T-CELL-MEDIATED MATERNAL IMMUNE ACTIVATION DURING PREGNANCY:
EFFECT ON COGNITIVE FUNCTION IN OFFSPRING
VARYING IN GENETIC BACKGROUND

By

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ABSTRACT OF THE DISSERTATION

T-cell-Mediated Maternal Immune Activation During Pregnancy: Effect on Cognitive Function in Offspring Varying in Genetic Background

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Stimulation of the immune system during pregnancy, known as maternal immune activation (MIA), can cause long-lasting neurobiological and behavioral changes in the offspring. This phenomenon has been implicated in the etiology of developmental psychiatric disorders, such as autism and schizophrenia. Much of this evidence is predicated on animal models that rely on activation of the innate immune system using bacterial agents such as LPS and/or viral mimics such Poly I:C, both of which act through toll-like receptors. Fewer studies have examined the role of direct activation of maternal T-cells during pregnancy and whether this also results in altered neurobiological and behavioral outcomes in offspring. Bacterial ‘superantigens’, such as Staphylococcal Enterotoxin A and B (SEA; SEB), are microbial proteins that activate CD4+ T-cells and cause prominent T-cell proliferation and cytokine production. We injected pregnant and non-pregnant adult female C57BL/6 and Balb/c mice with 5μg of SEA, SEB, or 0.9% saline, and measured splenic T-cell-derived cytokine concentrations (viz., IL-2, IFN-γ, IL-6, and IL-4) 2 hours later; animals injected with SEA were also measured for splenic
concentrations of TNF-\(\alpha\) and IL-17A. Half of the injected pregnant animals were brought to term, and their offspring were tested on a series of cognitive tasks starting at six weeks of age (postnatal day 42 [P42]). These tasks included a social interaction task, the elevated plus maze (EPM), an object recognition (OR) task, prepulse inhibition (PPI) of sensorimotor gating, and the Morris water maze (MWM). Results showed that SEA and SEB induced significant concentrations of all measured cytokines, and in particular IFN-\(\gamma\), in both strains of pregnant mice when compared to controls. While C57BL/6 animals responded with significantly greater concentrations of most proinflammatory cytokines to SEA exposure, Balb/c mice had greater cytokine concentrations to SEB exposure. In addition, pregnant animals exhibited reduced production of proinflammatory cytokines, and in the case of Balb/c SEB-injected mice, increased anti-inflammatory cytokine IL-4. Behavioral results showed distinct phenotypes among offspring from SEA- or SEB-injected mothers. C57BL/6 offspring from SEA-injected mothers displayed decreases in social behavior and spatial learning, and increases in anxiety, locomotion, interest in a novel object, short-term spatial memory, and depressive-like behaviors. Balb/c offspring from SEB-injected mothers displayed decreases in spatial learning, and increases in social behavior, anxiety, sensorimotor gating abilities, and depressive like-behaviors. Overall, through the novel use of SEA and SEB as prenatal immune challenges, we were able to elicit significant cytokine production in the mothers and distinct behavioral profiles in the offspring that both mirrors and diverges from previous models of maternal immune activation in important ways. We conclude that T-cell-mediated maternal immune activation is a valid and valuable model for studying the effects of prenatal
immune challenge on neurodevelopmental and behavioral alterations in offspring relevant to psychological diseases.
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CHAPTER 1: INTRODUCTION

1. General Overview of the Project

The immune system evolved to protect the body against potentially harmful microbial organisms, in particular bacteria and viruses. When microbes enter the body, the immune system mounts an immune response. There are multiple cellular and chemical components to this response. An essential piece is the production of chemical messengers called cytokines, which are generally categorized as either pro-inflammatory or anti-inflammatory, and serve to either enhance or dampen the immune response. The latter is sometimes referred to as an inflammatory reaction; since an ‘inflammatory response’ is a clinical manifestation of the immune response. However, inflammation does not always have to involve the immune system – although it is rare for inflammatory responses not to involve immune cells and their chemical products.

Most cells of the immune system produce cytokines, and in the present dissertation, focus is mainly on T cell-derived cytokines. Though an effective immune response consists of both pro- and anti-inflammatory cytokines, unregulated levels of either type can be dangerous not only to health, but also to normal cognitive functioning. This is especially true in pregnancy, during which by-products of maternal biological processes can be passed to the fetus. Therefore, when a heightened maternal immune response (known as ‘maternal immune activation’ or MIA) occurs during pregnancy, the pro-inflammatory cytokines produced by the maternal immune system can cross the placenta and impact the developing embryonic brain. It is believed that this can cause long-lasting effects on the development and functioning of the brain of the offspring, and in particular, a disruption of normal cognitive development.
There are several models of MIA, but these do not fully encompass the full range of the immune response. This is an important limitation, since it is important to understand how all the different elements of the maternal immune response can impact the neurobehavioral development of the offspring. Models of MIA to date utilize only one arm of the immune system’s wide-ranging response mechanisms. The majority of experimental studies investigating the consequences of MIA on offspring development utilize bacterial endotoxins, such as lipopolysaccharide (LPS) or viral mimics such as Polyinosinic:polycytidylic acid (poly IC). Both LPS and poly IC act through toll-like receptors (TLRs) found on macrophages, which are cells that serve a variety of immediate and so-called innate immune functions. Macrophage activation by poly IC or LPS does not recruit T cell involvement, as would normally occur during a normal immune response to a cellular (i.e. bacteria) or viral infection. In contrast, a class of T cell stimuli, referred to as ‘bacterial superantigens’, can bind directly to T-cell receptors (TCRs), cause activation of an exponential number of CD4+ T-cells and generate prominent T-cell proliferation, cytokine synthesis and cytokine release. By utilizing these superantigens, a new model of MIA can be developed and the effects of these bacterial agents on offspring development can be determined.

Therefore, the current project will make use of two bacterial T-cell superantigens, staphylococcal enterotoxins A and B, which will not only complement previous studies, but will also add an important new perspective on the immunological origins of psychiatric disease.
2. The Immune System

The immune response is made up of multiple, complex cellular and molecular elements that proceed through a well-defined series of stages, each comprised of unique effector mechanisms. The immune system refers to a heterogeneous group of specialized cells that circulate throughout the body and maintain the function of protecting against infectious disease. The host, or the organism, is protected from infection by bacterial and/or viral microorganisms through the ability of the immune system to recognize “self” versus “non-self” proteins. Protein-derived peptide sequences are considered ‘antigens’, and those expressed by invading pathogens are recognized by the immune system as “foreign” (i.e. non-self) and trigger an immune response. The ability to recognize foreign antigens is developed by means of negative and positive selection, in which immune components that recognize self-proteins are killed off early in development to prevent unnecessary self-directed responses later in the organism’s life. Problems in this selection process can be the basis for many immunological problems such as autoimmune diseases.

2.1. General Overview of the Immune System

The immune system is made up of different types of cells, but all develop through one of two main cell lineages: the myeloid cell lineage and the lymphoid cell lineage. The myeloid lineage is made up of three types of cells: monocytes, granulocytes, and mast cells. Monocytes circulate in the blood, and travel to most organs where they can be retained permanently to mature into tissue macrophages; as such, they serve to monitor and remove sequestered or proliferating pathogens, as well as dead tissue cells. Once a macrophage detects a foreign substance, it will engulf the cell or large protein using phagocytosis (literally “eating up”) and break it up so that it is no longer a threat to the
Phagocytosis is a key feature of cells of the myeloid lineage, especially the granulocytes, which are also called polymorphonuclear cells (PMNs), and can be further differentiated into basophils, eosinophils and neutrophils. The latter are the major PMNs and phagocytic cells, and like monocytes, they can circulate in the blood stream until they are activated and sent to the extracellular space to clear invading pathogens. Mast cells are found in tissues closest to the external environment, such as the mucosa of the gastrointestinal and respiratory tracts, and are also found throughout the brain (Abbas, Lichtman, & Pillai, 2011). These cells release cytokine chemical messengers and neurotransmitters like histamine and serotonin to facilitate an immune response and help clear out foreign debris. Clinically, mast cells are key to allergic responses, such as hay fever.

The lymphoid lineage is made up of lymphocytes that fall into three main cell types: B-cells, T-cells and Natural Killer (NK) cells. These cells reside mainly in tissues of the lymphatic system, including the reticular fibers within the organs that converge on lymph nodes, the spleen and the thymus (Kusnecov & Anisman, 2013). When lymphocytes encounter antigen, they respond in a number of ways, including destroying the invading microbe, duplicating itself (through mitosis) to seek out and destroy further pathogens, and creating memory cells with receptors specific for the target antigen (Abbas et al., 2011). Hence, the lymphoid lineage of cells is crucial in both clearing infected cells and also in creating specific immunoglobulins (the basis of antibody production) that can be saved and stored in case of future infection.

In response to injury, insult and foreign invaders, the immune system reacts in a systemized way to isolate and remove the danger. This response, known broadly as...
inflammation, is characterized by endocrine, autonomic and behavioral changes and is triggered by soluble mediators that are produced at the site of infection by activated accessory immune cells (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008). These mediators are known as pro-inflammatory cytokines, which coordinate the local and systemic inflammatory response to microbial pathogens. This response involves initiating tissue repair, maintaining basal cognitive functions, and preserving homeostatic function (Giunta, 2008). When inflammation occurs inside the brain - known as neuroinflammation – the same biological mechanisms occur in order to repair any damage that has resulted.

The above immune cell types and mechanisms will be explained in further detail below.

2.2. Innate and Adaptive Immunity

The immune system responds to any infectious challenge with a two-tiered approach: first, a generalized targeting and neutralizing of the pathogen, and second, a specialized attack on the specific invader. The former approach is mediated by the innate immune system, while the latter is mediated by the adaptive immune system. Both responses are necessary for an organism’s survival, and each relies and depends on the other for a complete and comprehensive immune response.

2.2.1. Features of the Innate Immune System

All multi-cellular organisms have some sort of innate immune defense. Innate immunity is built into an organism’s genome which codes for the ability to perfectly distinguish between self and non-self substances (Medzhitov & Janeway, 1997). There are three stages of defense in innate immunity: anatomical, cellular and humoral. The first stage of
the innate immune response is made up of the physical and biological surface barriers protecting an organism from external pathogens. For example, the skin acts as a mechanical shield for the body (Hazlett & Wu, 2011), while tears expel pathogens (Haynes, Tighe, & Dua, 1999; Hazlett & Wu, 2011), and saliva is secreted to immediately neutralize invading microbes using immunoglobulin A (Amerongen & Veerman, 2002). These initial defenses are at work continually, protecting the organism from the constant assault of microbial entities seeking access to our internal systems.

However, once a pathogen gains entry into the organism’s blood stream, the second and cellular stage of the innate immune system is activated. Until Charles Janeway Jr.’s revolutionary proposal at the 1989 Cold Springs Harbor Symposium on Immune Recognition (Janeway, 1989), very little was known about not only the cellular workings of the innate immune response, but about the concept of the innate immune system. Earlier studies had focused on the components of the adaptive immune system (discussed below), such as the major histocompatibility complexes (MHC) and lymphocyte activation, but little was known about how those responses were initiated (Medzhitov, 2009). Janeway theorized that it was not the antigen receptors of the adaptive immune system that detect the origins of an antigen, but cellular receptors in the innate immune system that identify pathogens and signal to the adaptive immune system the presence of microbial entities (Janeway, 1989). He proposed that these receptors are hard-wired to recognize conserved structures of microbial surface chemicals, now known as pathogen-associated molecular patterns, or PAMPs. This proposal was the first to indicate an ancient and germ-line-encoded system initiating an immune response, and one that connected animal immunity to that of non-vertebrates, which lack adaptive immunity.
Though Janeway had not identified these pattern recognition receptors (PRRs), it was only a matter of time before immunology research confirmed his theory.

The Toll-like receptor (TLR) family is the most highly studied and best characterized class of pattern recognition receptors in vertebrates. Toll-like receptors are found on many different types of cells, most notably professional phagocytes and antigen-presenting cells (APCs) like macrophages and dendritic cells (Kawai & Akira, 2010; Zaremba & Godowski, 2002), however they are found in B-cells, epithelial cells, and even natural killer (NK) cells (Rehli, 2002; Zaremba & Godowski, 2002). Though all TLRs detect molecular patterns unique to pathogens, each one is specialized for specific types of patterns. For instance, TLRs 1, 2, 4, 5, and 6 recognize bacterial products, while TLRs 3, 7, 8, and 9 focus on viral detection (Iwasaki & Medzhitov, 2004). In some cases, TLRs recognize the molecular structure of a pathogen itself as foreign, and in other cases they recognize an immune challenge by its accessibility to a TLR. Some TLRs are located in intracellular compartments (Matsumoto et al., 2003), and thus the nucleic acid they recognize and become activated by must always be from something foreign, since host nucleic acid is not found in intracellular compartments (Diebold, Kaisho, Hemmi, Akira, & e Sousa, 2004; Iwasaki & Medzhitov, 2004; Lund, Sato, Akira, Medzhitov, & Iwasaki, 2003).

Aside from the TLR family, other PRRs are present on the surfaces of innate immune cells to detect the presence of microbial invaders. Intracellular receptors found in the cytosol known as Nod-like receptors (NLRs) sense fragments of bacterial peptidoglycans that enter the cell through phagocytosis or pores in the cell wall (Girardin,
Boneca, Carneiro, et al., 2003; Girardin, Boneca, Viala, et al., 2003). Like TLRs, a family of receptors known as Dectins are transmembrane proteins that have been shown to play an important role in detecting bacterial and fungal components (G. D. Brown & Gordon, 2001; Gross et al., 2006; Taylor et al., 2007). Viruses, and specifically RNA viruses (Yoneyama et al., 2004), can be detected by intracellular sensors known as RIG-1 and MDA-5, which play a large role in antiviral immunity (Kawai & Akira, 2008). Other non-TLR PRRs including pentraxins (Bottazzi et al., 2006), collectins and ficolins (Holmskov, Thiel, & Jensenius, 2003) – all implicated in the humoral arm of the innate immune system – play a critical role in the innate immune system’s ability to sense invading pathogens and respond appropriately.

Depending on which type of TLR or other PRR is activated, a variety of different responses can occur. For almost all TLRs and many other PRRs, an intracellular protein known as MyD88 is used to activate transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinases (MAPKs) to generate the production of inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor (TNF) (Abbas et al., 2011; Kawai & Akira, 2010). Other TLRs, usually stimulated by viruses, ultimately produce type-I interferon cytokines interferon-α and interferon β (IFN-α and IFN-β), which are known for their anti-viral properties (Kawai & Akira, 2010). Dendritic cells (DCs) with different TLRs can also produce cytokines such as IL-12, TNF and IL-10 which aid in the protection against infection (Iwasaki & Medzhitov, 2004). In addition to inflammatory cytokine release, cells such as macrophages that detect an invader will also excrete proteins known as chemokines which attract leukocytes to the infected area (Janeway Jr, Travers, Walport, & Shlomchik,
Lastly, plasma proteins called complement activate a series of proteolytic reactions on microbial surfaces, coating them with fragments that are recognized and bound by phagocytic receptors on macrophages and other phagocytes (Janeway Jr et al., 2001).

All of the products of bacterial and viral recognition by the innate immune system lead to the induction of inflammation, characterized by swelling, heat, pain and redness at the site of infection. During inflammation, blood vessels dilate and allow for increased blood flow and increased permeability. Cytokines released by the cells of the innate immune system facilitate the binding of leukocytes to the endothelial walls of the blood vessels, while the release of chemokines draws them to the site of infection (Iwasaki & Medzhitov, 2004; Janeway Jr et al., 2001). Neutrophils are attracted to these areas in large numbers, and along with macrophages, detect, ingest and destroy harmful substances. By releasing inflammatory cytokines, chemokines and complement, the cellular stage of the innate immune system provides a critical and necessary stage in host defense and recovery from infection.

The last phase of the innate immune system, the humoral phase, is inextricably tied to the complex workings of the adaptive immune system. The adaptive immune system, which will be discussed in greater detail below, is designed to recognize specific fragments of antigen and respond in its own specialized way. The inflammatory response of the innate immune system enhances the movement of lymph (fluid comprised of white blood cells) containing antigen and antigen-bearing cells into lymphoid tissues, which is crucial for the eventual binding of T and B lymphocytes to antigen and their activation (Abbas et al., 2011; Banchereau & Steinman, 1998; Janeway Jr et al., 2001). In addition,
circulating dendritic cells can recognize and bind to various microbes, which they ingest, break down into smaller peptide fragments (which become the cognate antigens – i.e. which stimulate lymphocytes), and then present those pieces to T-lymphocytes in the lymphoid organs. The transit of DCs to the lymphoid organs and lymph nodes is mediated by TLR-induced downregulation of chemokine receptors and an increase in the receptors for lymphoid chemokines (Iwasaki & Medzhitov, 2004). Crucially, DCs also upregulate co-stimulatory molecules CD80 and CD86 (also known as B7-1 and B7-2) which are necessary for activation of T-cells once bound to an antigen (Banchereau & Steinman, 1998; Iwasaki & Medzhitov, 2004).

2.2.2. Features of the Adaptive Immune System

The adaptive immune system is unlike its innate counterpart, being present only in jawed mammalian vertebrates (Flajnik & Kasahara, 2010). It reacts in a highly specific and specialized manner, but to a diverse set of peptide fragments derived from the microbial world, and induces a powerful response involving cytotoxicity and regulation by T-cells, and activation of B-cells to produce antibody. In addition, the adaptive immune system creates memory cells that can be used to fight future infections of a similar nature, and creates optimal responses for those attacks. Without all of these features working perfectly together, a host will not be able to respond as efficiently and effectively as it needs to an immune challenge.

