 weeks after subchronic LPS challenge in males nor in females (ImmuneChallenge: $F(1,28) = 0.48$, $p = 0.50$; ImmuneChallenge x Sex: $F(1,28) = 1.69$, $p = 0.21$; Figure 2A). No differences in number of crossings into center between subchronic LPS challenge or vehicle treated males nor females

(ImmuneChallenge: $F(1,28) = 0.46$, $p = 0.51$; ImmuneChallenge x Sex: $F(1,28) = 0.26$, $p = 0.62$; Figure 2B). While there were no sex differences in time spent in center (Sex: $F(1,28) = 0.71$, $p = 0.41$), there were differences in number of center entries (Sex: $F(1,28) = 017.29$, $p < 0.01$), with greater number of center entries in females. We also determined whether subchronic LPS challenge resulted in long-lasting changes in locomotor behavior by assessing total distance traveled and velocity (cm/s). We did not observe any differences in total distance traveled between vehicle and treated groups in males nor females (ImmuneChallenge: $F(1,28) = 0.76$, $p = 0.39$; ImmuneChallenge x Sex: $F(1,28) = 0.67$, $p = 0.42$; Figure 2C) nor in velocity (ImmuneChallenge: $F(1,28) = 0.09$, $p = 0.76$; ImmuneChallenge x Sex: $F(1,28) = 0.12$, $p = 0.73$; Figure 2D). However, there were sex differences in total distance traveled (Sex: $F(1,28) = 10.15$, $p < 0.01$; Figure 2C), as females traveled greater distance than males and a trend towards higher velocity in females (Sex: $F(1,28) = 0.01$, $p = 0.92$).

3.2 No anxiety-like behaviors months after subchronic Poly I:C.

We observed no differences in anxiety-like behaviors months after subchronic Poly I:C challenge tested in the open field test. Males did not show differences in time spent in the center (t (14) = 1.14, $p = 0.27$; Figure 3A) nor in the number of entries into the center (t (14) = 0.12, $p = 0.83$; Figure 3B). Additionally, no long-lasting changes in locomotor activity were observed as no differences in total distance traveled (t (14) = 0.88, $p = 0.39$; Figure 3C) nor in velocity (t (14) = 0.88, $p = 0.39$; Figure 3D) were found between experimental groups.

Therefore, we observed no differences in anxiety-like behaviors at least 8 weeks after subchronic immune challenge. The time spent in the center of the open field and number of center entries is likely not confounded by alterations in locomotor behavior as no differences in

measures of locomotor activity, including distance traveled and velocity, were observed after subchronic LPS or Poly I:C challenge.

3.3 No despair-like behavior observed in forced swim test months after subchronic LPS.

We observed differences in time spent immobile (ImmuneChallenge = $F(1,28) = 5.55$, $p = 0.026$; ImmuneChallenge x Sex: $F(1,28) = 2.55$, $p = 0.12$; Figure 4A, with decreased time spent immobile in LPS-treated males ($p < 0.01$) but not females ($p = 0.56$). There were no sex differences in time spent immobile (Sex: $F(1,28) = 1.44$, $p = 0.24$; Figure 4A). No differences in times climbed were observed between males and females (ImmuneChallenge: $F(1,28) = 0.14$, $p = 0.072$; ImmuneChallenge x Sex: $F(1,28) = 3.49$, $p = 0.072$; Figure 4B), although a trend towards greater number of times climbed was observed in LPS-treated males and a trend towards decreased number of times climbed in LPS-treated females. There were no sex differences in climbing behaviors (Sex: $F(1,28) = 3.49$, $p = 0.072$; Figure 4B).

3.4 No despair-like behavior observed in forced swim test months after subchronic Poly I:C.

We observed differences in time spent immobile in males ($t(14) = 2.58$, $p = 0.022$; Figure 5A) but not females ($t(14) = 0.58$, $p = 0.58$; Figure 5B) months after subchronic Poly I:C challenge. Interestingly, Poly I:C treated males showed decreased time spent immobile during the test. No differences in number of times climbed were observed in males ($t(14) = 0.85$, $p = 0.41$; Figure 5C) nor in females ($t(14) = 0.65$, $p = 0.52$; Figure 5D).

3.5 No differences in sucrose preference months after subchronic LPS.

Since we observed no persistent increases in despair-like behavior, we also determined whether subchronic immune challenge induced long-lasting changes in another type of depressive-like behavior, anhedonic-like behavior. We tested anhedonic-like behavior using sucrose preference (using 1% sucrose solution) first in saline and LPS treated females 9 weeks after last injection. No differences in sucrose preference, either determined by comparison of number of water licks and sucrose licks in saline and LPS-treated females (Licks: $F(1,14) = 2.49$, $p = 0.14$; Licks x ImmuneChallenge: $F(1,14) = 0.20$, $p = 0.66$; Figure 6A) nor in percent sucrose preference ($t(14) = 1.08$, $p = 0.30$; Figure 6B) were observed between LPS and vehicle treated females.

There were differences in number of total licks between habituation days 1 and 2 (HabituationDay: $F(1,14) = 20.6$, $p < 0.01$; HabituationDay x ImmuneChallenge: $F(1,14) = 4.34$, $p = 0.056$; Figure 6C), with a greater total number of licks in saline treated females compared with LPS treated females ($p = 0.042$) on habituation day 2. There was also a trend towards lower total number of licks in LPS treated females on test day ($t(14) = 1.90$, $p = 0.072$; Figure 6D), both of which can serve as confounding variables in sucrose preference on test day.

3.6 No differences in sucrose preference months after subchronic Poly I:C.

In the previous sucrose preference experiment, we observed differences in total number of licks, potentially influencing sucrose preference. In this experiment, we optimized the sucrose preference protocol by varying sucrose concentrations (1%, 1.5%, 2%) for females to determine whether greater sucrose percentage will abolish differences in total number of licks that could influence sucrose preference testing. No differences in sucrose preference (with 1% sucrose)

between Poly I:C and vehicle treated females were observed, either determined by comparison of number of water licks and sucrose licks in saline and LPS-treated females (Licks x ImmuneChallenge: $F(1,14) = 0.20$, $p = 0.66$; Figure 7A) nor in percent sucrose preference ($t(14) = 1.34$, $p = 0.20$; Figure 7B) were observed between LPS and vehicle treated females. No differences between sucrose and water licks were observed in all animals (Licks: $F(1,14) = 0.52$, $p = 0.48$) nor differences in total licks averaged over the test days between saline or Poly I:C treated females ($t(14) = 1.34$, $p = 0.20$; Figure 7D).

Using the higher concentration sucrose (1.5% sucrose) resulted in no differences in sucrose preference between Poly I:C and vehicle treated females were observed, either determined by comparison of number of water licks and sucrose licks in saline and LPS-treated females (Licks x ImmuneChallenge: $F(1,14) = 0.87$, $p = 0.37$; Figure 8A) nor in percent sucrose preference between Poly I:C and vehicle treated females ($t(14) = 1.44$, $p = 0.72$; Figure 8B). There were differences in number of sucrose licks compared with water licks averaged across test days (Licks: $F(1,14) = 9.26$, $p < 0.01$; Figure 8A), with greater number of sucrose licks than water licks in saline ($p < 0.05$) but not Poly I:C- treated ($p = 0.16$) females. There was also a trend towards greater number of sucrose licks in saline females compared with Poly I:C- treated females ($p = 0.085$). There were differences in total number of licks averaged over the test days ($t(14) = 2.18$, $p = 0.047$; Figure 8D), with greater number of total licks in saline than in Poly I:C-treated females.

Similarly, using 2% sucrose resulted in no differences in sucrose preference. The number of water licks versus sucrose licks did not differ in saline and Poly I:C-treated females (Licks x ImmuneChallenge: $F(1,14) = 0.02$, $p = 0.89$; Figure 9A) nor did the percent sucrose preference between Poly I:C and vehicle treated females ($t(14) = 0.11$, $p = 0.92$; Figure 9B) as all females

preferred the sucrose solution over the water (Licks: $F(1,14) = 015.96$, $p < 0.01$). No differences in total number of licks averaged over the two test days were observed with this higher sucrose concentration ($t(14) = 0.23$, $p = 0.82$; Figure 9D). While no differences in sucrose preference were observed independent of the percentage sucrose used for females (1%, 1.5% 2%), sucrose preference was observed in all animals with the 2% sucrose solution, without any differences amongst experimental groups in total number of both sucrose and water licks.

No differences in habituation were observed with 1% sucrose solution (HabituationDay: $F(1,14) = 0.52$, $p = 0.48$; HabituationDay x ImmuneChallenge: $F(1,14) = 0.48$, $p = 0.52$; Figure 7C) nor with 1.5 % sucrose solution (HabituationDay: $F(1,14) = 0.02$, $p = 0.89$; HabituationDay x ImmuneChallenge: $F(1,14) = 1.34$, $p = 0.27$; Figure 8C). Using 2% sucrose solution resulted in increases in number of licks across the two habituation days for both saline and Poly I:C - treated females (HabituationDay: $F(1,14) = 25.01$, $p < 0.01$; HabituationDay x ImmuneChallenge: $F(1,14) = 0.67$, $p = 0.43$; Figure 9C). These data demonstrate that no long-lasting changes in anhedonic-like behavior is observed after subchronic Poly I:C challenge.

3.7 No despair-like behavior in forced swim test one week after subchronic Poly I:C.

We determined whether subchronic immune challenge resulted in short-term changes in affective processes by assessing changes in despair-like behavior in the forced swim test one week after immune challenge using the forced swim test. While no increased immobility was observed one week after Poly I:C injection, decreased time spent immobile was observed in females ($t(14) = 2.44$, $p < 0.05$; Figure 10B) but not in males ($t(13) = 0.64$, $p = 0.53$; Figure 10A). We observed differences in times climbed in males ($t(13) = 2.38$, $p = 0.033$; Figure 10C) but not females ($t(14) = 0.92$, $p = 0.38$; Figure 10D). Time spent immobile did not differ between

females in different stages of the estrous cycle, suggesting that estrous cycle stage does not impact behavior in forced swim test.

Discussion

These studies have examined long-lasting changes in affective processes, including anxiety-like and depressive-like behaviors. Overall, we have observed no long-lasting changes in anxiety-like behaviors at least eight weeks after subchronic immune challenge in the elevated plus maze and open field. Similarly, no increases in depressive-like behaviors, including both despair-like behavior in forced swim test as well as anhedonic-like behavior in sucrose preference test, were observed at least eight weeks after immune challenge. Subchronic immune challenge also did not induce short-term despair-like behavior as no increases in depressive-like behavior were observed at least one week after immune challenge. Therefore, my hypothesis that subchronic immune challenge will induce persistent anxiety-like and depression-like behaviors was not supported. These findings are contradictory with the research on the long-lasting changes in affective processes after a systemic inflammatory event in both animal models of sepsis and in patients. Thus, our findings suggest that a *milder* systemic inflammatory insult using subchronic immune challenge does not alter affective processes weeks to months after the insult.

While we found no increases in depressive-like behaviors, we observed alterations in despair-like behavior at least one and eight weeks after subchronic immune challenge. In the forced swim test, however, increased immobility does not necessarily mean that the animal is experiencing despair-like behavior. Instead, this increased immobility can be used to preserve energy for an attempted escape, during which the animal tries to climb out of the forced swim

apparatus (Yankelevitch-Yahav *et al,* 2015). Therefore, animals stay immobile during the forced swim test and wait for the experimenter to take them out of the water. Rodents have been known to avoid expending energy in places from which they cannot readily escape, such as the Porsolt apparatus. Therefore, rodents that have learned the most energy efficient strategy in the task stay immobile the longest, a concept referred to as learned immobility (West, 1990). It is possible that animals that show decreased immobility in the forced swim test are not exhibiting despair-like behavior, but rather worse learned immobility. Given that males showed decreased immobility months after subchronic immune challenge and females only showed decreased immobility in the forced swim test only one week after immune challenge, it is possible that these animals show increased immobility to avoid unnecessary efforts to escape. If this is the case, the animals that spent the most time immobile and show the least climbs, the saline treated controls, may be doing so due to successful learned immobility. Thus, rather than showing greater (giving up) on escaping an aversive stimulus (the water), the saline treated animals might be taking the more cost-effective approach in this test. The poly I:C treated males, however, may not learn the more effective behavior in this task in a single session. Therefore, the increased time spent immobile may be a measure of learning rather than despair-like behavior in these studies.

Anxiety-like or depressive-like behaviors are not increased long after subchronic immune challenge. It is likely that the neural mechanisms underlying these affective processes, including the associated neural circuitry and molecular substrates, are also not persistently altered by subchronic immune challenge in males nor females. However, given that both males and females show differences in swimming duration or times climbing after subchronic immune challenge, it is possible that immune challenge induces both short-term and long-term alterations in the neural substrates important for these behaviors. Previous studies suggest that the swimming and

climbing behaviors may be driven by neurotransmitter systems. For example, antidepressants increasing serotonergic neurotransmission also result in longer swimming durations whereas those that increase catecholaminergic neurotransmission result in longer climbing episodes (Cryan and Holmes, 2005). Similar serotonin mechanisms may be dysregulated long after subchronic immune challenge, leading to increased immobility in the forced swim test. Therefore, it is possible that there long-lasting dysregulation of the serotonergic system in males after subchronic immune challenge leading to the decreases in immobility in the forced swim test months after the last injection. Similarly, there is dysregulation of the catecholaminergic system in males one week after subchronic Poly I:C challenge. In females, there may be shorter term serotonergic dysregulation in females leading to the decreases in immobility in the forced swim test one week after immune challenge, which recovers in the following weeks. As we observed no long-lasting anhedonia-like behavior observed in females after subchronic LPS or Poly I:C challenge, it is likely that the neural circuits and neurotransmitter systems important for anhedonic-like behaviors, including the NAc, OFC, VP regions and dopamine, glutamate, GABA, and serotonin systems (Der-Avakian and Markou, 2013), are likely not dysregulated after subchronic LPS or Poly I:C challenge. These behavioral findings give us insights into the neural mechanisms that may underlie long-lasting cognitive changes after subchronic, systemic immune challenge.

In the clinical literature, depression and anxiety are observed months after a systemic inflammatory event (Bienvenu *et al*, 2019; Wintermann *et al*, 2018). However, there are studies showing recovery from depression or anxiety a year after major surgery (Jakobsson *et al*, 2015; Poole *et al*, 2016), suggesting that emotional processes might be less dysregulated by a systemic inflammatory event than other cognitive processes such as memory. Previous animal studies also

show mixed findings on long-lasting changes in anxiety-like or depressive-like behaviors weeks and months after an inflammatory insult. In animal models of sepsis using cecal ligation and puncture, anxiety-like behavior in the elevated plus maze was observed one week but not ten days or eight weeks after surgery (Barichello *et al*, 2007; Leite *et al*, 2013). In sepsis models using a bolus injection of lipopolysaccharide (LPS; 5 mg/kg), increased anxiety-like behaviors are observed even one month after immune challenge (Anderson *et al*, 2015).

Unlike anxiety-like behaviors, increases in depression-like behaviors persist after systemic immune activation. Both increases in despair-like behavior in the forced swim test and in anhedonic-like behavior were observed ten days after cecal ligation and puncture induced sepsis (Barichello *et al*, 2007; Comim *et al*, 2011). In models of sepsis using a high dose LPS (dose), depressive-like behaviors are observed one month after injection, including despair-like behavior in the forced swim test and tail suspension test and anhedonic-like behavior in the sucrose preference test (Anderson *et al*, 2015). The discrepancies in the consequences of a systemic inflammatory insult on affective processes may be due to differences in rodent species (mouse vs rat), peripheral and neuroimmune responses induced by each type of inflammatory insult (immune challenge vs surgery), as well as differences in protocol used between different institutions/ laboratories. Alternatively, affective processes are less prone to disruption by systemic immune activation than are memory processes. Given that we also observe no persistent changes in anxiety-like or depressive-like behaviors after subchronic immune challenge, it is likely that as the animals recover from the inflammatory insult, the anxiogenic and depressive-like responses also improve. Thus, subchronic immune challenge, unlike other inflammatory insults, does not persistently alter affective processing.

The subchronic immune challenge model is a novel tool to assess persistent changes in affective processes in males and females without persistent sickness or physiological changes, such as organ dysfunction, that may induce depressive states (Bleck *et al*, 1993; Ward and Fattahi, Fate, 2019). As discussed in chapter 2, the subchronic immune challenge results in no persistent "lingering sickness". Given that certain depressive-like behaviors, such as anorexia and anhedonic-like behavior as observed along with the physiological changes that are can be part of the sickness response (Dantzer *et al*, 1998), it is difficult to parse apart the affective changes from the sickness responses in animal models of sepsis where "overwhelming immune activation" both physiological and cognitive outcomes (Fink, 2014; Huerta *et al*, 2016). As we have observed no persistent decreases in weights and differences in locomotor activity at least eight weeks after subchronic immune challenge, it is unlikely that lack of differences in anxiety-like behaviors at this time are due to long-lasting changes in locomotor responses (e.g. decreased motor activity of animals treated with subchronic immune challenge). Our mild systemic immune challenge model, therefore, is advantageous in that it does allow to assess long-lasting changes in the depressive-like behaviors, including anhedonia-like behavior, or anxiety-like behaviors without the confounding effects of sickness.

There may be a few other confounding variables for our studies on long-lasting changes in affective processes, however, including the validity of some of the behavioral tests. The forced swim test was originally designed to predict the clinical efficacy of antidepressant drugs, such as selective serotonin reuptake inhibitors. As the drugs that did have antidepressant effects in patients were also shown to alter certain parameters in the forced swim test, including time spent immobile and climbing (Bogdanova *et al,* 2013), these behavioral changes were initially interpreted as 'anti-depressant effects' and the forced swim as an effective test for depressive-

like behaviors in animal models (Kloet and Molendijk, 2016; Van der Meersch-Mougeot *et al*, 1993). However, as anti-depressants take months to become effective in humans due to their mechanisms of action (Baudry *et al*, 2010; Erb *et al*, 2016), the ability of the forced swim test to measure true depression-like behavior or states has been questioned (Anyan and Amir, 2017). Additionally, as locomotor responses in the water or learning immobility may impact the time spent immobile vs swimming, the forced swim test also shows poor face validity. Therefore, the decreases in time spent immobile in the Poly I:C treated animals may not be indicative of changes in despair-like behavior. Given that there are also no differences in anhedonic-like behavior between the experimental groups in the sucrose preference test, it is likely that the differences in time spent immobile are not indicative of depression-like behaviors. An alternative to the forced swim test as a measure of despair-like behavior is the tail suspension test.

There may also be confounding variables for the sucrose preference studies. For example, we tested the long-lasting changes in anhedonic-like behavior in females after LPS treatment using 1% sucrose solution. The sucrose preference protocol was later optimized in females but tested only on females treated with subchronic Poly I:C challenge or saline. The differences in number of licks between the experimental groups may be due to differences in how rewarding the 1% solution appeared to the animals rather than an indication of their depressive-like state. Therefore, it is possible that using a higher percent sucrose solution will elicit higher number of licks in LPS treated females, and allow to more accurately assess differences in sucrose preference between saline and LPS treated females.

Similarly, there are criticisms of the validity for the elevated plus maze and open field test. While elevated plus maze has been described as having face validity due to increased anxiety in open spaces, and predictive validity between tests of anxiety-like behaviors, including

open field test (Walf and Frye, 2009), there have been criticisms regarding its ability to test anxiety (Hogg, 1996). For example, rodents can spend a significant portion of the test time in the center area, impacting the measures used to assess anxiety-like behaviors, such as time spent in closed arms vs open arms (Rodgers and Dalvi, 1997). Time spent in the open arms may also be interpreted as risk-assessment behavior rather than anxiety-like behavior (Carobrez and Bertoglio, 2005). As for the open field test, the validity of certain measures, such as time spent in center in the open field test has been criticized in mice as anxiolytics did not increase center time (Thompson *et al*, 2015). We have observed no differences in time spent in center nor number of center entries in both the elevated plus maze and the open field, suggesting a predictive validity of the behavioral tests used to assess anxiety-like behaviors after subchronic immune challenge.

Our studies are one of the first to assess long-lasting changes in affective processes in both males and females after systemic inflammatory insult. Acute, systemic immune activation has been shown to induce anxiety-like and depressive-like behaviors in both sexes (Fields *et al*, 2018; Millett *et al*, 2019), with only few studies showing greater depressive-like behaviors, such as despair-like behavior, in males (Millett *et al*, 2019; Pitychoutis and Papadopoulou-Daifoti, 2010). Similarly, in humans, no sex differences are observed in mood disturbances or anxiety state (Engler *et al*, 2016). Therefore, our findings support the idea that immune challenge does not induce sex differences in persistent anxiety-like or depressive-like. Alternatively, as we observed decreased time spent immobile in males at least eight weeks after LPS and Poly I:C challenge and in females at least one week after Poly I:C challenge, it is possible that there are sex-specific alterations in learned immobility after subchronic immune challenge, which may impact their behavior in the forced swim test.

In summary, our results suggest that subchronic immune challenge does not induce long-lasting anxiety-like behaviors nor depressive-like behaviors, including both despair-like and anhedonic-like behavior. We also do not observe any depressive-like behaviors at the earlier timepoint. The behavioral differences observed in the forced swim test maybe due to learned immobility rather than differences in despair-like behavior. As we have assessed anhedonic-like behavior only in females, future work could determine whether short-term or long-lasting changes in anhedonic-like behavior is observed months after LPS or Poly I:C challenge in males. Alternative tests could also be used to determine changes in despair-like behavior, including the tail suspension test. Together, these studies provide insights into the types of cognitive processes that become persistently altered after recovery from a mild systemic inflammatory insult.

Figures

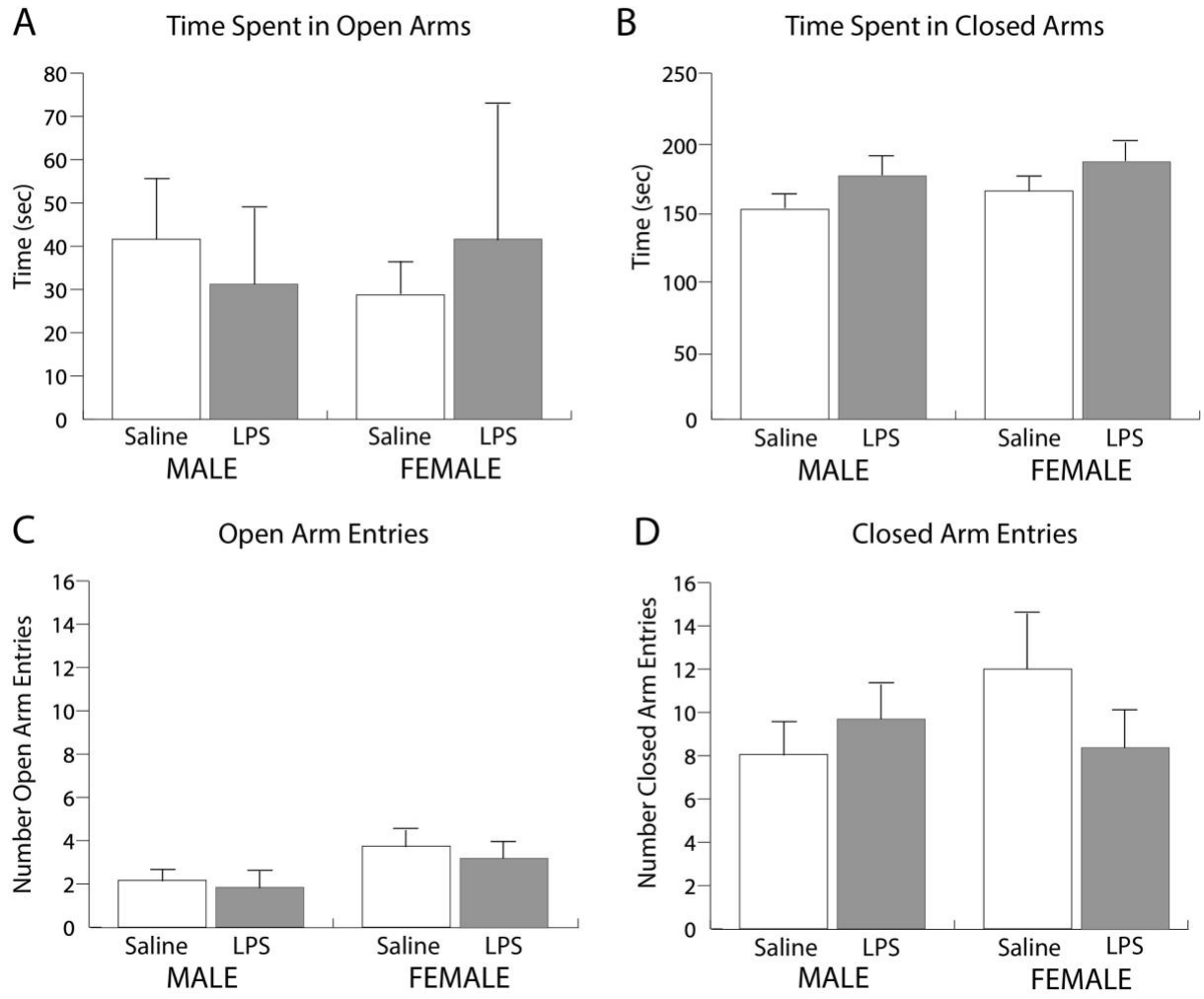


Figure 3.1. No differences in anxiety-like behaviors in elevated plus maze at least 8 weeks after subchronic LPS challenge. (A) Time spent in open arms in males and females. (B) Time spent in closed arms in males and females. (C) Number of open arm entries in males and females (D) Number of closed arm entries in males and females. These males and females are the same animals that had undergone foreground fear conditioning from Chapter 2, Figure 7. * $p < 0.05$ LPS *cf* saline.

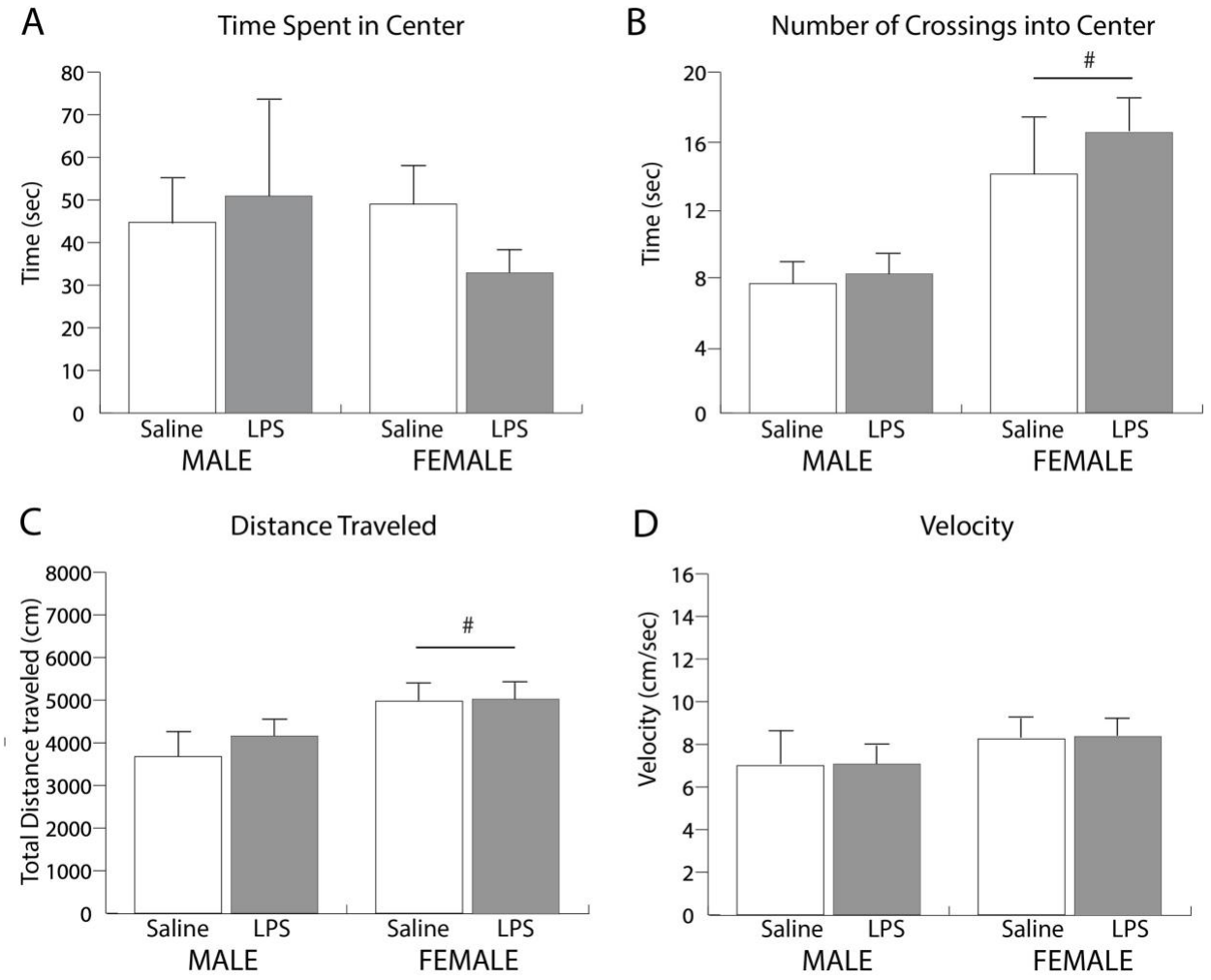


Figure 3.2. No differences in anxiety-like behaviors in open field test at least eight weeks after subchronic LPS challenge. (A) Time spent in open arms in males and females. (B) Time spent in closed arms in males and females (C) Number of open arm entries in males and females. (D) Number of closed arm entries in males and females. These animals are the same animals that had undergone foreground fear conditioning from Chapter 2, Figure 7. # $p < 0.01$ females *cf* males; * $p < 0.05$ LPS *cf* saline.

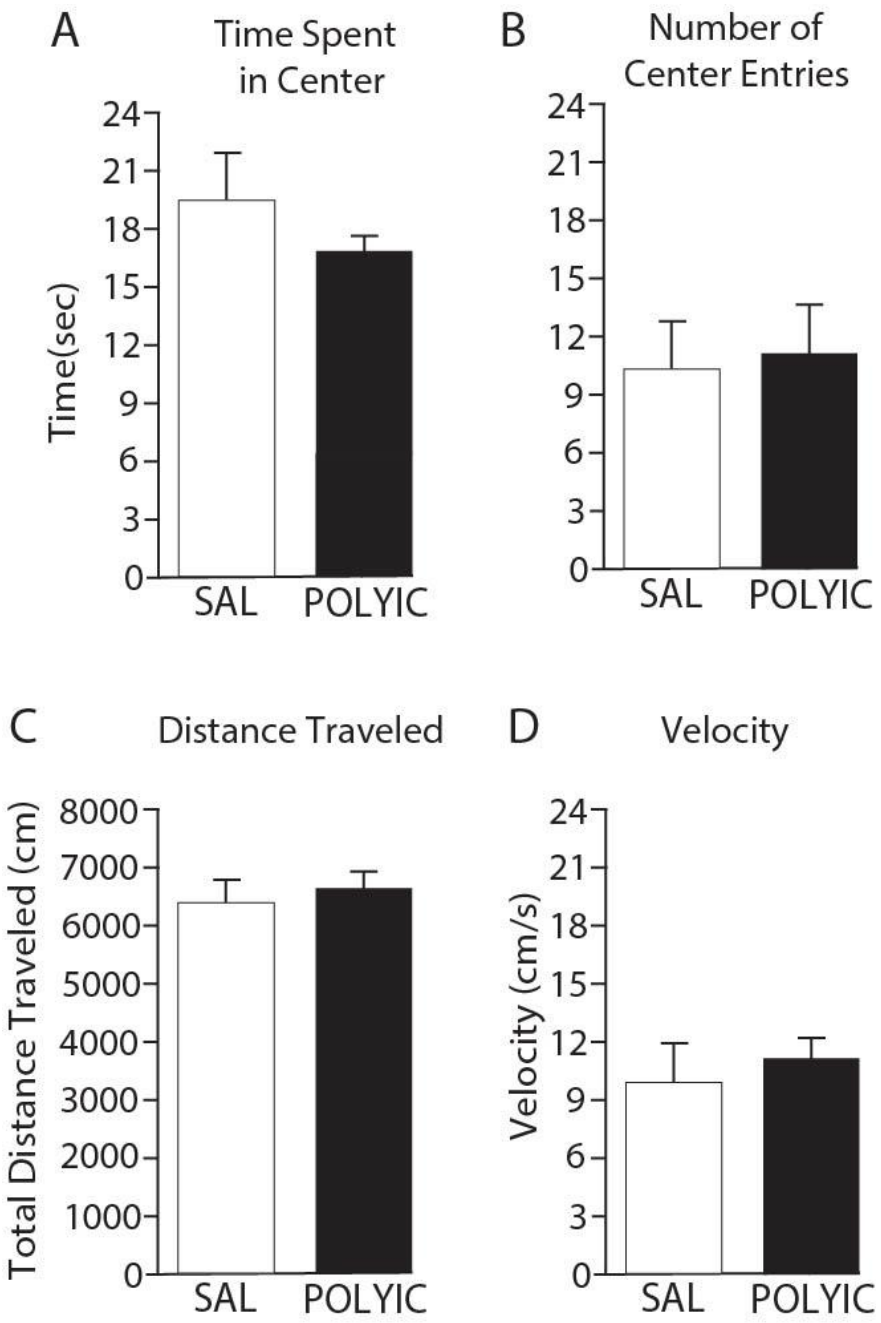


Figure 3.3. No differences in anxiety-like behaviors in open field test at least eight weeks after subchronic Poly I:C challenge. (A) Time spent in open arms in males and females. (B) Time spent in closed arms in males and females. (C) Number of open arm entries in males and females. (D) Number of closed arm entries in males and females. These males are the same animals that had undergone background fear conditioning from Chapter 2, Figure 5. * $p < 0.05$ Poly I:C *cf* saline.