The main class of cells necessary to mount an adaptive immune response is lymphocytes. Lymphocytes are the only cells in the body that recognize specific fragments of pathogens in order to respond in a specialized manner (Abbas et al., 2011). These “fragments” are called ‘antigens’ (i.e. “antibody-generating”). The term antigen, is
actually restricted to peptide fragments of 8-12 amino acids in length (which is what T and B cell receptors are specialized to recognize), although for practical purposes, most non-host proteins, bacteria and viruses are referred to as antigens, in order to denote their “foreignness” and ability to induce an immune response. Sometimes reference is made to “self antigens,” which implies that lymphocytes are capable of recognizing (but not necessarily responding to) peptides derived from host proteins. Less will be said about this, as it is a huge topic relevant to discussions of autoimmunity and autoimmune disease.

Both T- and B-lymphocytes begin development in the primary lymphoid organs, B-cells in the bone marrow and T-cells in the thymus. They then travel from those organs as naïve (or virgin) lymphocytes to the secondary lymphoid organs—the lymph nodes and spleen—where they encounter antigen from either tissues or from the blood (Butcher & Picker, 1996) and if activated by antigen, become effector cells (Abbas et al., 2011). As described earlier, antigens are displayed (or “presented”) by professional APCs, such as dendritic cells and/or macrophages. When B-cells bind antigen, they are activated, enter mitosis (i.e. proliferate) and mature into antibody-secreting cells with an increased cytoplasmic volume rich with protein synthesis (i.e. antibody production), and hence in this effector state are called ‘plasma cells.’ A subset of these plasma cells will cease antibody production and become dormant as memory cells. Memory B-cells are present in the body in order to rapidly mature and proliferate upon binding to a repeat encounter with a specific antigen (Abbas et al., 2011). The quality and quantity of antibody during this secondary response is much improved (has greater affinity and avidity to antigen) and more abundant. Moreover, the generation of antibody is more rapid.
An immature, or naïve, T-cell can differentiate into multiple types of effector T-cells. Cytotoxic T-cells (TC) destroy virally-infected self-tissue and tumor cells; Helper T-cells (Th) modulate both adaptive and innate immunity by facilitating the maturation of B-cells and by activating TC cells and macrophages; and regulatory T-cells (Treg) provide a shut-off mechanism for a T-cell-mediated immune response (Kusnecov & Anisman, 2013). Similar to B-cells, a portion of activated T-cells persist as memory cells, primed to respond more rapidly and robustly to repeat antigen encounters (Sallusto, Lenig, Förster, Lipp, & Lanzavecchia, 1999). The third lymphocyte cell type are the natural killer (NK) cells, which destroy virus-infected or cancerous cells by first releasing perforins, cytolytic enzymes that create holes in the membranes of cells, and then by emitting granzymes, which enter the punctured cell and cause apoptosis (Kusnecov & Anisman, 2013). The NK cells are larger than T and B lymphocytes, and are referred to as large granular lymphocytes (LGLs).

There are three types of receptor molecules in the adaptive immune system whose primary role is to recognize and bind antigen: antibodies, MHC molecules, and T-cell receptors. The first, antibodies, play a primary role in the humoral arm of the immune system, recognizing a diverse array of microbes and keeping a record of those interactions for future infections. Antibodies, also known as immunoglobulins (Ig), can either be situated on the membrane of a B-cell (where they serve as the primary cell surface antigen receptor for B cells) or exist as secreted antibodies (essentially soluble receptors) that reside in tissues or circulate in blood and lymph (Abbas et al., 2011). When membrane-bound Ig receptors recognize antigen, naïve B-cells are activated to not only create more B-cells with the same antigen-recognizing antibody, but to also
differentiate those new cells into multiple kinds of new B-cells that can secrete more antibodies or become circulating memory cells (Arpin, Dechanet, Van Kooten, & Merville, 1995). Antibodies themselves come in five different Ig forms–IgG, IgM, IgA, IgE, and IgD–and perform a variety of functions, from tagging an antigen to be phagocytosed by APCs, to activating NK cells to destroy the pathogen (Abbas et al., 2011; Burton & Woof, 1992). The memory cells and antibodies produced will not only respond quicker but with a more robust attack on subsequent encounters with the target antigen (Kimman, Westenbrink, & Straver, 1989). Indeed, in a memory response, the most abundant molecular form of antibody will be IgG, which, as stated earlier, will have greater affinity and avidity for antigen. Overall, generating antibodies is critical to host defense, as it is the primary means of ridding the body of infection. For example, death from AIDS is largely due to a failure of mounting adequate humoral immunity, since failure of T cell help (due to HIV infection of monocytes and T cells) weakens antibody production by B cells thereby compromising elimination of various environmental pathogens.

The second and third antigen-binding molecules in the adaptive immune response, the major histocompatibility complex (MHC)–or human leukocyte antigen (HLA) in humans–and the T-cell receptor (TCR) work in tandem to activate T-cells and stimulate another branch of the adaptive immune system. Antigen-presenting cells, like dendritic cells, have digested pathogen proteins (i.e. antigenic fragments) presented on their surface by proteins known as MHC molecules. MHC molecules present the antigen to T-cell receptors (TCRs), which bind to the antigen and ultimately activate the T-cell. (It should be noted, however, that a co-stimulatory signal is also needed for activation
There are two types of MHC molecules, MHC class I and MHC class II, that each interacts with specific cell surface molecules (called CD molecules) on T-cells (Abbas et al., 2011; Norment, Salter, Parham, Engelhard, & Littman, 1988). MHC I molecules, found ubiquitously on all nucleated cells in the body, present antigen to CD8+ T-cells, while MHC II molecules, expressed only on dendritic cells, B-cells, macrophages and a few other cell types, present antigen to CD4+ T-cells (Abbas et al., 2011; Engelhard, 1994). This specificity is due to the nature of the different T-cell responses. The CD4+ T-cells, also known as T-helper (Th) cells, once activated by antigen, facilitate the stimulation of antibodies, phagocytes and production of cytokines in the service of eliminating extracellular antigens. The CD8+ T-cells, on the other hand, mainly recognize cytosolic antigens and work to kill the host cells presenting the microbe (Engelhard, 1994). It is due to the specificity of the MHC molecules that display either extracellular or intracellular antigens to TCRs that the different types of T-cells know how to respond in the most effective way.

The adaptive immune system is an evolutionary development in the mammalian immune system designed not only to respond to specific microbes in specialized ways, but also designed to remember those interactions for future encounters. Circulating lymphocytes interact with antigen or APCs in order to activate their effector functions. With the help of MHC class I and II molecules and TCRs, T-cells are able to determine the origin of a pathogen and the response necessary for its elimination. Antibodies on B-cells and in the extracellular space bind to antigen and produce a myriad of responses from activating B-cells to tagging the antigen for removal. Between the production of T-
cells, cytokines, antibody, plasma cells and memory cells, the adaptive immune system provides thorough protection of the organism from present and future microbial challenges.

2.3. Cytokines: Chemical Messengers of the Immune System

The cognate processing and responding to microbial antigens discussed above would be fully limited, and ultimately ineffective, if it were not for chemical messaging between the various immune cells. As the chemical messengers of the immune system, cytokines and chemokines play a crucial role in initiating and mediating an immune response, and are essential to determining the various different ways in which the immune system can respond to invading pathogens. Cytokines are proteins and glycoproteins synthesized and secreted by various types of cells in order to act as signals to regulate the immune response to injury and infection (Clark, Michael, Keegan, & Tonelli, 2014). They act via functional cytokine receptors and influence cells that are distant from their source (an endocrine effect), adjacent to their source (a paracrine effect) and can even influence the producing cell itself in a feedback-like manner (an autocrine effect). Chemokines, a subclass of cytokines, are characterized by their ability to induce directed chemotaxis, which is the attracting of cells to or away from a given tissue site (Kusnecov & Anisman, 2013). This typically occurs during an immune response in a given area of the body, which requires that chemokines be released in order to “flag down,” as it were, leukocytes that are passing by and divert them to the site of inflammation. The recruited lymphocytes and monocytes will then be able to contribute to the elimination of the microbial antigen.
Like the hormones of the endocrine system, cytokines are frequently regulated in cascades or a step-wise manner, whereby initiation of the first wave of cytokines leads to the later production of other, and functionally different cytokines. Receptors for cytokines can also be found in a soluble form, which can inhibit the binding of the cytokine to its membrane-bound receptor through competitive ligand binding and prevent the intended activity of the cytokine (Cameron, 2003). This obviously serves a regulatory role when there is an over-abundance of a given cytokine. On the other hand, as in the case of a cytokine known as Interleukin-6 (IL-6), sometimes the binding of the cytokine to its soluble receptor can form a complex that enhances the biological activity of the cytokine (Scheller, Chalaris, Schmidt-Arras, & Rose-John, 2011). Overall, cytokines form a network with highly complex systems of interaction.

To differentiate between the varied biological activities of cytokines, they are usually described and characterized as either pro-inflammatory or anti-inflammatory. Pro-inflammatory cytokines stimulate and augment an inflammatory response and speed up the process of eliminating harmful substances and pathogens from the body by either activating cells to function more efficiently or altering physiological aspects of the host – such as increasing body temperature or body metabolism – to attain a more pro-inflammatory state (Abbas et al., 2011). Recruitment of immune cells such as neutrophils and macrophages is also an effect of the release of pro-inflammatory cytokines. Anti-inflammatory cytokines are released to decrease and eventually terminate an immune response. This occurs by either inhibiting cell function, decreasing the synthesis of other cytokines or blocking the production of cytokines by T-cells (Cameron, 2003). The right
balance between pro- and anti-inflammatory cytokines is necessary for a healthy and efficient immune response.

There are hundreds of identified cytokines, characterized by their function, structure and purpose. To simplify the nomenclature for these proteins, there are five major categories of cytokines, lending their initials to the cytokine names. These are interleukins (IL), interferons (IFN), tumor necrosis factors (TNF), transforming growth factors (TGF) and miscellaneous hematopoietins. In the present chapter, focus will be on seven specific cytokines from three of these categories: IL-1, IL-2, IL-4, IL-6, IL-17, TNF-α, and IFN-γ. These cytokines play key roles in either pro- or anti-inflammatory immune reactions.

2.3.1. Interlukin-1

The IL-1-related group of cytokines is made up of a number of different molecules, most notably IL-1α and IL-1β, both of which are generated by mononuclear and epithelial cells. The former alpha-form of IL-1 is typically membrane bound, and rarely secreted in high amounts; the beta-form is readily secreted, and functions to exert strong paracrine and endocrine functions. Early expression of brain IL-1 mainly takes place in activated microglia and perivascular macrophages, whereas later expression occurs in astrocytes. However, all resident brain cells, including neurons and vascular endothelial cells, can express IL-1 (Simi, Tsakiri, Wang, & Rothwell, 2007). Both IL-1α and IL-1β are crucial for initiating a fever response, as well as activating lymphocytes and inducing a wide variety of acute phase response genes (Cameron, 2003). They upregulate an immune response and aid in the migration of immunocompetent cells, such as phagocytes
and lymphocytes, to sites of infection (Cameron, 2003). IL-1α and IL-1β and the other members of the IL-1 family are crucial to a proper inflammatory response to immune challenges.

2.3.2. Interleukin-2 and Interleukin-4

Both IL-2 and IL-4 utilize a common gamma (γ) chain in their receptor and invoke lymphocyte activation and differentiation (Cameron, 2003). Interleukin-2, a proinflammatory cytokine, is mainly secreted by activated T-cells, can induce clonal expansion of T-cells, and it exerts effects on B-cells, NK cells, macrophages and neutrophils. Its presence can be considered a marker for T-cell activation. There is evidence to suggest that proper IL-2 signaling is required to induce regulatory T-cells and/or eliminate abnormally activated T-cells (Cameron, 2003).

Interleukin-4 is an anti-inflammatory cytokine recognized as being of T cell origin. However, it is also known to be produced by mast cells, basophils, and NKT cells. Moreover, IL-4 targets many cell types, including T-cells, B-cells and macrophages (Cameron, 2003). In particular, IL-4 is a necessary cytokine for regulating antibody production by B-cells. One feature of IL-4 is to switch production of antibody from IgG or IgM to the IgE form, which serves to mediate allergic reactions, inhibition of macrophage activation, and influences the effects of IFN-γ on macrophages. While both bind to the same γ receptor chain, IL-2 and IL-4 play vastly different roles in the process of inflammation. The former cytokine is considered pro-inflammatory, while IL-4 is thought to promote anti-inflammatory effects.
2.3.3. Interleukin-6
Interleukin-6 is another heavily studied cytokine, which utilizes the ubiquitously-expressed glycoprotein 130 (gp130) receptor (Scheller et al., 2011). It is produced by fibroblasts, endothelial cells, macrophages, T-cells and B-cells, mediating many critical immune-related functions, such as inducing fever, release of hormones and acute phase proteins, and promoting B- and T-cell expansion and differentiation upon injury or infection (Cameron, 2003). It also plays a pivotal role during the transition from innate to adaptive immunity and enhances monocyte recruitment (Scheller et al., 2011). Because of the above roles IL-6 plays in the immune system, it is generally regarded as a proinflammatory cytokine; strangely, however, IL-6 is also secreted by Th2 cells and has also been shown to mediate anti-inflammatory responses as well (Cameron, 2003; Scheller et al., 2011). This highlights some of the limitations that exist in categorizing cytokines as being inflammatory or proinflammatory.

2.3.4. Tumor necrosis factor-α and Interferon-γ
TNF-α is a member of the tumor necrosis factor family of cytokines, which is characterized by their ability to induce cell death. TNF-α, initially identified for its ability to kill tumor cells, can be found in either a membrane-bound or soluble form and its receptor is ubiquitously expressed on all cell types except for erythrocytes (Cameron, 2003). TNF-α has major pro-inflammatory properties and is produced mainly by activated macrophages, NK cells and Th1 cells. The secretion of TNF-α mediates endothelial activation and lymphocyte movement, and it is also one of the critical
intermediaries in acute and chronic inflammatory conditions (Cameron, 2003). Additionally, TNF-α can indirectly influence hormone and IL-1 secretion to induce a fever, and is also able to activate apoptosis (programmed cell death) in target cells.

Interferon-gamma (IFN-γ), or immune interferon, is secreted by activated T-helper cells and NK cells and plays a large role in antiviral activity in the body (Cameron, 2003). It is considered one of the hallmarks of a proinflammatory cytokine response, and is crucial in adaptive immunity due to its ability to increase antigen presentation and MHC molecule expression, promote Ig class switching to antibody secretion, and control the proliferation of transformed cells (Boehm, Klamp, Groot, & Howard, 1997). In addition, IFN-γ can stimulate macrophages for increased antigen capture and processing (Boehm et al., 1997) and its presence may also be necessary for the development of regulatory T-cells (Ivashkiv, 1995). The production of IFN-γ is essential for fighting intracellular pathogens and viruses, and may also act to downregulate the production of anti-inflammatory cytokines like IL-4 (Boehm et al., 1997).

2.3.5. Interleukin-17

Only discovered in 1996, IL-17 is a proinflammatory cytokine produced by T-cells (Miossec, 2009) that plays a crucial role in host defense against microbial infection (Gu, Wu, & Li, 2013). The IL-17 family is made up of six versions of the IL-17 cytokine, named IL-17A-F (Miossec, 2009). Cytokines from the IL-17 family are produced by a third CD4+ T-helper lineage known as Th17 cells, which also produce IL-17F, IL-22, TNF, and IL-6 (Islander et al., 2010). Through the production of a immune molecules such as cytokines, chemokines, acute phase proteins, and anti-microbial peptides, IL-17A can propagate cascades of events that lead to neutrophil recruitment, inflammation and
host defense (Gu et al., 2013). Overproduction of IL-17A leads to excessive inflammation and overt tissue damage, and has been implicated in rheumatoid arthritis, metabolic diseases, arthritis, and cancer (Gu et al., 2013; Miossec, 2009).

Overall, cytokines play a crucial and necessary role in the induction, maintenance, and termination of an immune response. Anti-inflammatory cytokines like IL-4 ensure tolerance to self-antigen by downregulating the immune response, and also ensure proper cessation of the immune response. Alternatively, pro-inflammatory cytokines such as TNF-α and IFN-γ are critical in sustaining innate and adaptive immunological responsiveness in the face of microbial immune challenges. Therefore, without the proper production and functioning of cytokines, there would be a total breakdown of immunologic action; this would lead to harmful and even fatal consequences.

3. Maternal/Prenatal Immune Activation and Neurobehavioral Abnormalities

Stimulation of the immune system during pregnancy, known as Maternal or Prenatal Immune Activation (MIA or PIA), can cause long-lasting neurobiological and behavioral changes in the offspring. Infection with bacteria or viruses causes a cascade of immunological events, including the production of cytokines. These cytokines, and particularly proinflammatory cytokines such as IL-6, IL-2 and IFN-γ which upregulate an immune response, may have the ability to cross the placenta connecting mother and fetus and infiltrate the developing fetal brain (Dammann & Leviton, 1997). Alternatively, their mere presence may cause the fetus itself to upregulate its own production of proinflammatory cytokines (U. Meyer et al., 2006). Since cytokines play a natural role in brain development – contributing to cortical migration and neuronal growth,
regeneration, development and survival (Bauer, Kerr, & Patterson, 2007; Burns, Clough, Klein, Wood, & Berman, 1993; Mehler & Kessler, 1997; Skundric & Lisak, 2003; Stolp, 2013) – the influx of proinflammatory cytokines can disrupt the delicate balance needed for normal neuronal development. This phenomenon has been implicated in the etiology of developmental psychiatric disorders, most prominently autism and schizophrenia (Boksa, 2010; Alan S Brown & Derkits, 2010; A. S. Brown & Patterson, 2011; Urs Meyer, 2013; U. Meyer, Feldon, & Dammann, 2011; Patterson, 2011). Both epidemiological (Alan S Brown & Derkits, 2010; Alan S Brown, Hooton, et al., 2004; Buka et al., 2001; Libbey, Sweeten, McMahon, & Fujinami, 2005) and experimental (U. Meyer, Feldon, & Fatemi, 2009; Urs Meyer, Spoerri, Yee, Schwarz, & Feldon, 2010; Patterson, 2009) studies have demonstrated significant associations between elevated levels of proinflammatory cytokines and long-lasting behavioral and neurobiological dysfunctions relating to schizophrenia-like and autism-like disorders.

3.1. Origins of MIA Research: Implications for Neurobehavioral Abnormalities

In 1957, the rapid global spread of the Asian flu virus (influenza A subtype H2N2) infected millions of people across several continents. Three decades later, a seminal discovery was made by Dr. Sarnoff Mednick and colleagues that changed the way the relationship between disease, pregnancy and neurodevelopmental disorders came to be viewed. They found that adults whose mothers had contracted the Asian flu in their second trimester of pregnancy were at a significantly higher risk of being diagnosed with schizophrenia in their adult lives (Mednick, Machon, Huttunen, & Bonett, 1988). This finding, and subsequent similar ones (Barr, 1990; E. O'Callaghan et al., 1994; Selten, Frissen, Lensvelt-Mulders, & Morgan, 2009)) led to an explosion of research on how
illness during pregnancy may affect the unborn fetus. Pregnancy is characterized by a shift to an anti-inflammatory state in the mother’s immune system (M. Marzi et al., 1996; D. P. Robinson & S. L. Klein, 2012). Nonetheless, the maternal immune system during pregnancy is not anergic, and will respond to a virus or bacteria, in order to prevent infectious disease. This stimulation of a pregnant woman’s immune system due to immune challenge is referred to as either maternal immune activation or prenatal immune activation. Importantly, it is the response of the mother’s immune system, and not the virus or bacteria itself that can cause grave effects on the developing brain of the fetus (Ashdown et al., 2006). Using various animal models, MIA has been shown to cause a host of alterations in the cytokine levels of the unborn fetal brain. This in turn can result in significant changes in cytokine levels, neuroanatomy, neurochemistry, neuropsychology, neurotransmitters, molecular makeup, and cognitive processes following birth and development over the postnatal period.

3.2. Contemporary MIA Research: Animal Research

Human studies on the effects of MIA on offspring are mainly done retrospectively or through correlational measures. However, to show cause and effect it has been necessary to use animal models, mainly using rats and mice. To model bacterial infections, lipopolysaccharide (LPS) is used, whereas to mimic a virus, influenza or polyinosinic: polycytidylic acid (poly IC) is administered. LPS and poly IC have been injected intraperitoneally (ip), intravenously (iv), or subcutaneously, while influenza is given intranasally.

As discussed earlier, LPS and Poly IC induce inflammation or MIA by interacting with the maternal innate immune system. LPS is a cell wall component of gram-negative
bacteria and leads to the activation of the innate immune response, including cytokine production and inflammation, fever, HPA axis activation and sickness behavior (Boksa, 2010). LPS binds to toll-like receptor-4 (TLR-4) on macrophages and triggers a cascade that leads to the production of transcription factors like nuclear factor kappa B (NFκB), which in turn activates genes for pro- and anti-inflammatory cytokines and chemokines (Aderem & Ulevitch, 2000). The surge of proinflammatory cytokines produced in response to LPS mainly consists of IL-6, TNF-α and IL-1 and these are released to create a feedback loop for further cytokine production (Luheshi, 1998) and induction of prostaglandin synthesis in the hypothalamus. This causes a rise in body temperature and eventual fever (Roth, Rummel, Barth, Gerstberger, & Hübschle, 2009). Poly IC is a synthetic double-stranded RNA analog that mimics the actions of a viral infection by binding to TLR-3 in the innate immune system and causing an induction of pro-inflammatory cytokines (Doughty et al., 2006; Koga et al., 2009). Like, LPS, poly IC mainly works by activating the innate immune system and taking advantage of its defense mechanisms against harmful pathogens. The influenza virus can also activate the innate immune system, although, it will also induce an adaptive immune response that elicits B and T cell effector responses (eg., antibody against the virus and cytotoxic T cell responses). Beyond the immune system, influenza virus can lower the amount of adrenocorticotropic hormone (ACTH) and cortisol in the body (Jefferies, Turner, Lobo, & Gwaltney, 1998), and in causing release of abundant pro-inflammatory cytokines, it results in common symptoms of fever, lethargy, muscle pain and headaches (Eccles, 2005). Though animal models of MIA may not always perfectly mimic virus and
bacterial infections in a natural setting (U. Meyer et al., 2009), they provide us with the best approximation for the effects diseases can cause on a mother and her unborn fetus.

3.3. Biological and Behavioral Effects of MIA

When a pregnant woman encounters an immune challenge, the products of her immune response not only work to rid her own body of the pathogen, but can also affect changes in the production of certain cytokines in crucial areas of fetal development. These changes may also underlie physical neurological differences, such as synaptic connections, observed in the offspring of MIA-affected mothers. In addition, behavioral differences in MIA offspring have been detected, including changes in levels of anxiety, social interaction and sensorimotor gating. The following will review much of the literature that addresses these changes in the offspring of mothers subjected to MIA.

3.3.1. Cytokine Changes in Fetal Brains

Infection of the pregnant mother can cause changes in cytokine concentrations present in the brain of the unborn fetus (Table 1). LPS injection of pregnant mice and rats at embryonic day 18 (E18) led to an increase in mRNA for the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 in the fetal brains between 1-24 hours after injection (Cai, Schools, & Kimelberg, 2000; Liverman et al., 2006; A. S. Paintlia et al., 2004). However there has also been evidence for a decrease in TNF-α after injection at E16 (Urakubo, Jarskog, Lieberman, & Gilmore, 2001), as well as evidence for no changes in any of the pro-inflammatory cytokines when injection took place at E18 (Ashdown et al., 2006). These differences in the findings may be due to the variable timings of injections, and also due to differing amounts of LPS injected into the rat. In mice, LPS injections at E17
increased TNF-α and IL-6 in the mouse fetal brain (H. M. Golan, V. Lev, M. Hallak, Y. Sorokin, & M. Huleihel, 2005; Ning et al., 2008). Only one study has looked into cytokine changes in fetal brain following poly IC injections, but the results are complex and change depending on the day of gestation and how long after the injection the brains were assayed (U. Meyer et al., 2006). In E9 mice, IL-1β decreased three hours after injection but then increased six hours later, while in E17 mice, it increased at three hours. Whereas injection of poly IC at E9 and E17 both increased fetal brain IL-6, at E9, IL-10 decreased, but at E17 it increased (U. Meyer et al., 2006). Thus, while it is clear that changes in cytokine concentrations occur in fetal brain due to MIA, the timing of the maternal immune challenge seems to play a role as to the particular nature of the change, and consequently, what morphological and behavioral consequences may ultimately form in the offspring.

3.3.2. Postnatal Cytokine Changes in the Offspring

Maternal immune activation has been shown to produce a general increase in pro-inflammatory cytokine production in the postnatal brains of the offspring (Table 2). At one week after birth, postnatal day 7 (P7), it was shown that IL-1-β, IL-6 and TNF-α were increased compared to controls in whole brains of rats born to mothers injected with LPS at E18 and E19 (Kumral et al., 2007; Yesilirmak et al., 2007). In addition, mRNA for IL-1 β was increased at P1 in rats, though the mRNA for TNF-α was shown to be decreased compared to controls. However, because this data was collected at such an early time point, it may not be as strong an indicator of cytokine changes as that observed
at later ages, once the shock of birth has passed. At later times, TNF-α levels were significantly greater in the midbrains of MIA offspring at P21 (Z. Ling et al., 2002), P120 (Z. Ling et al., 2004) and P510 (Z. D. Ling et al., 2004), almost a year and a half after their birth.

In an extensive investigation of postnatal changes in cytokines, Garay et al. (2013) surveyed 23 different cytokines and chemokines in different regions of the brain and at different stages of development in MIA-born mice. The results displayed a unique pattern of region- and age-related changes. In both the frontal cortex and cingulate cortex many cytokines, such as IL-1β, IL-6 and IFN-γ were elevated at birth (P0) and in adulthood (P60) compared to controls. Interestingly, during the periods of synaptogenesis and circuit formation (P7, P14 and P30), these cytokines and others, like IL-4 and IL-2, were significantly decreased in these brain regions. In the hippocampus, only IL-6 was elevated at birth, while IL-1β, IL-4 and IL-2 were all decreased. Only IL-4 stayed at a significantly lower level during the synaptogenesis phase and IL-6, too, was decreased during this time. Unlike the other two brain regions, by the time the mice were adults, the hippocampus showed no differences in cytokine concentration levels for any of the measured cytokines (Garay, Hsiao, Patterson, & McAllister, 2013). Though these results indicate region-specific and age-specific changes in cytokine levels between MIA offspring and control offspring, the overall conclusion from this body of research is that throughout the lifetime of mice born to infected/immune-challenged mothers, cytokine concentrations in the brain vary greatly from the concentrations found in the brains of control mice.
3.3.3. Morphological and Anatomical Changes in the Offspring

Given that cytokines play a large role in neuroplasticity and neurogenesis in both adult and developing brains, it is reasonable to consider whether morphological and anatomical changes occur in the brains of offspring born to mothers exposed to infection and/or immune challenge during pregnancy. In the hippocampus, decreased neurogenesis (Cui, Ashdown, Luheshi, & Boksa, 2009; S. H. Fatemi et al., 2009), axonal size, myelin thickness (Makinodan et al., 2008), dendritic branching (Baharnoori, Brake, & Srivastava, 2009), and Reelin (U. Meyer, Nyffeler, Yee, Knuesel, & Feldon, 2008) (a regulator of neuronal migration) have all been described in offspring subjected to MIA. There has also been evidence for increased microglial density (Roumier et al., 2008), pyramidal cell density (S. H. Fatemi et al., 2002; S. H. Fatemi et al., 1999), neuronal cell density (H. M. Golan et al., 2005) and astrocyte number (Samuelsson, Jennische, Hansson, & Holmäng, 2006) in the CA1 of the hippocampus. Prenatal immune activation has also significantly increased the amount of activated microglia (Zager et al., 2015) and increase microglial density (Van den Eynde et al., 2014) in the brains of the offspring. In the prefrontal cortex (PFC), decreases in dendritic branching (Baharnoori et al., 2009) and Reelin (U. Meyer et al., 2008) were also found. Because cognitive functions like learning and memory and even social behavior are mediated by the hippocampus (Jarrard, 1993) and the PFC (Anderson, Bechara, Damasio, Tranel, & Damasio, 1999) changes in these areas can prove detrimental to these cognitive domains. Indicative of damage to white matter, increased cell death and decreased myelin basic protein (Kumral et al., 2007; Yesilirmak et al., 2007) (MBP) in white matter as well as a reduction in immature oligodendrocytes and oligodendrocyte precursors have been reported (Paintlia,
Paintlia, Barbosa, Singh, & Singh, 2004). In sum, it is very likely that the cytokine changes brought about by MIA are having a broad and serious effect on the anatomical makeup of the brains of the offspring of these pregnancies.

3.3.4. Behavioral Changes in the Offspring

Behavioral changes in MIA offspring are among the most studied, reported and robust set of findings related to this field (Table 3). The most commonly reported change in these offspring is a deficit in sensorimotor gating, as measured by prepulse inhibition (PPI). Sensorimotor gating is the mechanism by which excess or unimportant sensory influences are filtered out in order to focus on and process the most significant aspects of the environment. Deficits in PPI have been found in rats and mice anywhere from P35 to P400 in offspring from mothers injected with LPS (Borrell, Vela, Arévalo-Martín, Molina-Holgado, & Guaza, 2002; M. E. Fortier, Luheshi, & Boksa, 2007; Romero et al., 2007), influenza (Shi, Fatemi, Sidwell, & Patterson, 2003), and poly IC (Garay et al., 2013; Makinodan et al., 2008; U. Meyer et al., 2006; U. Meyer et al., 2008; Ozawa et al., 2006; Shi et al., 2003; S. E. Smith, Li, Garbett, Mirnics, & Patterson, 2007; Wolff & Bilkey, 2008) either throughout their pregnancies or in their second or third trimester. Similar to sensorimotor gating, latent inhibition (LI), the ability to ignore irrelevant stimuli previously presented without reinforcement, has also been shown to be robustly disrupted in MIA offspring. Offspring mice born to mothers injected with poly IC on E12.5 showed disrupted LI at six weeks of age (Garay et al., 2013; S. E. Smith et al., 2007), as well as when mothers were injected on E9 (U. Meyer et al., 2006). In rats, offspring showed deficits in LI when mothers were injected with poly I:C at E15 or E19 (Zuckerman & Weiner, 2003). Though neither LPS nor influenza injections have been
investigated in relation to LI deficits, there is extremely strong evidence to point to PPI and LI dysfunction in MIA offspring, both indicating a decreased ability to filter out and ignore superfluous and irrelevant information.

Anxiety and social behavior, spatial learning and memory deficits have all been reported in offspring whose mother either received LPS, poly IC or influenza injections while pregnant. Administration of poly IC (S. E. Smith et al., 2007) and influenza (Shi et al., 2003) in mice reduced social interactions with new sex-matched peers by almost 3-fold in the offspring, and offspring of mothers injected with poly IC in their second trimester displayed fewer ultrasonic vocalizations in isolation and during social encounters (N. V. Malkova, Yu, Hsiao, Moore, & Patterson, 2012). Additionally, significant decreases in open field exploration, a sign of increased anxiety, was observed in adult mice born to mothers injected with either poly IC (U. Meyer et al., 2006; S. E. Smith et al., 2007) or LPS (Wang et al., 2010) during their pregnancy. Spatial learning and memory in the MWM and RAM was impaired in rat offspring of LPS-injected (F. Lanté et al., 2008; F. Lanté et al., 2007) and poly IC-injected (Wang et al., 2010) mothers, as well as in mouse offspring of poly IC-injected mothers (U. Meyer et al., 2008; Ozawa et al., 2006). While one group did not find differences in spatial learning on the traditional MWM, they did find a decreased rate of learning and memory for MIA mice in an associative learning version of the task (H. M. Golan et al., 2005). Lastly, memory for a familiar object was shown to be impaired in both rat offspring born to mothers injected with poly IC (Wolff, Cheyne, & Bilkey, 2011) and mouse offspring born to LPS-injected mothers (Coyle, Tran, Fung, Summers, & Rofe, 2009), with animals exploring familiar and new objects at the same rate. Thus, MIA with LPS, poly IC and/or
influenza has been shown to impair social behavior, learning and memory in both adult rats and mice.

**Summary.** Maternal immune activation leads to a host of changes in the offspring. Many are born with significant and major changes in their circulating cytokine profiles, with pronounced differences in particular brain regions and the brain as a whole. Aside from chemical changes, anatomical differences in white matter, dendritic properties, and neurogenesis are also present in MIA offspring. Behaviorally, offspring show major deficiencies in sensory, sensorimotor, social, spatial and memory tasks, with the offspring demonstrating impaired abilities to filter out unnecessary information, communicate with others and remember objects they have encountered in the past. The consequences of MIA on the offspring are not only disadvantageous and possibly debilitating, but are also robust and long-lasting.

3.4. MIA Implications for Autism and Schizophrenia

Though maternal immune activation produces wide-ranging neurobiological and behavioral effects on offspring, most of the literature on the topic focuses on its implications for the development of autism and schizophrenia. Schizophrenia is characterized by either negative or positive symptoms: negative symptoms referring to the absence of typical behavior such as deficits in social functioning and apathy, and positive symptoms referring to an excess or distortion of typical behavior such as hallucinations and thought disorders (Association, 2013). Symptoms usually appear in early adulthood, but prodromal symptoms may be present before an official diagnosis. Autism is usually diagnosed in childhood and is characterized by three core symptoms:
impairments in social functioning, repetitive/stereotyped behaviors, and deficits in communication (Association, 2013). Though schizophrenia and autism may seem like two completely distinct disorders, they, in fact, share many similarities. Individuals with either disease display deficits in social interactions, cognitive dysfunction, issues with sensorimotor gating and emotional processing, and executive function impairments (Cheung et al., 2010; U. Meyer et al., 2011). Anatomically and chemically, both disease states are characterized by reduced levels of gray matter volume in the limbic-striato-thalamic circuitry (Cheung et al., 2010), reduced Reelin signaling (S Hossein Fatemi, 2005), and impaired serotonin synthesis and receptor activity (Abi-Dargham, Laruelle, Aghajanian, Charney, & Krystal, 1997; Lam, Aman, & Arnold, 2006; Winter et al., 2008). Thus, grouping schizophrenia and autism together in relation to MIA is a logical step that may help to shed light on two debilitating disease states.

Evidence for a connection between MIA and schizophrenia and/or autism mainly come from retrospective epidemiological studies. As mentioned above, this research area began by connecting infection with the 1957 Asian flu virus and the eventual development of schizophrenia in the offspring (Mednick et al., 1988). Since then, numerous epidemiological studies from all over the world have found links between maternal infections with influenza, Taxoplasma gondii, Herpes Simplex Virus-2, bronchopneumonia, respiratory infection, rubella and many other diseases with children having a higher risk of obtaining a diagnosis of schizophrenia later in life ((Alan S Brown & Derkits, 2010; Alan S Brown & Susser, 2002; Eadbhard O'Callaghan et al., 1994). Though there have also been studies that did not find these same associations (Selten et al., 2009; Susser, Lin, Brown, Lumey, & Erlenmeyer-Kimling, 1994), some have argued
that the methodology in these studies were faulty (Patterson, 2009). Thorough and extensive reviews have been written detailing the research on this topic (Alan S Brown & Derkits, 2010), and there is a strong consensus in the scientific community that prenatal infections and the later development of schizophrenia are inarguably linked. Similar studies have been conducted investigating the link between prenatal infection and autism and have found comparable results (Arndt, Stodgell, & Rodier, 2005; Patterson, 2011). In addition, data has suggested that the timing of the maternal immune challenge–namely, during which trimester it occurs–can influence whether the offspring will later develop schizophrenia or autism (U. Meyer et al., 2006; U. Meyer et al., 2008). The elevated risk of developing either schizophrenia or autism due to prenatal immune activation is a robust and universal conclusion for most scientists and researchers in the field.