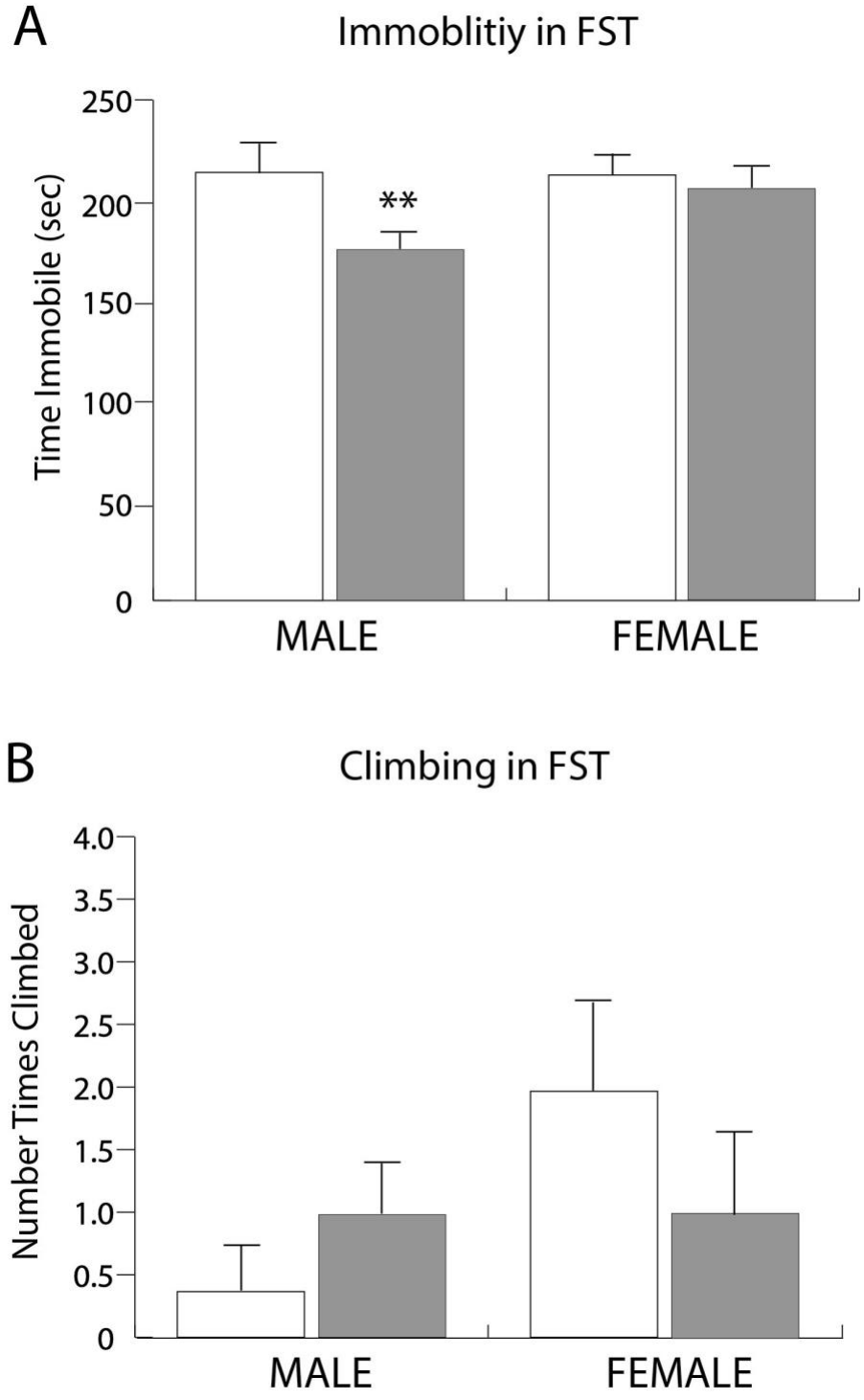


Figure 3.4. No despair-like behavior in forced swim test at least eight weeks after subchronic LPS challenge. (A) Time spent immobile (no moving paws except for floating) during 5 min. test. (B) Times climbed in forced swim test. These males and females are the same animals that had undergone foreground fear conditioning from Chapter 2, Figure 7. ** $p < 0.01$ females *cf* males.

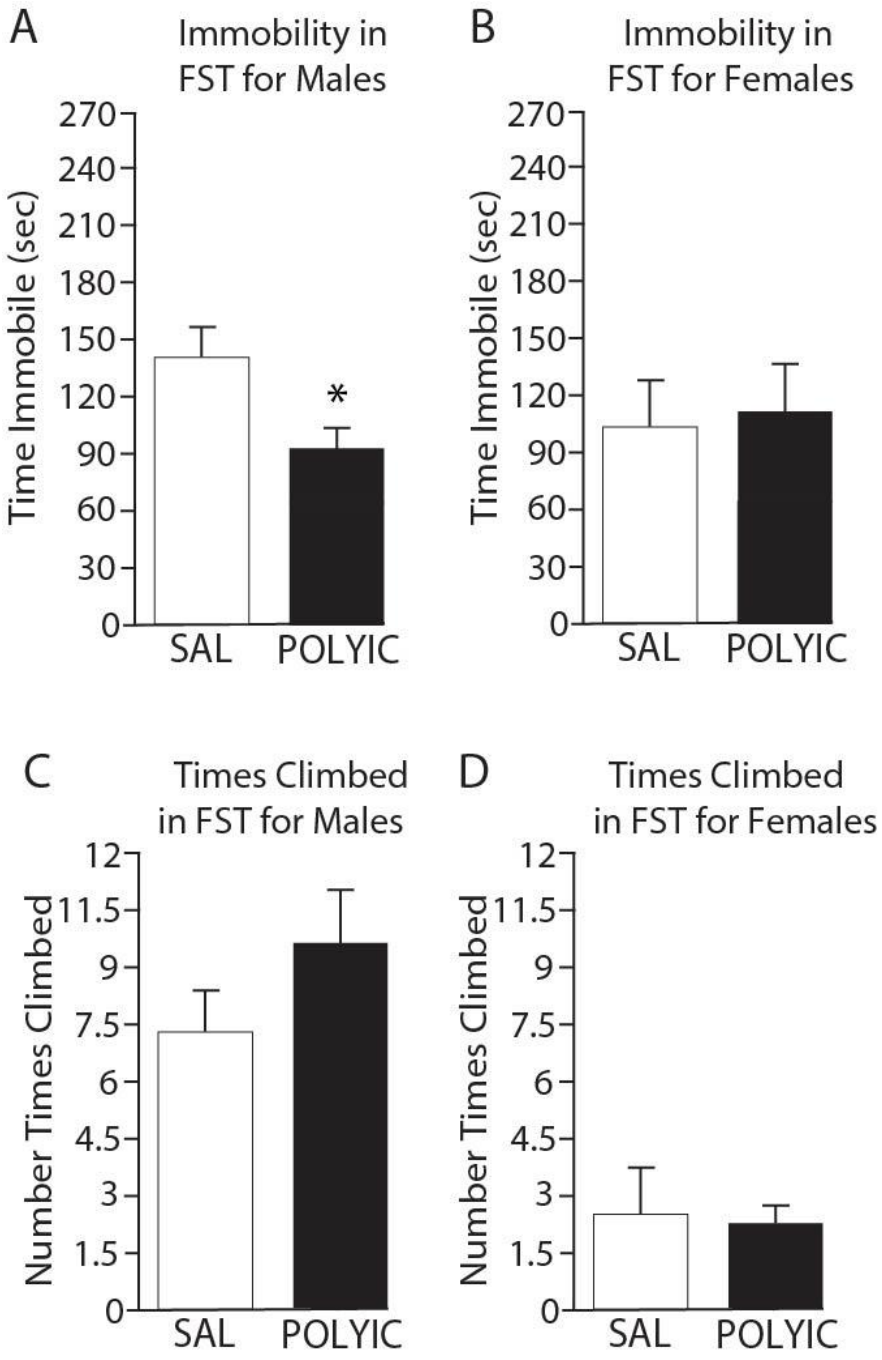


Figure 3.5. No despair-like behavior in forced swim test at least eight weeks after subchronic Poly I:C challenge. (A) Time spent immobile (no moving paws except for floating) during 5 min. test (real graphs will have # above Poly I:C). (B) Times climbed in forced swim test. ** $p < 0.01$ females *cf* males # $p < 0.05$ Poly I:C *cf* saline.

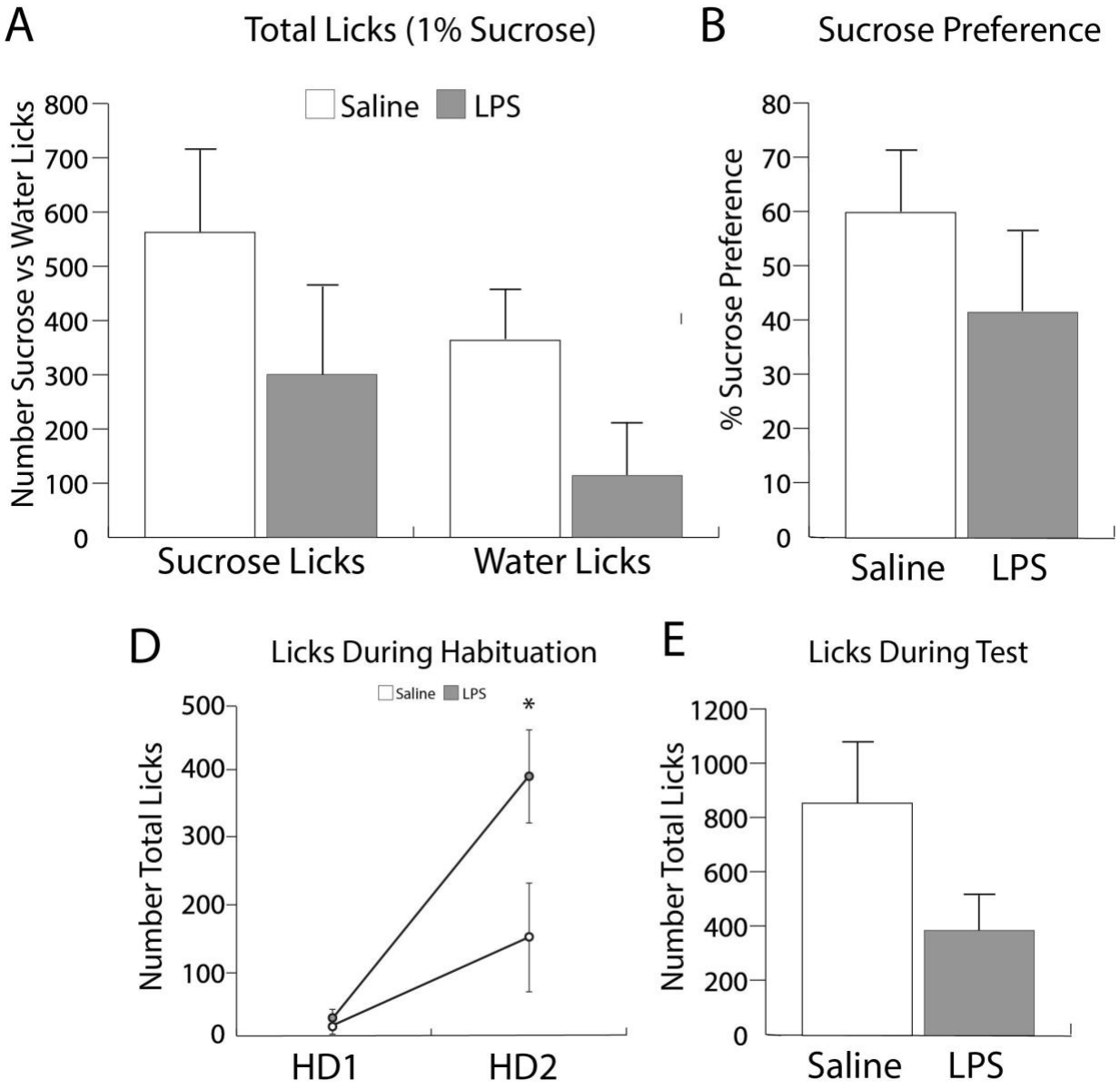


Figure 3.6. No andehonic behavior in sucrose preference test at least 8 weeks after subchronic LPS challenge. (A) Number sucrose and water in saline and LPS-treated females. (B) Percent sucrose preference. (C) Total number of licks during each habituation day. (D) Total number of licks during test day. These females are the same animals that had undergone novel object recognition from Chapter 2, Figure 7. * $p < 0.05$ LPS *cf* saline.

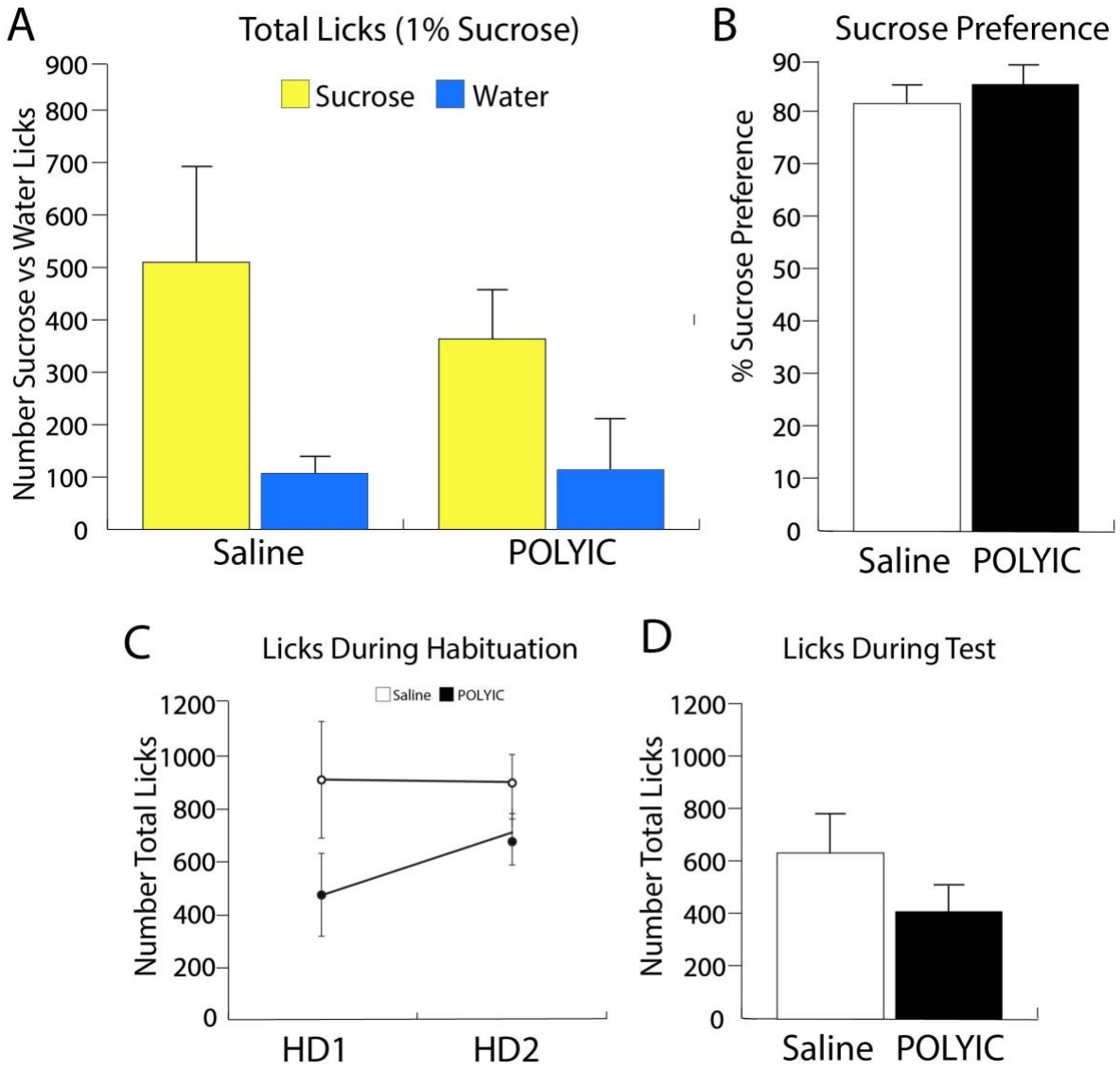


Figure 3.7. No andehonic behavior in sucrose preference test at least eight weeks after subchronic Poly I:C challenge: 1 % Sucrose solution. (A) Number sucrose and water in saline and Poly I:C-treated females. (B) Percent sucrose preference. (C) Total number of licks during each habituation day. (D) Total number of licks during test day. * $p < 0.05$ LPS *cf* saline.

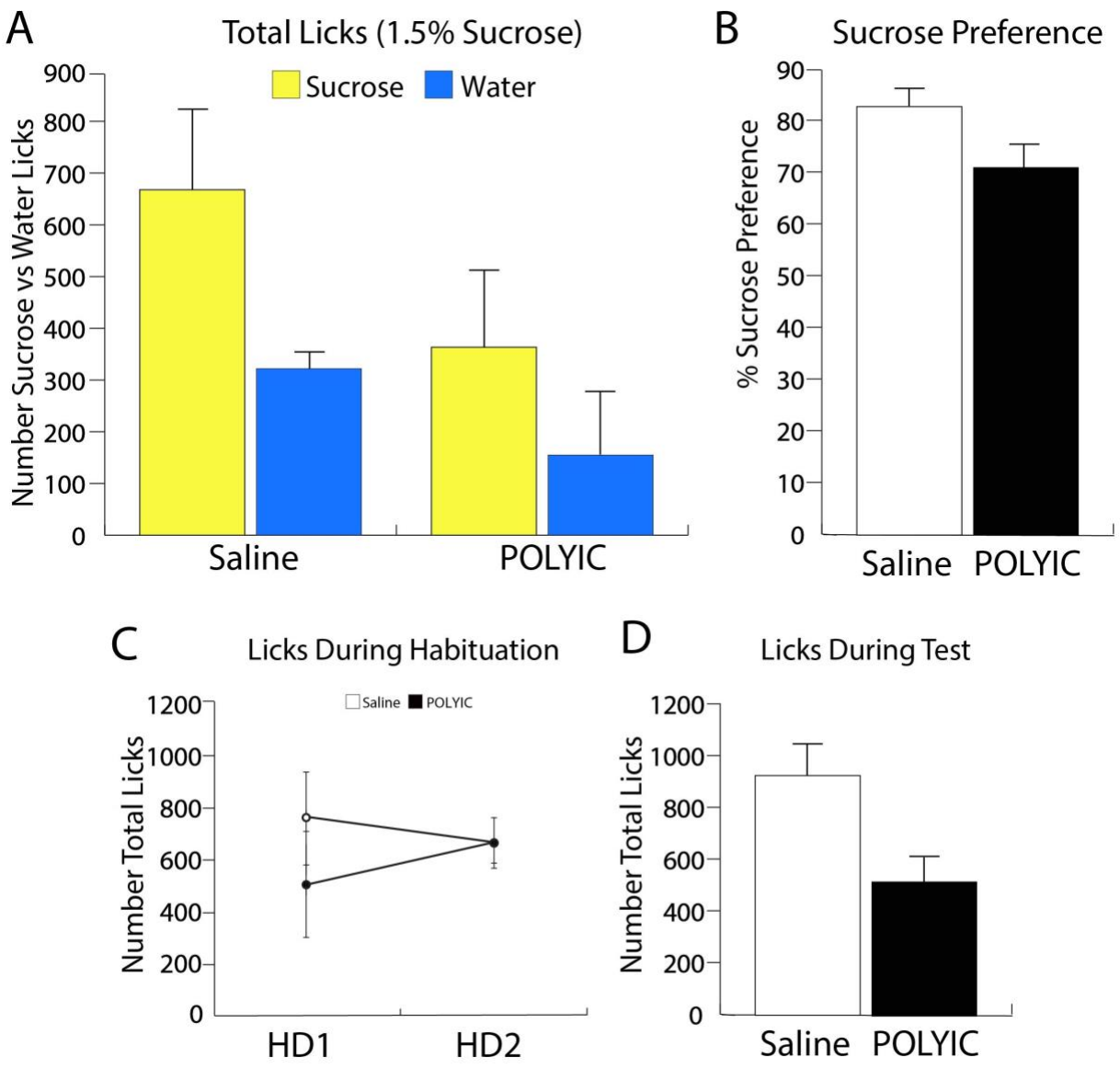


Figure 3.8. No andehonic behavior in sucrose preference test at least eight weeks after subchronic Poly I:C challenge: 1.5 % Sucrose solution. (A) Number sucrose and water in saline and Poly I:C-treated females (Real graphs will have a * on top of the water in saline animals). (B) Percent sucrose preference. (C) Total number of licks during each habituation day. (D) Total number of licks during test day (Real graphs will have a # above Poly I:C). # $p < 0.05$ LPS *cf* saline. $p < 0.05$ Sucrose vs water licks.

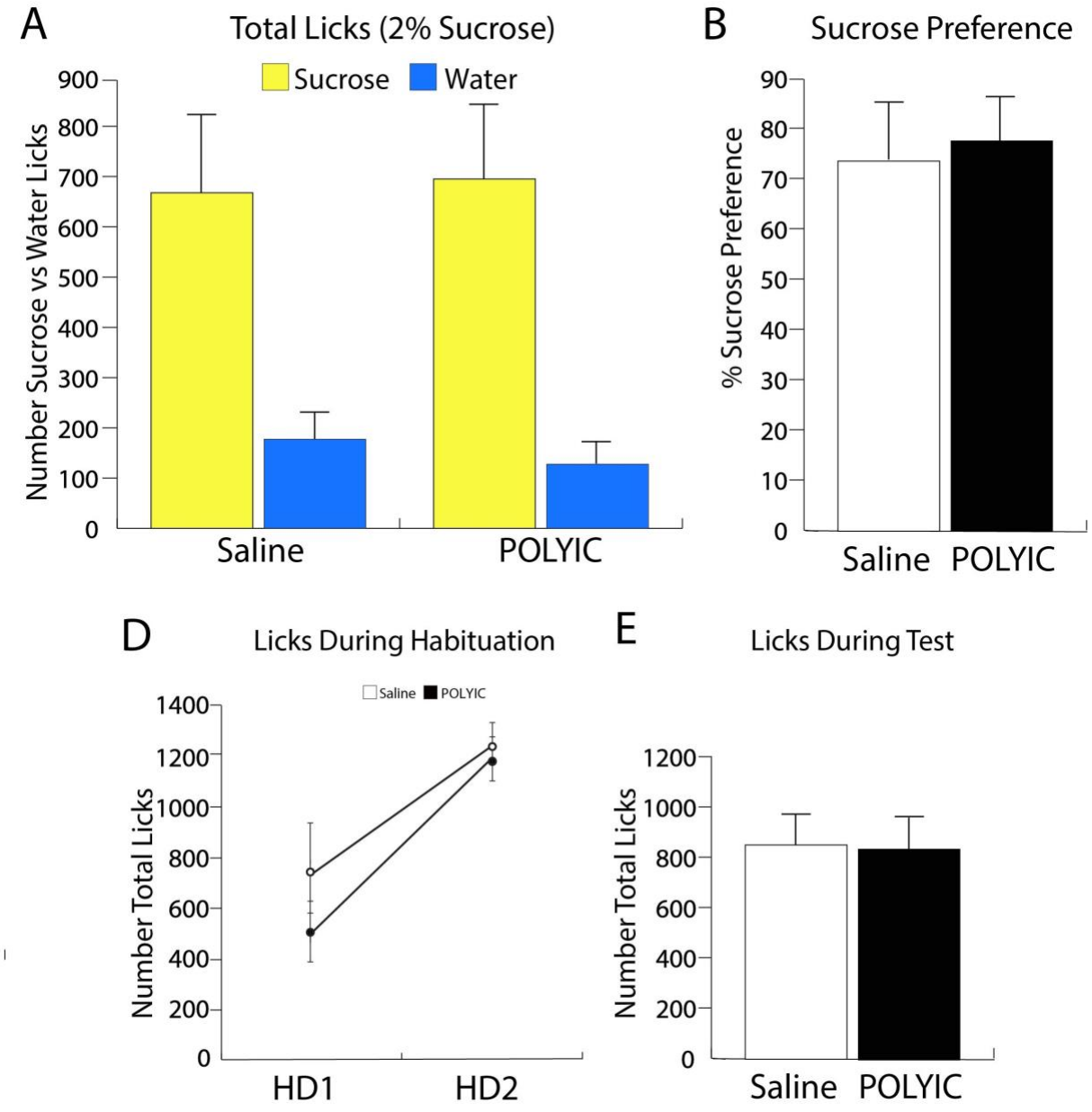


Figure 3.9. No andehonic behavior in sucrose preference test at least eight weeks after subchronic Poly I:C challenge: 2 % Sucrose solution. (A) Number sucrose and water in saline and Poly I:C-treated females (Real graphs will have a * on top of the water in saline animals). (B) Percent sucrose preference. (C) Total number of licks during each habituation day. (D) Total number of licks during test day (Real graphs will have a # above Poly I:C). # $p < 0.05$ LPS *cf* saline. $p < 0.05$ Sucrose vs water licks.

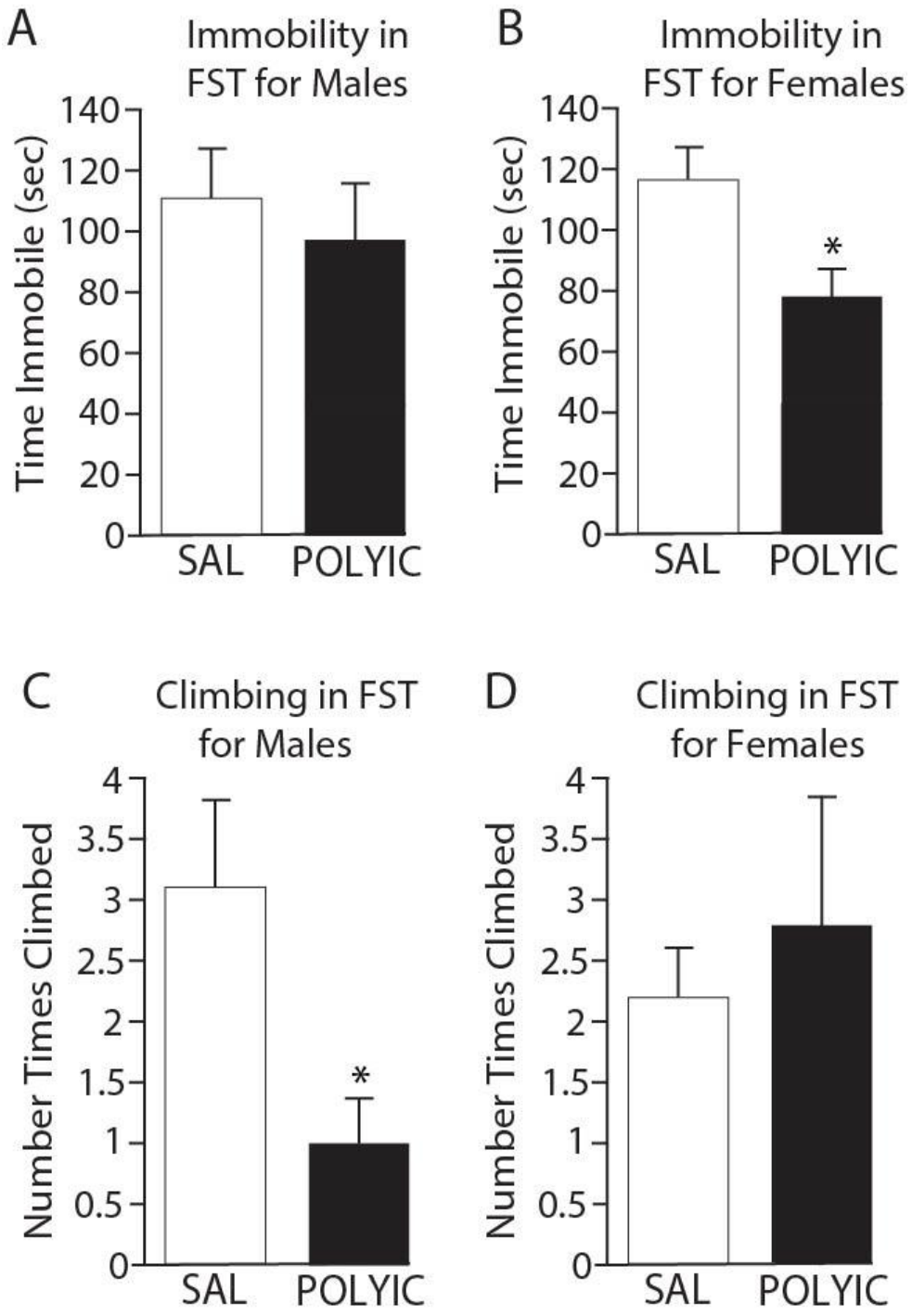


Figure 3.10. No despair-like behavior in forced swim test at least one week after subchronic Poly I:C challenge. (A) Time spent immobile (no moving paws except for floating) in males during 5 min. test. (B) Time spent immobile in females (real graphs will have # above Poly I:C). (C) Times climbed in forced swim test in males (real graphs will have # above Poly I:C). (D) Times climbed in forced swim test in females. # $p < 0.05$ Poly I:C *cf* saline

Chapter IV

Enduring and Sex-Specific Changes in Hippocampal Gene Expression after a Subchronic Immune Challenge

Abstract

Major illnesses, including major surgery, heart attack, and sepsis, can cause long-lasting cognitive impairments, depression, and progressive memory decline that persist well after recovery from the original illness. We have recently demonstrated that a series of intermittent injections of lipopolysaccharides over a two-week period resulted in sex-specific patterns of memory deficits that persist at long after the end of the immune challenge. Here, we used RNA-sequencing as a large-scale, unbiased approach to identify both persistent changes in gene expression long after an immune challenge, and changes in transcriptional response to a subsequent immune challenge. Males and females differed in number and patterns of gene expression in the hippocampus. In males, we observed enduring dysregulation of gene expression three months after the end of a subchronic immune challenge, in the absence of an additional insult. In contrast, females showed few persistent changes under basal conditions, but striking dysregulation of gene expression in response to an additional acute LPS injection. Striking sex differences in the specific genes, pathways, and biological processes affected were observed both after subchronic immune challenge and after a subsequent insult. Thus, subchronic systemic immune activation has enduring and sex-specific consequences for gene expression and response to subsequent stimuli. Such persistent changes in neural functions, together with

previous data from human and animal models showing memory deficits, demonstrate that in both males and females, neuroimmune signaling may contribute to subsequent vulnerability to cognitive decline, memory impairments, and affective disorders.

Introduction

The ability of biological systems to change as a consequence of experience is a fundamental feature of how individuals adapt to their current environment. In the brain, plasticity at multiple levels including behavior, circuits, synapses, and gene expression are core mechanisms of learning and long-lasting memory that allow animals to change their behavior in accordance with experience. Persistent changes in these mechanisms allow for adaptation to events including stress, drugs of abuse, or major illness may also contribute to vulnerability or resilience to disorders including depression, addiction, and cognitive decline. In chapter 2 and 3, I have shown long-lasting memory deficits in males and females without persistent changes in affective processing. In this chapter, I explore the role of subchronic immune challenge in mediating persistent changes in molecular substrates that may be important for hippocampal-dependent memory by determining enduring changes in hippocampal gene expression.

Gene expression is important for various cognitive functions, including learning and memory. Specific patterns of gene expression are required for memory (Igaz *et al*, 2004). Transcriptional studies of learning and memory show that particular neuroplasticity-related genes are expressed during specific memory processes such as consolidation and retrieval (e.g. *Fos*), at least in males (Peixoto *et al*, 2015). Alterations in molecular substrates, including expression of transcription factor CREB, have been implicated in changes in memory and cognitive functions (Alberini and Kandel, 2019; Hawk and Abel, 2011). As specific genes and patterns of gene

expression are important for proper memory functions, including formation and maintenance of memories, dysregulation of these patterns of gene expression may lead to memory dysfunction. It is possible, therefore, that persistent dysregulation of gene expression in the hippocampus may lead to long-lasting memory deficits, cognitive impairments, and brain dysfunction.

Recent work has demonstrated that various environmental experiences, including stress, drugs or inflammatory insults, induce persistent dysregulation of gene expression in the brain that alters an individual's responsiveness to subsequent insult. For example, prior exposure to stress can increase stress reactivity to subsequent stressors (Gray *et al*, 2014; Nestler, 2014; Sterlemann *et al*, 2008; Tafet and Nemeroff, 2016). Similalry, enduring changes in transcriptional responses occur after inflammatory insults. These enduring transcriptional changes are a hallmark of immune system function. During acute activation of the peripheral immune and neuroimmune systems, cytokines increase transcription and production of other cytokines and immune mediators. Activation of the inflammatory response results in persistent transcriptional changes, which encode new antibodies in the adaptive immune system, and training of the innate immune system (Netea and van der Meer, 2017). There is also evidence for such "training" of the innate immune system in the brain, with lasting changes in neuroimmune reactivity after multiple immune challenges (Wendeln *et al*, 2018). Enduring changes in cognition and memory after immune challenge may result from persistent alterations in gene expression and transcriptional regulation after illness or injury.

Interestingly, there are striking sex differences in the precise patterns of gene expression in the brain in the days and weeks after environmental insults, including stress (Hodes *et al*, 2015; Mychasiuk *et al*, 2016). For example, in a chronic unpredictable stress paradigm, males show more changes in gene expression and regulation of different gene networks compared with

females (Hodes *et al*, 2015). Changes in transcriptional regulation as a consequence of adverse events contributes to altered responses to later insults. If gene expression is differentially regulated in males and in females, then this may mediate sex-specific vulnerability or resilience to affective and cognitive disorders.

Here we examined enduring changes in hippocampal gene expression after a systemic, subchronic immune challenge in both sexes, and discuss the implications for persistent dysregulation of cognition, emotion, and memory. We determined whether changes in hippocampal gene expression persist long after an immune challenge in males and in females. Given that recent work from our laboratory has demonstrated deficits in object recognition in both males and females but only deficits in context and cued-fear conditioning in males as a consequence of mild subchronic immune challenge (Tchessalova and Tronson, 2019), we hypothesized that this challenge would also cause enduring and sex-specific changes in gene expression and transcriptional regulation in the hippocampus. We used a next generation RNA-sequencing as a large-scale, unbiased approach to identify long lasting changes in gene expression. We examined both persistent changes in gene expression long after an immune challenge and changes in the transcriptional response to a subsequent immune challenge. We demonstrate that males and females differ in number and patterns of gene expression in the hippocampus both three months after a subchronic immune challenge and in response to a subsequent, acute LPS injection.

Materials and methods

***2.1 Animals*:** Male and female 8-9 week old C57BL/6N mice were purchased from Envigo (Indianapolis, IN). All mice were individually housed with *ad libitum* access to standard mouse

chow and water as individual housing in mice prevents fighting-induced stress in males (Meakin *et al*, 2013) and is ethologically appropriate for males and females(Becker and Koob, 2016). The facility is ventilated with constant air exchange (60 m^3/ h), temperature (22 ±1°C), and humidity (55±10%) with a standard 12 h light-dark cycle (Keiser *et al*, 2017). One month prior to collection of tissue, all animals were tested on context fear conditioning anxiety-like behavior, and depression-like behavior. The results from context fear conditioning are published elsewhere (Tchessalova and Tronson, 2019), and we observed no effects of prior LPS challenge on anxiety or forced swim test. All experimental methods used in these studies were approved by the University of Michigan Committee on the Use and Care of Animals. Sample size was based on minimum number of animals needed per experimental condition for RNA sequencing (3 per condition).