Although one can never label a mouse or rat as having schizophrenia or autism, certain behavioral tests are used to illuminate behavioral similarities between humans and their animal counterparts. For signs of schizophrenia-like and/or autism-like behavior, social impairments are measured by comparing the number of social interactions subjects make with conspecific animals (Boksa, 2010; Tordjman et al., 2007). Sexual behavior, grooming and aggression are also used as measures of social behavior. For schizophrenic-like behavior, animals are tested on prepulse inhibition tasks and latent inhibition tasks to detect possible deficits in sensorimotor gating and information filtering, respectively (Tordjman et al., 2007). Pup distress calls and ultrasonic vocalizations are measured to determine communication dysfunctions in animals thought to display autism-like behaviors (N. V. Malkova et al., 2012; Tordjman et al., 2007). Stereotyped and repetitive behaviors are measured by marble burying tasks, grooming tasks, and digging tasks,
while working memory tasks can be incorporated into the Morris Water Maze and Object Recognition tasks which assess cognitive functioning and executive functioning abilities (Tordjman et al., 2007). The effects MIA has on these different behavioral dimensions of animals has been discussed in detail above, and provide us with further evidence of a connection between maternal immune challenge and two very difficult disorders. Though animal models have been highly debated and their validity questioned (Tordjman et al., 2007), as of now they are the best approximation available to inferring schizophrenic and autistic behaviors using animal models.

4. Alternative MIA Models: T Cell Superantigens

The term ‘superantigen’ (SAg) refers to a unique category of immune-activating viral and bacterial proteins that cause robust and prolonged T-cell activation and proliferation, and a quick and strong cytokine production. Superantigens bind TCRs and APCs without the need for specific antigen recognition, causing activation of an exponential number of CD4+ T-cells. The activation of these T-cells leads to prominent T-cell proliferation and cytokine production and release. This quick and extreme T cell reaction is not found in response to immune stimuli such as LPS or poly I:C, which act through the innate immune system (i.e. monocytes, neutrophils, macrophages) and not directly on T-cells. Superantigens such as staphylococcus aureus enterotoxins can present in multiple forms, each binding to different regions on the TCR, causing gastrointestinal and other bodily harm. Because of their unique effector functioning, their direct activation of T-cells and robust production of T-cell-derived cytokines, SAgs represent an interesting area of study in regard to their effects on the immune system.
4.1. Superantigens and the Immune System

Superantigens, unlike conventional antigens, interact with the immune system in a unique way. There are about 40 different molecules that have SAg properties, and are produced by two main types of bacteria: Staphylococcus aureus and Streptococcus pyogenes. These bacteria secrete protein toxins that ultimately interact, in a unique manner, with the adaptive immune system to activate T-cells (Fraser & Proft, 2008; B. Torres, Soos, Perrin, & Johnson, 2000; B. A. Torres, Kominsky, Perrin, Hobeika, & Johnson, 2001) (Figure 1). Unlike conventional antigens, SAgs are not pieces of antigen broken down into smaller fragments and presented to T-cells. They initially interact with immune cells as whole, intact molecules, binding directly to MHC II molecules outside the peptide antigen-binding groove (Dellabona et al., 1990), and also in the external portion (once again away from the peptide antigen-binding groove) of the variable region of the beta chain (\(v\beta\)) on TCRs (Fraser & Proft, 2008; McCormick, Yarwood, & Schlievert, 2001; B. A. Torres et al., 2001). Many kinds of cells with MHC class II molecules can present SAgs to TCRs, including macrophages, B-cells and even NK cells (D'orazio & Stein-Streilein, 1996; B. Torres et al., 2000), and when the \(v\beta\) region is bound, and the MHC molecule and the TCR are cross-linked, the T-cell is activated (Figure 1).

Because SAgs bind outside of the peptide-binding groove, T-cells can be activated without specific recognition of antigen, thus activating ten-fold greater numbers of T-cells that would normally be activated in a conventional immune challenge. When specific antigen recognition is needed to activate T-cells, only about .01% of the body’s total T-cell pool is activated (Abbas et al., 2011). However, when superantigens are present, about 20% or 1 femtogram/ml (10-15 g/ml) of the body’s T-cells are stimulated.
(Fraser & Proft, 2008). The robust stimulation of CD4+ T-cells causes an extreme production of T-cell-derived cytokines such as, IL-2, IFN-γ and TNF-α, which can accumulate to toxic levels (Fraser & Proft, 2008; B. Torres et al., 2000). Antibodies have the ability to block the binding of SAgs to MHC class II molecules, and are a main weapon of defense when presented with SAg challenge, and are crucial in blocking the lethal production of inflammatory cytokines (Fraser & Proft, 2008; B. Torres et al., 2000). The non-specific binding ability of SAgs to TCRs, and the potency of T-cell activation, work together to produce exceptionally higher amounts of T-cell activation, proliferation and cytokine production than conventional antigen immune challenges. In this way, superantigens hijack the adaptive immune system and exploit it to a dangerous degree.

4.2. Staphylococcal Aureus and Enterotoxins

As one of the two major sources of superantigen-producing infection, Staphylococcus aureus is a dangerous pathogen commonly found in the general population. *Staphylococcus aureus* (*S. aureus*) is a gram-positive bacterium that colonizes both humans and animals, and has been shown to be present in 20-30% of the general population (Control, 2011; Kluytmans, Van Belkum, & Verbrugh, 1997), while 60% are recurrent carriers (Kluytmans et al., 1997). The bacteria readily colonize the skin and mucosal surfaces, most frequently colonizing the nasal cavities (Krakauer & Stiles, 2003; Pinchuk, Beswick, & Reyes, 2010). Once they are activated, they have the ability to downregulate the immune system and ensure their survival in the host by producing protein A, coagulases, hemolysins, leukocidins, and inactive complement (Krakauer & Stiles, 2003). Infection with *S. aureus* can cause fever, food poisoning, skin infections,
pharyngitis, and life-threatening toxic shock. Many strains of \textit{S. aureus} have become resistant to antibiotics like methicillin and vancomycin and the incidence of these resistant bacteria is increasing worldwide (Appelbaum, 2006).

\textit{Staphylococcus aureus} produces virulent exotoxins that target the intestines and gut to cause a host of diseases and illnesses. Staphylococcal enterotoxins (SEs) are single-chain proteins that present in over 20 serologically distinctive forms and are named SEs A through V (e.g., SEA, SEB, SEC, etc.) and toxic shock syndrome toxin-1 (TSST-1). Each of these is serologically distinct, meaning that they express unique antigenic sites that induce specific antibodies (i.e. antibody to SEA will not cross-react to SEB, and vice versa). This explains their differential binding properties to immune surface molecules, as stated below. Staphylococcal enterotoxins contain between 220 and 240 amino acids, are about 25-30 kD in size, and have similar 3-dimensional structures (Pinchuk et al., 2010; Schlievert et al., 1995). All SEs bind to MHC/HLA II molecules, but not all bind to them in the same manner; some bind to both the \( \alpha \) and \( \beta \) domains of the molecule, while some SEs bind to only one or the other (Bohach, 1997). In addition, the potency of certain SEs is dependent on the isotype of the MHC II molecule, with some SEs producing greater effects for specific haplotypes of the molecule (Taub, Newcomb, & Rogers, 1992). Once bound to the MHC molecule, SEs are able to crosslink the \( \gamma \beta \) region on TCRs and activate the T-cells to produce massive amounts of proinflammatory cytokines. In this way, staphylococcal enterotoxins are a leading cause of food-borne illnesses, nosocomial infections, and gastrointestinal inflammatory injury, causing millions of infections and hospitalizations each year (Pinchuk et al., 2010). SEs are also notoriously resistant to heat, salt and acid and thus cannot be destroyed by mild
salting and cooking of food. The most widely studied of the SEs are SEA and SEB, similar in many ways, but also crucially different in others.

4.3. *Staphylococcal Enterotoxin A and B*

Both SEA and SEB have the ability to bind to MHC II molecules, however SEA binds to both the α and β domains of the molecule, while SEB binds only to the β chain (Pinchuk et al., 2010). Similarly, both superantigens crosslink the MHC II molecule to T-cells but are specific for certain \(v\beta\) regions on the TCR: SEA mainly for regions 6.3, 7.3, and 7.4, SEB for regions 3, 7, 8.1, 8.2, 8.3 (Taub et al., 1992). SEA and SEB share only 40-60% sequence homology (Krakauer & Stiles, 2003; Pinchuk et al., 2010), but their physical structures are conserved and reveal very similar arrangements. In both SEs, there are two distinct domains with a groove in between them, acting as the interaction site for the TCR \(v\beta\) chain (Hurley et al., 1995; Mehindate et al., 1995). Although SEA and SEB compete for binding to MHC II molecules, there seems to be at least two binding sites for SEs, and thus the binding of one does not inhibit the binding of the other (Chintagumpala, Mollick, & Rich, 1991). Crucially, SEA and SEB differentially bind to distinct MHC allele polymorphisms, such that SEA is more potent in animals expressing the H-2b isotype—for instance, C57BL/6 mice—and SEB is more potent in animals expressing the H-2d isotype—like Balb/c mice (B. G. Stiles, Campbell, Castle, & Grove, 1999; Taub et al., 1992). These preferences are present in human HLA-DR polymorphisms as well (Mollick, Chintagumpala, Cook, & Rich, 1991). SEA and SEB are both similar to one another and also unique in critical ways, at once classifying them both as potent superantigens but also providing them with their own unique fingerprint.
Both SEA and SEB have been implicated in a host of illnesses and diseases, and stimulate the immune system by increasing the production of proinflammatory cytokines to dangerous and even fatal levels. Because superantigens bind to and activate T-cells in a non-specific way, T-cell-derived cytokines are produced at increased rates. Between two and four hours after intraperitoneal injections of SEA or SEB in mice, concentrations of IL-1, TNF-\( \alpha \) and IFN-\( \gamma \) have all been shown to increase dramatically (Hayworth et al., 2009; Norrby-Teglund, Norgren, Holm, Andersson, & Andersson, 1994; B. Stiles, Bavari, Krakauer, & Ulrich, 1993; Uchiyama et al., 1990). The accumulation of these cytokines causes inflammation of the surrounding area, usually the stomach and intestines. Through this, SEs are powerful inducers of stomach pain, diarrhea, nausea, and vomiting. Only small amounts (0.004\( \mu \)g/kg) of SE are required to induce symptoms, and higher doses (0.02\( \mu \)g/kg) can be lethal (Pinchuk et al., 2010).

Though SEA and SEB cause similar biological and physical symptoms, each have also been researched in association with a unique set of illnesses. SEA is the superantigen most associated with food-borne illnesses, and may cause vomiting by increasing release of serotonin in the intestine and by activating enteric neurons (Hu et al., 2007). It has also been suggested that SEA may bind to intestinal myofibroblasts and cause the release of monocyte chemoattractant protein-1, ultimately producing production of proinflammatory cytokines (Barrera et al., 2004). The overabundance of proinflammatory cytokines causes severe inflammation of the gastrointestinal tract. As of yet, however, the exact mechanism of SEA food poisoning is unknown. SEB causes many of the same symptoms as SEA, but is the only Staphylococcus enterotoxin that has been studied as a potential biological weapon. During the Cold War, SEB was formed into an aerosol
version and researched as a possible means of compromising the enemy because of its potency to produce shortness of breath, chest pain, fever, pulmonary edema, and/or septic shock (Pinchuk et al., 2010). Work on SEB as a biological weapon is still ongoing. Aside from gastrointestinal illnesses, SEA and SEB may also play a role in non-menstrual toxic shock syndrome, atopic dermatitis, and allergic rhinitis (Fraser & Proft, 2008). Both SEs and their products have been implicated in these various disease states. Thus, SEA and SEB are uniquely identified with specific illnesses, but their similar course of action implicates them in a shared group of disorders as well.

In summary, superantigens as a whole represent a dangerous category of pathogens that bypass antigen specificity and directly bind MHC class II molecules with T-cells to produce an overwhelming production of T-cell-derived proinflammatory cytokines. Bacteria like *Staphylococcal aureus*, which naturally colonize human bodies, can secrete superantigenic exotoxins that target specific areas of the body such as the intestines. These enterotoxins come in many forms, the most studied of which are Staphylococcal enterotoxins A and B. Though similar in their mechanisms and outcomes, SEA and SEB also function in distinct manners, making their potency different for different hosts. In addition, their differences have made them uniquely suited for specific areas of study. All in all, both SEA and SEB are dangerous pathogens that provide a unique window into how the adaptive immune system works and the dangers of letting it run wild.

5. Project Objective

It is important to determine whether maternal immune activation with bacterial T-cell superantigens is a risk factor for neurodevelopmental alterations and emergence of
behavioral deficits relevant to psychological diseases. Until this point, the dominant immunological argument for the developmental origins of autism and schizophrenia in humans is maternal exposure to influenza virus, against which successful protection requires a T-cell response. Therefore, superantigens represent a convenient and explicit model for studying T-cell involvement in the effects of MIA on subsequent brain and behavioral development. Though the current experimental methods of LPS and poly IC stimulate the innate immune response, they are highly restricted and fail to capture the full extent of immunological involvement in a wide range of possible immune challenges. In this way, they minimize the generality of the MIA model of autism and schizophrenia. The current project makes use of bacterial T-cell superantigens in a new model of MIA that will not only complement previous studies, but will also add an important new perspective on the primary origins of psychiatric disease.
CHAPTER 2: GENERAL METHODOLOGY

**Subjects:** Adult male and female C57BL/6J (C57BL/6) and Balb/cJ (Balb/c) mice were group-housed (3-4 per cage) and given access to food and water *ad libitum*. Breeding males ranged in age from 7 weeks to 12 weeks, while the age of pregnant females ranged from 7 weeks to 10 weeks. Animals were maintained on a constant 12:12h light:dark cycle with lights on at 0600 h. Mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and allowed at least one week acclimation to the facilities prior to the start of experimentation. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health, and approved by the Rutgers Institutional Animal Care and Guidance Committee.

**Breeding/Injections:** Two females were placed with a single male overnight and returned to their home cages the following morning, which was considered embryonic day 0.5 (E.5). Presence of a sperm plug was assessed and each female’s weight was taken. On day E12.5, those females who had gained at least 10% of their body weight from E.5 were marked as “pregnant” and were randomly assigned to one of six groups: (1) SEB-injected, sacrificed, spleens used for ELISAs; (2) SEA-injected, sacrificed, spleens used for ELISAs; (3) Saline-injected, sacrificed, spleens used for ELISAs; (4) SEB-injected, brought to full term, pups tested; (5) SEA-injected, brought to full term, pups tested; (6) Saline-injected, brought to full term, pups tested. In addition, to act as controls for cytokine assays, non-pregnant females were also randomly assigned to one of three groups: (7) SEB-injected, sacrificed, spleens used for ELISAs; (8) SEA-injected, sacrificed, spleens used for ELISAs; (9) Saline-injected, sacrificed, spleens used for
ELISAs. All injections were intraperitoneal (ip) injections, and consisted of 5μg SEB or SEA (Toxin Technology, Sarasota, FL) or 0.9% saline.

**Spleen Extraction and ELISAs:** Two hours post-injection, animals assigned to groups designated for ELISA cytokine assays were sacrificed in a standard CO₂ chamber. The spleens were removed, and pregnancy status was confirmed by checking the abdomen for embryos. Spleens were homogenized with 1mL of 1x phosphate-buffered saline (PBS), and spun in glass vials for 10 minutes at 10,000 revolutions per minute. The supernatant of the vials were used for all immunoassay analyses.

An enzyme-linked immunosorbent assay (ELISA) as previously described in detail (Urbach-Ross, Crowell, & Kusnecov, 2008) was used to assess cytokine concentrations in the spleens. The spleen, a secondary lymphoid organ, is the body’s largest filter of blood and contains lymphoid tissue that is made up of specialized macrophages, T-cells and B-cells that respond to infection (Mebius & Kraal, 2005). In addition, superantigens have been shown to elicit T-cell and cytokine responses in the spleen (Bette, Schäfer, Van Rooijen, Weihe, & Fleischer, 1993). The ELISA assays for the cytokines IL-6, IL-2, IL-4, and IFN-γ were conducted for all samples, and TNF-α and IL-17A assays were performed on a selection of spleens. Unfortunately, TNF-α and IL-17A assays could not be performed on spleens from animals injected with SEB, as logistical issues were encountered and there was not enough sample left to analyze.

To perform the ELISAs, a 96-well microtiter plate was coated with antibody specific for the cytokine of interest and left to rest overnight. After carefully washing the plate six times with washing buffer (note, all washes involved this buffer made from mixing 9L of water with 5mL Tween 20 and 1L of 10x PBS), samples were diluted 1:4
(25µl sample in 75µl buffer) and placed in each of duplicate wells. Twenty-four hours later, the plate was washed thoroughly six times and a capture antibody specific for the cytokine of interest was mixed with horseradish peroxidase (HRP) - an enzyme that amplifies the signal of the detection antibody - and added to all wells. The concentration of capture antibody was prepared according to the manufacturer’s recommendation. An hour later, a substrate solution consisting of 1x TMB (also known as: 3,3',5,5'-Tetramethylbenzidine) was added in order to react with the HRP enzyme. The subsequent chromogenic reaction (due to chromophores in the substrate solution) produces a blue signal, which subsequently turns yellow when 50µl Sulfuric acid (1M H2SO4) is added to each well. The optical densities (expressed as absorbance units) of the wells were then read at a wavelength of 450 nm using a universal microplate reader (Bio-Tek Industries, ELx800) and accompanying software (KC Junior software; BioTek). For each ELISA a standard curve is established, with absorbance values being greater when there is more detection antibody (and hence cytokine) present in the well. Calculating the concentration of target cytokine in each spleen sample involved extrapolation from the standard curve. The standard curve was prepared in the same plate that received samples, and was established by making serial two-fold dilutions of a high starting concentration of a recombinant cytokine standard (supplied by the manufacturer). The typical range of the standard curves were between 2000 pg/ml to 10 pg/ml, with sample dilutions falling on the linear, readable portion of the curve.

The cytokine determinations using ELISA were corrected for the total protein concentration of the sample, such that cytokine concentrations were expressed per µg of protein. Total spleen protein was quantified using the BCA protein assay kit as per
manufacturers’ instructions (Pierce, Rockford, IL, USA). Samples were diluted in a 1:20 ratio (1µg sample to 19µg diluent) and concentrations were compared to a standard curve prepared in the same plate using bovine serum albumin (BSA). The optical densities of the wells were read at a wavelength of 562nm as described above.

**Behavioral Testing**

Summary of Testing Schedule: Two days before a pregnant female was due to give birth, she was separated into her own cage. Once the pups were born, the animals were not disturbed except for once weekly cage change (week 1, week 2). Pups were weaned three weeks after their birth date and put into their own cages. Males and females were housed separately.

At six weeks of age, the offspring began behavioral testing (Figure 2), which commenced at approximately the same time each day, between 9 and 10 AM. In addition, experimenters ran all the tests blind to the group of the subject: experimenters were unaware of which type of injection the pups’ mothers received. On day one, animals performed the social interaction test for a total of ten minutes (600s), followed the next day, with testing in the Elevated Plus Maze (EPM) for a total of five minutes. To minimize the mild stressful effects of sequential daily testing, the animals were rested on day three. On Days 4-8, the animals performed the object recognition task which consists of a single five-minute trial given each day. On days 9 and 10, the animals were rested, and on Day 11, the animals underwent a single 45-minute prepulse inhibition (PPI) session. Following the PPI test, all animals were rested for three weeks, since this was most likely the most stressful procedure. By the end of this rest period, animals were approximately ten weeks old, and were subjected to a two-day Morris Water Maze.
(MWM) task. On the first day the animals were given ten one-minute learning trials to find a hidden platform, and an hour after the final trial, they were administered a single one-minute probe trial in which the platform was removed. Twenty-four hours later, the animals were given another single probe trial. Data in all tests of learning and/or exploration (including social interaction test) was recorded using a ceiling-mounted camera connected to a computer using the AnyMaze animal tracking software program (Stoelting). Extracted parameters (e.g., locomotor activity) were statistically analyzed using SPSS (IBM) and JMP software (SAS).

**Behavioral Testing Procedures**

**Social Interaction Test**

The social interaction test is designed to analyze the number and amount of time a subject mouse spends interacting with a novel (stranger) mouse confined in an inverted wire cylinder, versus an empty cylinder (see Figure 3a). The cylinders are in a 38 x 38 cm (width x length) chamber, enclosed by 60 cm high Plexiglas walls. The floor of the chamber is made of stainless wire mesh, and in diagonally opposed corners, contains the wire cylinders (Figure 3a).

One cylinder is left empty (the control cup) while the other cylinder houses a novel mouse of similar age and matching sex (the target cup). The subject was placed in the center of the grid, and the number of contacts and amount of time spent touching each cylinder was recorded for a 600 second trial. The number of times the target cylinder was touched and the total amount of time spent touching the target cylinder were divided by the total number of touches and the total time spent touching both cylinders. Therefore,
the dependent variables were the percent number of contacts and contact time of the target cylinder.

Elevated Plus Maze (EPM)

The elevated plus maze is a cross-shaped elevated apparatus made up of four arms that are elevated 82 cm above the floor. All arms were 25 cm long and 7.62 cm wide. Two opposing arms had high walls (21.6 cm high), and were designated as ‘closed,’ while the other two opposing arms had no walls, and were designated as ‘open.’ A center area—simply designated ‘center’—represented the access region to all four arms (Figure 3b). Testing in the EPM was for 5 minutes, and commenced with the subject being placed at the end of a closed arm. The amount of time the animal spent in the closed arms and the open arms was recorded, and various parameters were calculated, including percent time in the open arms, closed arms, and center area, and the number of transitions into the various arms. The main variable commonly used to infer an ‘anxiety-like’ state is the percent time spent in the open arm relative to the total time spent in the closed and open arms. The center area is a decision point, and time spent in this region is excluded from calculation of open/closed arm ratio (since the center is neither open nor closed).

Object Recognition Test (OR)

The object recognition (OR) test consisted of five 5-minute trials, each given on succeeding days. On the first day, subjects were placed in a 56x62 cm empty open field (OF) and allowed to explore the arena (Figure 3c). On succeeding days 2-4, two matching objects were placed on opposite sides of the OF, and at the start of the trial, the subject was placed in the center, equidistant from the two objects. The objects consist of white
golf balls mounted with glue to a plastic base derived from the caps of 50 ml Falcon tubes. On each day, the number of object contacts and amount time spent contacting each object was recorded. On the final day (Day 5), one of the golf balls (designated as ‘familiar’ objects) was replaced with a round, metal tube (the novel object) of approximate size to the golf ball. The replaced object (left or right golf ball) was chosen at random and was counterbalanced among the animals. The number of object contacts and amount of time spent contacting the familiar object relative to the novel object were recorded and used as dependent variables for this test.

*Prepulse Inhibition (PPI)*

Testing was conducted in four SR-Lab Systems startle chambers (San Diego Instruments, San Diego, Calif., USA). Each chamber contains a 5.1-cm (outside diameter) Plexiglas cylinder mounted on a plastic platform (20.4 cm length × 12.7 cm width × 0.4 cm thick) with a piezoelectric accelerometer unit attached below the Plexiglas cylinder (Figure 3d). The piezoelectric unit transduces vibrations into signals that are rectified and stored by a microcomputer interface. Each chamber is sound-attenuated and contains, above the Plexiglas cylinder, a loudspeaker (28 cm above the cylinder) fitted into the ceiling of the chamber. Each test session began by placing a subject in the cylinder where it was left undisturbed for 2 min. After the 2-min acclimation, each subject was presented with 80 acoustic noise trials given over a 45-min period. The noise trials were of seven distinct types, in which some trials involved a prepulse+pulse combination, while others involved pulse alone or prepulse alone. For the prepulse+pulse trials, a low intensity noise (the prepulse) played for 40ms and preceded a high intensity noise (the pulse) by 100 ms. There were three types of prepulse+pulse trials, characterized by variations in the
magnitude of the prepulse. That is, the 40ms prepulse stimulus consisted of white noise that was either 2, 4, or 8 dB above background (measured to be 60dB), while the startle pulse was 50dB above background (i.e.110 dB). In addition to these three types of prepulse+pulse trials, there were three trials in which each of the prepulse intensities was also presented alone to measure baseline startle to the prepulse stimuli per se. Finally, there were trials where only the pulse alone was presented, and which provided a measure of the maximal startle response. Therefore, there were a total of seven trial types. The seven trial types were presented in pseudorandom order such that each trial type was presented once within a block of seven trials, and an equal number of each trial type was presented across the entire session (except for the pulse alone trials, which were presented as a string of 5 trials in the beginning, and 5 trials presented at the end of the session). The average inter-trial interval was 15s (ranged from 10 to 20s). The startle response was measured in arbitrary units by the software and recorded over a 250ms (measuring the response every 1 ms) period, starting with the onset of the startle stimulus. The highest value recorded over this 250 ms period was given as Vmax, and this was adjusted by subtracting the baseline values (designated Vinput) recorded immediately prior to the onset of the pulse. This value was the startle amplitude for any given trial. To calculate prepulse inhibition for each prepulse+pulse trial type (prepulse 2, 4 or 8 dB above background), the following formula was applied: \[\frac{(\text{pulse alone} - \text{prepulse+pulse})}{(\text{pulse alone})}\times 100.

Morris Water Maze (MWM)

The MWM task is a standard test of spatial learning and reference memory. As described here, an expedited form of the test was run over two days, and was performed three
weeks after the end of the last behavioral test in order to allow any lingering effects of previous tests to subside. The MWM pool was 101.6 cm in diameter and filled with water kept between 18° and 20° centigrade. During the acquisition phase, a small, round platform was hidden about 1-2 cm below the surface (Figure 3e). The water was made opaque with non-toxic colored paint, and the location of the platform was hidden. In addition, the main room light was turned off, with only a desk lamp and string of Christmas lights to illuminate the room. The tub was divided into four quadrants, and each subject was randomly assigned a specific quadrant in which the platform was hidden for all the trials. Throughout all phases of training and testing, the maze remained in a fixed position and was surrounded by several salient, extra-maze cues. A blue tarp was hung around the pool, with openings on the north side, revealing a string of lit Christmas lights, and on the south side to allow the experimenter to place the animal in the pool. On the west side of the pool, tape was placed on the tarp in a T-shaped design, while on the east side, a white piece of paper with dark black horizontal lines was taped to the tarp. The tarp also served as a visual barrier, concealing the experimenter from the mice. Each subject underwent ten trials, a maximum of 60s each, in which the subject was started from a random quadrant that did not house the platform; therefore, for three consecutive trials the animal began in a different empty quadrant. If the subject found the platform within 60s, it was left to sit on the platform for five seconds and was then removed. If the subject did not find the platform, at the end of the 60s trial it was placed on the platform and allowed to sit for 15 seconds before being removed. Latency to reach the platform was recorded for each subject in each trial. There was a 5-minute inter-trial interval between each of the ten trials.
One hour after the last trial, the platform was removed and each subject underwent one 60s short-term memory probe trial. The subject was placed in a randomly-selected quadrant (that did not house the platform), and was allowed to swim in the pool for a full 60s. When the trial ended, it was removed from the pool. The percent time spent in the quadrant where the platform was hidden during acquisition trials was recorded and used as the dependent variable for this probe test. Twenty-four hours later, a second 60s long-term memory probe trial was performed and time spent in the platform quadrant was used as the dependent variable for this probe test.

Data Analysis

All data was analyzed using JMP software (SAS). A combination of tests, including t-tests, ANOVAs, chi-square tables of frequency, and correlations were used throughout the analyses. For the ELISA data, 2x2x2 (strain x treatment x pregnancy status) ANOVAs and correlations were used to look for significant main effects, interactions, and correlations in the data. The behavioral data was analyzed on 2x2x2 (strain x treatment x sex) ANOVAs, repeated measure ANOVAs, chi-square tables of frequency, and correlation tests.

All outliers were removed from the data a priori to analysis and were determined as being more than two standard deviations away from the mean of each group (characterized by strain, treatment and sex). There was a large amount of variability within and between the groups resulting in differing sample sizes for many analyses. In no instance were more than 2% of animals excluded from any group. However, due to technical errors, the data for twenty animals in the SEB study is missing for some of the days of the Object Recognition Task. Table 12 below has the number of animals missing
for each group and the new Ns for those groups. Whether this missing data influenced the outcome of the analysis will be discussed in Chapter 5 below.
CHAPTER 3: CYTOKINE RESPONSES TO THE BACTERIAL SUPERANTIGENS
STAPHYLOCOCCAL ENTEROTOXINS A AND B (SEA AND SEB) DURING PREGNANCY

Relevant Background and Rationale

Superantigens are potent T-cell activators that have the ability to promote production and release of substantial amounts of proinflammatory cytokines, that normally would not be easily measureable in response to benign protein antigens (eg., bovine serum albumin, which is foreign in a mouse). About 20% of an organism’s T-cells can be activated by superantigens (Fraser & Proft, 2008), causing significant increases in T-cell-derived cytokines. Both SEA and SEB have been shown to be able to increase cytokines such as IL-2, TNF-α and, IFN-γ in the spleen or serum two to four hours after injection (Hayworth et al., 2009; B. Torres et al., 2000; B. A. Torres et al., 2001; Uchiyama et al., 1990), while other studies have shown significant peaks in proinflammatory cytokines just 30-60 minutes after injection (Bette et al., 1993; Miethke et al., 1992a). However, SEA and SEB might have differing effects on different mouse strains due to each SAg’s proclivity for binding to distinct MHC alleles. In this regard, SEA is more potent in animals expressing the H-2b haplotype, as found in C57BL/6 mice, and SEB is more potent in animals expressing the H-2d isotype found in Balb/c mice (B. G. Stiles et al., 1999; Taub et al., 1992). Because of this, injections with both SEA and SEB into the same mouse strain (but into different individual animals) might produce diverse results. For this reason, the present study used mice from both the C57BL/6 strain and the Balb/c
(Balb) strain, and predicted different concentrations of cytokine production after SEA and SEB injections.

These strains also differ fundamentally in terms of other types of immune responses. The C57BL/6 strain of mouse has a naturally different immune response to Balb/c mice that suggests a potentially more complex cytokine outcome. The C57BL/6 mouse contains more mature subsets of dendritic cells (DC) than do Balb/c mice, inducing different TLR expression between the strains in response to microbial pathogens; this leads to differing levels of susceptibility to infections such as *Listeria monocytogenes*, with Balb/c mice succumbing more easily to infection (Liu, Matsuguchi, Tsuboi, Yajima, & Yoshikai, 2002). The strains also differ in the frequency and function of CD4⁺CD25⁺ T-regulatory cells that suppress immune responses when faced with immune challenge, such that Balb/c mice have more CD4⁺CD25⁺ T-regulatory cells which exert greater levels of suppression (X. Chen, Oppenheim, & Howard, 2005). In addition, C57BL/6 mice generally respond to immune challenge with T-helper Type I cells (Th1) that produce elevated levels of IFN-γ and cause macrophage activation, whereas Balb/c mice are known to have a Th2 cell response bias that releases anti-inflammatory cytokines like IL-4, which regulates antibody production (Mills, Kincaid, Alt, Heilman, & Hill, 2000; Watanabe, Numata, Ito, Takagi, & Matsukawa, 2004). These differences in immune response can lead to differing disease profiles in each strain. The C57BL/6 mice are more susceptible to induction of experimental, organ-specific autoimmune diseases, such as experimental autoimmune myasthenia gravis (Graus, BREDA VRIESMAN, & BAETS, 1993) and experimental autoimmune uveitis (Sun et
In contrast, BALB/c mice display increased susceptibility to tumorigenesis (Medina, 1974) and colon tumors (Kuraguchi, Cook, Williams, & Thomas, 2001).

Though both C57BL/6 and Balb/c mice have displayed increased production of proinflammatory cytokines in response to SEA and SEB (Hayworth et al., 2009; B. Torres et al., 2000; B. A. Torres et al., 2001; Uchiyama et al., 1990), direct comparison of cytokine production to these two SAgS between the two strains has yet to be examined. By analyzing cytokine production between SEA or SEB-injected females from both strains who were not pregnant, we were able to study this comparison. Because C57BL/6 mice have a more robust response to SEA than do Balb/c mice, and because they respond in a Th-1-skewed manner under other antigenic challenge conditions (Mills et al., 2000; Watanabe et al., 2004), we hypothesized that the levels of all measured proinflammatory cytokines (IFN-γ, IL-2, IL-6, and TNF-α) would be greater in the C57BL/6 strain. However, because Balb/c mice normally initiate a Th2-skewed response, we expected to see greater levels of IL-4 in this strain in response to SEA. Similarly, because Balb/c mice react more strongly to SEB when compared with C57BL/6 mice, and because of their Th2 bias, we predicted higher levels of IL-4 in Balb/c mice in response to SEB. In fact, we did not hypothesize any detectable concentration of anti-inflammatory cytokines in C57BL/6 mice in response to either SEA or SEB. Lastly, saline-injected non-pregnant females from both strains acted as controls in order to provide basal measures of any constitutive production of cytokines. Concentrations for all cytokines for all cytokines are expected to be negligible in saline-treated animals.

The most important goal of the current chapter is the measurement of cytokine responses to the SAg injections in pregnant females of both strains. Aside from some of
the strain differences we expect to observe, it is of importance to confirm that treatment with SEA or SEB induces a maternal cytokine response. Maternal immune activation occurs because of the mother’s immune response to the immune challenge and not due to direct effects of the pathogen on the fetus (Ashdown et al., 2006). The cytokines and their concentration produced by the mother will precede the neurobehavioral outcome of the offspring. Because of this, it is of utmost importance to understand the products of the mother’s immune system to SEA and SEB before analyzing any behavioral consequences of the offspring.

Given what is known about murine responses to SAgS (discussed above), it would seem simple to predict what the cytokine production would be like in the pregnant dams, as we had predicted above for the non-pregnant females. However, the state of pregnancy is characterized by a functional shift in the immune system and can alter its normal responses. Over the course of the three trimesters in human and murine pregnancy, in conjunction with an increase in estradiol, estriol and progesterone, a female’s immune system shifts to a more anti-inflammatory mode of responding to immune challenge (Raghupathy, 2001; Dionne P Robinson & Sabra L Klein, 2012). In this way, the maternal immune system is better able to tolerate not only the fetus itself (partly made up of “foreign” paternal DNA), but also allows for the transference of antibodies through the placenta (D. P. Robinson & S. L. Klein, 2012). Because of this, when presented with an immune challenge, a pregnant female will produce Th2 cytokines, like IL-4 and IL-10, instead of the usual proinflammatory cytokines like IFN-γ and IL-2 (M Marzi et al., 1996; Piccinni, Scaletti, Maggi, & Romagnani, 2000; D. P. Robinson & S. L. Klein, 2012). While this is necessary for a successful pregnancy, it also comes with some risks.
For instance, when pregnant C57BL/6 mice were infected with *Leishmania major*, they displayed increased severity of infection along with decreased levels of splenic IFNγ and increased levels of splenic IL-4, IL-5 and IL-10 as compared to non-pregnant females (Krishnan et al., 1996). This suggests that even when faced with serious immune challenges, a pregnant female’s immune system may prioritize the survival of the fetus over protection of the mother.