***2.2 Subchronic Immune Challenge*:** Lipopolysaccharide (LPS, *Escherichia coli*, serotype 0111:B4; Sigma-Aldrich, St. Louis) was dissolved in saline for a final concentration of 12.5μg/mL. All LPS injections were given intraperitoneally (i.p.) at a dose of 250μg/kg. Vehicle control mice received an equivalent volume of saline (20mL/kg, i.p.) (Cloutier *et al*, 2012; Tchessalova and Tronson, 2019). The subchronic immune challenge consisted of five injections, spaced three days apart (days 1,4,7,10,13). All injections were administered in the morning between 9 and 10am. In the Long-Term condition, tissue collection occurred 12 weeks after the final LPS injection. Separate animals received a subsequent LPS (250μg/kg; or vehicle) injection 12 weeks after the subchronic immune challenge and tissue was collected 6 hours after injection (Long-term + Acute condition). For the Acute condition, animals received a single LPS (250μg/kg, or vehicle) injection and hippocampi were collected 6 hours later.

***2.3 RNA extraction and sequencing*:** To maintain RNA quality, whole hippocampi were collected and immediately placed in RNALater until processing (Bagot *et al*, 2016, 2017, Cates *et al*, 2017, 2018; Hodes *et al*, 2015; Lorsch *et al*, 2018; Walker *et al*, 2018). One hemisphere (counterbalanced by side between sex and experimental conditions) was selected for RNA extraction while the other was collected for protein analysis. RNA was isolated using Life Technologies PureLink RNA Mini kit (cat. no. 12183018A). Relative RNA quantity and integrity were first analyzed using NanoDrop (ThermoFisher) and gel electrophoresis. Quality and integrity checks were then completed on the Bioanalyzer by the University of Michigan DNA sequencing core, with acceptable RIN values greater than 7. Sequencing was performed using Illumina 4000 High-Seq platform, using single-end, non-strand, Ribo depletion with read lengths of 50 and sequencing depth of 40 million reads per sample. Total RNA (20ug) was used to construct the mRNA libraries. Barcoded cDNA libraries were constructed from polyadenylated transcripts that were purified, fragmented, and reverse transcribed using random hexameters. Three independent biological replicates were used per experimental condition.

***2.4 Differential gene expression analyses*:** Alignment, differential expression analysis, and post-analysis diagnostics were analyzed using the Tuxedo Suite software package. Reads were aligned to the Ensembl *Mus musculus* NCBIM37 reference genome using TopHat and Bowtie. The quality of the raw reads data for each sample was assessed using FastQC to exclude any reads with quality problems. Expression quantitation, normalization, and differential expression analyses were conducted through Cufflinks/CuffDiff with UCSC mm10.fa reference genome sequence. Differentially expressed genes (DEGs) were determined by multiple comparison correction using FDR > 0.05 cutoff.

Visualization differentially expression genes: DEGs in males and females were visualized using Venn diagrams through Venny 2.1.0 (http://bioinfogp.cnb.csic.es/tools/venny/). Volcano plots generated through Advaita's iPathway guide (https://www.advaitabio.com/ipathwayguide) were used to view DEGs by fold change and significance (p-value).

Biological pathways: Metascape (http://metascape.org/gp/index.html#/main/step1) was used to generate gene annotation and gene list enrichment analysis, with a focus on biological pathways and processes. Significance of biological processes was determined through P-values calculated on a hypergeometric distribution (log10). Reference gene lists and annotated information were obtained from the Enrichr web page. Metascape was also used to conduct meta-analysis, with generation of Circos plots to visualize shared genes and pathways amongst the conditions and heatmaps of gene ontology (GO) terms that hierarchically cluster together amongst experimental conditions.

Protein-protein interactions: Clusters of functional protein-protein interactions (existing and predicted) between targets in experimental conditions of interest were visualized using the STRING 10.5 software (https://string-db.org/).

Statistical analysis: Criteria for a differentially expressed gene included a fold change greater than 1.5, and false discovery rate greater than 5% (fold change $\geq \pm 1.5$ and FDR ≤ 0.05). All comparisons of DEGs between control and experimental conditions were made within sex using unpaired *t* tests (two-tailed) with Benjamini correction to account for multiple comparisons to determine the effect of immune challenge in males and in females separately. DEGs in the hippocampus of males and females at baseline were compared using unpaired *t* tests (two-tailed) between vehicle treated males and vehicle treated females. Groups were compared as shown in Table 1.

Data availability: To allow all interested parties to explore and utilize our processed data, we have made our data publicly available through user-friendly databases, including the Gene Expression Omnibus (GEO), with accession number GSE126678, and Sequence Read Archive (SRA), with SRA number SRP186132 and BioProject number PRJNA522922.

Analysis transcription factors: Potential transcription factors regulating were determined using an integrative, unbiased approach, TRANSFAC software. TRANSFAC gene sets were downloaded from the inventory of gene sets (Molecular Signature Database, MSigDB version 6.2). The files were downloaded as c3.tft.v6.2.symbols.gmt, with 615 gene sets each representing one transcription factor and the genes that have that binding site. The human geneset was converted into mouse genes using the MGI human to mouse table from http://www.informatics.jax.org/ downloads/reports/HOM_MouseHumanSequence.rpt. Signaling factors that were associated with the transcription factors were identified using The Signaling Pathways Project query tool (https://beta.signalingpathways.org/ominer/query.jsf). The mouse genes from the consensomes file were mapped to human genes using the Jackson laboratory HomoloGene tables (http://www.informatics.jax.org/downloads/reports/HOM_MouseHumanSequence.rpt). The final gene sets included genes in the top 1% of each consensome.

Results

3.1 Subchronic immune challenge induces persistent changes in gene expression

We observed striking changes in gene expression in the hippocampus 12 weeks after subchronic immune challenge in males, with fewer changes observed in females. Of over 20,000 genes detected, there were 230 DEGs in the hippocampus of males and 26 DEGs in the

hippocampus of females. In males, 183 genes were significantly upregulated and 47 significantly downregulated. In females, 7 genes were significantly upregulated and 18 downregulated. Five of these genes were differentially expressed in both males and females, with *Npas4* and *fos* downregulated in males and upregulated in females, and *Ifit1, Spp1* (*Opn*), and *Coch* upregulated in males and downregulated in females (Figure 1A; Table 1; Table 2). Overall, in males, neuroimmune-related genes showed persistent upregulation, whereas neuroplasticity-related genes showed downregulation months after the subchronic immune challenge. The 10 most upregulated genes, included *Wdr72, Prdm6, Slc16a8, Tmem72, Wfdc2, Cldn2, Kcne2, Steap1, Ttr,* and *Aqp1* while the 10 most downregulated genes included immediate-early genes and transcription factors *Egr2, Fosb, Fos, Npas4, Npas4, Egr4,* and *Junb* as well as genes related to immune signaling and transcription, *Btg2* and *Ccl3*, and extracellular matrix associated *Cyr6* (Figure 1B,C). Other upregulated genes of interest, with lower log2 fold change values included plasticity-related *Cdh3,* transmembrane proteins such as *Tmem184a,* tight-junction proteins such as *Cldn9,* and targets related to G-protein signaling (e.g. *Ccdc135).* Additional downregulated genes included other neuroplasticity related genes, such as *Nr4a1* and *Dusp6*, and cytokine *Ccl3* (Figure 1C) as well as immune-related *Ier2* and *Apold1* (not shown). Genes that almost reached significance for the Log2 fold change criteria of 0.5 included *Fbxo33* and *Fxyd1*. In females, although few DEGs were identified, they were consistently related to dopaminergic/monoaminergic signaling. The top upregulated genes included *Npas4, Dio3, Mid1, Fos, Gpr101, and Dlk1* while the top 10 downregulated genes included immune-related and monoaminergic-associated *Adora2a, Cd4, Drd2, Gbp4, Gpr88, Ifit1, Foxp2, Myl4, Scn4b*, and *Adra1b* (Figure 1B,D). Other dysregulated targets of interest included neuropeptide *Penk* and those involved in metabolic functions (e.g. *Xdh)*. Targets with the lowest log2 value and or

adjusted p-value included *Fmod*, and *Gbp5*.

Biological pathway and process enrichment analysis revealed top clusters in males were: extracellular matrix organization (log10(P) = -15.41), vascular development (log10(P) = -13.85), response to growth factor (log10(P) = -11.53), regulation of MAPK signaling (log10(P) = -10.2), and response to hormone (log10(P) = -8.87) (Figure 2A). In females, top biological processes included response to amphetamine (log10(P) = -8.49), regulation of defense response (log10(P) = -4.76), and regulation of membrane potential (log10(P) = -3.06) (Figure 2B). Importantly, DEGs in males and females pertain to distinct biological pathways, with neuroplasticity- and MAPK-associated signaling in males and monoaminergic signaling and innate immune-related signaling in females.

Functional protein-protein interaction analysis (STRING) identified groups of DEGs with functional relationships. In males, these clusters included synaptic plasticity and memory-related genes, extracellular matrix targets including collagens and matrix breaking enzymes, growth factors and their receptors, and immediate early genes. There were also several immune-related targets involved in innate immune responses, including regulators of MAPK signaling, complement genes and their regulators, and MHC II-related targets. Notably, some regulators of immune signaling, including *NR4a1/2, Spp1,* and *Dusp1/6* are also plasticity related genes. Smaller clusters of genes included targets associated with organic anion/cation solute carriers, and potassium and chloride channels involved in inhibitory transmission (Figure 2C; Table 1).

In females, STRING analysis identified two clusters: one including dopaminergic signaling, adenosine signaling, adrenergic signaling, and G protein-coupled signaling; and other genes involved in interferon-mediated signaling (Figure 2D; Table 2). Importantly, the clusters identified in females did not overlap with those identified in males.

3.2 TRANSFAC analysis reveals potential sex differences in potential transcription factor activity months after subchronic immune challenge

We determined transcription factors associated with the experimentally derived transcriptional changes by comparing our ranked gene expression sets to computationally derived gene sets matching transcriptional motifs to nearby genes using gene set enrichment analyses in males and in females. The male gene dataset contained 15,584 genes. There was an enrichment of SRF motif in the long-term condition containing genes with a net enrichment score (NES) of -2.54 with an adjusted p-value of 5.7e-03. Genes that drive the greatest association with SRF include immediate-early genes, such as *Fos, EGR1/3/4*, and *Npas4*. There was also enrichment of many CREB related motifs, including CREBP with a net enrichment score of -2.4, ATF with a net enrichment score of -2.3, and CREB with a net enrichment score of -2.23, all with adjusted p-values of 5.7e-03. CREB-dependent genes included transcription factors *Fosb, Nr4a2, Atf3*, transcriptional repressor of TGFB transcribed genes (*Tgif2*), solute carriers *Slc18a2* and *Slc35f5*, and regulators of phosphorylation *Dusp1* and *Ppp1r15a*. Some genes enriched early while others are enriched late, suggesting that CREB-dependent genes may be upregulated or downregulated months after LPS injection (Table 10).

To empirically compare pathways enriched by SRF and CREB we also compared our results to consensus gene sets derived from meta-analyses of transcriptional data sets obtained from the Signaling Pathways Project. The gene sets glucocorticoid receptor (NES:-1.52, p-value: 2.2e-02) and mineralocorticoid receptor (NES: -1.38, p-value: 4.0e-02) were downregulated while some upregulated gene sets included peroxisome proliferator-activating receptors (NES: 1.52, p-value: 2.2e-02), Farnesoid X receptor (NES:1.48, p-value 4.7e-02), and retinoid acid receptors (NES:1.45, p-value 4.7e-02). These data demonstrate that genes that are affected by SRF and CREB are enriched in inactivation of glucocorticoid, peroxisome proliferator-

activating, and retinoid acid receptors.

The female gene dataset contained 15,584 genes, of which 33 were differentially expressed. There was an enrichment of STAT5-related motifs in the long-term condition, including STAT5B containing genes with a net enrichment score of -1.65 and STAT5BA motif containing genes with a net enrichment score of -1.62, both with an adjusted p-value of 8.2e-02. Genes predicted to have the greatest STAT5 interaction included immune-related (e.g. *Ccl2/5, Socs2, Irf9*), extracellular-matrix associated (e.g. *AdamtsL3, Pcolce*), and growth factor-related (e.g. *Bmp6, Wnt10a*) amongst other available in Table (tbd). There was also enrichment of OCT1 motif, which contained genes with a net enrichment score of 1.51 with an adjusted p-value of 1.7e-01, and AP1 motif with a net enrichment score -1.60 with an adjusted p-value 9.8e-02. Genes predicted to have the greatest OCT1 interactions were related to solute carriers (e.g. *Slc24a3, Slc6a15*), histones (e.g. *Hist2h2ac, Hist1h2bc*), and nuclear receptors (e.g. *Nr6a1, Nr2f2*) amongst others in Table (tbd). Enriched glucocorticoid receptor-dependent genes included cytokine-related targets (e.g. *Cxcl10, Il6r, Ifit2*), regulation proliferation (e.g. *Cdnkn1a/n2b*), transcription factors induced by cellular activity or stress (e.g. *Per1/2, Ier2*) (Table 11).

To empirically compare pathways enriched by SRF and CREB we also compared our results to consensus gene sets derived from meta-analyses of transcriptional data sets obtained from the Signaling Pathways Project. The gene sets glucocorticoid receptor (NES:-1.72, p-value: 1.5e-03), estrogen receptor (NES:-1.59, p-value:8.0e-03), mineralocorticoid receptor (NES:-1.41, p-value: 4.7e-02), and retinoid acid receptors (NES:-1.40, p-value 5.5e-02) were all downregulated. Together, this data suggests that, in males, there may be changes in regulation of CREB and SRF transcriptional activity and in females, in regulation of STAT5 and OCT1months

after subchronic immune challenge.

Along with the TRANSFAC data showing enrichment of CREB-related genes in the male dataset from the hippocampus, we also observed changes in one CREB-related target, the NR4a family nuclear receptor NR4a1, in the male hippocampus months after subchronic immune challenge. NR4a1 was persistently decreased in the dorsal hippocampus of males but not females after subchronic immune challenge (Appendix Figure 1). Yet, subchronic immune challenge did not alter protein levels of another cAMP signaling associated targets, including the dopamine and c-AMP regulated protein phosphoprotein (DARPP-32), which was downregulated in the hippocampus of females.

3.3 Prior subchronic immune challenge alters hippocampal gene expression in response to a later acute LPS injection in a sex-specific manner

We examined the long-lasting effect of prior subchronic immune challenge on transcriptional regulation to a subsequent acute LPS injection (Long-term+Acute condition). In males, we observed 58 DEGs, whereas in females, 432 genes were differentially expressed compared with acute immune challenge in previously naïve mice. Twenty-one genes showed differential expression in both sexes under these conditions, with 15 genes (e.g. *Gpr151, Chrna/b3, Ptprv, Slc5a7, Tac, Six3, Penk, Drd2, Adora2a, Foxp2, Syt6, Lrrc55, Drd1a, Susd2*) upregulated in both sexes, one gene (*Lcn2)* downregulated in both sexes, and five were dysregulated in opposite directions (e.g. *Slc35a3, AW551984, Arhgap6, Dlk1, Gpx3*) (Figure 2A). The top 10 upregulated genes in males included *Isl1, Prdm2, Gpr151, Eomes, Barhl2, Chrna3, Slc10a4, Sstr5, Chrnb3, Sln* and the only two downregulated genes included *Fermt1 and Lcn2* (Figure 3B,C). Other upregulated targets with lower log2 fold change include

neurotransmitter-associated *Drd1, Chrnb4*, insulin-related *Irs4*, and transcription factor *Foxp2*. The genes with the lowest log2 fold change include G-protein signaling *Gpr153, Adcyap1*, and TGF-beta associated, DNA-binding *Peg10*. The only downregulated gene that almost reached significance is immune-associated *Erdr1* (Figure 3C). In females, the 10 most upregulated genes have immune-related, RNA-binding, and hormone-related functions and included *Cga, Sperina9, Cd4, Nxf7, Ntrk1, Adora2a, Ptprv, Chat, Drd2, Cd6*. The 10 most downregulated targets were related to extracellular receptor or transporter activity and hormonal or metabolic functions and included *Dpep1, Sele, Oca2, Cldn2, Tmprss11a, Sult1c2, Rrh, Slc4a5, Fol1r*, and *Ttr* (Figure 3B, D). Other upregulated targets of interest included neurotransmitter-associated *Htrd1*, hormone-related *Trhr*, immune-associated *Ccbp2*. Additional downregulated genes of interest include metabolism-associated *Gytl1b*, and methylation-associated *Bhmt1*, as well as immune-related *Il-31ra* and *Tgfbi*, tight junction associated *Cldn9*, and hormone-associated *Mc3r* (not shown). Several genes almost reached the log2 fold change and significance threshold, including for example, transmembrane protein *Fam70a*, immunoglobulin superfamily member 9B (*Igsf9b*) and asparaginase (*Aspg*) (Figure 3D).

Biological pathway and process enrichment analysis revealed diverging pathways in males and females. In males, we observed changes in gene expression related to cholinergic synaptic transmission (log10(P) = -11.94), hormone secretion (log10(P) = -10.09), neuropeptide signaling (log10(P) = -7.45), response to nicotine (log10(P) = -6.01), and organic hydroxy compound transport (log10(P) = -6.00) (Figure 4A). In females, the top biological pathways and processes included regulation of hormone levels (log10(P) = -15.39), extracellular matrix organization (log10(P) = -8.1), hormone metabolic processes (log10(P) = -8.41), regulation of cell adhesion (log10(P) = -7.91), and MAPK cascade (log10(P) = -6.68) (Figure 4B).

Functional protein-protein interaction analysis (STRING) in males identified clusters associated with neurotransmission and neuroplasticity, including receptors important for dopaminergic and adenosine receptor-associated signaling and neuropeptides/neuropeptide receptors, a large cluster of transcription factors, and smaller clusters of calcium-activated chloride channels, and neural differentiation (Figure 4C; Table 3). In females, we found more immune-related clusters than in males, including MHC II signaling, interferon-mediated signaling, cholinergic signaling, and stress hormones. There were also immune and plasticity-related targets, including extracellular matrix and cell adhesion molecules. There were clusters with monoaminergic signaling including dopamine, serotonin, noradrenaline, and adenosine receptors, neuropeptide, and hormone receptors. Smaller clusters included channels, solute carriers important for neuronal inhibition, and metabolic functions (Figure 4D; Table 4).

3.4 Hippocampal gene expression in response to an acute immune challenge

We examined the hippocampal transcriptional response to a single, peripheral acute immune challenge in males and females. In males, we found 176 DEGs, and in females, 406 genes were differentially expressed (Figure 5A). The top 10 upregulated genes included mainly immune, hormone, and extracellular matrix associated genes, including *Defb9, Lcn2, Trh, Ttr, Wdfc2, Aqp1, Slc16a8, Tmprss11a, Steap1,* and *Sult1c2*. The top 10 most downregulated genes included immediate early genes, such as *Fos, Egr2, Fosb, Npas4, Egr4, Arc* and neurotransmitter associated *Gabra6*, and extracellular matrix-related *Cyr61* (Figure 5B,C). Additional upregulated targets of interest included those with metabolic functions (e.g. *Aldh1a2, Steap4, Sult1c2, Slco1a5*), immune function (e.g. *H2-Aa),* and transmembrane proteins *Tmem27* and *Tmem184*. Additional downregulated genes included immune and plasticity-related *Ccl3* and *Dusp5*,

extracellular matrix related *Col6a3*, and G-protein signaling associated *Gem*. Genes with the lowest log2 and p-value included *Col4a4* and *Cccdc37*. In females, the top 10 upregulated genes included targets related to extracellular matrix, tight/gap junction, hormone, and metabolic functions, such as *Saa3, Lcn2, Tmem72, Tmprss11a, Kcne2, Slc4a5, Cldn2, Aqp1, Folr1*, and *Steap1*. The 10 most downregulated genes included mainly metabolic, nuclear, and immune functions, such as *Cpn11, Gkn3, Nxf7, Cd4, Aldh1a3, Opalin, Alox12, Slc47a1, Fermt1*, and *Cited4* (Figure 5B,D). Additional upregulated targets included those with metabolic functions (e.g. *Sult1c1, Sult1c2, Cndp1*), hormone functions (e.g. *Mc3r*), and neuroplasticity functions (e.g. *Calml1, Sylt1*), and downregulated genes included immune-associated *H2-Q1* and *Ier5l*, extracellular matrix associated *Enpp2*, and transcription factor *Crabp2*. Genes with the lowest log2 and p-value included *Ccdc135* and *Krt12*.

Biological pathway and gene enrichment analysis in males revealed gene categories for vascular development (log10(P) = -12.6), MAPK cascade (log10(P) = -11.02), anion transport (log10(P) = -10.63), response to growth factor (log10(P) = -10.43), and negative regulation of MAPK cascade (log10(P) = -7.65) (Figure 6A). In females, most of the DEGs were related to cell adhesion and immune function. These included targets involved in extracellular matrix organization (log10(P) = -11.65), cell chemotaxis (log10(P) = -9.34), cytokine production (log10(P) = -8.86), regulation of cell adhesion (log10(P) = -8.57), copper ion transport (log10(P) = -8.08), positive regulation of MAPK cascade (log10(P) = -7.31), and acute inflammatory response (log10(P) = -5.78) (Figure 6B). Protein-protein interactions from STRING analysis in males revealed associations between mostly plasticity-related genes including a large cluster related to neuroplasticity with activity-dependent transcription factors and kinases, and solute carriers. There were also changes in targets related to neurotransmission, containing

monoaminergic, neuropeptide, and hormone targets; in collagens; and in growth factors including insulin-like growth factor and bone-morphogenetic signaling. There were also immune-associated clusters, including genes related to MHC II signaling, and regulation of immune signaling (Figure 6C; Table 5). In females, we observed clusters of interferon-mediated signaling, negative regulation of cytokine signaling, complement signaling, and immune cell activation. Plasticity-related targets included clusters of cell adhesion molecules and neurotransmission (Figure 6D; Table 6).

3.5 Differential gene expression in male versus female hippocampus

We also examined sex differences in baseline gene expression in the hippocampus. We observed 220 genes differentially expressed in the hippocampus of males compared and females, 95 of which are more strongly expressed in males and 125 in females. As expected, Y-chromosome genes including *Ddx3y* were abundant in males but not evident in females, whereas genes that escape from x-inactivation, including *Xist,* were more abundant in females. The top 10 highest genes in males included Y-linked *Ddx3y*, and *Ei2s3y*, and transcription factors *Fos, Fosb, Npas4, Btg2, Egr4,* and others such as *Tmem72, Ttr, Slc4a5* (Figure 7A,C) while the top 10 highest genes in females included *Sv2c, Coch, Bmp6, H2-D1, Kdm6a, Nid1, Serpinf1, Tmem90a, Ranbp3l*, and *Plxdc1* which have various functions, including metabolic, immune, and transcriptional roles (Figure 7B,C). Other genes of interest higher in males included extracellular matrix associated *Krt18* and *Serpine1*, neurotransmitter-associated targets (e.g. *Chrm5*), transcription factors (e.g. *Maff*), and those with metabolic functions (e.g. *Steap1*), while other genes higher in females included MHC class II targets (e.g. *H2-Aa, H2-Q6, Cd4*), interferon signaling-related *ifi47*, and solute carriers (e.g. Slc26a7). Genes with the lowest log2 fold change

and p-value that were higher in males at baseline included *Adams14* and higher in females at baseline included *Isg15* (Figure 7C).

Biological processes for male-biased gene expression included response to hormone (log10(P) = -9.1), response to growth factor (log10(P) = -8.1), negative regulation of catalytic activity (log10(P) = -7.5), cellular response to calcium ion (log10(P) = -6.9), and negative regulation of nuclear transcribed mRNA poly(A) tail shortening (log10(P) = -5.1) (Figure 8A). Female-biased gene expression included biological processes such as extracellular matrix organization (log10(P) = -10.1), neurotransmitter transport (log10(P) = -9.3), response to interferon gamma (log10(P) = -7.5), cell adhesion (log10(P) = -7.5), drug transport (log10(P) = -5.2), regulation of defense response (log10(P) = -4.5), complement and coagulation cascades (log10(P) = -4.1), and cellular metabolic process (log10(P) = -3.9) (Figure 8B).

Protein-protein interaction analysis (STRING) revealed that targets more strongly expressed in the hippocampus of males are involved in G protein and calcium signaling, protein phosphorylation, as well as immediate early genes and DNA-methylation modifiers (Figure 8C; Table 7). Targets that more strongly expressed in the hippocampus of females included extracellular matrix receptor genes, growth factor, neurotransmitter receptors, neurotransmitter transporter, protein phosphorylation, immune signaling, and complement-associated genes (Figure 8D; Table 7).

To examine the contribution of initial sex differences in gene expression on the sex-specific changes after subchronic immune challenge, we examine the impact of prior immune challenge on genes more strongly expressed in males at baseline (“male-biased” genes) and those more strongly expressed in females (“female-biased” genes). Overall, in males, male-biased genes tended to be downregulated and female-biased genes upregulated long after immune

challenge. Here, of the 46 genes higher in males at baseline, 34 were downregulated and only 12 upregulated; and of the 42 genes higher in females at baseline 41 were upregulated in males and only one downregulated long after subchronic immune challenge. Conversely, female-biased genes tended to be downregulated (13 of 13) and male-biased genes were more likely to be upregulated (4 of 4) in females long after subchronic immune challenge.

Specifically, the male-biased genes downregulated in males long after immune challenge represented biological pathways including long-term memory (*Arc, Egr1, Npas4*), p38MAPK cascade (*Gadd45b/g, Per2*), and positive regulation of cell death (e.g. *Atf3, Dusp1, Ptgs2*). Female-biased genes upregulated in males included those belonging to biological pathways transport of bile salts and organic acids, metal ions, and amine compounds (e.g. *Slc6a12, Slc22a6, Sphk1*), collagen chain trimerization (e.g. *Bmp7, Col9a2*), and prostaglandin biosynthesis (e.g. *Cd74, Ptgds*) (Table 8).

In the Long-term+Acute group, female-biased genes were more likely to be upregulated in males and downregulated in females. Interestingly, whereas female-biased genes were strongly differentially expressed in both sexes, male-biased genes were not differentially expressed in either sex. In males, 6 of 6 female-biased genes were upregulated. In females, 53 of 75 were downregulated, including MHC class II (e.g. *H2-Aa, Cd74, Spp1*), cellular hormone metabolism (e.g. *Bmp6, Ttr*), and extracellular matrix organization (e.g. *Cdh1, Col8a1, Enpp2*) (Table 9).

3.6 Shared targets and pathways amongst experimental conditions:

We conducted meta-analyses for both males and females to examine similarities and differences between persistent, acute, and long-lasting transcriptional changes in the

hippocampus. In males, 103/230 the DEGs (purple lines) in the Long-term condition were also differentially expressed in response to an acute LPS injection, demonstrating a persistent change in immune processes 3 months after subchronic immune challenge. In contrast, only one DEG was common to both Long-term condition and animals in the Long-term+Acute, demonstrating that the DEGs that are altered at baseline, and those that are differentially regulated in response to another immune challenge reflect different pathways or processes. In addition, 6/230 genes were dysregulated in both the Long-term+Acute and Acute conditions, demonstrating that the acute neuroimmune response in hippocampus is largely unchanged by prior subchronic immune challenge (Figure 9A).

In females, only 1/26 DEGs in the Long-term condition were also dysregulated after acute immune challenge. One gene is also commonly dysregulated between Long-Term and Long-term+Acute conditions suggesting that baseline changes in gene expression likely contribute to dysregulation of subsequent neuroimmune response. Consistent with this idea, more than 188 DEGs in the Long-term+Acute condition were shared with those in the Acute conditions, demonstrating an exaggerated – or dysregulated – acute immune response long after a previous subchronic immune challenge (Figure 9C).

This analysis also compares biological pathways and processes between groups (blue lines). In males, 15 of the top 20 biological pathways are shared between the Long-term and Acute conditions. Most of these shared pathways show similar degrees of enrichment in both conditions. The exception here is extracellular organization processes, which is more highly enriched in the Long-term vs Acute conditions, indicating more changes in extracellular organization processes in long-term conditions. Between Long-term and Long-term+Acute conditions, only 3 biological pathways are shared, again demonstrating that baseline changes in

gene expression long after immune challenge regulate processes other than response to acute challenge. Similarly, only 3 biological processes substantially differ between Long-term+Acute and Acute groups, demonstrating the overwhelming similarity of the acute response, with or without prior subchronic immune challenge. (Figure 9B).

In females, 6 biological pathways are shared between Long-term and Acute conditions. Interestingly, even in overlapping pathways, the Acute condition is much more enriched for immune-related processes including cytokine production, whereas Long-term condition results in enriched categories related to catecholaminergic signaling. Unlike males, females showed a strong overlap between Long-Term and Long-term+Acute conditions, with 9 overlapping pathways; and 15 shared pathways in Long-term+Acute and Acute conditions, demonstrating the strongly increased transcriptional response to an acute challenge after prior immune experience. (Figure 9D).

Comparing these males and females in these analyses demonstrates the diverging impact of prior immune challenge on different components of immune function. Whereas males show persistent alterations in baseline expression of immune-related genes also activated during acute immune challenge, females show a fewer DEGs and less overlap with acute immune regulation. In contrast, males show little impact of a prior immune challenge on the acute transcriptional response in the hippocampus, whereas females that have experienced a prior immune challenge show a grossly exaggerated response to an acute inflammation.

Discussion

Here we demonstrated long-lasting consequences of subchronic immune challenge on gene expression in the hippocampus of males and females. In males, we observed enduring

dysregulation of gene expression three months after the end of a two-week immune challenge. In contrast, females showed few persistent changes at baseline, but striking changes in gene expression in response to an additional acute LPS injection three months after the subchronic immune challenge. These findings suggest that sex-specific changes in transcriptional regulation may mediate sex- and gender-differences in vulnerability to cognitive decline (Lobo *et al,* 2018) and affective dysregulation (Bjerkeset *et al*, 2005) long after illness or injury. For example, in patients of heart attack, women were at increased risk of anxiety or depression (Bjerkeset *et al*, 2005). Animal models have also shown persistent emotion and memory, and recent findings from our laboratory demonstrate sex differences in vulnerability to memory impairments long after subchronic immune challenge (Tchessalova and Tronson, 2019). The findings described in this paper therefore set the foundation for future studies of how the specific genes, pathways, and processes dysregulated long after subchronic immune challenge contribute to cognitive and affective processes and to vulnerability to further insult in males and females.

Males and females differed in both the magnitude of gene expression changes, and in the specific genes and pathways differentially expressed in the hippocampus long after immune challenge. In males but not females, we observed clear changes in both immune-related pathways and in genes and clusters related to neural plasticity. Immune-related pathways and genes, including MAPK signaling (e.g., *Spp1*, *Dusp1/6*), response to interferon-gamma (e.g., *Ifitm3, Ifit1, Oasl2*), complement signaling (e.g., *C2, Cfh, Col8a1, Col8a2*), and MHC II signaling (e.g., *H2-Eb1, Cd74*) were significantly dysregulated in males. We also observed changes in expression of several pathways that are important for neuroplasticity. This includes extracellular matrix organization (e.g., *Col4a1, Pcolce, Sulf1*) and cell adhesion molecules (e.g., *Cdh1/3*) that are important for synaptic organization, as well as immediate early genes (e.g., *Fos*,

Arc, Egr1, Nr4a1, Nr4a2, Junb, Atf3) that are involved in activity-dependent transcriptional changes necessary for synaptic plasticity (Hawk and Abel, 2011; Minatohara *et al*, 2016). Several genes (*Fos, NR4a1/2, Egr, Spp1, Dusp1/6*) and pathways (MAPK signaling) are notably required for both immune- and neuroplasticity-related functions (Borja-Cacho and Matthews, 2008; Donzis and Tronson, 2014; Nisticò *et al*, 2017; Stephen *et al*, 2017). Alterations in these and other immune-associated targets may therefore also contribute to long-lasting changes in neural function.

In females, the dominant changes were pathways and genes related to monoaminergic signaling and its regulation, including adenosine, dopamine, and adrenergic receptor (e.g., *Adora2a, Drd2, Adr1b*), and its downstream signaling (e.g., *Ppp1r1b*). Given the importance of dopaminergic and adrenergic signaling for memory modulation, motivational processes, and affective responses (Badgaiyan *et al*, 2010; Stone *et al*, 1999; Strange and Dolan, 2004; Wassum *et al*, 2011). These findings demonstrate striking sex differences in the persistent changes in basal gene expression after a subchronic immune challenge. Importantly, this suggests that differential vulnerability to cognitive decline, memory impairments, and affective disorders (Himanen *et al*, 2006; Hogue *et al*, 2003; Lavoie *et al*, 2017; Léveillé *et al*, 2019; Liossi *et al*, 2009; Niemeier *et al*, 2007; Rainville and Hodes, 2018; Suarez *et al*, 2015) after illness or injury may be mediated by sex differences in the changes in gene expression and transcriptional regulation that persist long after immune challenge.

Together with the gene expression data, the sex-specific transcription factors identified through TRANSFAC studies suggest that subchronic immune challenge differentially impacts hippocampal transcription in males and females months after the inflammatory insult. For example, CREB was identified as a top transcription factor for the male DEGs while STAT5A/B

was identified as was the top transcription factor for the female DEGs. Given the role of cAMP signaling and CREB regulation of plasticity-related transcription factors and genes (Hawk and Abel, 2011; Vecsey *et al*, 2007) and the striking observations that several plasticity related genes, including activity-dependent transcription factors, were downregulated in the male hippocampus 12 weeks after LPS, it is possible that systemic immune challenge induces persistent changes in molecular substrates important for hippocampal function through dysregulation of plasticity related targets. The decreased protein levels of CREB-related nuclear receptor and transcription factor NR4a1 months after subchronic immune challenge suggest an importance of long-lasting regulation of CREB related targets at the mRNA and protein level.

In females, the few changes in DEGs that include immune-related targets such as *ifit1* and *Spp1*, may be due to transcriptional activity of the immune-related transcription factors STAT5A/B. As many monoaminergic genes are dysregulated in females, it is possible that changes in expression of dopaminergic and adrenergic receptors are also due to transcriptional activity of STAT5. While CREB activation has been well documented to enhance memory in males (Vecsey *et al*, 2007), the role of STAT5 in memory modulation is still largely unknown. One study has shown that decreased levels of STAT5 expression have been associated with deficits in memory formation in several hippocampal-dependent memory tests, including novel object recognition and context fear conditioning (Furigo *et al*, 2018). These findings suggest a potential role of STAT5 in long-lasting regulation of hippocampal gene expression after subchronic immune challenge, which may have functional implications for changes in memory observed in females months after LPS.

Differences in persistent changes in gene expression are likely mediated, in part, by sex differences in the initial cascade of events during the acute immune response. We observed that

females showed more changes in hippocampal gene expression after an acute LPS injection compared with males. These findings are consistent with previous findings of sex differences in the neuroimmune response, with differential activation of glial cells (Acaz-Fonseca *et al*, 2015; Santos-Galindo *et al*, 2011), and differences in pattern and timing of cytokine production in the hippocampus (Acaz-Fonseca *et al*, 2015; Santos-Galindo *et al*, 2011; Speirs and Tronson, 2018; Tonelli *et al*, 2008). Surprisingly, stronger regulation of gene expression during acute immune challenge in females did not correspond to more enduring dysregulation of baseline gene expression in females. Nevertheless, three months after subchronic immune challenge, females showed strikingly different transcriptional responses to an acute challenge compared with previously naïve mice. In contrast, males showed similar responsiveness to an acute challenge regardless of prior immune experience. Together, these findings demonstrate that a subchronic immune challenge results in a persistent shift in hippocampal gene expression in males, with few changes in response to a subsequent immune challenge. In contrast, females show little change in gene expression under baseline conditions months after a subchronic immune challenge, but an exaggerated transcriptional response to a subsequent immune challenge.

There were also changes in gene expression that showed notable similarities between the sexes across several conditions. One example is extracellular matrix-related genes, including collagens (e.g., *Col4a1, Col8a1*), matrix regulatory enzymes (e.g., *Pcolce, Sulf1, Adamts1*), and others such as *Otx2*, *Spp1*, and *Cyr61*. Extracellular matrix, and the more specialized perineuronal nets, are of particular interest for their regulation by immune stimuli and their permissive or limiting roles in neuronal plasticity (Beurdeley *et al*, 2013; Carulli *et al*, 2010). As such, extracellular matrix proteins and perineuronal nets contribute to enduring changes in plasticity associated with memory and affective behaviors (Riga *et al*, 2017; Thompson *et al*,

2018). Several of the extracellular matrix genes were differentially regulated months after subchronic immune challenge in males (e.g., *Otx2, Cyr61*) and in females both after a secondary insult and after an acute challenge (e.g. *Otx2, Col9a3*). Such similarities across conditions and sex suggests that extracellular matrix organization may be fundamental to mediating persistent effects of immune challenge and other environmental stimuli, including stress, (Li *et al*, 2013) on neuronal plasticity and function.

Our findings on enduring, sex-specific patterns of gene expression are consistent with previous demonstrations that males and females show strikingly different patterns of gene expression in the brain after stress or drugs of abuse (Finn *et al*, 2018; Hodes *et al*, 2015; Randesi *et al*, 2018) further demonstrate that the consequences of environmental insults including immune challenge have sex-specific implications for enduring changes in gene expression, neural function, and vulnerability of resilience to subsequent stressors. The changes in gene expression that persist long after an environmental event mediate adaptive responses to later experiences. Here we observed sex-specific transcriptional changes to a subsequent immune challenge. Males showed similar patterns of gene expression after an acute LPS injection regardless of whether or not they had previously experienced a subchronic challenge. In contrast, females previously exposed to a subchronic immune challenge showed markedly different patterns of gene expression after an acute injection compared with previously naïve mice. Together with the sex-specific changes in baseline gene expression, where males but not females showed changes 12 weeks later, these findings suggest that males and females have different patterns of vulnerability and resilience to future stressors, including immune challenge, and other environmental events (Tchessalova *et al*, 2018).

Sex-specific patterns of differential gene expression are consistent with the differential

patterns of memory deficits observed in males and females after immune challenge or illness. We have recently demonstrated that males but not females show deficits of fear memory, but that both sexes exhibit impairments of object recognition memory several months after subchronic immune challenge (Tchessalova and Tronson, 2019). Sex-specific patterns of memory deficits are also observed in patients, where women are more vulnerable to disruption of visuospatial tasks months after surgery or injury (Hogue *et al*, 2003; Liossi *et al*, 2009) whereas men show more progressive memory decline over the following years (Himanen *et al*, 2006). Determining the long-lasting changes in gene expression in males and in females is therefore important for identifying sex differences in the contribution of environmental insults to the vulnerability or resilience to cognitive decline, memory impairments, and affective disorders.

How baseline sex differences in hormonal levels (Koss and Frick, 2017; McEwen and Milner, 2017), memory processes (Keiser *et al*, 2017; Keiser and Tronson, 2015), emotion (Pitychoutis and Papadopoulou-Daifoti, 2010), and gene expression (Vied *et al*, 2016) mediate differential vulnerability to immune-triggered memory decline remains unknown. Here, we examined the relationship between sex-biased gene expression at baseline and differential regulation of those genes after immune challenge in males and females. We observed that most of the genes that were higher in the hippocampus of males at baseline were downregulated in the male hippocampus and upregulated in the female hippocampus long after immune challenge. Similarly, we found that female-biased genes were downregulated in females and upregulated in males after immune challenge. This pattern suggests that baseline differences in hippocampal gene expression contribute to their regulation after immune challenge. The pattern of regulation may be important for predicting which genes and pathways may be differentially vulnerable between the sexes to changes as a consequence of illness or stress.

There are a few limitations of these gene expression studies. First, as the animals were not perfused prior to hippocampal dissections, it is possible that contamination of peripheral immune cells from the blood may have contributed to the changes in hippocampal gene expression. Second, we did not assess cell-type specific patterns of gene expression in males and females after immune challenge. Future work using cell-type specific RNA-sequencing methods will inform contribution of peripheral immune cells, neurons, astrocytes, and microglia to gene expression in the brain after systemic immune challenge. Single-cell RNA sequencing experiments will provide insights into the functions of the targets of interest based on cell type and will help to understand the role of these targets in dysregulation of memory and cognitive processes by immune challenge. Third, gene expression was not assessed in peripheral immune organs, and future studies could determine whether enduring hippocampal gene expression are associated with long-lasting changes in function of peripheral immune organs. Fourth, we assessed gene expression changes only in the hippocampus and it is intriguing whether/how subchronic immune challenge alters transcription in multiple brain regions important for memory and affective processes. Determining long-lasting gene expression changes in these regions will provide critical insights into how mild systemic immune activation alters brain-wide transcriptional networks relevant for sex-specific disorders of memory and cognition.

The findings described in this chapter provide critical new insight into the long-lasting impact of immune-related signaling on gene expression in the brain. Understanding the acute and long-lasting contributions of neuroimmune signaling to neuromodulation and plasticity is particularly important given the growing recognition that many environmental events, including stress (Frank *et al*, 2017; McKim *et al*, 2016; Serrats *et al*, 2017; Weber *et al*, 2015; Wohleb *et al*, 2015) and drugs of abuse (Crews *et al*, 2015; Hofford *et al*, 2018) both recruit neuroimmune

signaling pathways. Here we demonstrated sex-specific patterns of gene expression months after a subchronic immune challenge, where males showed a persistent shift in baseline gene expression and females showed a markedly different response to subsequent stimulation. Enduring changes in genes and pathways that mediate plasticity-related processes, in addition to immune-related genes, in the hippocampus suggests that transient inflammatory signaling in the brain has important implications for neural function and hippocampal-dependent processes. To determine the functional impact of the changes in hippocampal gene expression after subchronic immune challenge or after multiple inflammatory insults on hippocampal-dependent memory processes, such as memory formation, future work could assess activity-dependent transcriptional changes in the hippocampus and other memory-relevant brain regions after training using an approach that examines activity-dependent gene transcription, such as Bru-Sequencing. These studies and future work will identify the sex-specific changes in transcriptional regulation important for predicting vulnerabilities to memory decline and provide essential tools for identifying new, sex-specific biomarkers and therapeutic targets.

Tables

Table 1. Clusters of functional protein-protein interactions (PPI) between targets in males 3 months after subchronic immune challenge (from Figure 4.2C).

PPI Cluster	Gene ID	Gene name	Log2 Fold Change	FDR
Neuroplasticity				
	Fos	FBJ osteosarcoma oncogene	-4.76	0.0038
	Fosl2	fos-like antigen 2	-1.14	0.0038
	Junb	jun B proto-oncogene	-2.28	0.0038
	Nr4a1	nuclear receptor subfamily 4, group A, member 1	-1.96	0.0038
	Nr4a2	nuclear receptor subfamily 4, group A, member 2	-1.38	0.0038
	Atf3	activating transcription factor 3	-1.8	0.0038
	Egr1	early growth response 1	-1.91	0.0038
	Egr2	early growth response 2	-4.92	0.0038
	Egr3	early growth response 3	-1.51	0.0038
	Egr4	early growth response 4	-2.6	0.0038
	Gadd45b	growth arrest and DNA-damage-inducible 45 beta	-0.75	0.0038
	Gadd45g	growth arrest and DNA-damage-inducible 45 gamma	-1.64	0.0038
	Spp1	secreted phosphoprotein 1	1.16	0.0038
	Dusp1	dual specificity phosphatase 1	-1.61	0.0038
	Dusp6	dual specificity phosphatase 6	-0.78	0.0038
	Cdh1	cadherin 1	1.44	0.0038
	Cdh3	cadherin 3	1.67	0.0151
Extracellular matrix organization				
	Col3a1	collagen, type III, alpha 1	1.04	0.0038
	Col4a3	collagen, type IV, alpha 3	1.53	0.0038
	Col4a4	collagen, type IV, alpha 4	1.24	0.0038
	Col4a6	collagen, type IV, alpha 6	0.9	0.0327
	Col8a2	collagen, type VIII, alpha 2	1.56	0.0038
	Col9a3	collagen, type IX, alpha 3	1.23	0.0038
	Col1a1	collagen, type I, alpha 1	0.98	0.0038
	Col1a2	collagen, type I, alpha 2	0.87	0.0038
	Col17a1	collagen, type XVII, alpha 1	1.77	0.0038
	Krt8	keratin 8	1.56	0.0038
	Krt18	keratin 18	1.69	0.0038
	Fbln1	fibulin 1	0.69	0.0038
	Pcolce	procollagen C-endopeptidase enhancer protein	1.39	0.0038
	Pcolce2	procollagen C-endopeptidase enhancer 2	0.68	0.0099

	Adamst1	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1	-0.9	0.0038
Interferon-mediated signaling				
	Ifit1	interferon-induced protein with tetratricopeptide repeats 1	0.84	0.0286
	Ifitm3	interferon induced transmembrane protein 3	0.70	0.0038
	Islr	immunoglobulin superfamily containing leucine-rich repeat	0.97	0.0038
	Oasl2	2'-5' oligoadenylate synthetase-like 2	0.80	0.0307
	Bub1b	BUB1B, mitotic checkpoint serine/threonine kinase	-0.91	0.0038
	Cenpa1	centromere protein A	-0.95	0.0175
	Tuba1c	tubulin, alpha 1C	1.33	0.0175
Growth factor signaling				
	Igf2	insulin-like growth factor 2	1.55	0.0038
	Igfbp2	insulin-like growth factor binding protein 2	1.33	0.0038
	Tgfbi	transforming growth factor, beta induced	1.10	0.0038
	Bmp6	bone morphogenetic protein 6	1.04	0.0038
	Bmp7	bone morphogenetic protein 7	0.97	0.0038
MHC Class II signaling				
	H2-Aa	histocompatibility 2, class II antigen A, alpha	1.11	0.0038
	H2-eb1	histocompatibility 2, class II antigen E beta	1.1	0.0125
	H2-q1	histocompatibility 2, Q region locus 1	1.22	0.0366
	Cd74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	1.08	0.0038
	Fap	fibroblast activation protein	1.14	0.007
Complement signaling				
	C2	complement component 2 (within H-2S)	0.98	0.0099
	Cd59a	CD59a antigen	1.09	0.0038
	Cfh	complement component factor h	0.6	0.0038
	Serping1	serine (or cysteine) peptidase inhibitor, clade G, member 1	0.92	0.0038
Solute carriers				
	Slc13a3	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3	0.78	0.0038
	Slc22a2	solute carrier family 22 (organic cation transporter), member 2	1.45	0.0347
	Slc22a6	solute carrier family 22 (organic anion transporter), member 6	1.03	0.0038
	Slc47a1	solute carrier family 47, member 1	1.19	0.0099
Tight-junctions				
	Cldn1	claudin 1	1.06	0.0038
	Cldn2	claudin 2	2.18	0.0038
	Cldn9	claudin 9	2.09	0.0403

Table 2. Clusters of functional protein-protein interactions (PPI) between targets in females 3 months after subchronic immune challenge (from Figure 4.2D).

PPI Cluster	Gene ID	Gene name	Log2 Fold Change	FDR
Monoaminergic signaling				
	Ppp1r1b	protein phosphatase 1, regulatory (inhibitor) subunit 1B	-0.69	0.0038
	Adora2a	adenosine A2a receptor	-1.57	0.0038
	Drd2	dopamine receptor D2	-1.10	0.0125
	Adra1b	adrenergic receptor, alpha 1b	-0.76	0.0125
	Gpr88	G-protein coupled receptor 88	-0.88	0.0038
Immune signaling				
	ifit1	interferon-induced protein with tetratricopeptide repeats 1	-0.85	0.0175
	Irgm2	immunity-related GTPase family M member 2	-0.64	0.0476
	Gbp4	guanylate binding protein 4	-1.02	0.0038

Table 3. Clusters of protein-protein interactions (PPI) differentially regulated after an acute LPS challenge in males previously exposed to a subchronic immune challenge (from Figure 4.4C).

PPI Cluster	Gene ID	Gene name	Log2 Fold Change	FDR
Neurotransmission				
	Adora2a	adenosine A2a receptor	0.85	0.0286
	Drd1a	dopamine receptor D1	0.69	0.0099
	Drd2	dopamine receptor D2	0.94	0.0440
	Penk	preproenkephalin	0.96	0.0038
	Sstr5	somatostatin receptor 5	1.80	0.0265
	Tac1	tachykinin 1	1.05	0.0038
	Tac1r	tachykinin receptor 1	0.79	0.0385
Transcription factor				
	Isl1	ISL1 transcription factor, LIM/homeodomain	3.63	0.0385
	Eomes	eomesodermin	2.24	0.0038
	Barhl2	BarH-like 2 (Drosophila)	2.16	0.0221
	Six3	sine oculis-related homeobox 3	0.99	0.0385
	Foxp2	forkhead box P2	0.81	0.0151
Cholinergic signaling				
	Chrna3	cholinergic receptor, nicotinic, alpha polypeptide 3	1.84	0.0038
	Chrmb3	cholinergic receptor, nicotinic, beta polypeptide 3	1.78	0.0038
	Crhmb4	cholinergic receptor, nicotinic, beta polypeptide 4	1.06	0.0307
Calcium activated chloride channels				
	Ano1	anoctamin 1, calcium activated chloride channel	0.93	0.0038
	Ano2	anoctamin 2	0.74	0.0151
Neural differentiation				
	Dlk1	delta-like 1 homolog (Drosophila)	0.65	0.0038
	Peg10	paternally expressed 10	0.59	0.0038

Table 4. Clusters of protein-protein interactions (PPI) differentially regulated after an acute LPS challenge in females previously exposed to a subchronic immune challenge (from Figure 4.4D).

PPI Cluster	Gene ID	Gene name	Log2 Fold Change	FDR
Extracellular matrix proteins				
	Col4a3	collagen, type IV, alpha 3	-1.89	0.0038
	Col4a4	collagen, type IV, alpha 4	-2.17	0.0038
	Col8a1	collagen, type VIII, alpha 1	-2.02	0.0038
	Col8a2	collagen, type VIII, alpha 2	-1.71	0.0038
	Col9a3	collagen, type IX, alpha 3	-1.28	0.0038
	Col18a1	collagen, type XVIII, alpha 1	-0.76	0.0038
	Col23a1	collagen, type XXIII, alpha 1	-0.67	0.0038
	Col24a1	collagen, type XXIV, alpha 1	1.34	0.0038
	Col17a1	collagen, type XVII, alpha 1	-1.46	0.0038
	Fbnl1	fibulin 1	-0.77	0.0038
	Krt8	keratin 8	-1.48	0.0038
	Lgals1	lectin, galactose binding, soluble 1	-0.67	0.0070
	Pcolce	procollagen C-endopeptidase enhancer protein	-1.16	0.0038
Monoaminergic signaling				
	Adora2a	adenosine A2a receptor	2.95	0.0038
	Adra1b	adrenergic receptor, alpha 1b	1.23	0.0038
	Drd1a	dopamine receptor D1	1.38	0.0038
	Drd2	dopamine receptor D2	2.54	0.0038
	Gpr6	G protein-coupled receptor 6	2.33	0.0038
	Gpr88	G-protein coupled receptor 88	2.32	0.0038
	Oxtr	oxytocin receptor	-0.71	0.0038
	Trhr2	thyrotropin releasing hormone receptor 2	1.79	0.0070
Immune signaling				
	Ifg2	insulin-like growth factor 2	-1.69	0.0038
	Igfbp2	insulin-like growth factor binding protein 2	-1.47	0.0038
	Igfbp3	insulin-like growth factor binding protein 3	-0.75	0.0038
	Igfbp6	insulin-like growth factor binding protein 6	0.63	0.0070
	Spp1	secreted phosphoprotein 1	-1.03	0.0038
	Tgfbi	transforming growth factor, beta induced	-0.69	0.0125
MHC Class II signaling				
	Cd4	CD4 antigen	3.59	0.0038
	Cd74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	-1.15	0.0038

	H2-Aa	histocompatibility 2, class II antigen A, alpha	-1.28	0.0038
	H2-Ab1	histocompatibility 2, class II antigen A, beta 1	-1.22	0.0038
	H2-Eb1	histocompatibility 2, class II antigen E beta	-1.05	0.0099
	H2-Q1	histocompatibility 2, Q region locus 1	-1.45	0.0458
Cell adhesion molecules				
	Icam	intercellular adhesion molecule 1	-1.10	0.0038
	Vcam1	vascular cell adhesion molecule 1	-0.71	0.0038
	Cdh1	cadherin 1	-1.28	0.0038
	Cdh3	cadherin 3	-1.83	0.0038
Interferon-mediated signaling				
	Ifi44	interferon-induced protein 44	-1.20	0.0038
	Ifitm1	interferon induced transmembrane protein 1	-1.36	0.0038
	Ifitm3	interferon induced transmembrane protein 3	-0.81	
	Irf7	interferon regulatory factor 7	-0.98	0.0038
	Oasl2	2'-5' oligoadenylate synthetase-like 2	-0.68	0.0265
Neuronal inhibition				
	Best3	bestrophin 3	-1.13	0.0243
	Clic6	chloride intracellular channel 6	-2.09	0.0038
	Gabrd	gamma-aminobutyric acid (GABA) A receptor, subunit delta	0.83	0.0038
	Slc6a13	solute carrier family 6 (neurotransmitter transporter, GABA), member 13	-0.63	0.0125
Tight junction				
	Cldn1	claudin 1	-1.66	0.0038
	Cldn2	claudin 2	-2.94	0.0038
Gap junction				
	Gjb1	gap junction protein, beta 1	0.87	0.0038
	Gjb2	gap junction protein, beta 2	-0.67	0.0038
	Gjc2	gap junction protein, gamma 2	0.78	0.0038
Stress hormone				
	Crhr2	corticotropin releasing hormone receptor 2	-1.28	0.0038
	Pomc2	pro-opiomelanocortin-alpha	2.14	0.0038

Table 5. Clusters of functional protein-protein interactions (PPI) in males after an acute LPS injection (from Figure 4.6C).

PPI Cluster	Gene ID	Gene name	Log2 Fold Change	FDR
Neuroplasticity				
	Camk2d	calcium/calmodulin-dependent protein kinase II, delta	0.83	0.0038
	Gadd45b	growth arrest and DNA-damage-inducible 45 beta	-0.87	0.0038
	Gadd45g	growth arrest and DNA-damage-inducible 45 gamma	-1.86	0.0038
	Clic6	chloride intracellular channel 6	1.67	0.0038
	Kcne2	potassium voltage-gated channel, Isk-related subfamily, gene 2	1.73	0.0038
	Kcnj2	potassium inwardly-rectifying channel, subfamily J, member 2	-1.69	0.0038
	Slc37a2	solute carrier family 37 (glycerol-3-phosphate transporter), member 2	0.88	0.0038
	Slc17a6	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6	0.84	0.0038
	Tmem27	transmembrane protein 27	1.73	0.0476
Regulation immune signaling				
	Ccl3	chemokine (C-C motif) ligand 3	-2.14	0.0327
	Nfkbiz	nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, zeta	-0.80	0.0038
	Ppp1r15A	protein phosphatase 1, regulatory (inhibitor) subunit 15A	-0.91	0.0038
	Ptgs2	prostaglandin-endoperoxide synthase 2	-1.80	0.0038
	Krt8	keratin 8	1.18	0.0038
	Krt18	keratin 18	1.24	0.0038
Growth factor				
	Igf2	insulin-like growth factor 2	1.08	0.0038
	Igfbp2	insulin-like growth factor binding protein 2	0.96	0.0038
	Igfbp6	insulin-like growth factor binding protein 6	0.62	0.0198
	Bmp6	bone morphogenetic protein 6	0.71	0.0070
	Bmp7	bone morphogenetic protein 7	0.75	0.0038
Extracellular matrix protein				
	Col4a4	collagen, type IV, alpha 4	0.89	0.0494
	Col6a3	collagen, type VI, alpha 3	-0.75	0.0175
	Col8a1	collagen, type VIII, alpha 1	1.59	0.0038
	Col8a2	collagen, type VIII, alpha 2	1.28	0.0038
	Col9a3	collagen, type IX, alpha 3	0.91	0.0038
Immune cell activation				
	Cyr61	cysteine rich protein 61	-2.43	0.0038

	Lbp	lipopolysaccharide binding protein	1.01	0.0038
	Ly6a	lymphocyte antigen 6 complex, locus A	0.80	0.0038
MHC Class II signaling				
	H2-Aa	histocompatibility 2, class II antigen A, alpha	0.97	0.0243
	H2-Ab1	histocompatibility 2, class II antigen A, beta 1	1.15	0.0038
	Cd74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	1.07	0.0038
Complement signaling				
	A2m	alpha-2-macroglobulin	0.77	0.0198
	Cd59a	CD59a antigen	0.97	0.0038
Neurotransmission				
	Gabra6	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 6	-2.53	0.0038
	Htr2c	5-hydroxytryptamine (serotonin) receptor 2C	0.76	0.0038
	Ntsr1	neurotensin receptor 1	0.62	0.0458

Table 6. Clusters of protein-protein interactions (PPI) between targets in females after an acute LPS injection (from Figure 4.6D).

PPI Cluster	Gene ID	Gene name	Log2 Fold Change	FDR
Immune cell activation				
	Lbp	lipopolysaccharide binding protein	2.00	0.0038
	Tlr2	toll-like receptor 2	1.25	0.0038
	Lgals3	lectin, galactose binding, soluble 3	1.00	0.0366
	Lgals3bp	lectin, galactoside-binding, soluble, 3 binding protein	0.89	0.0038
	Lgals9	lectin, galactose binding, soluble 9	0.72	0.0038
	Cd4	CD4 antigen	-1.45	0.0038
	Bcl3	B cell leukemia/lymphoma 3	1.26	0.0347
	Socs3	suppressor of cytokine signaling 3	0.86	0.0151
Interferon-mediated signaling				
	Ifi35	interferon-induced protein 35	0.81	0.0265
	Ifi44	interferon-induced protein 44	1.42	0.0038
	Ifim1	interferon induced transmembrane protein 1	0.87	0.0151
	Ifitm3	interferon induced transmembrane protein 3	1.15	0.0038
	Irf7	interferon regulatory factor 7	1.67	0.0038
	Oas2	2'-5' oligoadenylate synthetase 2	0.96	0.0366
	Oasl2	2'-5' oligoadenylate synthetase-like 2	0.69	0.0175
	Zbp1	Z-DNA binding protein 1	2.61	0.0038
Monoaminergic signaling				
	Htr2c	5-hydroxytryptamine (serotonin) receptor 2C	1.40	0.0038
	Cckbr	cholecystokinin B receptor	-0.67	0.0038
	Trhr	thyrotropin releasing hormone receptor	1.17	0.0038
	Enpp2	ectonucleotide pyrophosphatase/phosphodiesterase 2	2.75	0.0038
	Enpp6	ectonucleotide pyrophosphatase/phosphodiesterase 6	-0.76	0.0151
Complement activation				
	A2m	alpha-2-macroglobulin	2.96	0.0038
	Ccr2	chemokine (C-C motif) receptor 2	1.12	0.0476
	Ccr5	chemokine (C-C motif) receptor 5	0.68	0.007
	Cd59a	CD59a antigen	1.41	0.0038
	Cd55	CD55 molecule, decay accelerating	0.75	0.0198
Cell adhesion molecules				
	Cldn1	claudin 1	0.89	0.0038
	Cldn2	claudin 2	4.55	0.0038
	Col4a4	collagen, type IV, alpha 4	2.17	0.0038
	Col8a1	collagen, type VIII, alpha 1	3.35	0.0038

	Col8a2	collagen, type VIII, alpha 2	2.45	0.0038
Metabolism				
	Steap1	six transmembrane epithelial antigen of the prostate 1	4.01	0.0038
	Steap2	six transmembrane epithelial antigen of prostate 2	1.69	0.0038
	Ucp2	uncoupling protein 2 (mitochondrial, proton carrier)	0.90	0.0038
	Xdh	xanthine dehydrogenase	1.1	0.0038
Negative regulation cytokine signaling/growth factor signaling				
	Igf2	insulin-like growth factor 2	1.06	0.0038
	Igfbp2	insulin-like growth factor binding protein 2	1.14	0.0038
	Slc22a8	solute carrier family 22 (organic anion transporter), member 8	-0.83	0.0038

Table 7. Clusters of protein-protein interactions (PPI) between targets that show differential expression in male and female hippocampi (from Figure 4.8C-D).

PPI Cluster	Gene ID	Gene name	Log2 Fold Change	FDR
MALE BIASED GENE EXPRESSION				
Neuroplasticity				
	Fos	FBJ osteosarcoma oncogene	-5.57	0.0038
	Fosl2	fos-like antigen 2	-1.44	0.0038
	Atf3	activating transcription factor 3	-1.74	0.0038
	Nr4a2	nuclear receptor subfamily 4, group A, member 2	-1.20	0.0038
	Jun	jun proto-oncogene	-0.69	0.0038
	Junb	jun B proto-oncogene	-2.48	0.0038
	Egr1	early growth response 1	-2.12	0.0038
	Egr3	early growth response 3	-1.48	0.0038
	Egr4	early growth response 4	-2.85	0.0038
	Ier2	immediate early response 2	-1.73	0.0038
	Arc	activity regulated cytoskeletal-associated protein	-2.44	0.0038
	Gadd45b	growth arrest and DNA-damage-inducible 45 beta	-0.98	0.0038
	Gadd45g	growth arrest and DNA-damage-inducible 45 gamma	-2.04	0.0038
	Per1	period circadian clock 1	-0.83	0.0038
	Irs2	insulin receptor substrate 2	-0.61	0.0038
	Nfil3	nuclear factor, interleukin 3, regulated	-0.69	0.0070
	Kl	Klotho	-0.91	0.0038
	Cyr61	cysteine rich protein 61	-2.42	0.0038
	Dusp1	dual specificity phosphatase 1	-1.73	0.0038
	Dusp6	dual specificity phosphatase 6	-1.10	0.0038
	Ppp1r15a	protein phosphatase 1, regulatory (inhibitor) subunit 15A	-0.86	0.0038
	Sgk1	serum/glucocorticoid regulated kinase 1	-0.70	0.0038
	Serpine1	serine (or cysteine) peptidase inhibitor, clade E, member 1	-1.23	0.0265
	Tiparp	TCDD-inducible poly(ADP-ribose) polymerase	-1.27	0.0038
Neural inhibition				
	Gabra6	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 6	-2.29	0.0038
	Clic6	chloride intracellular channel 6	-1.08	0.0038
	Kcne2	potassium voltage-gated channel, Isk-related subfamily, gene 2	-2.52	0.0038
	Kcnj2	potassium inwardly-rectifying channel, subfamily J, member 2	-1.35	0.0038
Neurotransmission				
	Chrm5	cholinergic receptor, muscarinic 5	-1.49	0.0175
	Trhr	thyrotropin releasing hormone receptor	-0.81	0.0099
Coagulation				
	F5	coagulation factor V	-1.93	0.0038
	Folr1	folate receptor 1 (adult)	-2.11	0.0038

FEMALE BIASED GENE EXPRESSION				
MHC II signaling				
	H2-Aa	histocompatibility 2, class II antigen A, alpha	1.01	0.0198
	H2-Ab1	histocompatibility 2, class II antigen A, beta 1	1.20	0.0038
	H2-D1	histocompatibility 2, D region locus 1	0.63	0.0038
	H2-K1	histocompatibility 2, K1, K region	0.71	0.0038
	H2-Q1	histocompatibility 2, Q region locus 1	1.50	0.0038
	H2-Q4	histocompatibility 2, Q region locus 4	0.93	0.0038
	H2-Q6	histocompatibility 2, Q region locus 6	2.57	0.0070
	Cd4	CD4 antigen	1.38	0.0175
	Cd74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	1.10	0.0038
	Cyp1b1	cytochrome P450, family 1, subfamily b, polypeptide 1	0.68	0.0327
	Myoc	Myocilin	0.66	0.0038
	Mrc1	mannose receptor, C type 1	0.93	0.0038
	Mrc2	mannose receptor, C type 2	0.75	0.0038
Interferon signaling				
	Ifi44	interferon-induced protein 44	1.30	0.0099
	ifi47	interferon gamma inducible protein 47	1.36	0.0307
	ifit1	interferon-induced protein with tetratricopeptide repeats 1	0.94	0.0099
	Ifitm3	interferon induced transmembrane protein 3	0.73	0.0038
	Isg15	ISG15 ubiquitin-like modifier	0.95	0.0440
	Irgm2	immunity-related GTPase family M member 2	0.76	0.0125
	Gbp2	guanylate binding protein 2	1.10	0.0038
	Gbp3	guanylate binding protein 3	0.88	0.0038
	Gbp4	guanylate binding protein 4	1.47	0.0038
	Oasl2	2'-5' oligoadenylate synthetase-like 2	0.88	0.0038
Monoaminergic signaling				
	Adora2a	adenosine A2a receptor	0.86	0.0099
	Adra1b	adrenergic receptor, alpha 1b	0.72	0.0366
	Cckbr	cholecystokinin B receptor	0.78	0.0038
	Drd1a	dopamine receptor D1	0.87	0.0038
	Drd2	dopamine receptor D2	1.17	0.0038
	Gpr88	G-protein coupled receptor 88	0.97	0.0038
	Ntsr1	neurotensin receptor 1	0.66	0.0286
	Ptgds	prostaglandin D2 synthase (brain)	1.40	0.0038
	Ptgfr	prostaglandin F receptor	1.39	0.0070
Extracellular matrix organization				
	Aebp1	AE binding protein 1	1.01	0.0038
	Col3a1	collagen, type III, alpha 1	1.45	0.0038
	Col4a6	collagen, type IV, alpha 6	0.94	0.0243
	Col1a1	collagen, type I, alpha 1	1.38	0.0038
	Col1a2	collagen, type I, alpha 2	1.07	0.0038
	Efemp1	epidermal growth factor-containing fibulin-like extracellular matrix protein 1	0.93	0.0038
	Fmod	Fibromodulin	1.50	0.0038
	Islr	immunoglobulin superfamily containing leucine-rich repeat	1.05	0.0038

	Ogn	Osteoglycin	0.89	0.0038
Solute carriers				
	Slc13a3	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3	0.99	0.0038
	Slc22a2	solute carrier family 22 (organic cation transporter), member 2	2.06	0.0038
	Slc22a6	solute carrier family 22 (organic anion transporter), member 6	1.33	0.0038
	Slc22a8	solute carrier family 22 (organic anion transporter), member 8	0.69	0.0038
	Slc47a1	solute carrier family 47, member 1	1.62	0.0038
Complement signaling				
	C2	complement component 2 (within H-2S)	1.26	0.0038
	Cfh	complement component factor h	0.72	0.0038
	Spp1	secreted phosphoprotein 1	1.71	0.0038
	Serping1	serine (or cysteine) peptidase inhibitor, clade G, member 1	0.94	0.0038
	Serpinf1	serine (or cysteine) peptidase inhibitor, clade F, member 1	0.62	0.0151
Growth factor				
	Cdh1	cadherin 1	1.71	0.0038
	Bmp6	bone morphogenetic protein 6	0.64	0.0125
	Bmp7	bone morphogenetic protein 7	0.89	0.0038
	Foxc2	forkhead box C2	1.69	0.0038

Table 8. Impact of subchronic immune challenge on expression of genes more strongly expressed in males ("male-biased") or females ("female-biased") at baseline.

MALE-BIASED GENES				
	MALES		**FEMALES**	
	Pathway	**Genes**	**Pathway**	**Genes**
Upregulated	**Inorganic cation transport**		n/a	
		Steap1		
		Slc39a4		
		Kcne2		
Downregulated	**Positive regulation of cell death**			
		Atf3		
		Egr1		
		Fos		
		Nr4a1		
		Ccn1		
		Gadd45b		
		Ptsg2		
		Dusp1		
		Gadd45g		
		Dusp6		
		Per1		
		Pppr1r15a		
		Sik1		
	p38 MAPK cascade			
		Gadd45b		
		Gadd45g		
		Dusp1		
		Per1		
	Long-term memory			
		Arc		
		Egr1		
		Npas4		

FEMALE-BIASED GENES				
	MALES		**FEMALES**	
	Pathway	**Genes**	**Pathway**	**Genes**

Upregulated	**Collagen chain trimerization**		n/a
		Aebp1	
		Aldh1a2	
		Bmp7	
		Col1a1	
		Col1a2	
		Col3a1	
		Col4a6	
		Col9a2	
		Cd74	
		Efemp7	
		Emilin1	
		Ifitm3	
		Islr	
		Mrc2	
		Ptgdr	
		Serping1	
		Slc22a6	
	Immunoglobulin mediated immune response		
		Sned1	
		Bmp7	
		C2	
		Col3a1	
		Emilin1	
		H2-Ab1	
		Ifit1	
	Transport of bile salts and organic acids, metal ions and amine compounds		
		Serping1	
		Slc6a12	
		Slc6a13	
		Slc22a6	
		Slc47a1	
		Slc6a20a	
		Slc13a3	
	Prostaglandin biosynthesis		
		Sphk1	

		Aldh1a2		
		Bmp6		
		Cd74		
		Ptgds		
		Sphk1		
Downregulated	n/a		**Response to amphetamine**	
				Adora2a
				Adra1b
				Cckbr
				Coch
				Drd2
				Gbp4
				Irgm2
				Scn4b
				Spp1
			Response to virus	
				Coch
				Gbp4
				Ifit1

Table 9. Impact of subchronic + Acute immune challenge on expression of genes more strongly expressed in males ("male-biased") or females ("female-biased") at baseline.