Because of the change in a pregnant female’s immune system, we were unsure of what to expect in terms of cytokine production to SEA or SEB. We hypothesized high levels of proinflammatory cytokine production to the SAgs, however, we expected to see a smaller concentration than in those females who were injected with SEA or SEB but were not pregnant, due to the downregulation of Th1 responses during pregnancy. Our predictions regarding differences between the C57BL/6 and Balb/c strains remained consistent with the non-pregnant animals: we anticipated higher proinflammatory concentrations to SEA in the C57BL/6 strain, around equal amounts to SEB in both strains, and higher levels of the anti-inflammatory cytokine IL-4 in Balb/c mice regardless of the type of SAg injected. Differing from our predictions in non-pregnant animals, we did expect to see some detectable concentrations of IL-4 in the C57BL/6 mice to SEA. Because SEA strongly affects C57BL/6 mice, and because pregnancy is known to induce a Th2-skewed response, we hypothesized an increase in IL-4 production compared to saline-injected animals. As before, saline-injected pregnant females served as controls in order to highlight significant production of cytokines to the SAgs in both strains.
**Materials and Methods**

*Experimental groups and procedure*
Similarly-aged female C57BL/6 and Balb/c mice were split into two groups based on pregnancy status (pregnant vs non-pregnant) on embryonic day 12.5 (E12.5) of pregnancy. Animals from each group were randomly assigned to be injected with either 5μg (approximately 250 μg/kg) of SEA, SEB (Toxin Technology, Sarasota, FL) or 0.9% saline. We split the SEA-injected animals and SEB-injected animals into two groups such that separate saline-injected animals were used to compare against each group. This was because immunoassays were performed first on the SEB-injected animals and then on the SEA-injected animals, and we wanted to keep everything consistent with the saline groups. Therefore, there were four total injection groups: SEA, Saline (vs SEA), SEB, and Saline (vs SEB). The breakdown of the number of animals per group can be seen in Table 4. It should be noted that more animals than are listed were injected with SEA and SEB, but only animals that showed significant production of IL-2, an indicator of T-cell activation (K. A. Smith, 1988), were included in the analysis.

Two hours after injection, animals were placed in a sealed CO₂ chamber for approximately five minutes and then removed and monitored for another three minutes to ensure death. Their spleens were removed and stored at -80°C until ready for homogenization. During the spleen removal, pregnancy status was confirmed by checking the abdomen for the presence of embryos.

Spleens were homogenized in 1mL of 1x phosphate-buffered saline (PBS) and spun in glass vials for 10 minutes at 10,000 revolutions per minute. The supernatant of each vial was collected and stored at -80°C until the time of assay. Enzyme-linked immunosorbent assays (ELISAs) were performed on each sample, targeting four or six...
different cytokines: IL-2, IL-6, IFN-γ, IL-4, and in some cases TNF-α and IL-17A. Because of timing issues and technical difficulties, we were only able to assay a selection of saline-injected and the SEA-injected mouse spleens for TNF-α and IL-17A, but we were not able to analyze SEB-injected mouse spleens for these cytokines. We do not believe this will significantly impact our analysis since the other three proinflammatory cytokines—IL-2, IL-6, IFN-γ—should still be able to give us a good perspective on how the immune system is responding to SEB. The ELISAs were performed over a three-day period as described above (see Chapter 2). Total protein was quantified for each individual spleen (see Chapter 2), and all cytokine concentrations are expressed as picogram (pg) of cytokine per microgram (µg) of protein.

Data Analysis

Concentrations for each cytokine were retrieved from the ELISA and protein assay analyses and used as the dependent variables in the data analysis. Concentrations are expressed as picograms of cytokine per microgram of protein (i.e. pg/µg protein). The main effects of interest for the measured cytokines were the treatment group (SEA, SEB or saline), the strain of the animal (C57BL/6 or Balb/c) and pregnancy status (pregnant or not pregnant). Therefore, a 2x2x2 ANOVA was run for each cytokine of interest. However, if one factor (toxin treatment, for example) did not produce any measureable cytokine concentrations, it was removed from the analysis and a 2x2 (strain x pregnancy status) ANOVA was used. In addition, correlations were run with all cytokines tested, analyzing relationships between the concentrations for each cytokine. Main effects, interactions, and correlations were considered significant at a p<0.05 level. All outliers were removed from the data a priori to analysis and were determined as being more than
two standard deviations away from the mean of each group (characterized by strain, treatment and pregnancy status). No more than one data point was excluded from each ANOVA, and each of those outliers was removed from the correlation analysis.

**Results**

*SEA Group*

Six cytokines were measured for all spleens: IL-4, IFN-γ, IL-6, IL-2, TNF-α, and IL-17A. Saline-injected animals did not produce detectable levels of cytokine concentrations for IFN-γ, IL-6, IL-2, and TNF-α, and so 2x2 (strain x pregnancy status) ANOVAs were run to analyze those data. This in itself is significant in that SEA injections, in comparison to saline injections, were effective in eliciting IFN-γ, IL-6, IL-2, and TNF-α responses that would otherwise not be present. Only four of the 19 saline-injected animals produced detectable levels of IL-17A, and two of these were deemed outliers *a priori* to data analysis. All but one saline-injected animal produced detectable levels of IL-4, but two of these were deemed outliers *a priori* to data analysis. We are unsure why we detected baseline IL-4 levels, as usually they are undetectable, but after running the ELISA multiple times and getting similar results, we decided to use the data as it is. Therefore, for both the IL-17A and IL-4 data analysis, 2x2x2 (strain x treatment x pregnancy status) ANOVAs were used.

**Interleukin-4.** Even though most of the saline-injected animals produced detectable levels of IL-4, there was a main effect of treatment, $F_{(1,33)}=22.78$, $p<0.0001$, such that SEA-exposed mice produced significantly higher concentrations of IL-4 than controls (Figure 4). A one-way ANOVA also revealed that there was a strong trend for non-pregnant SEA-
exposed C57BL/6 mice to produce significantly more IL-4 than pregnant mothers, $F_{(1,12)}=4.34$, $p=0.0594$ (Figure 4). There were no strain differences between the Balb/c and C57BL/6 mice in IL-4 production ($F_{(7,33)}=0.83$, $p=0.3692$).

**Interferon-γ.** For IFN-γ, there was a main effect of strain on production in response to SEA, $F_{(1,36)}=4.14$, $p=0.0493$, indicating that C57BL/6 mice produced significantly higher concentrations than Balb/c mice (Figure 5). Though non-pregnant mothers from both strains appeared to produce higher concentrations of IFN-γ than pregnant animals (Figure 5), this difference did not come out significant, $F_{(1,20)}=2.76$, $p=0.1122$.

**Interleukin-2.** For IL-2 production, there was no main effect of strain, $F_{(1,20)}=2.72$, $p=0.1145$, indicating equal production in both strains in response to SEA exposure (Figure 6). There was, however, a main effect of pregnancy status, such that both C57BL/6 and Balb/c non-pregnant mice produced significantly higher concentrations of IL-2 than pregnant mothers, $F_{(1,20)}=7.02$, $p=0.0154$ (Figure 6).

**Interleukin-6.** There was a main effect of strain on IL-6 concentrations, with C57BL/6 mice exhibiting increased IL-6 production after SEA injections, as compared to Balb/c mice ($F_{(1,20)}=26.98$, $p<0.0001$) (Figure 7). In addition, a one-way ANOVA revealed a very strong trend for non-pregnant C57BL/6 mice to produce increased concentrations of IL-6 relative to pregnant females ($F_{(1,12)}=4.22$, $p=0.0623$) (Figure 7). No IL-6 was found in saline-injected mice.

**Tumor necrosis factor-α.** The results showed that C57BL/6 mice produced significantly higher TNF in response to SEA, as compared to Balb/c mice injected with SEA
There was also a main effect of pregnancy status, independent of strain, that showed higher concentrations of TNF-\( \alpha \) in non-pregnant animals relative to pregnant animals \((F_{1,20}=14.55, p=0.0011)\) (Figure 8). No saline-injected animals produced detectable concentrations of TNF-\( \alpha \).

**Interleukin-17A.** Lastly, four saline-injected animals exhibited detectable concentrations of IL-17A, but two of these were deemed outliers and removed from data analysis. Despite this, there was a main effect of treatment, such that SEA-injected animals produced significantly more IL-17A than controls \((F_{1,33}=27.18, p<0.0001)\) (Figure 9). There was also a main effect of pregnancy status, with non-pregnant females producing significantly increased concentrations of IL-17A than pregnant females \((F_{1,33}=4.97, p=0.0327)\), although this was only true for the SEA-exposed mice and not for the saline-injected controls \((F_{1,33}=5.79, p=0.0219)\) (Figure 9).

**Spleen Total Protein.** Protein assays, measured in micrograms of protein, were performed for all subjects and compared between groups on a 2x2x2 (strain x treatment x pregnancy status). There was a main effect of pregnancy status on total protein amount, with pregnant spleens, regardless of strain, containing increased levels of protein than non-pregnant spleens, \((F_{1,35}=25.50, p<0.0001)\) (Figure 10). This did not affect the relative amount of cytokine concentrations since all of the above concentrations are expressed in pg of cytokine per \(\mu \)g of protein. Correlations were run for the concentrations of each of the six cytokines, and the results can be seen in Table 5. Concentrations for all six cytokines were significantly correlated with one another, with proinflammatory cytokines IFN-\( \gamma \), IL-2, IL-6 and TNF-\( \alpha \) having very strong correlations with one another, while
anti-inflammatory cytokine IL-4 and Th17 cytokine IL-17A having moderate correlations with proinflammatory cytokines and each other.

**SEB Group**

Because the SEB experiments were carried out first, only four cytokines were measured in the samples: IL-4, IFN-γ, IL-6, and IL-2. It was only later in the study that TNF-α and IL-17A were added to the analysis, but not enough SEB sample was left to use for these assays. None of the saline-injected animals produced detectable levels of IL-4, IFN-γ, IL-6 or IL-2, and so a 2x2 (strain x pregnancy status) ANOVA was run to analyze those data. This in itself is significant in that SEB injections, in comparison to saline injections, were effective in eliciting IL-4, IFN-γ, and IL-2, responses as would be expected from the immunological literature (Fraser & Proft, 2008).

**Interleukin-4.** For IL-4 concentrations, there was a main effect of strain \( F_{(1,18)}=19.52, p=0.0003 \), indicating overall higher concentrations of IL-4 in Balb/c animals compared to C57BL/6 animals (Figure 11). In fact, it is questionable if SEB readily induces IL-4 in C57BL/6 mice. In response to SEB, only one of five non-pregnant, and only one of eight pregnant mice showed detectable IL-4 levels after SEB injection. There was also a significant strain x pregnancy interaction, in which pregnant Balb/c animals produce significantly more IL-4 than non-pregnant Balb/c animals (Figure 11). Given the poor IL-4 response by C57BL/6 mice, it is evident that the statistical results are driven by the Balb/c IL-4 response to SEB.

**Interferon γ.** For IFN-γ, there was a similar main effect of strain, \( F_{(1,18)}=36.10, p<0.0001 \), with higher concentrations present in Balb/c mice, regardless of pregnancy
status (Figure 12). In this case, four of the five non-pregnant C57BL/6 mice produced detectable levels, while only three of eight pregnant C57BL/6 were detectable for IFN-γ, one of which was deemed an outlier. Though there was no significant strain x pregnancy status interaction in the 2x2 ANOVA, a one-way ANOVA revealed a significant difference between pregnant and non-pregnant C57BL/6 mice, showing a significantly higher concentration of IFN-γ in non-pregnant females, $F_{(1,10)}=7.25$, $p=0.0226$ (Figure 12).

Interleukin-2. The same main effect of strain could be seen for IL-2 concentrations, $F_{(1,19)}=8.21$, $p=0.0198$, indicating higher levels of IL-2 in Balb/c mice (Figure 13). There was also a main effect of pregnancy status for IL-2, $F_{(1,19)}=8.21$, $p=0.0099$, with non-pregnant animals producing higher concentrations of IL-2 than pregnant animals (Figure 13). Though there was no significant strain x pregnancy status interaction in the 2x2 ANOVA, this main effect of pregnancy status was heavily weighted by the C57BL/6 animals, which according to a one-way ANOVA, does reflect higher levels in the non-pregnant animals compared to the pregnant animals, $F_{(1,11)}=7.74$, $p=0.0178$ (Figure 13).

Interleukin-6. For IL-6, four outliers were identified a priori. Two of these outliers belonged to the Balb/c pregnant group, while the other two belonged to the C57BL/6 pregnant group. Because each of these groups only had five animals in each of them, it seemed that removing the outliers would impact the outcome of the statistical analysis. An analysis was run with the four outliers included and another analysis was run without them; both analyses produced the same outcome. The 2x2 ANOVA for strain and pregnancy status did not reach significance for main effects or an interaction in either
case, $F_{(3,19)}=1.40$, $p=0.2707$ (including outliers; Figure 14), $F_{(3,15)}=0.44$, $p=0.7277$ (not including outliers). Thus, it seems that SEB did not produce a significant IL-6 response in the animals.

**Spleen Total Protein.** Protein assays, measured in micrograms of protein, were performed for all subjects and compared between groups. There was a main effect of pregnancy status on the amount of protein, indicating increased amounts of protein in pregnant animals compared to non-pregnant animals, $F_{(1,31)}=5.58$, $p=0.0005$ (Figure 15). This did not affect the relative amount of cytokine concentrations since all of the above concentrations are expressed in pg of cytokine per $\mu$g of protein. Correlations were run for the concentrations of each of the four cytokines, and the results can be seen in Table 6. Concentrations for IL-4, IFN-$\gamma$, and IL-2 were all significantly positively correlated with one another. Concentrations of IL-6 were only correlated with concentrations of IL-2 and IFN-$\gamma$.

**Discussion**

The above results show that staphylococcal enterotoxins A and B are potent activators of the immune system, but their effects on cytokine production can be moderated depending on the strain of mouse into which it is injected and whether that mouse is pregnant (Table 7). Not surprisingly, saline injections did not produce any detectable levels of IFN-$\gamma$, IL-2, IL-6 or TNF-$\alpha$ in either strain of mouse. However, as expected, SEA and SEB caused significant elevations in cytokine concentrations in most animals. Staphylococcal enterotoxins A and B are superantigens capable of activating a large number of T-cells by crosslinking MHC class II molecules with T-cell receptors, without the need for specific
recognition of the toxin in the peptide-binding region of the T-cell receptor (Fraser & Proft, 2008; Pinchuk et al., 2010). This activation of T-cells has been shown to be able to increase all of the above tested cytokines in the spleen or serum two to four hours after injection (Hayworth et al., 2009; Lagoo et al., 1994; Miethke et al., 1993; B. Torres et al., 2000; B. A. Torres et al., 2001; Uchiyama et al., 1990), a time period chosen for analysis in the current experiments.

There were clear strain differences in the production of cytokines to both SEA and SEB. An important indication of T cell activation is the production of IL-2. Both strains of mice showed similarly strong IL-2 responses to SEA and SEB. This provided confirmation that the T cell arm of the immune system was activated by these toxins. However, variations in the magnitude of cytokine responses was observed between strains and across the two SEs. In response to SEA injections, C57BL/6 mice produced significantly higher concentrations of proinflammatory cytokines IFN-γ, IL-6 and TNF-α, while SEB injections produce higher concentrations of IL-4, IFN-γ and IL-2 in Balb/c mice, all regardless of pregnancy status. Different isotypes of the MHC II molecule exist, and each type can be more efficacious for T cell activation when binding to a specific type of Staphylococcal enterotoxin (Taub et al., 1992). The C57BL/6 mice contain the H-2b isotype, responding more strongly to SEA, while Balb/c mice contain the H-2d isotype, which has been shown to produce a more robust immune response when bound by SEB (B. Stiles et al., 1993; Taub et al., 1992). Therefore, it makes sense that each strain responded more robustly with certain cytokines to its respective preferential SAg. In terms of the poor IL-4 and IL-17A responses to SEA, it is possible that the
concentrations of these cytokines were too low in both strains to detect any noticeable strain differences.

It was surprising, however, that IL-2 concentrations in response to SEA were not different between the strains and that IL-6 concentrations in response to SEB were also not different between Balb/c and C57BL/6 mice. However, even though the production of other cytokines is different between the two strains in response to SEA, the impact on T-cell proliferation itself, as suggested by IL-2, may not be different (Taub et al., 1992). This is supported by an older report that these strains do not differ in their proliferative response to SEA (Taub et al., 1992). This is in contrast to the effects of SEB, which in Balb/c mice, increased T-cell proliferation more significantly than in C57BL/6 mice (Taub et al., 1992). Thus, possibly explaining the discrepancy in IL-2 production to SEA and SEB exposure in both strains.

Measures of IL-6 did not show a difference between the two strains after SEB injections. One explanation for this could be that IL-6 was not yet at its peak level when the spleens were removed. IL-6 has been shown to be at its highest concentration 4 hours post-injection (Miethke et al., 1993), whereas in the current study spleens were removed at 2 hours. Another explanation might be the source of IL-6 following an SEB injection. Superantigen-reactive T-cells are the source of IL-2, IL-4 and IFN-γ, but IL-6 could be coming from other cells aside from T-cells, such as macrophages, endothelial cells and fibroblasts (Carlsson & Sjögren, 1985; Miethke et al., 1993). Thus, while SEB produces increased concentrations of IL-2, IL-4 and IFN-γ in BALB/c mice, perhaps C57BL/6 animals produce comparable amounts of IL-6 from other cells being activated by the SEB injection. In fact, C57BL/6 mice produced more IL-6 in response to SEB than the other
proinflammatory cytokine, IFN-γ. Support for this explanation can come from the fact that while SAgs have been consistently shown to increase T-cell derived cytokines like IL-2, TNF-α and IFN-γ, significant increases in T-cell-derived IL-6 are reported less often (Fraser & Proft, 2008; Hayworth et al., 2009; Kohman, Crowell, Urbach Ross, & Kusnecov, 2009; B. Stiles et al., 1993).