MALE-BIASED GENES				
	MALES		**FEMALES**	
	Pathway	**Genes**	**Pathway**	**Genes**
Upregulated	n/a		n/a	
Downregulated	n/a		n/a	
FEMALE-BIASED GENES				
	MALES		**FEMALES**	
	Pathway	**Genes**	**Pathway**	**Genes**
Upregulated	**Regulation synaptic transmission**		**Neurotransmitter transport**	
		Adora2a		*Adora2a*
		Drd1		*Cckbr*
		Drd2		*Cd4*
		Sphk1		*Cobl*
	Learning			*Dpp4*
		Adora2a		*Drd1*
		Drd1		*Drd2*
		Drd2		*Gpr88*
		Foxp2		*Rims3*
				Scn4b
				Slc6a12
				Syt2
				Trhr2
			Regulation synaptic vesicle exocytosis	
				Adora2a
				Drd1
				Drd2
				Rims3
				Syt2
			Leukocyte cell-cell adhesion	
Downregulated	**n/a**			*Adora2a*
				Cd4
				Dpp4
			Antigen processing and	

		presentation of exogenous peptide antigen via MHC class II	
			Bmp7
			Cdh1
			Cd74
			Cldn2
			Col8a1
			Cyp1b1
			Enpp2
			Gbp2
			H2-Aa
			H2-Ab1
			H2-Q1
			Ifitm3
			Krt18
			Mpzl2
			Mrc1
			ASpp1
		Negative regulation cellular proliferation	
			Aldh1a2
			Bmp7
			Cdh1
			Cyp1b1
			Dlk1
			H2-Aa
			H2-Ab1
			Ifitm3
			Npr3
			Ptdgs
		Extracellular matrix organization	
			Bmp7
			Cdh1
			Col8a1
			Cyp1b1
			Fol1r
			Pcolce
			Spp1
			Ttr

		Cellular hormone metabolic process	
			Aldh1a2
			Bmp6
			Crym
			Cyt1b1
			KI
			Mc4r
			Spp1
			Ttr
		Regulation viral life cycle	
			Cd74
			Ifitm3
			Oasl2
		Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor	
			F5
			Itih2
			Spp1

Table 10. Top enriched transcription factors and genes that drove the association from TRANSFAC analysis in males. Dataset from long-term condition.

ENRICHMENT	ADJ. P-VALUE	NES	CATEGORY	DEPENDENT GENES
SRF	0.006	-2.54	Immediate-early genes	*EGR2*
				EGR3
				EGR4
				FOS
				FOSB
				IER2
				JUNB
				NPAS4
ENRICHMENT	**ADJ. P-VALUE**	**NES**	**CATEGORY**	**DEPENDENT GENES**
CREB	0.006	-2.23	Transcription factors	*ATF3*
				CREM
				FOSB
				JUND
				HDX
				MAFF
				NR4A2
				PEG2
				PER1
				ZBTB37
				ZNF184
			Protein phosphorylation	*DUSP1*
				MAP3K13
				MBIP
				PPP1R15A
				PTPRU
			Metabolism	*CBX8*
				DIO2
				KCTD8
				SLC18A2
				SRRM4
				TH
				USP48
			G-protein signaling	*ARL4D*
				GEM

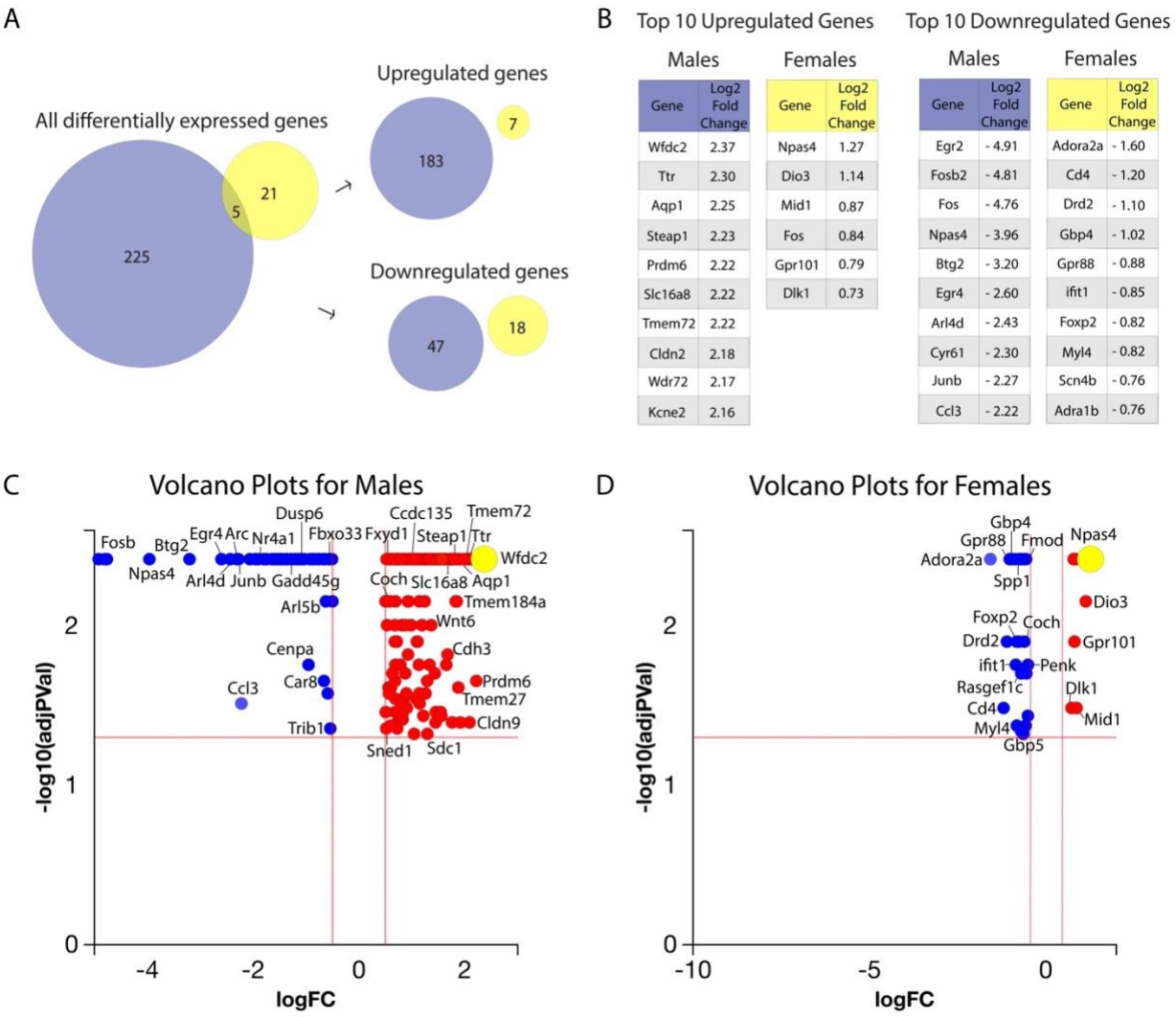
		GPR3
	Growth factor	*TGIFB*
	Cell cycle	*ANAPC10*
		CNTROB
	Extracellular matrix	*HS3ST2*
	Histone	*SUV39H2*

Table 11. Top enriched transcription factors and genes that drove the association from TRANSFAC analysis in females. Dataset from long-term condition.

ENRICHMENT	ADJ. P-VALUE	NES	CATEGORY	DEPENDENT GENES
STAT5B	0.082	-1.65	Immune-signaling	*CCL2*
				CCL5
				KLF4
				IRF9
				NFKBIA
				PDLIM1
				VASN
				SOSC2
			Extracellular matrix/ Cell adhesion	*ADAMSTL1*
				CDH1
				ICAM1
				PCOLCE
				LAMA1
				ENPP2
			Growth factors	*BMP5*
				FST
				PROK1
				WNT10A
			Complement System	*C7*
				C1QTNF1
				SERPIN1G
			GTPase	*EXPH5*
				RRAS
ENRICHMENT	**ADJ. P-VALUE**	**NES**	**CATEGORY**	DEPENDENT GENES
OCT1	0.064	0.441	Transcription	*EGR2*
				SP6
				NFIA
				NR2F2
				NR6A1
				POUF2
			Metabolic	*PFKFB1*
				Ces5a
				TBXAS1
				BCOC2
				NDUFAL2

		SLC24A3
		SLC25A35
	Extracellular matrix	*CD180*
		GNB3
		Col25a1
		Gcp4
		PCDH8
	G-protein	*GNB3*
		PROKR2
		PRKG1
		DUSP6
	Development	*MID1*
		VGLL3
		NRL
		TSPAN13
	Histone	*HIST1H2BC*
		HIST2H2AC
		HIST2H2BC

Figures



Gene	Log2 Fold Change
Wfdc2	2.37
Ttr	2.30
Aqp1	2.25
Steap1	2.23
Prdm6	2.22
Slc16a8	2.22
Tmem72	2.22
Cldn2	2.18
Wdr72	2.17
Kcne2	2.16

Gene	Log2 Fold Change
Npas4	1.27
Dio3	1.14
Mid1	0.87
Fos	0.84
Gpr101	0.79
Dlk1	0.73

Gene	Log2 Fold Change
Egr2	- 4.91
Fosb2	- 4.81
Fos	- 4.76
Npas4	- 3.96
Btg2	- 3.20
Egr4	- 2.60
Arl4d	- 2.43
Cyr61	- 2.30
Junb	- 2.27
Ccl3	- 2.22

Gene	Log2 Fold Change
Adora2a	- 1.60
Cd4	- 1.20
Drd2	- 1.10
Gbp4	- 1.02
Gpr88	- 0.88
ifit1	- 0.85
Foxp2	- 0.82
Myl4	- 0.82
Scn4b	- 0.76
Adra1b	- 0.76

Figure 4.1. Subchronic, peripheral LPS challenge induces changes in hippocampal gene expression 12 weeks after last injection. (A) Males (purple) show a greater number of DEGs than females (yellow). Males showed 183 upregulated and 47 downregulated genes, whereas females showed 7 and 18 up- and down-regulated genes, respectively. (B) Top upregulated and downregulated genes in males and females. (C, D) Volcano plots showing differentially expressed genes in males (C) and females (D) obtained from Advaita iPathway Analysis. Differentially expressed (DE) genes are represented in terms of their measured expression change (x-axis) and the significance of the change (y-axis), with upregulated genes shown in red and downregulated genes shown in blue.

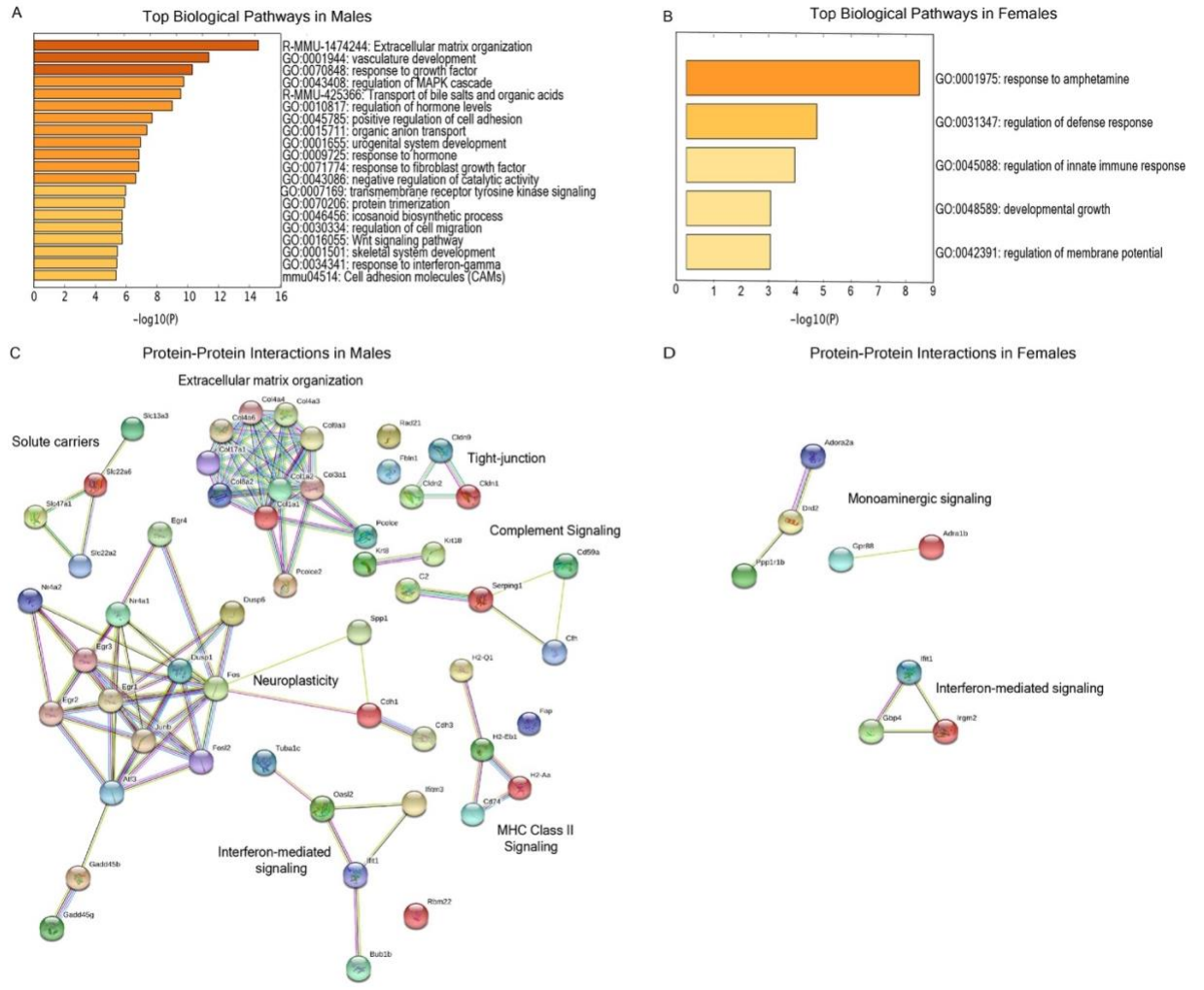
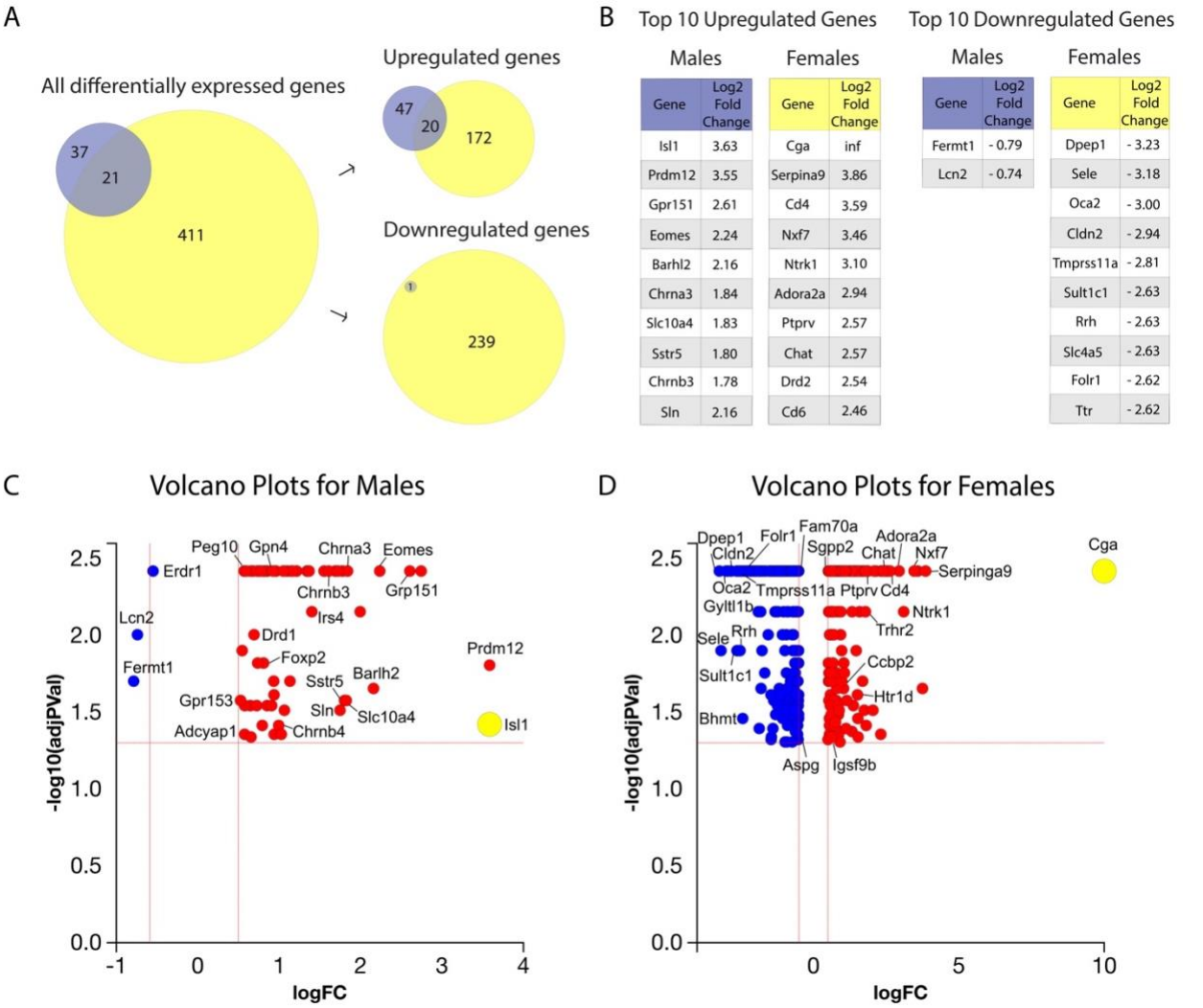
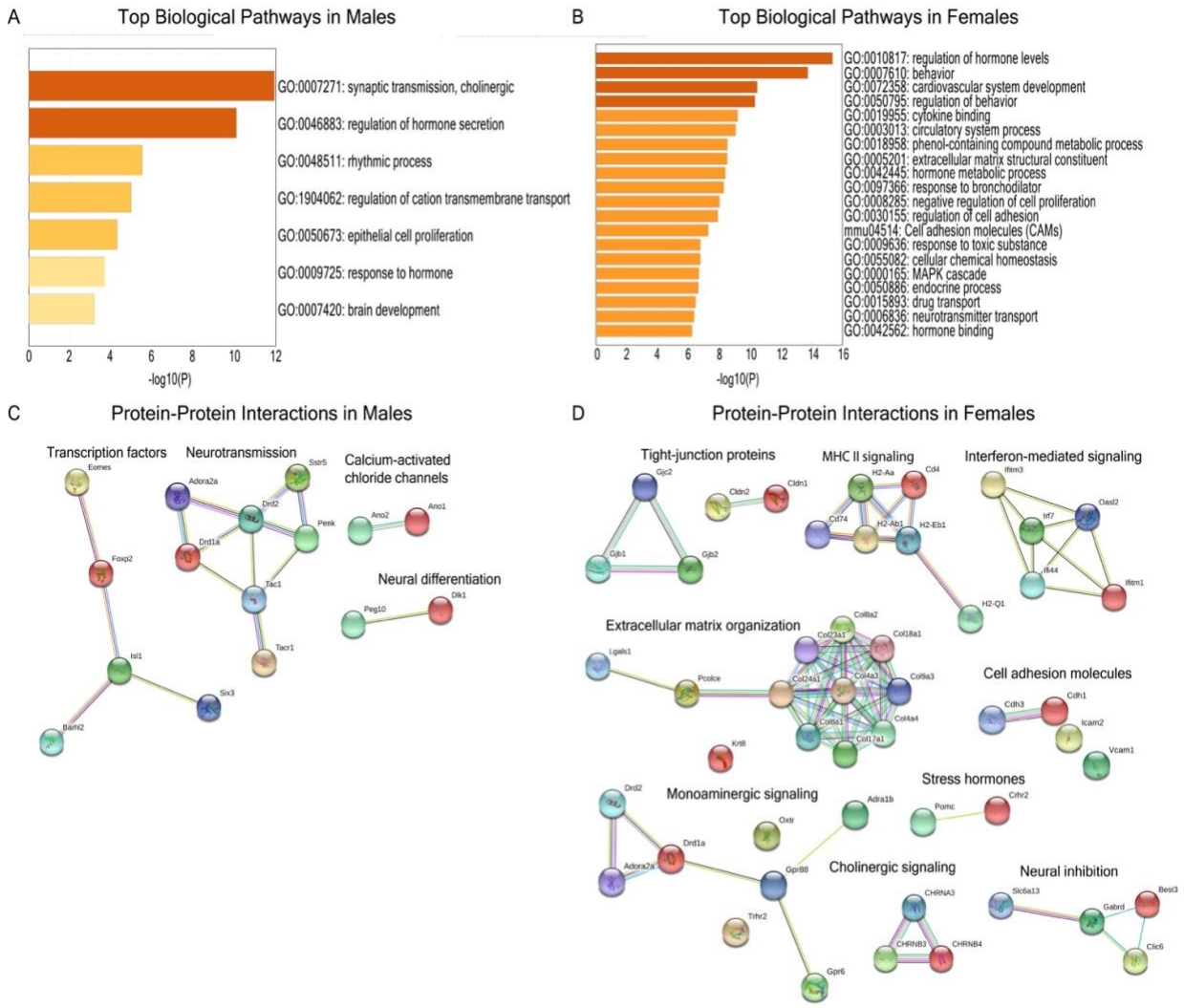


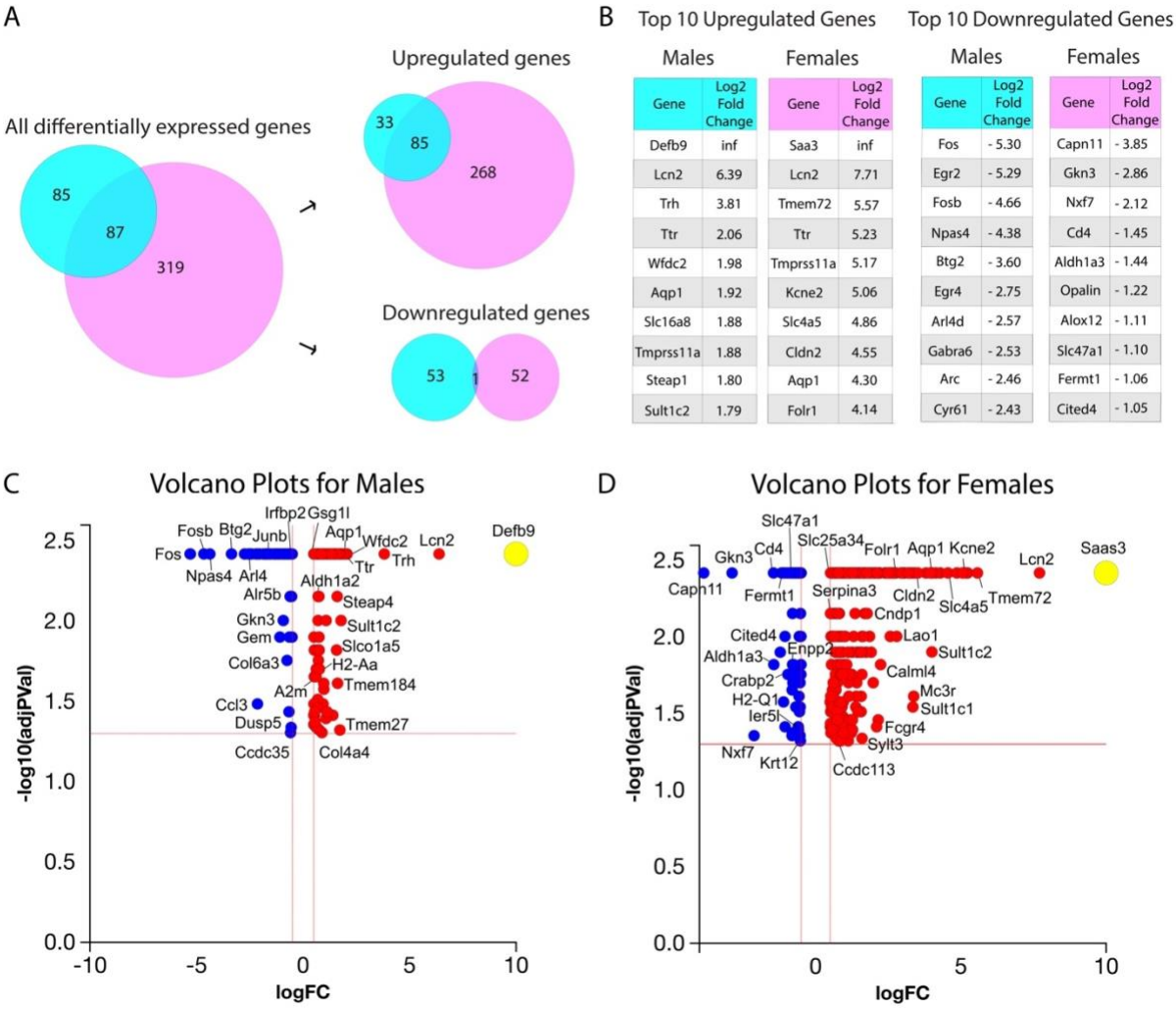
Figure 4.2. Sex-specific functions of differentially expressed genes 12 weeks after subchronic, peripheral LPS challenge induces. (A,B) Biological pathways and processes enriched in gene set (A) in males and (B) in females were generated through Metascape. Distinct biological pathways are observed in males and females, with greater plasticity and immune-related targets in males and greater monoaminergic signaling in females. (D, E) Protein-protein interaction (PPI) networks of targets were generated using STRING 10.5 and clustered by biological function (D) in males and (E) in females. Edges between nodes are color coded for relationship type. Blue: known interactions; Pink experimentally determined interactions; Black: Co-expression of targets; Purple: protein homology; Green, yellow, and dark blue: predicted interactions.



Figures 4.3. Prior subchronic, peripheral LPS challenge alters hippocampal gene expression in response to a subsequent, acute challenge in a sex-specific manner. (A) Females (yellow) show a greater number of differentially expressed genes than males (purple). Females show 192 upregulated and 240 downregulated genes, whereas males show 67 up- and 1 downregulated gene compared with the response to acute immune challenge in previously naïve animals. (B) Top upregulated and downregulated genes in males and females. (C, D) Volcano plots showing differentially expressed genes in males (C) and females (D) obtained from Advaita iPathway Analysis. Differentially expressed (DE) genes are represented in terms of their measured expression change (x-axis) and the significance of the change (y-axis), with upregulated genes shown in red and downregulated genes shown in blue.



Figures 4.4. Subsequent acute immune challenge leads to dysregulation of sex-specific targets in the hippocampus. (B,C) Distinct biological pathways and processes are enriched in DEGs of (B) males and (C) females who showed greater numbers of pathways and changes in immune-related pathways. (D,E) Protein interaction (PPI) networks of targets, clustered by biological function (D) in males and (E) in females.



Figures 4.5. Acute, peripheral immune challenge induces greater changes in the female hippocampus. (A) Previously naïve females (pink) exhibited greater differential gene expression in response to acute LPS challenge than do males (turquoise). Females had 353 genes upregulated and 53 downregulated genes compared with saline treated mice, and males showed 118 upregulated and 54 downregulated after acute immune challenge. (C, D) Volcano plots showing differentially expressed genes in males (C) and females (D) obtained from Advaita iPathway Analysis. Differentially expressed (DE) genes are represented in terms of their measured expression change (x-axis) and the significance of the change (y-axis), with upregulated genes shown in red and downregulated genes shown in blue.

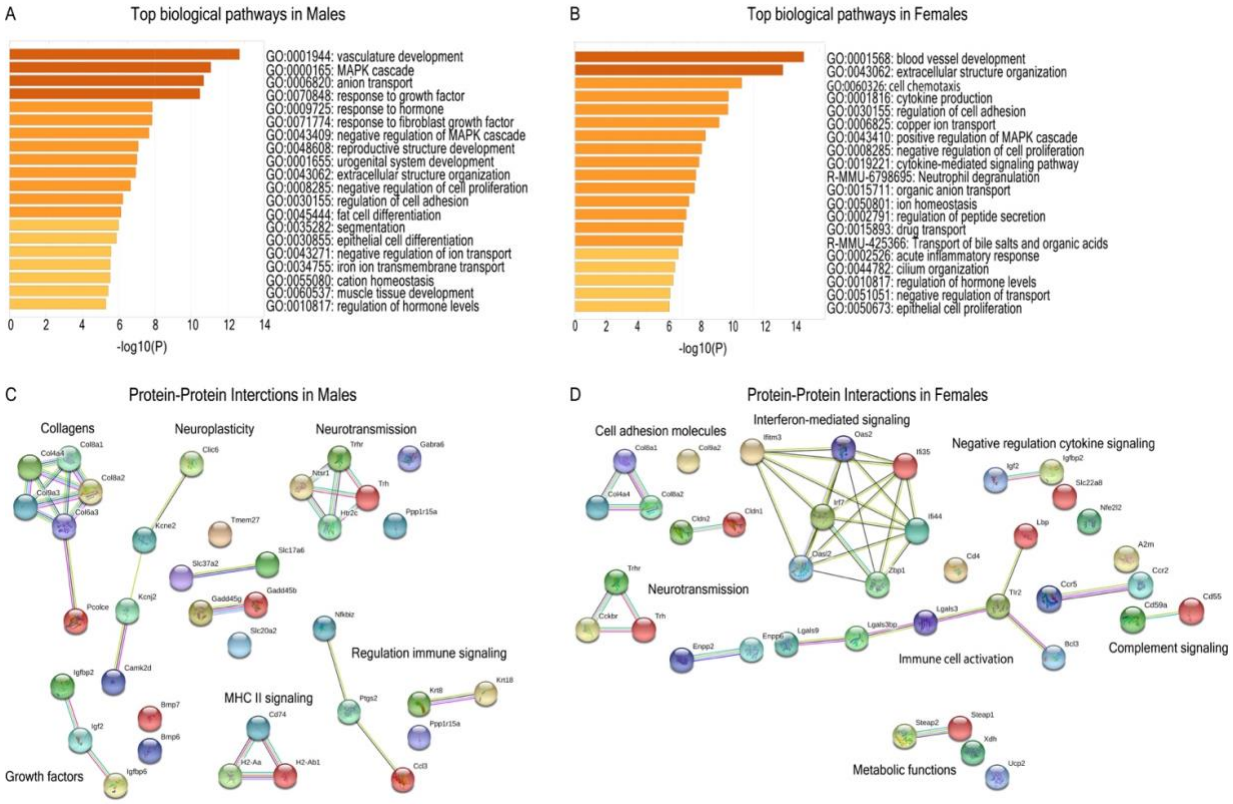


Figure 4.6. Sex-specific functions of differentially expressed genes after acute immune challenge. (A,B) Biological pathways and processes enriched in gene set (A) in males and (B) in females were generated through Metascape. Distinct biological pathways are observed in males and females, with greater plasticity and immune-related targets in males and greater monoaminergic signaling in females. (D, E) Protein-protein interaction (PPI) networks of targets were generated using STRING 10.5 and clustered by biological function (D) in males and (E) in females. Edges between nodes are color coded for relationship type. Blue: known interactions; Pink experimentally determined interactions; Black: Co-expression of targets; Purple: protein homology; Green, yellow, and dark blue: predicted interactions.

A

Top 20 Male Biased Genes

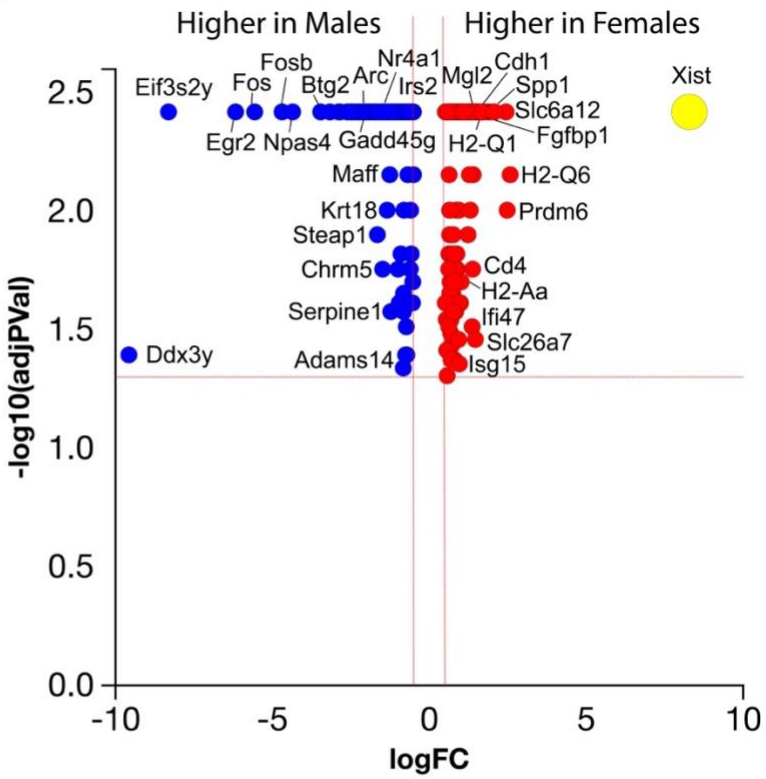
Gene	Log2 Fold Change
Ddx3y	9.58
Eif2s3y	8.31
Egr2	6.18
Fos	5.57
Fosb	4.70
Npas4	4.36
Btg2	3.47
Tmem72	3.17
Ttr	2.89
Egr4	2.85
Slc4a5	2.66
Kcne2	2.52
Junb	2.47
Arl4d	2.46
Arc	2.44
Cyr61	2.29
Gabra6	2.24
Nr4a1	2.12
Egr1	2.11
Folr1	2.06

B

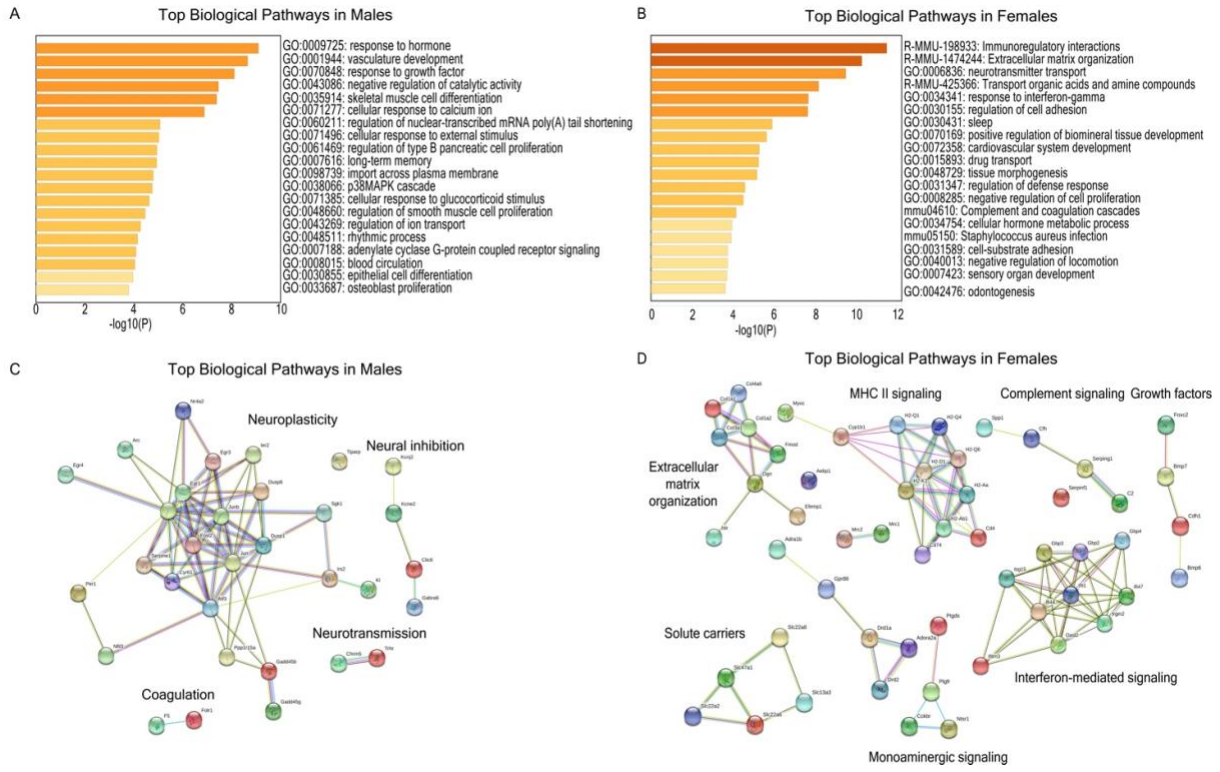
Top 20 Female Biased Genes

Gene	Log2 Fold Change
Xist	8.28
H2-Q6	2.57
Prdm6	2.48
Slc6a12	2.44
Mgl2	2.08
Slc22a2	2.06
Fgfbp1	1.89
Cdh1	1.72
Spp1	1.71
Wnt6	1.70
Foxc2	1.69
Fam180a	1.65
Slc47a	1.62
Ptgdr	1.61
Fmod	1.59
H2-Q1	1.50
Gbp4	1.47
Slc26a7	1.46
Col3a1	1.45
Sphk1	1.40

C



Figures 4.7. Differential gene expression in hippocampus of males *vs* females prior to immune challenge. (A) All differentially expressed (DEGs) in immune-naïve male *versus* female mice. 95 genes were more highly expressed in male hippocampi whereas 125 genes were more highly expressed in females. (C, D) Volcano plots showing genes that are higher in males (C) and higher in females at baseline (D) obtained from Advaita iPathway Analysis. Differentially expressed (DE) genes are represented in terms of their measured expression change (x-axis) and the significance of the change (y-axis), with upregulated genes shown in red and downregulated genes shown in blue.



Figures 4.8. Functions of differential gene expressed genes in hippocampus of males *vs* females prior to immune challenge. (B,C) Biological pathways and processes enriched in (B) males compared with females and (C) in females compared with males. (D,E) Protein interaction (PPI) networks of targets, clustered by biological function (D) in males and (E) in females.

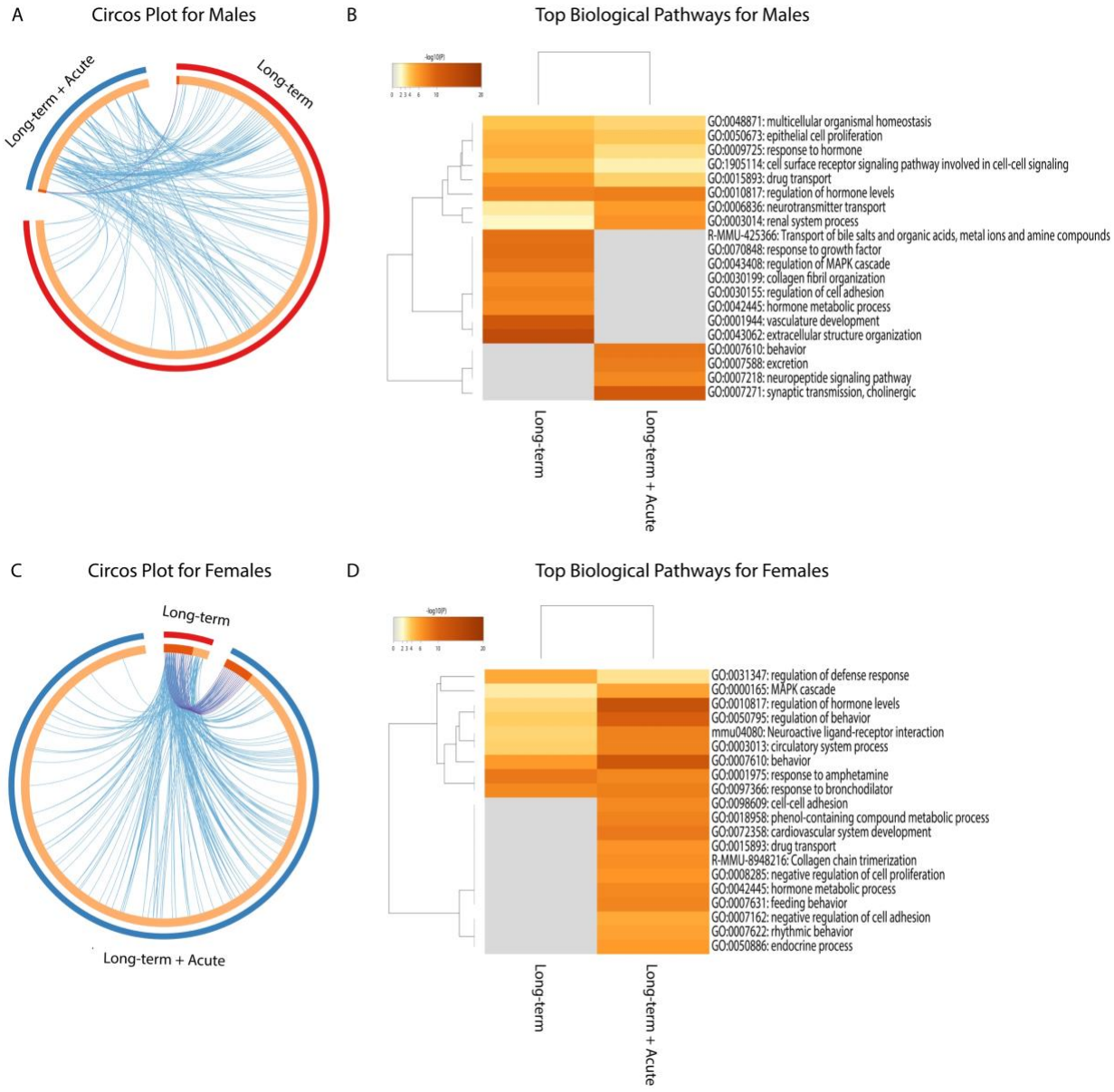


Figure 4.9. Meta-analysis of differentially expressed genes amongst long-term and acute conditions in males and females. (A) Males. Circos plot of differentially expressed genes amongst Long-term (3 months after subchronic LPS injections) Acute (6 hrs post single LPS injection), and Long-term+Acute (LPS injection 3 months after subchronic challenge) conditions. DEG links shared between experimental conditions are depicted by purple lines. while different genes that share similar biological pathways are depicted in blue. (B) Heatmap of selected enriched gene ontology (GO) terms compared between long-term and acute conditions in males. (C) Females. Circos plot of differentially expressed genes amongst Long-term, Acute, and Long-term+Acute conditions. (D) Heatmap of selected enriched gene ontology (GO) terms compared between long-term and acute conditions in females.

Chapter V

How does subchronic immune challenge cause lasting alterations of memory mechanisms?

Abstract

Persistent memory dysfunction persists for months to years in men and women, even after individuals recover from the systemic inflammatory event. How these memory deficits emerge and persist, however, remains unknown. Animal models have most often shown persistent memory deficits that co-occur with sustained changes in peripheral or neuroimmune activation. However, memory impairments have also been observed without changes in immune mediators and instead with changes in synaptic integrity or in plasticity related mechanisms. The studies in this chapter determine whether sustained changes in neuroimmune activation, as measured by microglial activation or blood-brain barrier permeability, or changes in neural plasticity, as measured by alterations in activity-dependent changes in c-Fos levels, are associated with the long-lasting changes in memory. We observed no sustained microglial activation nor increased blood-brain barrier permeability months after subchronic immune challenge in males nor females, suggesting no persistence of neuroimmune dysfunction after a subchronic systemic inflammatory insult. Instead, we observed reduced induction of c-Fos levels after contextual fear conditioning in the dorsolateral entorhinal cortex, a region important for fear memory. There were also differences in interregional c-Fos correlations between saline and Poly I:C treated males. The alterations of c-Fos induction in specific brain regions and interregional correlations between regions across the fear memory network after Poly I:C treatment suggests that immune

challenge persistently alters processes related to activity-dependent transcription and neuronal plasticity, which may underlie the long-lasting memory deficits.

Introduction

Long-lasting memory and cognitive deficits are evident months after a systemic inflammatory event. Survivors of a critical illness who display memory deficits also show increased plasma levels of markers of neuroinflammation, including cytokines such as Il-6, IL-10, and TNF-a as well as markers of blood-brain barrier and/or astrocytic injury and endothelial activation (Hughes *et al*, 2019; Maciel *et al*, 2019; Terrando *et al*, 2010). Additionally, sepsis patients show changes in acute neuronal damage, brain metabolism, as well as long-lasting reduction in hippocampal volume in the left hemisphere and frequency cortical activity that are associated with verbal learning and memory deficits (Hughes *et al*, 2019; Semmler *et al*, 2013). These studies suggest that a systemic inflammatory event induces peripheral immune activation and changes in brain morphology that are predictive of cognitive decline. The mechanisms by which a transient immune activation induces these long-lasting changes in the brain and in memory are unknown.

Persistent changes in blood-brain barrier are commonly observed after illness and injury and are proposed as a mechanism contributing to persistent neuroimmune changes and a variety of neurological and psychiatric disorders (Erickson and Banks, 2018; Prakash and Carmichael, 2017; Sakusic and Rabinstein, 2018; Saunders *et al*, 2008). Persistent neuroimmune activation has been observed months after the systemic inflammatory insult and correlates with memory deficits. Sustained neuroimmune mechanisms, specifically microglial activation and cytokine expression in brain regions important for memory, including hippocampus and frontal cortex, are

observed weeks and months after an overwhelming inflammatory insult and have been associated with memory deficits (Bossu *et al*, 2012; Giustina *et al*, 2017; Semmler *et al*, 2007; Weberpals *et al*, 2009). Therefore, one possibility is that long-lasting changes in peripheral immune activation, blood-brain barrier disruption, and subsequent neuroimmune activation mediate the long-lasting memory deficits.

Nevertheless, memory deficits have been observed months after immune challenge without persistent neuroimmune activation, including both changes in markers of microglial (Iba-1) and astrocytic activation (GFAP) as well as increased cytokines levels (Bian *et al*, 2013). Working memory impairments have been observed months after sepsis, without ongoing elevations of HMGB1 (Chavan *et al*, 2012). Similarly, microglial activation as determined by increased Iba-1 and Cd11b immunoreactivity have not been associated with deficits in novel object recognition (Anderson *et al*, 2015). These studies suggest that peripheral and neuroimmune activation are not necessary for persistence of memory deficits. Persistent memory deficits have also been observed with dysregulation of neural function months after a transient immune activation. For example, a single high dose of LPS results in persistent alterations of cholinergic function (Ming *et al*, 2015), decreased neurogenesis (Ormerod *et al*, 2013; Valero *et al*, 2014), and late-occurring striatal neurodegeneration (Liu *et al*, 2008). Other studies have demonstrated changes in synaptic integrity and plasticity, including decreases in spine density in hippocampus and amygdala months after sepsis (Huerta *et al*, 2016; Volpe *et al*, 2015) as well as decreases in dendritic spine turnover (Kondo *et al*, 2011). It is still unclear whether sustained neuroimmune dysregulation or immune-triggered changes in the brain, including disruption of neural processes, mediate memory deficits long after a systemic immune challenge. Inflammatory insults can induce long-lasting changes in neural plasticity (Maggio *et al*, 2013),

and neural substrates critical for neuronal plasticity and memory. Notably, activity-induced induction of immediate early genes, including c-Fos, Arc and Egr1, have been altered both hours (Czerniawski and Guzowski, 2014) and weeks after immune challenge (Anderson *et al*, 2015). As the induction of the immediate early genes is important for neural plasticity, activation within particular brain regions can be used for mapping brain activity patterns in response to memory tasks (Miyashita *et al*, 2009). Thus persistent changes after an inflammatory insult suggest that systemic immune activation induces persistent alterations in plasticity.

Changes in neuroplasticity are unlikely to occur in a single brain region, as learning and formation of long-term memories requires coordinated activity between multiple brain regions and circuits (Vetere *et al*, 2017). Induction of immediate early genes such as c-Fos in the hippocampus are important for recognition memory and fear memory (Mendez *et al*, 2015; Milanovic *et al*, 1998). Activation in the perirhinal cortex may be more important for recognition memory (Tanimizu *et al*, 2018) while activation in the amygdala may be more critical for fear memory formation (Vetere *et al*, 2017). This network approach to studying changes in neural systems underlying memory allows to better understand how specific circuits or processes are recruited during memory tasks. For example, dorsal hippocampus and dorsolateral entorhinal cortex connections mediate place encoding and visuospatial representations, which are important for object recognition and location as well as context fear conditioning, while ventral hippocampal to dorsolateral entorhinal cortex are also important for affective behaviors and defensive responses (Steffenach *et al*, 2005). Similarly, while the basolateral amygdala is crucial for fear memory (Maren and Fanselow, 1996), the basal and lateral amygdala play different roles in the formation *vs* expression of fear (Manassero *et al*, 2018). The circuit level approach also allows for understanding how particular neural systems become altered by particular

environmental experiences, such as subchronic immune challenge, and whether/how they alter specific memory processes.

In this chapter, I determined whether overt neuroimmune processes, including blood-brain barrier permeability or microglial activation, continue months after subchronic immune challenge, and the impact of prior immune challenge on context fear memory networks.

Materials and methods

***2.1 Animals and Subchronic immune challenge*:** Mice received five intermittent injections of LPS (250μg/kg; n = 4), Poly I:C (6mg/kg; n = 4), or saline control (n = 4), spaced three days apart as described in Chapter 2. Mice underwent novel object recognition 1 week and novel object location 2 weeks after last injection (males and females from Figs. 7-8 in Chapter 2).

***2.2 Blood-brain barrier permeability*:** Blood-brain barrier permeability was assessed in male and female mice (n = 3 (Wang et al. 2018) using the most sensitive method to detect disruption, sodium fluorescein (Birngruber et al., 2013; Kaya & Ahishali, 2011; Saunders et al., 2008). Sodium fluorescein (2%, i.p.) was injected 20 mins prior to blood collection and transcardiac perfusion with saline. Brain permeability index (BPI) was calculated comparing fluorescence (relative fluorescence units, RFU) in brain to fluorescence in serum [BPI = (RFU brain/brain weight)/(RFU Serum/serum volume)], and normalized to brain permeability index in control animals (Devraj, Guérit, Macas, & Reiss, 2018). We also used fluorescent microscopy to visualize qualitative differences in sodium fluorescein penetration in brain sections across multiple brain regions (20μM) (Nikon A1 laser scanning microscope (Devraj et al., 2018; Nikolian et al., 2018).

Data analysis and Statistics. Independent sample t-tests were used to compare brain permeability index for Poly I:C- and saline-treated animals.

2.3 Immunohistochemistry for Microglial Activation: Animals (n = 4/group (Morrison et al. 2017) were anesthesized (Avertin 480 mg/kg, i.p.) and transcardially perfused at 12 weeks post last immune challenge with 4% paraformaldehyde. 40 μM sections through the hippocampus were incubated with rabbit anti-mouse Iba-1 (1:10,000, WAKO), goat anti-mouse secondary (1:200, Vector Labs), and DAB chromagen (Sigma Aldrich, St Louis, MO). ImageJ (NIH, Bethesda, MD) was used to count microglia and a fractal analysis plugin in ImageJ was used to determine microglial morphology [FracLac V. 2.5, (Karperien, Ahammer, & Jelinek, 2013)]. Microglial activation state is commonly assessed using number and morphological changes where cells shift from a resting ramified state with extended, branching processes, to an activated ameboid state with a larger cell body and retraction of processes (Kondo, Kohsaka, & Okabe, 2011; Singer et al., 2016; Weberpals et al., 2009). Fractal analysis is a sensitive and quantitative method to assess small changes in microglial shape using measures of pattern complexity and self-similarity (fractal dimension) as well as heterogeneity (lacunarity) (Karperien et al., 2013; Young & Morrison, 2018). An experimenter blind to the treatment conditions performed microglial counts and morphology analyses in the dentate gyrus molecular layer and CA1.

Data Analysis and Statistics. Two-way ANOVA (Sex × ImmuneChallenge) were used to assess the effects of prior immune challenge on microglial activation for both microglia number and morphology. Post-hoc tests were used to further assess specific group differences.

2.4 Immunohistochemistry for c-Fos activation: Males were treated with intermittent Poly I:C (6 mg/kg) or saline injections (5 injections; one every 3 days) (Tchessalova & Tronson, 2019).

Eight weeks after the last injection, half of the animals treated with subchronic Poly I:C challenge or with saline were trained in context fear conditioning as described in Chapter 2 while half of the animals in both experimental conditions did not undergo fear conditioning. Animals were anesthesized (Avertin 480 mg/kg, i.p.) 1.5 hours after training (Strekalova *et al*, 2003) and transcardially perfused with 4% paraformaldehyde. 40 μM sections through the hippocampus were incubated with mouse anti-mouse c-Fos (1:2000, Abcam, Cat # ab208942), goat anti-mouse secondary (1:200, Vector Labs), and DAB chromagen (Sigma Aldrich, St Louis, MO). ImageJ (NIH, Bethesda, MD) was used to count cells positive for c-Fos staining (Keiser et al., 2017).

Data Analysis and Statistics. Two-way ANOVA (Training × ImmuneChallenge) were used to assess the effects of prior immune challenge on number of c-Fos positive cells 8 weeks after last injection, without training, or 1.5 hrs after context fear conditioning. Post-hoc tests were used to further assess specific group differences.

2.5 Generation c-Fos correlation matrix: A correlation matrix of c-Fos levels across multiple brain regions was generated using Pearson's r correlations for each of the experimental groups to generate an activation map of brain regions involved in context fear conditioning (Tanimizu et al., 2018). The corrplot Package from R Cran was used to organize data from the multiregional correlations (Tyebji, Seizova, Garnham, Hannan, & Tonkin, 2019), which were visualized in Adobe Illustrator. Correlations between c-Fos levels in brain regions in saline treated animals, termed as 'Saline Network', were compared with those for Poly I:C treated animals, or the 'Poly I:C Network'.

Results

3.1 No increases in blood brain barrier permeability months after subchronic immune challenge.

Blood-brain barrier showed no increased permeability long after Poly I:C treatment. Poly I:C did not alter the brain permeability (ImmuneChallenge: $t(1,12) = 1.06$, $p = 0.31$; Fig 1A), as indicated by the brain permeability index and there was no increases in sodium fluorescein staining of saline and Poly I:C treated males nor females in hippocampus (Figure 1B).

3.2 Microglia number in hippocampus, amygdala, and cortex months after subchronic immune challenge.

Subchronic immune challenge did alter microglial cell counts in the CA1 (all: $F(1,12) < 1$; Fig. 2A), CA2 (ImmuneChallenge: $F(1,12) = 3.35$, $p = 0.92$, Sex x ImmuneChallenge: $F(1,12) < 1$; Fig. 2B), CA3 (all: $F(1,12) < 1$; Fig. 2C) nor dentate gyrus (molecular layer) (all: $F(1,12) < 1$; Fig. 2D), of the dorsal hippocampus three months after last injection. There were significantly higher microglial cell counts in the basolateral amygdala (ImmuneChallenge: $F(1,12) = 12.89$, $p < 0.01$, Sex x ImmuneChallenge: $F(1,12) = 0.45$, $p = 0.52$, Males $p < 0.05$ *cf* saline, Females $p = 0.061$ *cf* saline; Figure 2E) as well as in the perirhinal cortex (ImmuneChallenge: $F(1,12) = 4.96$, $p = 0.046$; Sex x ImmuneChallenge: $F(1,12) < 1$; Figure 2F). No differences in microglial cells counts were observed in these hippocampal regions between males and females (CA1 Sex: $F(1,12) < 1$; CA2 Sex: $F(1,12) < 1$; and CA3 Sex: $F(1,12) < 1$); dentate Sex: $F(1,12) = 2.30$, $p = 0.16$; nor perirhinal cortex (Sex: $F(1,12) < 1$). A trend for lower microglial counts were observed in basolateral amygdala of females (Sex: $F(1,12) = 3.73$, $p = 0.09$).

3.3 Microglia morphology is not altered months after subchronic immune challenge

Subchronic immune challenge did not alter microglial morphology, including measures of pattern complexity and self-similarity (fractal dimension) in the CA1(ImmuneChallenge: $F(1,12) = 2.63$, $p = 0.13$, Sex x ImmuneChallenge: $F(1,12) < 1$; Fig. 2A), CA2 (all: $F(1,12) < 1$; Fig. 2B), CA3 (ImmuneChallenge: $F(1,12) = 1.11$, $p = 0.31$, Sex x ImmuneChallenge: $F(1,12) < 1$; Fig. 2C), and dentate (all: $F(1,12) < 1$; Fig. 2D) of the dorsal hippocampus. Similarly, patterns of heterogeneity (lacunarity) were unaltered in the CA1 (ImmuneChallenge: $F(1,12) = 1.78$, $p = 0.21$, Sex x ImmuneChallenge: $F(1,12) < 1$; Fig. 2A), CA2 (ImmuneChallenge: $F(1,12) = 1.02$, $p = 0.34$; Sex x ImmuneChallenge: $F(1,12) < 1$; Fig. 2B), CA3 (ImmuneChallenge: $F(1,12) = 1.95$, $p = 0.19$; Sex x ImmuneChallenge: $F(1,12) < 1$); Fig 2C), and dentate (ImmuneChallenge: $F(1,12) = 2.16$, $p = 0.17$; Sex x ImmuneChallenge: $F(1,12) < 1$; Fig. 2D) of animals three months after subchronic immune challenge. No differences in measures of microglial morphology were observed in the BLA (fractal dimension: ImmuneChallenge: $F(1,12) < 1$, lacunarity ImmuneChallenge: $F(1,12) < 1$; Sex x ImmuneChallenge: $F(1,12) = 2.74$, $p = 0.12$; Fig. 2E) nor perirhinal cortex (all: $F(1,12) < 1$; Fig. 2F).

Microglial morphology (fractal dimension and lacunarity) also did not differ between males and females in any of the brains regions of interest, including dentate (Sex: $F(1,12) < 1$), CA1 (fractal dimension Sex: $F(1,12) = 1.09$, $p = 0.32$; lacunarity Sex: $F(1,12) < 1$), CA2 (all: $F(1,12) < 1$), CA3 (all: $F(1,12) < 1$), perirhinal cortex (fractal dimension Sex: $F(1,12) = 2.43$, $p = 0.15$; lacunarity Sex: $F(1,12) < 1$), and BLA (fractal dimension Sex: $F(1,12) = 3.34$, $p = 0.09$; lacunarity Sex: $F(1,12) = 3.02$, $p = 0.11$).

3.4 c-Fos induction after context fear conditioning is altered by subchronic Poly I:C challenge.

Context fear conditioning significantly increased the number of c-Fos + cells in memory-relevant regions. A significant effect of training was observed in all memory-relevant brain regions that were assessed, including dorsal hippocampal subregions such as dentate gyrus (Training: $F(1,28) = 22.83$, $p < 0.01$), CA1 (Training: $F(1,27) = 12.17$, $p < 0.01$), CA2 (Training: $F(1,27) = 7.00$, $p < 0.05$), CA3 (Training: $F(1,27) = 18.98$, $p < 0.01$) (Figure 4), amygdalar subregions (basal amygdala: Training: $F(1,22) = 32.00$, $p < 0.01$ and lateral amygdala: Training: $F(1,22) = 14.93$, $p < 0.01$; Figure 5), and cortical regions including the retrosplenial cortex (Training: $F(1,32) = 11.13$, $p < 0.01$), dorsolateral entorhinal cortex (Training: $F(1,27) = 25.61$, $p < 0.01$), perirhinal cortex (Training: $F(1,27) = 27.7$, $p < 0.01$), and piriform cortex (Training: $F(1,26) = 11.93$, $p < 0.01$) (Figure 6).

Subchronic Poly I:C challenge persistently dysregulated c-Fos levels in the dorsolateral entorhinal cortex (ImmuneChallenge; $F(1,27) = 7.33$, $p < 0.05$) in naïve animals. Activity-dependent c-Fos induction after context fear conditioning in the dorsolateral entorhinal cortex (ImmuneChallenge x Training; $F(1,27) = 14.51$, $p < 0.01$). The number of c-Fos + cells were significantly increased in the dorsolateral entorhinal cortex of saline treated males ($p < 0.01$), but not Poly I:C treated males ($p < 0.38$) after training, and the number of c-Fos + cells differed between trained animals ($p < 0.01$), but not untrained animals ($p = 0.48$). Poly I:C treatment persistently altered activity-dependent c-Fos levels also in the piriform cortex (ImmuneChallenge x Training: $F(1,26) = 5.54$, $p < 0.05$), with saline treated males ($p < 0.01$) but not Poly I:C treated males ($p = 0.41$) showing increases in number of c-Fos positive cells ($p < 0.01$).

Subchronic Poly I:C challenge did not persistently alter number of c-Fos + cells in the dorsal hippocampal regions (dDG: ImmuneChallenge: $F(1,28) < 1$, CA1: $F(1,27) = 0.66$, $p =$

0.42), CA2: $F(1,27) = 0.74$, $p = 0.39$, CA3: $F(1,28) = 1.26$, $p = 0.27$), lateral amygdala ($F(1,22) = 1.19$, $p = 0.29$), retrosplenial cortex (ImmuneChallenge; $F(1,32) = 1.2$, $p < 0.28$), perirhinal cortex (ImmuneChallenge; $F(1,27) = 2.55$, $p = 0.12$), piriform cortex (ImmuneChallenge; $F(1,26) < 1$) at least eight weeks after last injection. There was a trend towards decreased number c-Fos positive cells with Poly I:C treatment in the basal amygdala (ImmuneChallenge; $F(1,22) = 3.32$, $p = 0.082$).

3.5 c-Fos induction after context fear conditioning is altered by subchronic Poly I:C challenge.

We observed distinct patterns of inter-regional correlations between number of c-Fos positive cells across the dorsal hippocampal subregions (dDG, dCA1, dCA2, dCA3), amygdala subregions (BA, LA), and cortical regions (RSC, dlEC, PERI, PIRI). The 'Saline Network' contained significant positive correlations in number of c-Fos + cells between the perirhinal cortex and dorsal dentate gyrus (r = 0.631) and negative correlations between the dorsolateral entorhinal cortex and the retrosplenial cortex (r = -0.66) and the dorsolateral entorhinal cortex and piriform cortex (r = -0.54) (Figure 7, A). The 'Poly I:C Network' contained positive correlations in number of c-Fos + cells between the dorsal CA1 and basal amygdala (r = 0.80), dorsal CA2 and dorsolateral entorhinal cortex (r = 0.61), dorsal CA3 and basal amygdala (r = 0.84), basal amygdala and lateral amygdala (r = 0.880, and dorsolateral entorhinal cortex and perirhinal cortex (r = 0.61) (Figure 7B). There were negative correlations in number of c-Fos + cells between the dorsal dentate gyrus and dorsal CA2 (r = -0.69), dorsal dentate gyrus and dorsolateral entorhinal cortex (r = -0.62), and dorsal dentate and perirhinal cortex (r = -0.47).

Discussion

Overall, we found no sustained activation of neuroimmune signaling long after immune challenge, as measured by microglial activation, including no changes in microglia number in hippocampal regions and no changes in microglial morphology in any of the brain regions assessed. Persistent increases in microglia number have been observed in the perirhinal cortex of both sexes while persistent increases in the amygdala (BLA) were only found in males. Similarly, no sustained increases in blood-brain barrier permeability were observed months after last Poly I:C injection in the whole brain; and, specifically, no increases in permeability were observed in the hippocampus. These results suggest that subchronic, systemic immune challenge does not induce persistent neuroimmune activation.

Together with the data from chapter 2, these data demonstrated that overt neuroimmune activation is not correlated with persistent memory deficits after repeated administration of a lower dose of Poly I:C. In our model of subchronic immune challenge, no long-lasting changes in blood-brain barrier permeability nor microglial activation were observed. Since blood-brain barrier is important for protecting the brain from toxic substances in the periphery that may damage neural function and memory processes (Stranahan *et al*, 2016; Wardill *et al*, 2016), the lack of blood-brain barrier permeability in our studies suggest that the long-lasting memory deficits are not due to sustained brain damage from these toxic mediators. As we did observe greater number of microglia in the amygdala (BLA) months after subchronic immune challenge, it is possible that these differences in numbers of microglia play a role in the deficits observed in the amygdala-dependent tone fear conditioning months after subchronic immune challenge (Tchessalova and Tronson, 2019). Similarly, the differences in microglia number in a region important for object recognition memory, the perirhinal cortex, may suggest a contribution of

sustained increased microglia number to object recognition impairments. Given that only differences in microglia number, but not microglia morphology, were observed in the BLA and perirhinal cortex, we cannot consider these changes as persistent microglial activation. A notable finding here is the lack of differences in microglial number and microglial morphology observed in the hippocampus. This finding is inconsistent with previous studies, including studies with animal models of sepsis, in which persistent changes in microglia shape and number within the hippocampus are observed along with the long-lasting memory deficits, and therefore have been suggested as a major contributor to the memory deficits (Kondo *et al*, 2011; Singer *et al*, 2016; Weberpals *et al*, 2009). To better understand the functional significance of these changes in microglia number months after systemic immune activation, future studies will need to explore the role that microglia number play in memory and cognition. Overall, our findings suggest that overt microglial activation are not necessary for enduring or emerging memory deficits after immune challenge. Therefore, it is likely that transient immune activation causes changes in neural function that are independent of ongoing neuroimmune activity. Given that the memory deficits we observed in males and females persisted for months after immune challenge, it is likely that these changes in neural function are key mechanisms for long-lasting memory dysfunction.

We also showed that subchronic immune challenge correlates with long-lasting changes in activity-dependent induction of c-Fos, an immediate early gene important for neuroplasticity and for activation of memory-relevant networks (Tanimizu *et al*, 2018; Vetere *et al*, 2017). Notably, activity-dependent c-Fos induction is persistently altered by subchronic immune challenge in the dorsolateral entorhinal cortex, in that context fear conditioning does not increase c-Fos levels in the Poly I:C treated animals while c-Fos induction is observed in the saline-

treated controls. These reduced numbers of c-Fos + cells after training in Poly I:C treated males suggest that the subchronic immune challenge results in long-lasting changes in neuroplasticity in the dorsolateral entorhinal cortex. As expected, the saline treated controls showed an increased number of c-Fos + cells after context fear conditioning across all brain regions assessed, including the dorsal hippocampus (dDG, dCA1, dCA2. dCA3), amygdala (BA and LA), dorsolateral entorhinal cortex, perirhinal cortex, and piriform cortex. The Poly I:C treated animals surprisingly only showed c-Fos induction after training in all of the same regions except for piriform cortex, suggesting that neuroplasticity related processes may be intact in these animals except for a few select brain regions.

Prior exposure to Poly I:C did not change the number of c-Fos cells in the absence of context fear conditioning, suggesting that subchronic immune challenge does not dysregulates protein levels of immediate early genes in most brain regions without training. The reduced activity-dependent induction of c-Fos in memory-relevant brain regions in Poly I:C-treated males along with studies from Chapter 2 showing the deficits in fear memory formation in males two months after last Poly I:C injection, suggest that subchronic immune challenge alters activity-dependent mechanisms crucial for memory formation.

The interregional correlation matrixes show that saline and Poly I:C treated males exhibit distinct patterns of c-Fos levels across multiple brain regions. This network analysis may suggest that the patterns of brain regions activated after context fear conditioning may differ between Poly I:C and saline treated males. As the 'Saline Network' represents multiregional c-Fos correlations for the control group, a group that has been shown to freeze to the context during testing and therefore successfully formed an association between the context and aversive stimulus (shock) (Chapter 2, Figure 5 B,I), the difference between the 'Saline Network' and

'Poly I:C Network' may suggest dysregulation of the 'Saline Network' in the Poly I:C treated males. As such, the positive and negative correlations between the memory-relevant brain regions in the Poly I:C network that are not present in the Saline network may suggest a memory network by which memory is dysregulated in males months after subchronic immune challenge. The positive correlation between the perirhinal cortex and dorsal dentate gyrus in the saline-treated males and negative correlation between these two brain regions in Poly I:C treated males may be a specific example of this and of great interest for further studies. Additionally, the presence of positive dlEC-PERI c-Fos correlations as well as negative dlEC-PIRI and dlEC-RSC cortex c-Fos correlations that are observed in the Saline network but not Poly I:C network may suggest a breakdown of Saline network that is important for fear memory formation. Therefore, both the dysregulation and breakdown of the 'Saline Network' may have functional implications for the memory impairments are observed after Poly I:C challenge.

This data is preliminary and further examination of changes in c-Fos levels in additional brain regions important for fear memory, including ventral hippocampus, infralimbic cortex, as well as several thalamic nuclei and septal nuclei (Wheeler *et al*, 2013) is necessary for understanding how Poly I:C alters activation of brain regions and networks important for fear memory. As these experiments were completed only in males, future work could determine whether similar changes are observed in females, which do not show impairments in context nor tone fear conditioning. Such studies would strengthen the possibility that memory deficits observed in males two months after subchronic Poly I:C challenge are mediated by decreased c-Fos induction in brain regions important for context and auditory-cued fear conditioning. Future experiments may use a more network-scale approach. The network analysis could include connectivity "wheels" based on the interregional c-Fos correlations to understand how the

network engaged by fear memory is altered by subchronic immune challenge. Additionally, using network analyses to explore how certain memory-relevant "hub" regions are altered after subchronic immune challenge will give us insights into the regions that are most critical for fear memory or other types of memory and its modulation by subchronic immune challenge in males and in females. These "connectivity wheels" and "hub" regions will identify the brain regions recruited for specific memory tasks or processes in males and females and will provide insights into how these comprehensive brain activation maps are persistently altered by subchronic immune challenge. To determine the causal role of specific brain regions in long-lasting memory dysfunction after subchronic immune challenge, future work could examine whether increasing activity in regions that have shown persistent decreases in activity-dependent c-Fos induction (e.g. dorsolateral entorhinal cortex) will rescue the memory deficits. These studies could use optogenetics, DREADDs, or transgenic mice with spatiotemporal control of c-Fos induction, to increase activity within particular brain regions in a time-dependent manner, such as during training.