The strain differences in the concentrations of IFN-γ were dependent on the type of toxin used. For SEA, C57BL/6 mice were higher, whereas for SEB, the BALB/c mice had a stronger response. Once again, this could be due to MHC class II haplotype differences. Interestingly, C57BL/6 mice are known to have a Th-1 pro-inflammatory skew when infected with a pathogen (Mills et al., 2000; Watanabe et al., 2004). However, this skew was not enough to compensate for the affinity of SEB to the H-2d isotype, leading to increased production of IFN-γ in Balb/c mothers. It is important to note that though C57BL/6 mice do not contain the H-2d isotype of the MHC II molecule, the females did still produce significant detectable concentrations of IL-2, IL-6, and IFN-γ to SEB injections as compared to the saline injections. Whether these levels of cytokines were enough to produce changes in the offspring will be discussed in chapters below.

The Balb/c mice produced high amounts of detectable IL-4 to SEB, whereas C57BL/6 mice did not. In addition, there were no differences between the strains in IL-4 concentrations in response to SEA. As mentioned above, SEB reacts more potently with an MHC II allele found in Balb/c mice but not in C57BL/6 mice, producing increased levels of cytokines in response to infection. In addition to this, C57BL/6 mice tend to produce Th1 cytokines when challenged by pathogens, producing low levels of anti-inflammatory cytokines like IL-4 and IL-10 (Mills et al., 2000; Stanford & McFadden,
2005; Watanabe et al., 2004). Thus, SEB increased production of IL-4 in Balb/c mothers but caused very low levels in C57BL/6 mice. Along the same lines, even though C57BL/6 mice respond stronger to SEA, because they respond in a proinflammatory-type way, IL-4 production is comparable to the Th-2-skewed Balb/c mice.

Interestingly, there were clear differences in cytokine production between pregnant animals and non-pregnant virgin females. In SEB-injected mice, pregnant Balb/c mice produced significantly more IL-4 than non-pregnant mice, while for, pregnant C57BL/6 mice, IFN-γ and IL-2 were lower relative to non-pregnant mice. In SEA-injected animals, IL-2, IL-6, TNF-α, and IL-17A concentrations were all greater in non-pregnant animals than pregnant ones, irrespective of strain. As we predicted, these results might reflect the anti-inflammatory nature of pregnancy, which can shift T cells to a more Th-2 type cytokine production, such as IL-4 and IL-10, with less production of proinflammatory cytokines like IFN-γ (M Marzi et al., 1996; Raghupathy, 2001; D. P. Robinson & S. L. Klein, 2012). This serves to protect the fetus, which contains foreign paternal DNA, and aids in the passage of necessary antibodies across the placenta (D. P. Robinson & S. L. Klein, 2012). Even though the animals were challenged with potent pathogenic toxins like SEA and SEB, the inflammatory responses were dampened, but not extinguished by pregnancy.

Overall, it is possible that there were no differences in pro-inflammatory cytokines in the between pregnant and non-pregnant Balb/c mice given SEB, because Balb/c mice are already characterized as an anti-inflammatory-skewed strain (Watanabe et al., 2004). Immune challenge plus pregnancy does not doubly reduce the Balb/c pro-
inflammatory response (by reducing IFN-γ and IL-2), but could doubly increase the anti-inflammatory reaction (by increasing IL-4, as was observed).

In general, many of the predicted outcomes for this study proved correct. That is, C57BL/6 animals responded with significantly greater concentrations of most proinflammatory cytokines to SEA exposure, while Balb/c mice had greater cytokine concentrations to SEB exposure. Most interestingly, pregnant animals exhibited reduced production of proinflammatory cytokines, and in the case of Balb/c SEB-injected mice, increased anti-inflammatory IL-4.
CHAPTER 4: BEHAVIORAL PHENOTYPE OF MICE BORN TO MOTHERS TREATED DURING PREGNANCY WITH STAPHYLOCOCCAL ENTEROTOXIN A

Relevant Background and Rationale

Staphylococcus aureus is a gram-positive bacterium that produces virulent exotoxins that target the intestines and gut to cause an array of diseases and illnesses. Exotoxins of the bacteria, known as staphylococcal enterotoxins (SEs), can present in one of over 20 forms (SEA-V) and target the intestines and stomach (Pinchuk et al., 2010). Because of their ability to cross-link APCs and T-cells without the need for specific antigen recognition—hence, being a “superantigen”—a prolific number of T-cells are activated and release exponential amounts of proinflammatory cytokines (Fraser & Proft, 2008; B. A. Torres et al., 2001). Staphylococcal enterotoxin A (SEA) is one of the most widely studied SEs because of its role in many infections and diseases, especially food-borne illnesses (Hu et al., 2007). Within just four hours of injection with SEA, robust increases in proinflammatory cytokines such as IFN-γ, IL-6, and TNF-α have been seen, in addition to inflammation of the intestines and stomach (Norrby-Teglund et al., 1994; Uchiyama et al., 1990). Though not a conventional immune challenge nor antigen, SEA is a potent method of activating the immune system.

Activating the immune system of a pregnant female at different time points during gestation has been shown to lead to behavioral deficits in the offspring. Current research mainly utilizes influenza, lipopolysaccharide (LPS), or the synthetic single-stranded RNA virus polynosinic:polycytidylic acid (poly IC) as a means for inducing MIA. It is believed that these immune challenges cause a surge in production of proinflammatory
cytokines such as IL-6 and IFN-γ, which ultimately cross the placenta and interfere with fetal brain development (Ashdown et al., 2006; Urs Meyer, Yee, & Feldon, 2007; Patterson, 2011). Behavioral abnormalities seen in MIA offspring mainly reflect symptomatology that characterizes the neuropsychological disorders of autism and schizophrenia, such as social dysfunction and sensorimotor gating deficits.

One of the core symptoms of autism, MIA offspring consistently display deficits in social behavior from an early age. Social behavior can be tested in a number of ways, from the number of interactions an animal makes with an age-matched, sex-matched peer, to the amount of time it spends making those contacts, to the number of ultrasonic vocalizations a pup makes both in isolation and during social encounters. Administration of poly IC in the second or late second trimester has shown to reduce both the number of interactions and the time an offspring spends interacting with a container a peer is in as compared to an empty container ((Lipina, Zai, Hlousek, Roder, & Wong, 2013; Schwartzer et al., 2013; S. E. Smith et al., 2007). Injection with LPS during the second trimester also reduced social behaviors and exploration in mice as young as three weeks old (Oskvig, Elkahloun, Johnson, Phillips, & Herkenham, 2012), while pups from influenza-injected mothers displayed similar behavioral results (Shi et al., 2003). It has also been shown that maternal injections with poly IC may cause a reduced number of ultrasonic vocalizations when pups are in isolation from as young as eight days old up until adulthood, and adult male offspring vocalize significantly less when encountering female counterparts (N. Malkova, 2010). In addition, the quality of the male vocalizations are different to saline offspring, and males display reduced scent marking in response to female urine (N. V. Malkova et al., 2012). Lastly, it is interesting to note that social
abnormalities in connection with MIA has recently been seen in rhesus macaques whose mothers were injected with poly IC toward the end of their first trimester (Bauman et al., 2014).

Increased anxiety and information processing changes have both been shown to be present in those diagnosed with autism and/or schizophrenia and have both also been linked with MIA. Though not a core symptom of either autism or schizophrenia, both disorder have high comorbidity rates with anxiety and anxiety-like symptoms (Cosoff & Julian Hafner, 1998; Kim, Szatmari, Bryson, Streiner, & Wilson, 2000; Seedat, Fritelli, Oosthuizen, Emsley, & Stein, 2007; White, Oswald, Ollendick, & Scanhill, 2009). In mice and rats anxiety is measured by the amount of time in which they spend venturing out into open or elevated spaces as opposed to the time they spend in closed-off or shaded areas. Maternal injections with poly IC in mid or late second trimester were related to increased anxiety levels in the adult pups as compared with pups of saline-injected mothers (Golan, Lev, Mazar, Hallak, & Huleihel, 2006; U. Meyer et al., 2006; S. E. Smith et al., 2007). Adult mice whose mothers were injected with LPS throughout their second trimesters also showed markedly increased anxiety levels in open field tests (Wang et al., 2010). Therefore, it is common to find increased anxiety in MIA pups. In addition, dysfunctions in information processing is a key feature of schizophrenia that contribute to a wide range of psychological problems present in the disorder (Braff, 1993). Animals can be tested on the efficiency of information processing through the use of a novel object task: an object unfamiliar to the animal is placed in an open field with a familiar object to the animal, and the subject is given time to explore both objects. Healthy animals investigate the novel object at an increased rate relative to the familiar
object, demonstrating a memory for the familiar object and a willingness to explore a new one. However, as demonstrated by mid-pregnancy maternal LPS and poly IC injections, novel object exploration is significantly decreased in pups of MIA mothers relative to saline mothers (Coyle et al., 2009; Ozawa et al., 2006; Wang et al., 2010; Wolff et al., 2011). Furthermore, pups whose mothers were injected with influenza virus in the second trimester of pregnancy also displayed deficiencies in novel object exploration (Shi et al., 2003). Conversely, there is also evidence for increased exploration of a novel object in MIA pups (H. Golan, V. Lev, M. Hallak, Y. Sorokin, & M. Huleihel, 2005; Ito, Smith, Hsiao, & Patterson, 2010). Either way, significant changes in information processing and increased anxiety are present in rodent offspring of MIA mothers.

The most frequent, robust and consistent behavioral alteration in rodent offspring of MIA mothers is a deficit in prepulse inhibition (PPI) of the acoustic startle response. Prepulse inhibition is viewed as a measure of sensorimotor gating by which excess or unimportant sensory influences are filtered out in order to focus on and process the most significant aspects of the environment. An initial, weaker stimulus temporarily dampens the processing system so that when another stimulus immediately follows it the second input does not evoke as heightened a response as it normally would. A deficiency in PPI is so prevalent in schizophrenia patients, it has even been suggested that it is an endophenotype of the disease (Braff & Freedman, 2002). Likewise, deficits in PPI have been robustly reported in adult offspring of rodent mothers injected with LPS during their pregnancy (Borrell et al., 2002; M.-È. Fortier, Joober, Luveshi, & Boksa, 2004; M. E. Fortier et al., 2007; Romero et al., 2007). Similarly, more than four separate research
groups has found significantly reduced PPI in offspring of mothers injected with poly IC in their second trimesters of pregnancy (Makinodan et al., 2008; Urs Meyer, 2013; U. Meyer et al., 2009; U. Meyer et al., 2008; Urs Meyer et al., 2007; Ozawa et al., 2006; Vorhees et al., 2015; Yee, Ribic, de Roo, & Fuchs, 2011). Therefore, deficiencies in PPI in MIA mice and rats is extremely common and consistent.

The Morris water maze (MWM) is a test of spatial learning for rodents that relies on distal cues to navigate from start locations around the perimeter of an open swimming arena to locate a submerged platform. Spatial learning is assessed across repeated trials and reference memory is determined by preference for the platform area when the platform is absent. Both learning and memory for this task are disrupted in MIA offspring. Adult offspring of LPS-injected mothers display longer latencies over repeated trials to find the platform, indicating aberrant learning, while spending less time in the area of the platform once it is removed, suggesting diminished memory for its spatial location (H. Golan et al., 2005; Golan et al., 2006; Hao, Hao, Li, & Li, 2010; F. Lanté et al., 2008; F. Lanté et al., 2007). Similarly, adult pups from poly IC-injected mothers display these same results (Ito et al., 2010; Khan et al., 2014; U. Meyer et al., 2006; U. Meyer et al., 2008). In addition, MIA pups showed decreased flexibility in the MWM: when the platform was moved to a new location, MIA offspring could not learn its new location as compared to the control offspring who could (Ito et al., 2010; Khan et al., 2014; U. Meyer et al., 2006; U. Meyer et al., 2008). This implies a difficulty in adapting to a modification introduced to previously-acquired information. Taken together, adult offspring from MIA mothers display not only impaired spatial learning and reference
memory, but also impaired cognitive flexibility when compared to their peers from saline-injected mothers.

The timing of the injection into the mother may play an important role in the behavioral outcome of the offspring. There is much variation in the MIA literature as to when maternal injections take place, with most research focusing on either the late first/early second trimester (E12.5 in mice and rats) or the late second/early third trimester (E17). Each time point represents a distinct period in fetal rodent brain development, with second trimester representing a time point characterized by period of major neuronal proliferation and migration, and the third trimester representing a time point characterized by tangential growth of the cortex and by the spreading and subdivision of the central cerebellar nuclei (Goffinet, 1984; Miska et al., 2004). There is evidence that injection with LPS and poly IC at each time point will produce distinct immunological responses in both the mother and fetus, as well as different behavioral profiles (Cui et al., 2009; M. E. Fortier et al., 2007; U. Meyer et al., 2006; U. Meyer et al., 2008). However, all of the above-mentioned behavioral deficits have been indicated in adult offspring of mothers injected with an immune challenge during their second trimester of pregnancy (for review, see (Boksa, 2010)), with many focusing on E12.5 (Ito et al., 2010; S. E. Smith et al., 2007). In addition, there is also epidemiological evidence highlighting this time period of human pregnancy as a particularly vulnerable time of increasing the risk of offspring developing autism or schizophrenia as a result of maternal infection (Atladóttir et al., 2010; Alan S Brown, Begg, et al., 2004). Because this time period is crucial for developing cells destined for cortical migration (Gillies & Price, 1993), and because previous research has demonstrated this time as significant in
influencing behavioral outcomes of the offspring, the present study injected mothers
during the critical time of E12.5.

Crucially, SEs present in over 20 serologically distinctive forms (SEA-V), each
with unique binding properties so that they do not cross-react with one another (Pinchuk
et al., 2010). In addition, MHC II molecules can have varied allele polymorphisms such
that different animals contain differing isotypes of the molecules (Taub et al., 1992).
Staphylococcal enterotoxin A is a more potent T-cell activator in animals that express the
H-2b isotype of MHC II (B. Stiles et al., 1993; Taub et al., 1992). In mice, the C57BL/6
strain is characterized by the expression of this isotype, whereas the Balb/c strain is not.
As shown above in Chapter 3, SEA produced higher levels of circulating pro-
inflammatory cytokines in C57BL/6 mothers as opposed to their Balb/c counterparts.
Because of this, we anticipate differing behavioral results between the two strains of
offspring. We expect C57BL/6 pups to display a more severe behavioral phenotype
associated with maternal immune activation than the Balb/c pups. In particular, we
anticipate lower levels of social interaction, novel object exploration and prepulse
inhibition, higher levels of anxiety, and increased cognitive impairment in regards to the
MWM. While we may see some of the same behavioral patterns in the Balb/c mice (as
increased levels of all cytokines measured was produced in response to SEA), we do not
expect as strong a result.

**Material and Methods**

*Experimental groups and procedure*

Similarly-aged female C57BL/6 and Balb/c mice were injected on embryonic day 12.5
(E12.5) of pregnancy. Animals from each strain were randomly assigned to be injected
with either 5μg (approximately 250 μg/kg) of SEA (Toxin Technology, Sarasota, FL) or 0.9% saline. The number of mothers per group, with average litter size in parentheses, can be seen in Table 8. There were no significant differences in litter size ($F_{(3,14)}=0.1255$, $p=0.9435$). Pups were weaned three weeks after birth, and were separated based on sex at five weeks after birth. The numbers of male and female pups per behavioral group are listed in Table 9.

At six weeks old, the offspring began the behavioral testing schedule (Figure 2). Testing took place at approximately the same time every day, starting between 9 and 10 AM. Each animal underwent five total tests in the same order and at the same age as one another. The tests consisted of a social interaction task, the elevated plus maze (EPM), and object recognition (OR) task, a prepulse inhibition (PPI) session, and a two-day Morris water maze (MWM) task. As a means of reducing test order effects, fatigue, and stress, the animals were given a one day rest between the EPM and OR tasks, two days between the OR and PPI tests, and three weeks between the PPI and the MWM. A detailed description of the behavioral tests can be seen in Chapter 2.

**Data Analysis**

The behavioral data was analyzed on 2x2x2 (strain x treatment x sex) ANOVAs, repeated measure ANOVAs, chi-square tables of frequency, and correlation tests. All outliers were removed from the data *a priori* to analysis, if values were more than two standard deviations away from the mean of each group (characterized by strain, treatment and sex). In instances when there was a main effect of strain differences, planned one-way ANOVAs by strain were conducted to parse out differences within one strain that may have been affecting overall treatment effects. There was a large amount of variability
within and between the groups resulting in differing sample sizes for many analyses. In no instance were more than 2 animals excluded from any group. Behavioral data for the EPM, OR and MWM was recorded using the Any-Maze software program (San Diego Instruments). Social interaction data was recorded through IBM compatible computer "basic" software, and PPI data was collected through the SR-Lab startle response system (San Diego Instruments). All data was analyzed using JMP software (SAS).

**Results**

**Social Interaction**

The numbers of touches made for each cup was recorded, and the percent of total touches made to the target cup ($\frac{\# \text{ touches target cup}}{\# \text{ touches target cup} + \# \text{ touches to control cup}}$) were analyzed. According to a 2x2x2 (strain x treatment x sex) ANOVA, there were no significant main effects or interactions between the different variables, all p’s > 0.1997. However, there did seem to be a trend for C57BL/6 SEA offspring to have touched the target cup less then saline offspring, and because our main interest was analyze treatment differences within strains, we performed a planned one-way ANOVA which confirmed this trend, F$_{(1,47)}$=3.67, p=0.0614 (Figure 16a). The total time each animal spent touching the cups was recorded and a percentage of time spent touching the target cup ($\frac{\text{time touching target cup}}{\text{time touching target cup} + \text{time touching control cup}}$) was calculated and analyzed with a 2x2x2 (strain x treatment x sex) ANOVA. There were no significant main effects or interactions, all p’s > 0.215. However, for C57BL/6 mice, a planned one-way ANOVA revealed a significant difference between the SEA offspring and saline offspring,
F_{(1,47)}=5.18, p=0.0274, such that the SEA offspring spent significantly less time touching the target cup than the controls (Figure 16b). Taking these two analyses together, the C57BL/6 SEA offspring touched and spent less time touching the target cup with a novel mouse than their C57BL/6 counterparts from saline-injected mothers.