In this subchronic immune challenge model, we show that a lack of sustained neuroimmune activation, but rather dysregulation activity dependent induction of c-Fos months after immune challenge that correlates with the long-lasting memory deficits. Yet, systemic immune activation has been shown to increase neuroimmune mediators at earlier time points that lead to memory dysfunction (Comim *et al*, 2011; Danielski *et al*, 2018; Giustina *et al*, 2017; Michels *et al*, 2014). Future work could determine the neuroimmune mechanisms triggered soon after subchronic immune challenge to understand how short-term neuroimmune activation contributes to persistent changes in memory. These studies could assess increases in blood-brain barrier permeability, microglial activation, and increases in cytokine levels in the periphery and

brain hours after first injection and last injections of the subchronic immune challenge. As systemic inflammatory insults have been shown to induce both disruptive and non-disruptive changes in blood brain barrier, both leading to a dysfunctional barrier (Varatharaj and Galea, 2017), it would be worthwhile to explore whether immune challenge induces non-disruptive blood-brain barrier changes that not readily observed using an inert tracer, including changes in cellular traffic, and upregulation of endothelial receptors and transporters or cytokine production. Additionally, we have shown different patterns of memory deficits in males and in females after subchronic LPS or Poly I:C treatment. Determining the differences in strength of activation, type of peripheral immune responses, and specific neuroimmune mediators induced by either type of immune stimulant may provide insights into how the differences in transient peripheral and neuroimmune responses after LPS or Poly I:C lead to deficits in specific types of memory (e.g. object recognition memory, fear-associated memory). Future work could also explore the role of another neuroimmune cell, the astrocyte, in long-lasting memory dysfunction after immune challenge. In the context of recent data demonstrating the critical role of astrocytes in memory processes and synaptic plasticity (Adamsky *et al*, 2018; Guo *et al*, 2017; Suzuki *et al*, 2011), it is possible that enduring changes in astrocyte function (e.g. metabolism, growth factor production, and modulation of neuronal activity) and interaction with neurons may mediate the emergence of memory deficits observed weeks and months after subchronic immune challenge.

Thus our findings on the long-lasting decreases in immediate early genes in multiple memory-relevant brain regions and differences in c-Fos interregional correlations between saline and Poly I:C-treated males, along with data showing no persistent microglial activation nor blood-brain barrier permeability months after immune challenge, suggest that neuroimmune activation as a consequence of systemic immune challenge has long-term impact on neuronal

function, even after neuroimmune activation has resolved. Together, these studies and proposed future directions will help to delineate the mechanisms by which a systemic inflammatory insult induces persistent neural dysfunction and long-lasting memory deficits in males and females.

Figures

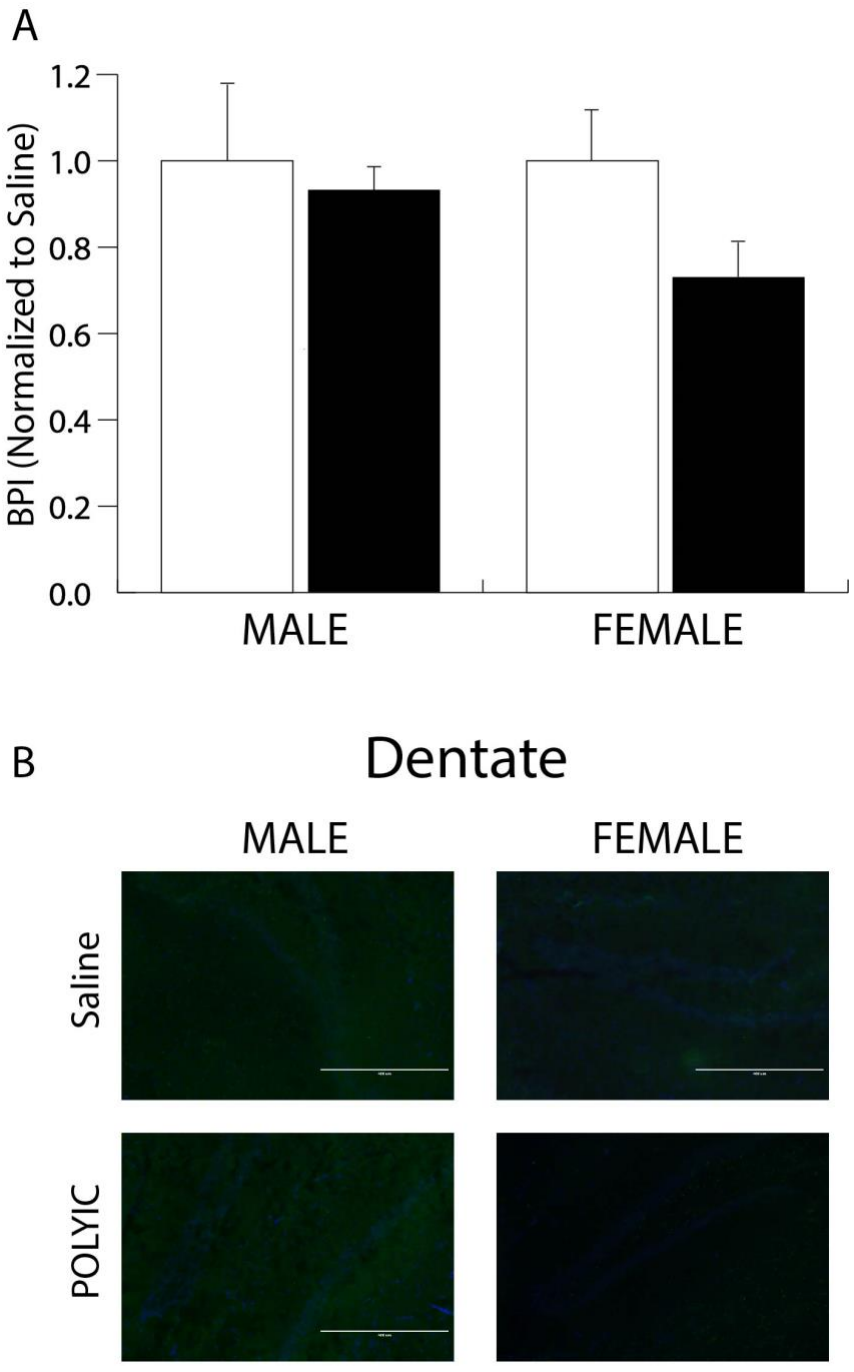


Figure 5.1. No sustained blood-brain barrier permeability is observed months after systemic, subchronic Poly I:C. (A) Prior Poly I:C did not induce sustained blood-brain permeability in males (n = 3 per group) and females (n = 3 per group). (B) Representative images sodium fluorescein (green) and DAPI (blue) staining in the hippocampus.

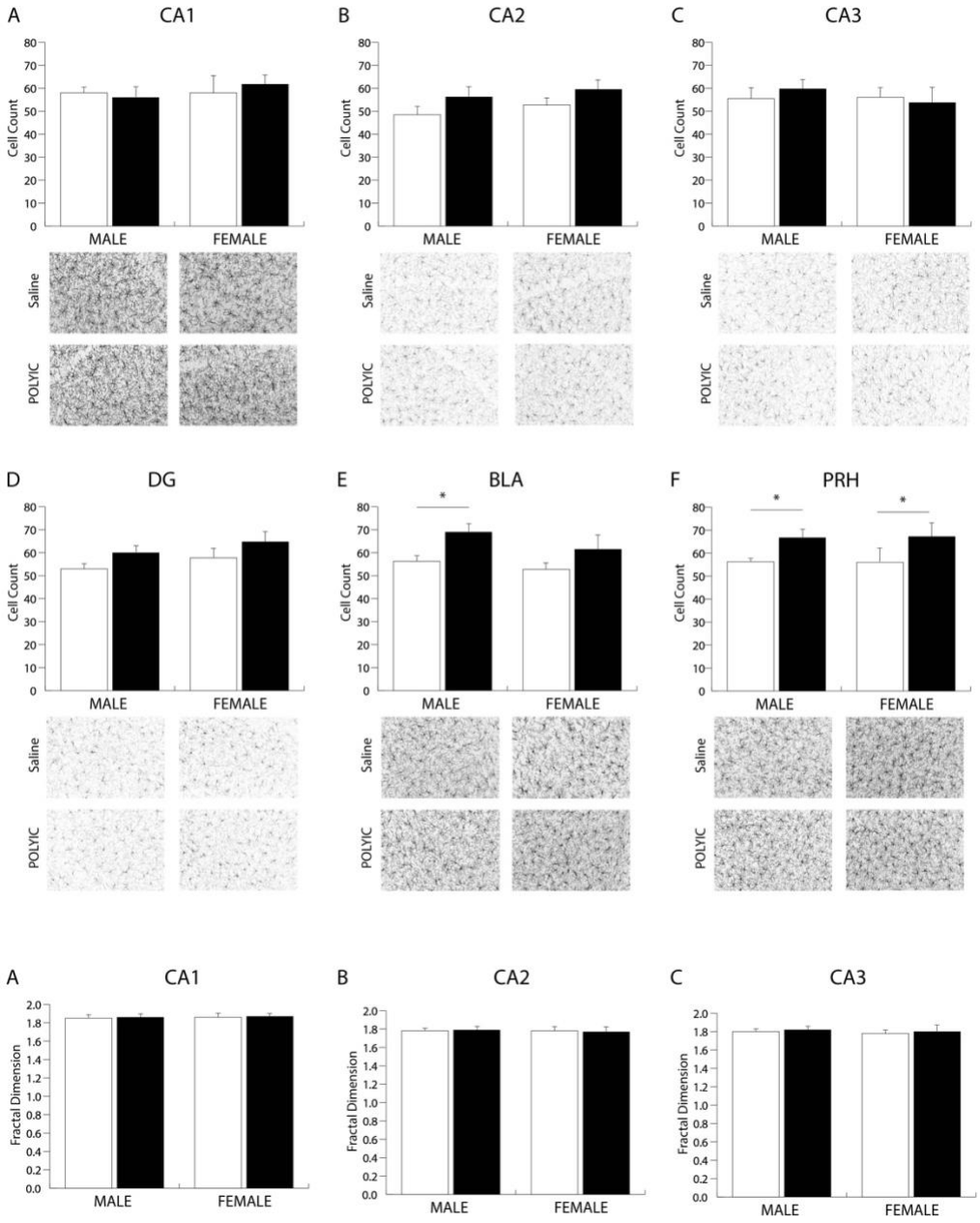


Figure 5.2. Microglial cell counts 12 weeks after systemic, subchronic Poly I:C. Microglial numbers are unaltered in the CA1 (A), CA2 (B), CA3 (C), or dentate gyrus (molecular layer) (D) of males (n = 4 per group) nor females (n = 4 per group) months after Poly I:C challenge. (E) Increased microglial cell counts were observed in the basolateral amygdala (BLA) of males ($p < 0.01$ *cf* saline) but not females ($p = 0.061$). (F) Poly I:C induces persistent increases in microglial cell counts in the perirhinal cortex of both sexes ($p < 0.05$). Representative images of the microglial cell counts (20x) in males and females are below each quantification. Data is presented as group means with error bars representing SEM.

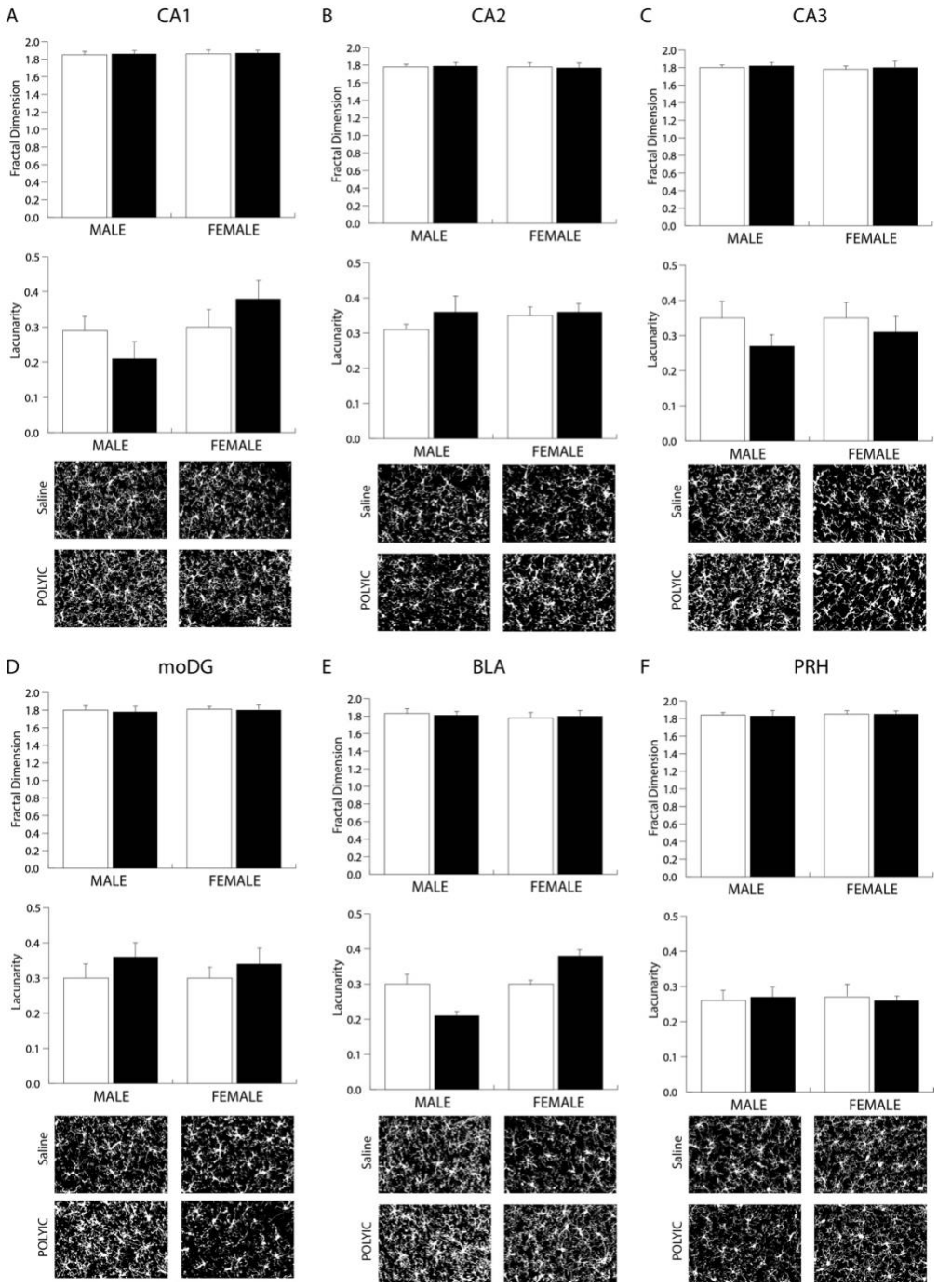


Figure 5.3. Microglial cell morphology is not altered 12 weeks after systemic, subchronic Poly I:C. Prior Poly I:C did not alter microglial morphology, as measured by decreased self-similarity (fractal dimension) and increased heterogeneity (lacunarity) months after immune challenge in the CA1 (A), CA2 (B), CA3 (C), dentate gyrus (molecular layer) (D), basolateral amygdala (E), nor perirhinal cortex (F) of males (n = 4 per group) nor females (n = 4 per group) months after Poly I:C challenge. Representative images of the microglia (40x) in males and females are below each quantification. Data is presented as group means with error bars representing SEM. Representative images used for FracLac analysis areshown for each brain region.

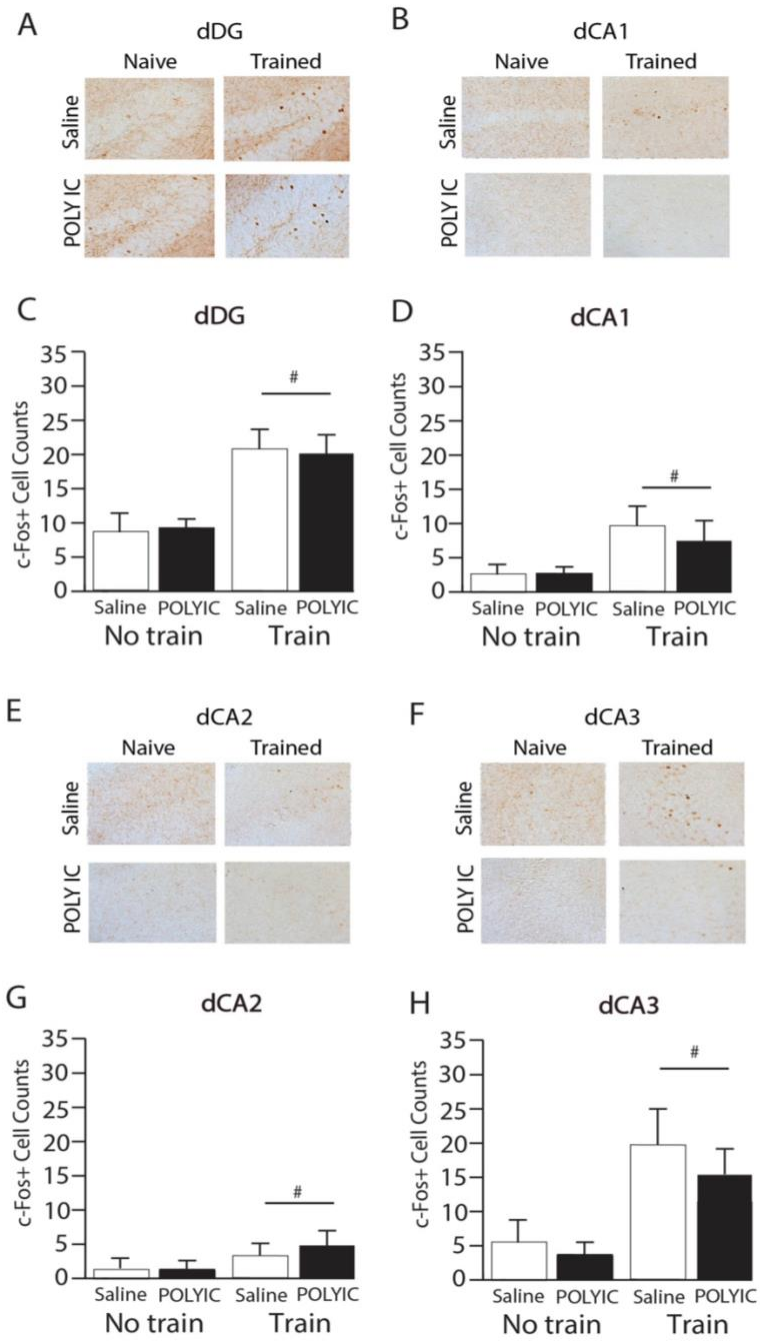


Figure 5.4. Context fear conditioning increases c-Fos levels in the dorsal hippocampus eight weeks after subchronic Poly I:C. (A,B,E,F) Representative images (10X) for saline and Poly I:C naïve and trained animals (n = 7 per group). c-Fos protein levels in (A) dorsal dentate gyrus (B) dorsal CA1, (C) dorsal CA2, and (D) dorsal CA3 of saline and Poly I:C treated animals either with training (trained) or without training (naïve). # $p < 0.05$ Main effect training; *post doc trained vs naïve* * $p < 0.05$

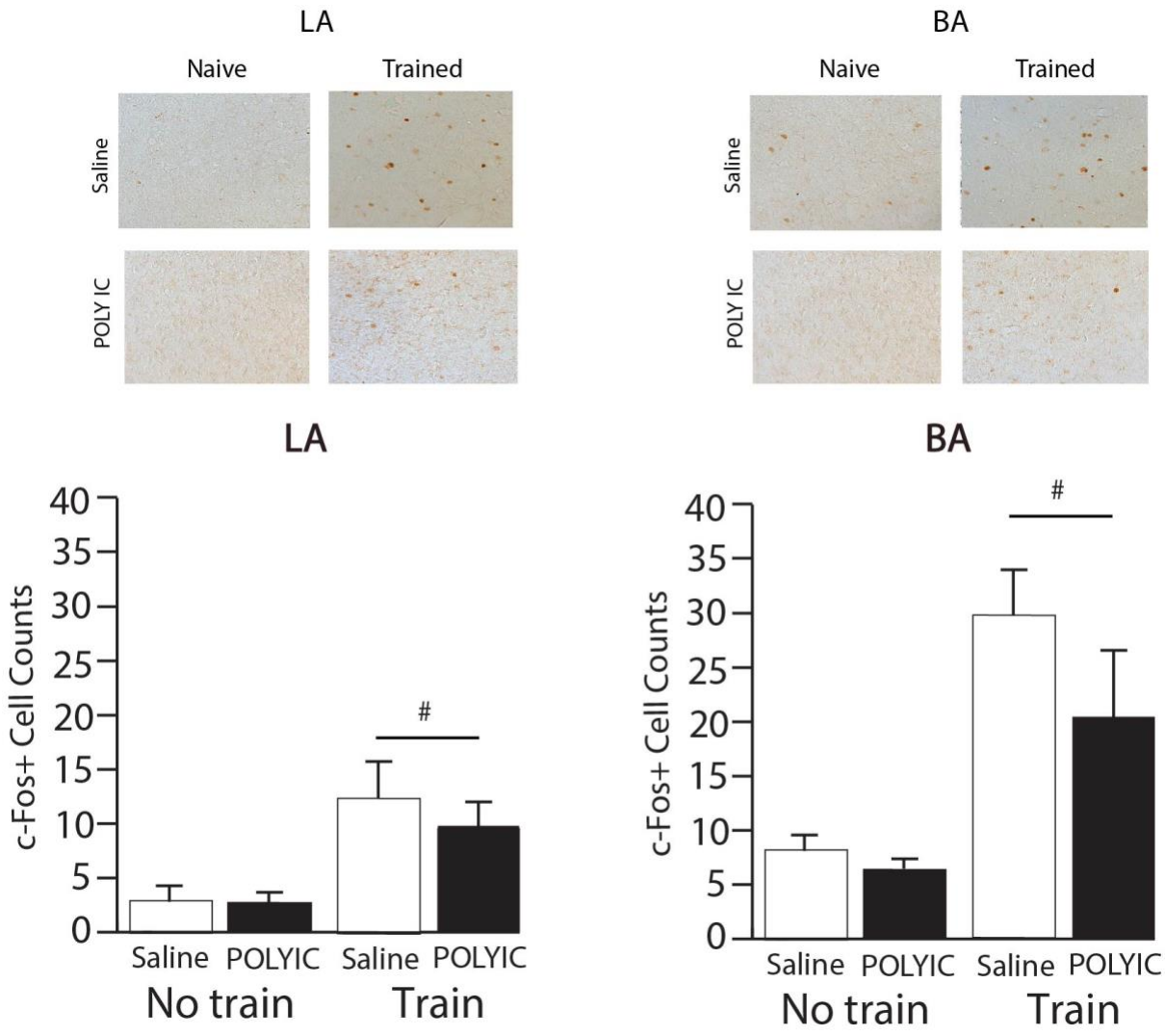


Figure 5.5. Context fear conditioning increases c-Fos levels in the amygdala eight weeks after subchronic Poly I:C. (A,B) Representative images (10X) for saline and Poly I:C naïve and trained animals (n = 7 per group). Quantification c-Fos protein levels in (A) lateral amygdala (B) bsal amygdala (of saline and Poly I:C treated animals either with training (trained) or without training (naïve). # $p < 0.05$ Main effect training; *post doc trained vs naïve* * $p < 0.05$

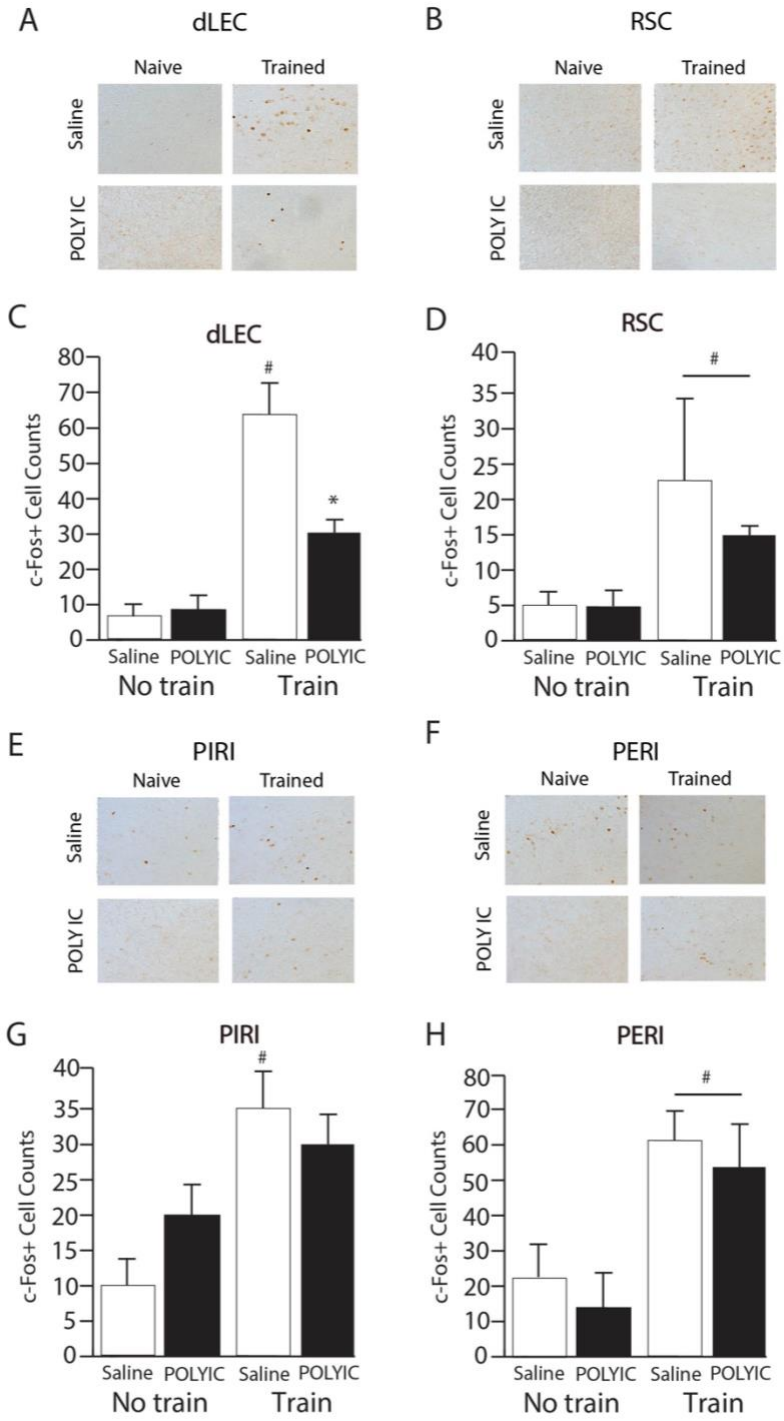


Figure 5.6. c-Fos induction in memory-relevant cortical regions eight weeks after subchronic Poly I:C. (A,B, E, F) Representative images (10X) for saline and Poly I:C naïve and trained animals (n = 7 per group). (A) c-Fos protein levels are increased in the dorsolateral entorhinal cortex after training in saline, but not Poly I:C animals. There is a significant decrease in c-Fos induction in dlEC in Poly I:C treated animals after training. Context fear conditioning increased c-Fos levels in (D) Retrosplenial cortex (RSC), (G) Piriform cortex (PIRI), and (H) Perirhinal Cortex (PERI) of saline and Poly I:C treated animals. # $p < 0.05$ Main effect training; Saline vs Poly I:C * $p < 0.05$

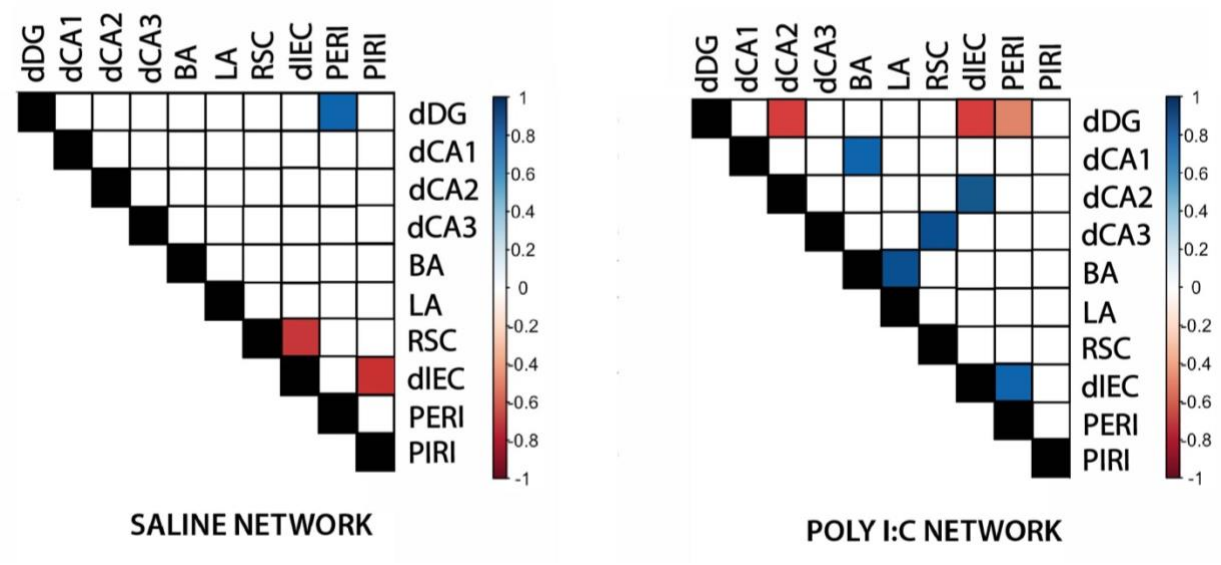


Figure 5.7. c-Fos interregional correlations network in Saline and Poly I:C animals. (A) c-Fos interregional correlations matrix in saline animals, referred to as 'Saline Network'. (B) c-Fos interregional correlations matrix in Poly I:C animals, referred to as 'Saline Network'. Blue represents positive correlations and red negative correlations based on Pearson's r correlations.

Chapter VI

Discussion

Synopsis

This dissertation research identifies a novel mouse model to study how memory and affective processes are altered in both males and females in the weeks and months after subchronic, systemic immune challenge. I have presented studies showing that immune challenge induces changes in memory in both sexes, without increasing anxiety or depressive-like behaviors. Yet, males and females differed in the emergence and persistence of memory deficits, with females showing deficits in novel object recognition both weeks and months after subchronic immune challenge, and males showing impairments only months after immune challenge, with disruptions of object recognition, location, and context fear conditioning. Along with these sex-specific patterns of memory deficits, we observed differential gene expression patterns in the hippocampus of males and females with respect to the number and types of targets that are persistently dysregulated. Interestingly, persistent changes in neuronal rather than neuroimmune mechanisms may mediate the memory deficits observed after subchronic immune challenge, with changes in activity-dependent mechanisms (e.g. c-Fos induction) after context fear conditioning in males.

I have shown that males and females show differential patterns of memory deficits after subchronic immune challenge (Chapter 2). Females showed early and late memory deficits, but only in object recognition after immune challenge, while males showed deficits in broader types

of memory, including object recognition and context fear conditioning months after immune challenge. Interestingly, while object recognition memory was impaired months after subchronic LPS or Poly I:C treatment, fear-associated memory (context and tone) was dysregulated only in males after Poly I:C challenge.

While subchronic immune challenge induces long-lasting memory impairments, I showed no persistent increases in anxiety-like or depression-like behaviors, including despair-like and anhedonia-like behaviors, months after the inflammatory insult (Chapter 3). Subchronic immune challenge also did not induce short-term increases in depression-like behaviors one week after last injection. The alterations in measures of depressive-like behaviors observed, including immobility and climbing, could be more suggestive of learned immobility rather than depressive-like states in males and females. Altogether, the findings from chapter 2 and 3 suggest that subchronic immune challenge results in long-lasting memory dysfunction without alterations in affective processes.

Along with the long-lasting memory dysfunction, I showed persistent changes in molecular substrates in the hippocampus. Sex-specific patterns of gene expression in hippocampus hours and months after subchronic immune challenge, with differences in magnitude and categories of targets differentially expressed (Chapter 4). Specifically, we observed long-lasting alterations in plasticity-related targets in males (e.g. immediate early genes, extracellular matrix proteins) and changes in expression of monoaminergic receptors and associated signaling in females. Prior subchronic immune challenge alters hippocampal transcriptional processes to a secondary, acute immune challenge in both males and females, with females showing a greater transcriptional response and changes in immune-related and monoaminergic genes and males showing a blunted transcriptional response with primarily

changes in neurotransmitter receptors and associated signaling. Acute immune challenge also had sex-specific consequences on the hippocampal transcriptome, with greater dysregulation of genes expression in females. Baseline differences in genes expressed between the hippocampus of males and females also impacted their dysregulation by subchronic immune challenge, with several of the male-biased targets downregulated and several of the female-biased targets upregulated in the male hippocampus months after immune challenge, and *vice versa* for females.

Lastly, I determined mechanisms of long-lasting memory dysfunction and observed no sustained changes in neuroimmune activation, including microglial activation or blood-brain barrier permeability, months after subchronic immune challenge. Instead, subchronic immune challenge may induce long-lasting memory deficits through persistent changes in activity-dependent processes important for memory formation (Chapter 5).

Subchronic Immune Challenge Impacts Different Memory Types

Our studies show that subchronic immune challenge induces long-lasting changes in memory. Subchronic immune challenge impaired specific types of memory, including recognition, spatial, and fear-associated memories. Given that the different types of memory employ different brain regions/ neuronal circuits (Henry *et al*, 2014; Nummenmaa *et al*, 2017), it is possible that subchronic immune challenge induces long-lasting/late-occurring changes in these different memory circuits. Both types of subchronic immune challenge (LPS or Poly I:C) have been shown to disrupt object recognition memory tested both 24 hr and 3 hr after training. This suggests that subchronic immune challenge could disrupt hippocampal-entorhinal-perirhinal networks mediating short-term object recognition memory (Barker and Warburton, 2011) as well

as the hippocampal networks important for the 24 hr novel object recognition test (Vogel-Ciernia and Wood, 2015).

Long-lasting hippocampal dysfunction is likely in the model of subchronic immune challenge as memory tests that require strong hippocampal recruitment such as fear conditioning, at least in males (Keiser *et al*, 2017; Maren *et al*, 2013), are impaired months after subchronic immune challenge. Impairments in hippocampal-dependent fear-associated memory, including contextual fear conditioning and inhibitory avoidance, have also been observed in multiple animal models of sepsis, weeks to months after surgery (Barichello *et al*, 2007; Huerta *et al*, 2016; Singer *et al*, 2016; Tuon *et al*, 2008). Similarly, hippocampal-dependent working memory and reference memory deficits are also observed in the radial arm maze months after immune challenge (Weberpals *et al*, 2009), suggesting that hippocampal-dependent memory processes are particularly vulnerable to various types of inflammatory insults.

Our previous studies suggested that a mild systemic inflammatory event does pose long-lasting negative consequences on amygdala-dependent memory processes, along with changes in hippocampal-dependent memory formation. These data on the mild impairments in auditory-cued amygdalae-dependent memory differs from previous studies showing no changes in amygdalae-dependent auditory-cued memory months after sepsis (Huerta *et al*, 2016; Singer *et al*, 2016). Similar results on the modulation of amygdala dependent processes have also been found using acute immune challenge, where auditory-fear conditioning not affected (Barrientos *et al*, 2002; Pugh *et al*, 1998). Given that the animals show mild impairments in auditory-cued fear conditioning (Chapter 2, Figure 6), it is possible that amygdala-dependent processes and their associated neural circuits are not as resilient to repeated systemic inflammatory insults and that there may be hypofunction or dysregulation of circuit important for auditory cued memory.

While we did not observe differences in neuronal activity in the amygdala after subchronic immune challenge (as assessed by c-Fos induction), it is still possible that amygdala recruitment during fear conditioning is altered after immune challenge. Future studies using alternative markers of neuronal activity may provide insights into whether and how the amygdala and associated circuits are persistently altered by subchronic immune challenge.

Since subchronic immune challenge impacts memory but not affective processes, it is possible that either the neural circuits and/or molecular substrates important for emotional regulation are not persistently altered by subchronic immune challenge. For example, acute immune challenge has been shown to alter c-Fos levels in the hippocampus, hypothalamus, and amygdala and increase depressive-like behaviors (Frenois *et al*, 2007). This suggests the importance of the hippocampal-amygdalar-hypothalamic circuit in regulating affective processes in mice and the potential role of the immune challenge in dysregulation of this circuit, leading to increased depressive-like behaviors. As we observed no increases in depressive-like behaviors, it is possible that the hippocampal-amygdalar-hypothalamic connections are not dysregulated long after subchronic immune challenge. Alternatively, given that chronic depressive-like behaviors are observed after a repeated immune challenge where injections are spaced a week rather than a few days apart (Kubera *et al*, 2013), it is possible that a specific schedule and doses of immune stimulants results in long-lasting depressive-like behaviors and dysregulation of their circuits. Another possibility is that there are compensatory mechanisms in the emotional circuits that prevent subchronic immune challenge from altering these circuits as much as the memory circuits. Future work determining how the circuits, rather than distinct brain regions, are altered by immune challenge may provide insights into whether there are circuits and neuronal networks that are most vulnerable to dysregulation by inflammatory insults.

Differential Effects LPS and Poly I:C on Memory?

We observed differences in memory deficits after LPS and Poly I:C challenge in males, with novel object recognition deficits observed after both LPS and Poly I:C challenge and impairments of context fear conditioning only after Poly I:C challenge. The differences in hippocampal-dependent, contextual fear memory formation we have observed in males but not females could be due to differential effects of these two types of immune challenges on alterations in cell signaling pathways, activation of specific cell types, neuroinflammatory responses relevant to memory formation long after subchronic immune challenge, or potential sex-differences in long-lasting consequences of a systemic inflammatory event on fear-associated memory in males and females. Lipopolysaccharide (LPS) signals using both TRIF and MYD88 dependent signaling, leading to activation of transcription factor NF-κB and mitogen-activated protein kinases (MAPKs) to induce inflammatory cytokines (Kawai and Akira, 2006), Poly I:C binds TLR3 and preferentially promotes the production of both type I interferon and inflammatory cytokines (Kawai and Akira, 2010). As the effects of neuroimmune signaling differ depending on the presence of other cytokines and specific cell types (Norden *et al*, 2016), it is possible that the transient activation of these different types of cell signaling pathways in neuroimmune cells may lead to alternative long-lasting changes in the neural networks important for memory formation.

LPS and Poly I:C are present on different types of neuroimmune cells. LPS is a ligand for till-like receptor 4 (TLR4), mainly present on microglia, while Poly I:C binds toll-like receptor 3 (TLR3), present on many more neuroimmune cells, including microglia, astrocytes, and neurons (Okun *et al*, 2012). Activation of TLR3, which can be found on many more cells, could lead to a stronger neuroinflammatory response during the injections, and potentially long-term

consequences on neural/molecular mechanisms important for hippocampal-dependent memory formation. Additionally, Poly I:C could preferentially activate specific neuroimmune cells, such as astrocytes, that are crucial for modulating processes underlying memory formation, such as changes in synapse formation and pruning, synaptic plasticity, and neurotransmission (Clarke and Barres, 2015; Court and Alvarez, 2016; Perez-Alvarez *et al*, 2014), thereby rendering neural and molecular mechanisms underlying hippocampal-dependent memory processes particularly vulnerable to disruption long after subchronic Poly I:C challenge. Given that subchronic Poly I:C has been shown to disrupt more types of memory, it is possible that this type of systemic inflammatory insult also disrupts a greater proportion of neural networks important for memory. Different types of memory, including spatial working memory, recognition memory, and contextual fear conditioning, have been shown to improve with decreases in TLR3 presence in the brain (Okun *et al*, 2010). As low levels of receptors or activation of TLR3 may be important for optimal performance in hippocampal-dependent memory tests, greater levels or activation of TLR3 by Poly I:C during the subchronic immune challenge could induce impairments in these hippocampal-dependent memory tests greater than 8 weeks after immune challenge.

One lingering question is whether the subchronic LPS or Poly I:C challenge induces differences in strength of peripheral or neuroimmune responses that may differentially impact sickness behaviors, physiological responses, and eventually memory deficits. Interestingly, both LPS and Poly I:C induce transient changes in fever and other "sickness behaviors" (Hopwood *et al*, 2009) that are similar in time course and magnitude (Fortier *et al*, 2004). We also observe no differences in sickness behaviors after LPS or Poly I:C challenge in males and females, suggesting that the impairments observed in in contextual fear conditioning after Poly I:C but not LPS challenge are likely due to differential effects of these two types of subchronic immune

challenge on memory networks rather than differences in immune activation or sickness after immune challenge. However, given that the dose of Poly I:C needed to induce similar changes in body temperature, body weight, food intake, and cage activity as LPS is 50 times higher in some rodents species, such as rats (Hopwood *et al*, 2009), it is possible that when choosing doses of LPS and Poly I:C for mice, we did not select a dose was that equal in magnitude. Therefore, future studies will need to characterize the peripheral and neuroimmune responses after subchronic LPS or Poly I:C challenge to determine the type of immune response and strength of immune activation that each immune simulant triggers, and whether there are doses and/or protocols that trigger similar immune responses. These findings will provide insights into the mechanisms by which transient immune activation leads to long-lasting changes in memory and cognition.

Impact of Sex on Long-Lasting Changes in Memory and Cognition after Subchronic Immune Challenge

Prior studies have focused on the impact of a systemic inflammatory event on memory, cognition, and emotion only in males. The studies outlined in this thesis provide insights into how a mild, systemic inflammatory event alters memory and cognitive processes in both *males and female*s. We have shown differential trajectory of memory deficits in males and in females after subchronic immune challenge with females showing memory deficits both at least one week and eight weeks after subchronic immune challenge, but only in object recognition, and males showing memory deficits in object recognition and fear-associated memory, at least eight weeks after the last injection. Interestingly, these sex-specific patterns of memory deficits occurred without increased anxiety-like behaviors or depression-like behaviors.

Sex differences in peripheral immune and neuroimmune activation as well as signaling pathways (Gresack *et al*, 2009; Kudo *et al*, 2004) and related gene expression important for memory formation (Antunes and Biala, 2012; Mizuno and Giese, 2010) all contribute to the observed sex-specific changes in memory deficits. Recent studies show that there are sex differences in the activation of microglia (Bodhankar *et al*, 2015; Morrison and Filosa, 2016; Schwarz and Bilbo, 2011) and astrocytes (Acaz-Fonseca *et al*, 2015; Santos-Galindo *et al*, 2011) as well as neuroimmune signaling in the hippocampus (Speirs and Tronson, 2018). These differential patterns of neuroimmune cell activation and neuroimmune signaling likely contributes to the sex-specific patterns of memory deficits in males and in females after subchronic immune challenge.

In the periphery, there are sex differences in immune responses to bacteria or viruses, with males developing a Th1 inflammatory response associated with greater negative consequences of immune activation, and females developing a Th2 response associated with greater dampening of the damaging inflammatory signals (Roberts *et al*, 2014). In the brain, males show greater damaging neuroinflammatory responses while more protective responses are observed in females after inflammatory insults (Bodhankar *et al*, 2015; Santos-Galindo *et al*, 2011). These differences in acute immune activation could impact neuronal processes differently in males and in females and pose different consequences on synaptic and memory processes in males and females. With the greater protective mechanisms against immune and neuroimmune signaling, females could be less likely to develop memory deficits long after a subchronic immune challenge. A more severe systemic inflammatory event may therefore be required to disrupt these memories in females. While both sexes show sickness behaviors during the subchronic immune challenge, their peripheral immune and neuroimmune responses may differ

during peripheral intermittent injections of both LPS or Poly I:C, contributing to the sex-specific changes in memory weeks to months after the inflammatory insult.

There are also sex differences in neuroimmune cell distribution across the brain, with fewer astrocytes in the amygdala of females (Johnson *et al*, 2008), and differences in morphology of these cells, with males showing longer processes and greater complexity of branching in the amygdala (McCarthy *et al*, 2003). It is possible that decreased number of the neuroimmune cells could contribute to lower activation and a decreased neuroinflammatory response in the amygdala of females during the subchronic Poly I:C challenge. Given the important role of the amygdala in contextual fear conditioning and for astrocytes in memory formation (Ledoux, 2000; Steinman *et al*, 2016), decreased activation of astrocytes in the amygdala could contribute to less alterations in contextual fear conditioning in females long after subchronic Poly I:C challenge. While we examined changes in microglia number and activation across multiple memory-related brain regions, sex-specific changes in contextual fear conditioning long after Poly I:C challenge may be due to changes in another type of neuroimmune cell, the astrocytes.

Males and females also differ in behavioral strategies and molecular mechanisms important for memory modulation (Keiser *et al*, 2017; Keiser and Tronson, 2015). It is possible that certain behavioral strategies and their cellular and molecular correlates are more adaptive to inflammatory insults than others, leading one sex to be less vulnerable to certain types of memory impairments, such as the deficits observed in context fear conditioning in males only after Poly I:C challenge. Yet, much more work needs to be accomplished on the mechanisms that underlie not only sex differences in learning and memory, but also sex differences in vulnerability to different types of memory disorders to truly understand how systemic immune

activation impacts these processes and leads to sex-specific patterns of memory deficits or sex differences in memory dysfunction.

As most studies previous studies on memory and affective processes have focused on males, little is known about the factors that could contribute to long-lasting emotional regulation in females, and lack of their dysregulation by environmental insults such as immune challenge. Therefore, this subchronic immune challenge model will allow future research to explore how sex differences in peripheral and immune responses contribute to sex-specific patterns of memory deficits. Future studies on the characterization of peripheral immune responses and neuroinflammatory profiles of males and females both shortly after the first and last injection will determine how the immune responses differ between the sexes and provide insights into their implications for sex-specific memory impairments.

Impact of Age on Long-Lasting Changes in Memory and Cognitive Functions after Systemic Immune Activation

Clinical studies have observed long-lasting memory deficits and cognitive decline after a systemic inflammatory event in men and in women. These persistent memory deficits and cognitive impairments have been observed mainly in older patients (Kulason *et al*, 2017; Langa *et al*, 2012; Luo *et al*, 2019). Yet, the elderly may have other health complications by the time they undergo surgery or experience a critical illness that may further exacerbate the brain dysfunction after a systemic inflammatory event (Langa *et al*, 2012; Iwashyna *et al* 2010). For example, the elderly do not recover from a systemic inflammatory insult as do young individuals, and the non-resolving inflammation after the systemic inflammatory insult turns into chronic low-grade inflammation that has been proposed as a driver of their cognitive decline

(Cunningham and Hennessy, 2015). While few studies show memory and cognitive decline in younger individuals (Monk *et al*, 2008; Semmler *et al*, 2013), these long-lasting memory deficits can be observed in at least 30% of young (18-39 years old) and middle-aged (40-59 years old) patients and have not been shown to be correlated with non-resolving inflammation (Monk *et al*, 2008). Therefore, our model of subchronic immune challenge, in which no persistent neuroimmune activation is observed, may be used to model the changes in neural and cognitive functions that occur in middle age adults after a systemic inflammatory event.

Novel Mechanisms of Memory Modulation after a Mild Systemic Inflammatory Event

This subchronic immune challenge model has yielded long-lasting sex-specific patterns of memory deficits months after insult without sustained neuroimmune activation. The long-lasting nature of the memory deficits without ongoing neuroimmune activity is surprising given the multitude of previous studies suggesting the importance of neuroimmune mechanisms in mediating memory dysfunction after a systemic inflammatory event. In this model, we observed dysregulation of several plasticity related genes, including immediate-early genes (e.g. *Fos, Egr1, Arc*) and extracellular matrix related genes in males, and targets associated with neurotransmission and associated signaling, such as monoaminergic signaling, in females. While we have very few genes that are dysregulated in both sexes, there is a common neuroplasticity-related pathway that some of the targets pertain to: the cAMP associated signaling pathway. It is possible that dysregulation of cAMP-related signaling and associated changes in activity-dependent mechanisms and neuroplasticity may mediate long-lasting memory deficits.

A striking observation using the large-scale and unbiased approach at examining gene expression after subchronic immune challenge showed dysregulation of genes associated with

structures that mediate long-lasting alterations in neural plasticity, such as perineuronal nets (e.g. *Otx2*). Perineuronal nets are long-lasting extracellular matrix structures that alter synaptic interactions and function in several brain regions critical for neuronal plasticity, including the hippocampus (Sorg *et al*, 2016). These perineuronal nets may also be important for activity-dependent processes, such as induction of c-Fos and neuroplasticity (Morikawa *et al*, 2017). Changes in levels of another class of extracellular matrix proteins in the brain, collagens, have also been shown to improve memory and reduce neuronal processes associated with memory dysfunction, such as neuronal loss in hippocampus (Shin *et al*, 2015). As such, changes in expression of extracellular matrix related proteins may serve to protect the brain from injury or inflammatory insults and allow the brain to adapt to subsequent insults. The delayed memory deficits observed in males may be associated with or even driven by enduring alternations to these extracellular matrix structures weeks to months after subchronic immune challenge. As extracellular matrix organization associated genes are dysregulated in the hippocampus of males but not females three months after subchronic immune challenge, it is possible that the perineuronal nets or other collagen proteins contribute to the memory deficits observed in males while a different mechanism, a mechanism that remains to be explored, may underlie memory deficits in females.

Interestingly, several of our genes also pertain to MHC class II proteins, such as *CD74, C3, Serping, H2-ab, H2-Aa* in males and *CD4* in females. MHCII is a major histocompatibility complex that is present on surface of antigen presenting cells. It binds to molecules including toll-like receptors (*Tlr2, Tlr4, Tlr7*) and complement components (*C1s, C3, C4a, Serping1*), allowing immune cells to recognize potentially threatening pathogenic peptide sequences (Vanguilder *et al*, 2011). While the role of MHC class II in memory and plasticity is largely

unknown, MHC class I is important for hippocampal-dependent memory and underlying neural processes. For example, animals with non-functional MHC I show deficits in context fear conditioning, object recognition, social recognition (Nelson *et al*, 2013) and synaptic plasticity and excitatory neurotransmission (Fourgeaud *et al*, 2010). MHC class II targets may remain dysregulated months after systemic immune activation as part of a neuroprotective mechanism, which may also alter hippocampal function and associated cognitive functions.

Although we did not observe ongoing microglial activation nor blood-brain barrier permeability, it is likely that more subtle neuroimmune changes, including changes in gene expression persist long after a subchronic immune challenge (Tchessalova *et al*, 2018; Tchessalova and Tronson, 2019). Previous studies have shown that there are persisting changes in expression of immune mediators, such as iNOS2 expression in the hippocampus and frontal cortex after 2 months (Weberpals *et al*, 2009) as well as increased TNF-a in the hippocampus 10 months (Bossu et al., 2012) after a systemic inflammatory event. Given that we observe no persistent increases in these cytokines, but instead dysregulation of other taregts related to interferon mediated signaling (e.g. *ifit1*), MHC class II signaling (e.g. *H2-Eb1*), complement signaling (e.g. *Cfh*) and blood-brain associated cell adhesion molecules (e.g. *Cldn9*) in whole hippocampus months after an immune challenge (refer to Chapter 4), it is possible that these enduring changes in expression of neuroimmune substrates in glia, neurons, or cells making up the blood-brain barrier, rather than sustained cytokine signaling, are important for modulating memory processes long after systemic immune challenges.

Neuroimmune Activation Drives Multiple Brain States

Systemic immune activation can lead to persistent changes in memory and cognitive functions. Prior studies have focused on the persistent peripheral or neuroimmune activation that is observed concurrently with memory deficits (Olivieri *et al*, 2018; Singer *et al*, 2016; Weberpals *et al*, 2009). In animal models of sepsis, it is thought that immune activation resulting from an illness or stressor does not resolve and can result in persistent inflammation or state of chronic low-grade inflammation that negatively impacts neural and cognitive functions through dysregulation of brain function by overactive immune signaling. The non-resolving inflammation has also been shown impacts also cognitive functions in patients. Patients who have diseases associated with chronic low-grade inflammation, of arthritis and periodontal disease, also show cognitive deficits, depression and an increased risk for Alzheimer's disease (Chou *et al*, 2017; Simos *et al*, 2016). Together, these persisting immune processes lead to a hypofunctional brain state (Figure 1).

However, ongoing neuroimmune activation is not necessary for persistence of memory deficits. In our model, we observe long-lasting memory deficits *without* sustained in neuroimmune activation. Instead, we observed long-lasting neuronal changes related to alterations in protein levels of the immediate early gene c-Fos, which is crucial for neuroplasticity and memory processes. Similarly, a few animal models have shown long-lasting memory deficits with delayed or persistent changes in neuronal processes important for cognitive functions (Huerta *et al*, 2016; Ming *et al*, 2015). A question that arises from these studies is how important persistent neuroimmune activation is for mediating long lasting changes in memory and cognition. Is ongoing neuroimmune signaling critical for changes in memory and cognition weeks to months after a systemic inflammatory event? Or could transient interactions between

neuroimmune signaling and the neural systems/substrates important for memory during the acute phase of the systemic inflammatory event lead to their long-lasting dysregulation?

Transient systemic immune activation, whether by illness, injury, or experimental administration of LPS or other immune trigger, has been shown to induce broad networks of cytokines and immune molecules and downstream signaling pathways in the brain (Tchessalova *et al*, 2018). This neuroimmune signaling then interacts with well-known learning and memory pathways such as ERK and modulates memory and cognitive processes (Donzis and Tronson, 2014). While neuroimmune signaling resolves within days after peripheral immune challenge (Speirs and Tronson, 2018), we show that both memory deficits and dysregulation of hippocampal gene expression persist for months after the inflammatory insult (Tchessalova *et al*, 2018; Tchessalova and Tronson, 2019). Therefore, it is likely that the memory deficits observed are mediated by long-lasting changes in neural function, which could include changes in neuronal processes and neuroimmune cell functions.

Long-lasting alterations in neural function and memory processes are mediated by persistent alterations in molecular substrates, including transcriptional changes and associated epigenetic regulatory modifications, including decreased histone H3/H4 acetylation and alterations in DNA methylation (Peleg *et al*, 2010; Rudenko and Tsai, 2014). As distinct patterns of gene expression are important for specialized neural function (Igaz *et al*, 2004) and may govern susceptibility or resilience to environmental experiences, such as stress (Hodes *et al*, 2015), it is likely that alterations in these transcriptome profiles also mediate the long-lasting changes in memory functions observed after inflammatory insults. For example, these persistent changes in gene expression have been correlated with impaired working memory months after immune insult (Weberpals *et al*, 2009). As immune-induced, sustained changes in gene

expression have been observed along with alterations in chromatin modifications, such as H3K9 and H3K27 acetylation and H3K4 trimethylation (Choi *et al*, 2017; Schaafsma *et al*, 2015), as well as DNA methylation (Grassi *et al*, 2017), the epigenetic modifications could regulate persistent changes in gene expression mediating cognitive function and memory processes long after an inflammatory event. Therefore changes in neural function that persist long after the resolution of immune challenge, including the decreased neuronal connectivity, synaptic spines and plasticity months after inflammatory insult (Huerta *et al*, 2016; Kondo *et al*, 2011; Maggio *et al*, 2013), may be due to dysregulation of gene expression necessary for plasticity-related processes rather than ongoing neuroimmune activation.

Long-lasting changes in gene expression and neural function are mediated by epigenetic modifications. Sustenance of particular epigenetic marks is important for regulating many of the long-lasting changes not only in neurons but also in neuroimmune cells such as microglia and astrocytes. A defining feature of the peripheral immune system is that acute activation results in permanent changes to immune function that persist after resolution of inflammatory signaling. The innate immune system also shows ‘‘trained’’ immunity, in which a transient immune challenge results in increased responsiveness to subsequent immune stimuli (Netea and van der Meer, 2017) by altering interactions between neurons and glial cells and the quantity and specific patterns of cytokines produced (Šišková and Tremblay, 2013; Wendeln *et al*, 2018). Specifically, microglial activation after immune insults can sensitize with repeated exposure, producing exaggerated inflammatory responses a concept referred to as “priming” (Perry and Holmes, 2014). Priming effects after immune challenge in adults occur with changes in microglial and astrocytic function and behavior (Fenn *et al*, 2014; Liddelow and Barres, 2017; Muccigrosso *et al*, 2016; Norden *et al*, 2015; Wendeln *et al*, 2018). For example, prior neuroinflammatory insult

can significantly exaggerate future glial activation and worsen not only cognitive outcomes, such as hippocampal-dependent memory but also affective outcomes, such as depressive-like behaviors in the subsequent months (Fenn *et al*, 2014; Muccigrosso *et al*, 2016). In other instances, however, immune a prior inflammatory insult can serve as a neuroprotective barrier against the neuropathology induced by future immune insults, such as decreasing instances of immune-induced neurodegeneration, a concept referred to as preconditioning (Pardon, 2015). Together, these findings demonstrate that a prior immune challenge causes persistent changes in glial function. These long-lasting functional changes in glial cells may be mediated by enduring chromatin modifications such as acetylation and methylation of H3 and associated changes in gene expression (Gandhi *et al*, 2007; Schaafsma *et al*, 2015; Wendeln *et al*, 2018). The altered changes in gene expression in glial cells mediate persistent changes in neuroimmune function even after recovery from an inflammatory event (Wendeln *et al*, 2018). Thus, transient illness may cause not only the initial acute immune state, but also a new permanently altered brain state that is marked by persistent changes in neuronal and glial cell functions.

The long-lasting changes in neuronal and glial functions may be thought of as a distinct, shifted baseline state that results in functional adaptation for subsequent illnesses, a brain state that can be referred to as "persistent' brain state. I propose that a milder systemic inflammatory insult, such as subchronic immune challenge, results in long-lasting alterations in neural function, including changes in activity of neuronal and glial cells and their interactions necessary for proper neurotransmission and/or neuroplasticity, rather than persistent neuroimmune activation. In this new "persistent" brain state, it is possible that other neuronal processes, including processes important for memory formation such as neurogenesis, are altered. These persistent alterations in neural function result in long-lasting memory and cognitive dysfunction.

As the persistent alterations in neural function are likely driven by long-lasting transcriptional dysregulation, future work could determine the dysregulated chromatin and DNA regulatory mechanisms that contribute to long-lasting changes in gene expression important for various neural processes underlying memory functions.

Future Directions

Our subchronic immune challenge model shows long-lasting memory deficits, distinct changes in transcriptional responses in the hippocampus, and persistent changes in activity-dependent c-Fos induction in a memory-relevant brain region. Future work will need to discover the mechanisms by which transient immune activation leads to persistent changes in neuronal processes, such as activity-dependent c-Fos induction, resulting in long-lasting memory dysfunction. This first includes characterization of peripheral immune responses and subsequent neuroimmune signaling induced by subchronic immune challenge that interact with neural processes important for memory. Additionally, even if we observed no sustained neuroimmune activity, such as blood-brain barrier permeability or microglial activation, that correlate with the long-lasting memory deficits, it is possible that enduring molecular changes in neuroimmune mediators, rather than over neuroimmune activation, contributes to the memory deficits observed in males and in females after immune challenge. Future cell-type specific RNA-sequencing analyses will provide insights into the long-lasting molecular substrates associated with memory deficits. As sex-specific patterns of memory deficits along with distinct gene expression changes in the hippocampus of males and females months after immune challenge, future studies will need to delineate the sex-specific mechanisms by which transient systemic immune activation induces changes in neural function, leading to long-lasting memory dysfunction.

Future work needs to focus on the long-lasting changes in neuroplasticity-related substrates after subchronic immune challenge. Given the long-lasting changes in *fos* mRNA levels and c-Fos protein levels are observed after subchronic immune challenge, it is possible that such long-lasting changes in gene expression may be mediated by molecular mechanisms that induce long-lasting transcriptional changes, such as epigenetic modifications. Future work could investigate whether subchronic immune challenge induces persistent changes in chromatin modifications (e.g. histone acetylation or methylation) at promoters or gene bodies of c-Fos targets after training in a memory paradigm (e.g. context fear conditioning) in males and in females. As expression of DNA modifying enzymes is dysregulated months after immune challenge (e.g. *Gadd45b, Gadd45g*), it would be interesting to determine whether subchronic immune challenge increases DNA methylation at the promoters of these targets thereby reducing their expression. As c-Fos induction is not the sole mechanism important for fear memory formation, future work could broaden the scope of research on the activity-dependent mechanisms underlying memory formation by determining whether subchronic immune challenge alters A) protein levels of other immediate early genes whose mRNA levels were decreased months after the inflammatory insult in Chapter 4, including Arc, Erg1, Nr4a1/2/3, Npas4, B) other plasticity-related synaptic proteins (e.g. GluA1, GluN2A/B), and C) changes in immediate early genes induction or in synaptic plasticity related proteins after other memory tests in which we observe impairments, including object recognition.

Sex-specific transcriptional changes are also observed in the hippocampus after a secondary, acute immune insult and may suggest that prior exposure to an inflammatory insult alters hippocampal function and responses to environmental changes, such as subsequent inflammatory insults, differently in males than in females. Given the differences in both

magnitude and functions of targets and pathways in the hippocampus of males and females described in Chapter 4, it is likely that a secondary immune challenge alters hippocampal function via sex specific mechanisms. Therefore, this subchronic immune challenge model may be used in future studies to determine how prior mild, systemic inflammatory insults induce changes in the brain, emotion, and cognition in males and in females after a secondary, acute immune challenge. As males show a reduced transcriptional response in the hippocampus when a secondary acute peripheral immune challenge is given months after subchronic immune challenge (Chapter 4, Figure 2), these multiple inflammatory insults may serve a neuroprotective role and prevent impairments in object recognition or fear conditioning in males. Given the enhanced transcriptional response in the hippocampus of females, multiple inflammatory insults may make females less resilient to memory deficits in fear conditioning.

We did not observe depression- or anxiety-like behaviors either shortly or long after immune challenge. Nevertheless, it is also possible that changes in anxiety-like or depressive-like behaviors may also be observed after a secondary insult. In prior animal models, affective outcomes, including depression-like behavior, become worsened in animals receiving an immune challenge a month after prior neuroinflammatory insult (Fenn et al., 2014). Therefore, it is likely that a prior inflammatory insult induces persistent changes in the brain that makes animals more vulnerable to subsequent exposure to inflammatory stimuli. As we performed subchronic immune challenge using only one type of immune stimulant, such as LPS or Poly I:C, and observed tolerance after multiple injections in both males and females (Chapter 2; Figure 1), it would be interesting to determine whether using repeated peripheral injections using different types of immune stimulants or different types of inflammatory insults (e.g. surgical interventions or heart attack model followed by immune challenge) would have similar consequences for

memory and affective processes. It would also be interesting to understand how prior subchronic immune challenge impacts memory or affective processes after exposure to other types of environmental insults, including stress or drugs.

Conclusions

The studies in this dissertation characterized a mouse model to understand how systemic immune activation leads to long-lasting changes in memory and cognition in males and in females. We have observed sex-specific patterns in the emergence and persistence and types of memory deficits after subchronic immune challenge, with females showing deficits in object recognition memory in the weeks and months after subchronic immune challenge and males showing deficits in object recognition and fear conditioning months after immune challenge. Specific memory processes are persistently altered by a mild, systemic inflammatory insult as memory formation, but not memory retrieval, is impaired after subchronic immune challenge.

Our model of subchronic immune challenge selectively impacts memory processes as affective behaviors, such as anxiety-like and depressive-like behaviors, are not observed months after subchronic immune challenge. While prior studies have focused on the role that neuroimmune activation plays in memory dysfunction after a systemic inflammatory event, our work suggests that plasticity-related mechanisms, such as alterations in c-Fos induction after training, may underlie the long-lasting deficits in memory formation, at least in males. In females, it is possible that dysregulation of monoaminergic signaling is responsible for the memory deficits. Our findings provide insights into the mechanisms that mediate long-lasting changes in memory and cognitive functions after a mild, systemic inflammatory event in males and in females and informs development of novel therapeutic strategies for cognitive and memory decline in men and in women after a mild systemic inflammatory event.

Figure

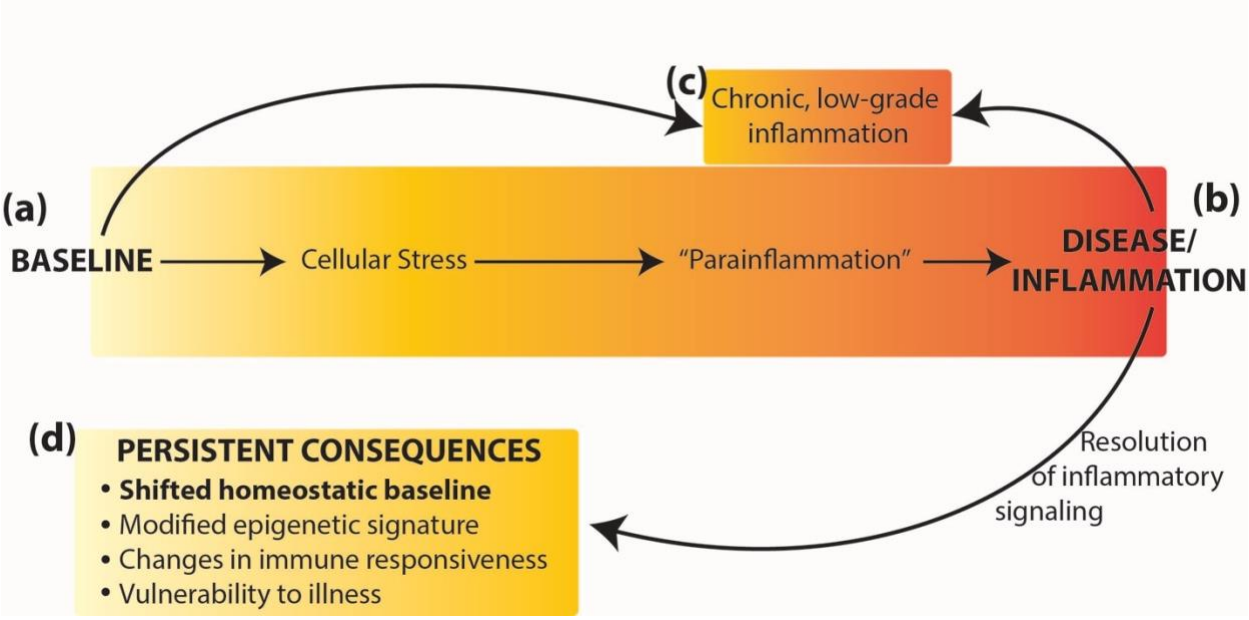


Figure 6.1 Neuroimmune activation occurs along a continuum from the naïve (homeostatic) baseline (A), to an active inflammatory state **(B)** or chronic inflammation **(C)**. We propose that resolution of inflammatory signaling does not result in return to the original baseline, but rather results in persistently altered homeostatic baseline **(D)** mediated by epigenetic changes in the brain. Figure adapted from Chovatiya and Medzhitov (2014).

APPENDIX

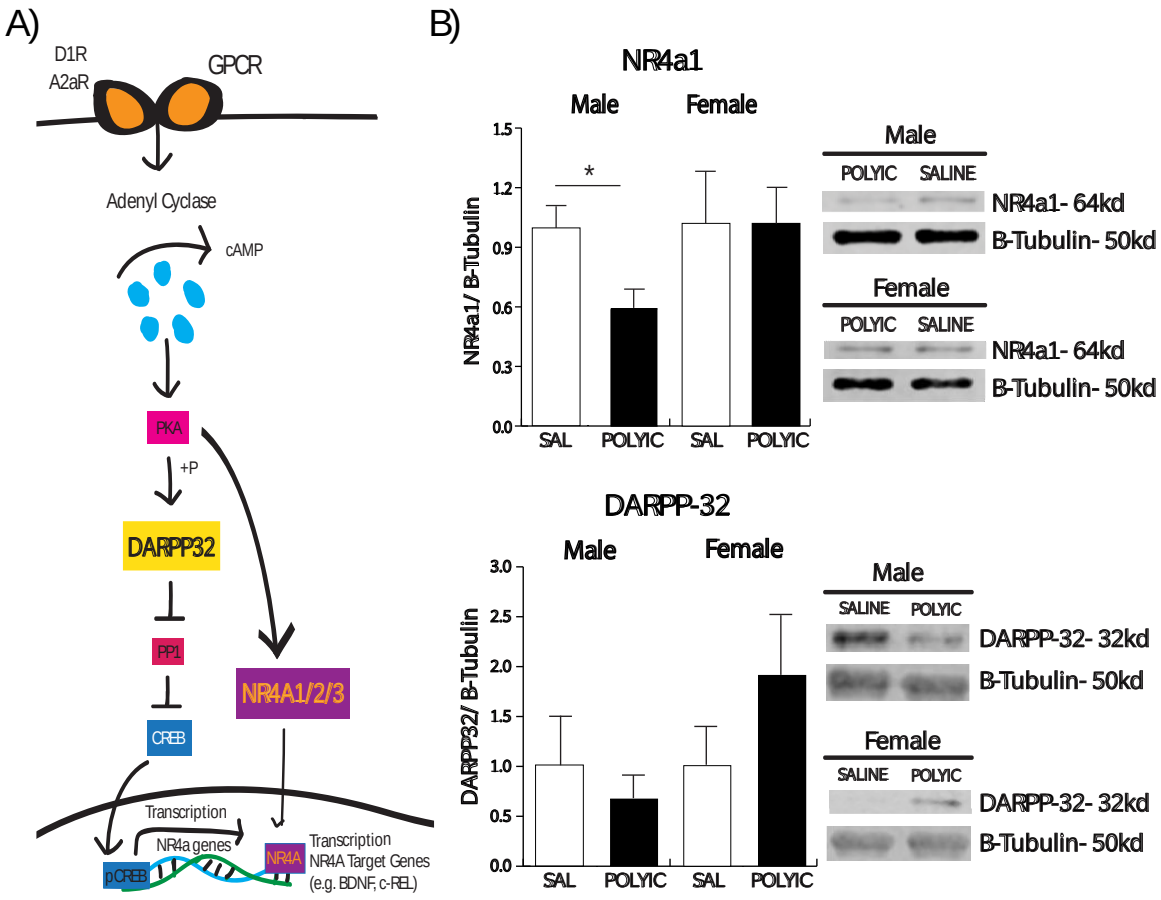


Figure A1. cAMP-associated targets in male and female hippocampus. A) cAMP-signaling with DARPP-32 and NR4a nuclear receptors. B) representative western blot and quantification of hippocampal NR4a1 and DARPP-32 levels 8 wks post immune challenge. $p < 0.05$

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