_Elevated Plus Maze (EPM)_

Animals were recorded in each part of the EPM apparatus, and the amount of time they spent in the closed arms, open arms, and center area was collected. A 2x2x2 (strain x sex x treatment) ANOVA was used to compare differences between the groups. There was a significant main effect of strain, F_{(1,87)}=119.92, p<0.0001, on the percent time the animals spent in the open arms, such that C57BL/6 mice spent relatively more time in the open arms (Figure 17a). There was a significant strain x sex interaction, F_{(1,87)}=4.76, p=0.0319, with C57BL/6 females spending more time in the open arms than C57BL/6 males. Lastly, there was also a strain x treatment interaction, F_{(1,87)}=5.08, p=0.0267. It was evident for the C57BL/6 mice that saline offspring spent significantly more time in the open arms than the SEA offspring (Figure 16a). For both the percent time spent in the closed arms and the center area, there were three outliers identified _a priori_ to data analysis and removed from the analysis. There was a main effect of strain for the percent time in the closed arms, F_{(1,84)}=233.34, p=<0.0001, and also for the percent time in the center area, F_{(1,84)}=117.15, p<0.0001. This was due to Balb/c mice, regardless of treatment or sex, spending more time in the closed arms and less time in the center area. There were no main effects of treatment or sex for either of these measures.

The strain x treatment interaction in percent open time for the C57BL/6 offspring, and the absence of this for percent time in the center area, implies that while the saline
offspring ventured into the center just as often as the SEA offspring, they crossed over into the open arms much less frequently. This was validated by two 2x2x2 ANOVAs comparing the number of entries into the open arms and the number of entries into the center. While there was no difference between the C57BL/6 SEA and saline offspring for the number of entries into the center, $F_{(1,85)}=0.8905$, $p=0.6973$, there was a significant increase in open arms entries for C57BL/6 saline offspring as compared to SEA offspring, $F_{(1,85)}=2.98$, $p=0.0038$ (Figure 17b). There were also main effects of strain on the number of entries into the center and open arms, with increased entries for C57BL/6 mice compared to Balb/c mice, $F_{(1,85)}=120.21$, $p<0.0001$; $F_{(1,85)}=107.93$, $p<0.0001$, respectively.

The distance traveled in the EPM was also measured, as was the latency to enter the center area and the open arms. Four outliers were determined a priori to data analysis for the distance, and two outliers were identified for the latency to enter the open arms. All three of these measures were analyzed using a 2x2x2 ANOVA. There were significant main effects of strain for each of these measures, such that C57BL/6 mice traveled overall further distances in the EPM (both in the closed and open arms), $F_{(1,83)}=46.81$, $p<0.0001$, and Balb/c mice had longer latencies to enter both the center area, $F_{(1,87)}=73.51$, $p<0.0001$, and the open arms, $F_{(1,85)}=38.07$, $p<0.0001$. There were no significant main effects of treatment or sex, nor were there any significant interactions, for all three measures. This demonstrates that locomotor behavior was not affected by MIA, but the cognitive aspects of the test – choosing to spend time in the different zones – did relate to MIA.
In sum, Balb/c mice displayed longer latencies to enter the center area and open arms, spent less time in the open arms, and traveled shorter distances overall in the EPM as compared to C57BL/6 mice. The behavior of BALB/c mice in the EPM was not influenced by maternal exposure to SEA. For C57BL/6 mice, however, while SEA offspring had comparable latencies to leave the closed arms and spent equal amounts of time in the closed arms and center area as saline offspring, they showed significantly fewer entries into the open arms and spent less time in the open arms than mice from saline-treated mothers.

**Object Recognition (OR)**

The OR task was a 5-day test, in which objects were placed at specific locations of an open field (OF), after an initial introductory day when animals were in an empty OF. Data were collected on each day of the 5-day test (Figure 2). For each day, an animal was considered “inactive” if it spent more than 50% of the 5-minute trial in a stationary spot. Almost all of the time this spot was the point at which the animal was placed in the OF (in the middle of the center area), essentially representing a freezing response that resulted in prolonged immobility. On the first day, when there were no objects present and animals were exposed to an empty open field (OF), a total of 16 subjects were inactive, all of which were Balb/c mice. According to a chi-square test for frequency, this constituted a significant difference in strain inactivity, $X^2=20.495$, $p<0.0001$ (Figure 18a), regardless of sex and/or treatment type. Though the number of inactive animals ranged from five to eleven between days 2-5, for each day there significantly more Balb/c subjects that were inactive than C5BL/6 mice: $X^2=13.52$, $p=0.0003$ (Day 2); $X^2=5.622$, $p=0.0177$ (Day 3); $X^2=6.822$, $p=0.0090$ (Day 4); $X^2=4.21$, $p=0.0402$ (Day 5). In addition,
of these Balb/c animals, significantly more males than females were inactive, as well, $X^2=9.94, p=0.0016$ (Day 2); $X^2=6.12, p=0.0134$ (Day 3); $X^2=7.42, p=0.0064$ (Day 4); $X^2=4.72, p=0.0299$ (Day 5). For Day 2, when the two similar objects were placed in the open field for the first time, significantly more SEA offspring were inactive as compared to control offspring, $X^2=7.31, p=0.006$ (Figure 18b). Because all of these animals were Balb/c mice, and because 10 of the 11 inactive subjects were males, this treatment difference applied only to this specific group of animals, namely Balb/c male offspring from SEA-treated mothers.

The amount of time the animals spent in the peripheral area of the OF (also known as thigmotaxis – in this case, “hugging” the wall) and the time spent in the center area were collected and transformed into percent of total time in the apparatus (300s). Though some subjects did not make the cutoff to be considered inactive, many Balb/c animals were observed to sit in their starting locations in the middle of the open field for a long stretch of time at the beginning of the trials on all five days (for examples of this, see Figure 19 for sample maps of animals’ movements). Because of this, it was not possible to accurately use the percent time in the open area nor the total distance traveled as reliable measures of anxiety-like behavior in this strain. Therefore, measures of percent time in the open area, and total distance were only analyzed for the C57BL/6 subjects, and 2x2 (sex x treatment) ANOVAs were used. Days 1, 2, and 5 were the main days of interest, as those were days that the animals were presented with a novel situation: Day 1, first exposure to the apparatus; Day 2, first exposure to two identical objects; Day 5, one of the objects presented on Days 2-4 was replaced with a new object.
Figure 18c shows the percent time in the open arena across all five days for the C57BL/6 mice. Analyses did not reveal significant main effects of either sex or maternal treatment, nor was there a significant sex x treatment interaction, all $p$’s $> 0.199$, with all groups slightly decreasing from Day 1–4 and then increasing from Day 4 to 5. On Day 1 (empty OF) and Day 5 (one familiar object and one new object), there was a main effect of treatment on the total distance traveled in the OF ($F_{(1,45)}=13.92, p=0.0065; F_{(1,45)}=7.83, p=0.0075$, respectively) (Figure 18d). This was reflected by SEA offspring travelling greater distances in both the walled and center areas. However, on Day 2 (two similar objects for the first time), there was a significant interaction between sex and treatment, such that male saline offspring had greater overall travel distances than male SEA offspring, $F_{(1,45)}=5.81, p=0.0201$ (Figure 18d). A repeated-measures ANOVA revealed that this difference was not due to a drop in activity by SEA male offspring, but rather an increase in activity by male saline pups from Day 1 to Day 2, $F_{(1,45)}=5.97, p=0.0186$.

Lastly, the number of interactions and amount of time the subjects spent touching the novel object as opposed to the familiar object was measured and transformed into a percentage of total interactions and total time. Some animals did not interact with either object on Day 5, with a chi-square analysis revealing that Balb/c mice ($N=17; 37\%$) interacted less than C57BL/6 mice ($N=1; 2\%$), $X^2=18.84, p<0.0001$. Because such a large number of Balb/c animals did not interact with the object on Day 5, the analyses for percent touch and percent time were performed only on the C57BL/6 subjects.

Therefore, for C57BL/6 mice, there were no significant main effects or interactions for the percent number of interactions with the novel object, $F_{(3,44)}=1.31, p=0.2817$. There was, however, a significant main effect of maternal treatment for the
percent time spent interacting with the new object, such that SEA offspring spent more time in contact with the object, when compared to control offspring from saline-injected mothers, $F_{(1,44)}=4.62$, $p=0.0372$ (18e).

We next determined whether interactions with the new object were due to interest in the new object and not simply due to an animal’s place preference. To do this, we compared the percent of total interactions and time with the novel object, on Day 5, with interactions on Day 4 with the familiar object that was in the same location. For this analysis, we included the Balb/c subjects, since not all of the animals that were inactive on Day 5 were inactive on Day 4, and vice versa. Using a repeated-measures ANOVA, we confirmed that there were no significant changes in percent interactions with the object in that location between the two days, $F_{(7,79)}=1.18$, $p=0.3221$, but that there was a significant main effect of treatment on the percent time spent interacting, $F_{(1,78)}=9.36$, $p=0.0030$. This was due to saline offspring showing a decrease in the time spent interacting with the object when compared to SEA offspring (Figure 18f).

Overall, Balb/c subjects displayed significantly higher levels of inactivity compared to C57BL/6 subjects, with male mice exhibiting the majority of this inactivity. There were no differences in the C57BL/6 strain for the percent time spent in the center area of the OF for any of the days of interest, but on Day 1 and Day 5, SEA offspring did have increased overall travel distances in the OF. Interestingly, C57BL/6 saline males significantly increased their distance traveled on Day 2, whereas SEA males did not. Though there were no differences between groups in the percent total number of
interactions with the novel object on Day 5, SEA offspring did have significantly increased times interacting with the novel object compared to saline offspring.

**Prepulse Inhibition (PPI)**

The data collected and analyzed in the PPI task were startle responses recorded by the SR-Lab Systems (San Diego Instruments, San Diego, Calif., USA) software. Startle responses were measured in arbitrary units by the software. Percent PPI of the prepulse+pulse trials was calculated using startle values and according to the following formula:

\[ \frac{(\text{pulse alone}) - (\text{prepulse+pulse})}{\text{pulse alone}} \times 100 \].

There were three types of prepulses: 2dB above background noise (+2dB), 4dB above background noise (+4dB) and 8dB above background noise (+8dB). Startle pulses were 50dB above background. Because startle reaction and startle inhibition are inextricably linked, any outlier found for one prepulse trial type or startle reaction was excluded from all of the analyses for percent PPI. In total there were seven outliers removed from the PPI analysis for displaying percent PPI levels at least two standard deviations away from the mean percent PPI for that group.

For startle responses to pulse alone, there was a significant main effect of strain, \( F_{(1,80)}=22.85, p<0.0001 \), whereby startle responses for Balb/c mice were greater than for C57BL/6 mice, regardless of sex and/or treatment (Figure 20a). There were also significant main effects of strain for percent PPI at each prepulse intensity, with C57BL/6 mice exhibiting significantly higher levels of percent PPI than Balb/c mice, \( F_{(1,78)}=28.62, p<0.0001 \) (+2dB); \( F_{(1,78)}=2.12, p=0.052 \) (+4dB); \( F_{(1,78)}=2.66, p<0.0160 \) (+8dB). At the highest prepulse intensity, +8dB, there was also a main effect of treatment, with SEA offspring exhibiting greater percent PPI than saline offspring, \( F_{(1,78)}=4.10, p=0.0463 \) (Figure 20b). A one-way ANOVA revealed that this difference was mainly due to a strong
trend in the Balb/c SEA animals to have increased percent PPI compared to controls, $F_{(1,43)}=3.61, p=0.0640$ (Figure 20b).

In addition, all %PPI at all three prepulse intensities were significantly correlated with one another, while startle was not correlated with any of the percent PPI levels. According to a repeated-measures ANOVA, there was a significant strain difference in percent PPI across prepulse intensity conditions. That is, Balb/c subjects increased their percent PPI as the prepulse intensity increased, whereas C57BL/6 increased from the +4dB to +8dB, but not from +2dB to +4dB, $F_{2,77}=7.53, p=0.0010$ (Figure 20c).

**Morris Water Maze (MWM)**

The MWM task involved three different stages. The first stage involved learning or acquisition, during which mice were required to find a hidden platform (see Chapter 2 for methods). Subjects were considered to have “learned” the MWM if three of the last four (of ten) trials averaged a latency of less than 20s and was at least 5s faster than the average of the middle three trials. Sample learning curves for “Learned” and “Not Learned” can be seen in Figure 21. A chi-square analysis was run comparing all groups on the frequency of “Learned” vs “Not Learned,” and there was a significant main effect of strain, such that significantly less Balb/c mice ($N=30, 61\%$) learned the MWM than C57BL/6 mice ($N=19, 38\%), X^2=6.64, p=0.0100$. Of the 19 C57BL/6 mice that did not learn the task, more than half ($N=13, 68\%$) were SEA offspring, although this was not significant ($X^2=2.94, p=0.0864$; see Figure 22a).

After stage 1 or acquisition training, all subjects were run through a probe trial both at 1 hour and 24 hours after the final learning trial. Probe data was analyzed on only the 46 animals that learned the task (Table 10). The critical measure was percent of total
time (60 seconds) that the animal spent in the target quadrant where the platform was normally present. There was a significant strain x treatment interaction, $F_{(1,38)}=6.71$, $p=0.0135$, that was due to C57BL/6 SEA offspring spending more time in the target quadrant than saline offspring (Figure 22b). Conversely, for Balb/c mice, a one-way ANOVA revealed a non-significant trend for SEA offspring to spend less time in the target quadrant than saline offspring, $F_{(1,14)}=3.35$, $p=0.0883$ (Figure 22b). At 24 hours, this interaction disappeared and there was no difference between groups in percent time spent in the platform quadrant, $F_{(7,38)}=0.532$, $p=0.804$. A repeated-measures ANOVA showed that all groups decreased in the percent time spent in the platform quadrant, regardless of strain, all $p$’s > 0.2674.

In addition to the percent time spent in the platform quadrant, the percent total distance traveled in the platform quadrant was measured as well. The percent distance in the platform quadrant was significantly correlated with percent time in the platform quadrant for each probe, $r=0.3860$, $p=0.0088$ (1-hour); $r=0.8550$, $p<0.0001$ (24-hour), but the correlation at 1-hour was weaker, while the correlation at 24 hours was stronger. In addition, there were no significant main effects or interactions for the percent distance at either probe, all $p$’s > 0.2611 (1-hour); all $p$’s > (1-hour).

Lastly, we measured the amount of time the subjects floated during the learning trials and the two probe trials. Floating times in another water task known as the forced swim test (FST) can be indicative of a depressed phenotype (Cryan & Mombereau, 2004). For the learning trials, we averaged all ten trials together to get an average floating time. There were no significant main effects or interactions for floating time during the learning trials or the 1-hour probe, but there was a main effect of treatment on floating
times during the 24-hour probe, such that SEA animals from both strains spent significantly more time floating, $F_{1,87}=7.21$, $p=0.0087$, averaging almost half (approximately 30 seconds) of the probe trial (Figure 22c).

In sum, significantly more Balb/c subjects failed to learn the MWM. And of the C57BL/6 mice that did not learn the task, a majority of them were SEA offspring. During the 1-hour probe trial, C57BL/6 SEA offspring spent more time in the platform quadrant than did saline offspring, although the distances they traveled while in the quadrant were equal. In the 24-hour probe trial, SEA offspring tended to float significantly more than saline offspring, perhaps and indication of depression-like helplessness behavior.

**Discussion**

Though not necessarily as predicted, it was apparent that the offspring of mothers injected with SEA displayed a distinct behavioral phenotype, when compared to offspring from saline-injected mothers (Table 11). These differences were more marked in the C57BL/6 strain. However, SEA offspring from BALB mothers also differed on selective behavioral tasks from their offspring of saline-injected mothers.

First, in every test except the social interaction task, there was a distinct behavioral separation between the C57BL/6 strain and the Balb/c strain, regardless of treatment and/or sex. The C57BL/6 mice appeared to be less anxious, more active, have greater PPI, and learned the MWM better than Balb/c mice. In addition, independent of strain, females displayed lower levels of anxiety-like behavior than males. As discussed below, these differences are not new, and have been reported previously.

For the EPM, Balb/c animals, when compared to C57BL/6 mice, displayed selective exploration by spending significantly more time in the closed arms, less time in
the open arms, and taking longer to leave the closed arms. This strain difference in the EPM has been reported (Francis, Szegda, Campbell, Martin, & Insel, 2003), and usually attributed to higher levels of anxiety-like behavior in the Balb/c mouse strain (Crabbe, 1986; Crawley et al., 1997; Francis et al., 2003). The difference in baseline anxiety levels might also explain the increased startle response in Balb/c mice during the PPI task. An increased startle reaction in Balb/c mice compared to C57BL/6 mice has also been reported before and seems to be a robust finding (Crawley et al., 1997; Paylor & Crawley, 1997). Furthermore, Balb/c animals were inactive each day of the OR task and interacted with the objects less on the fifth day, suggesting a higher anxiety-like state. Interestingly, as opposed to the reduced locomotor activity in task-specific situations, Balb/c mice have been shown to be more active than C57BL/6 mice when observed in their home cages (Lassalle & Pape, 1978). Therefore, the BALB/c behavioral phenotype is consistent with the notion that inhibition of exploratory behavior is commonly associated with increased anxiety (Archer, 1973).

In addition, the sex differences displayed in the EPM and OR tasks also correspond to previous literature on anxiety levels of males and females. Not only did females spend more time in the open arms in the EPM, but they were also significantly more active than males every day of the OR task. Female rodents generally display less fearful behavior than do males (Gray, 1971), and female rats and mice have been shown to display reduced anxiety-like behavior in the EPM test (Johnston & File, 1991; Zimmerberg & Farley, 1993) and the OR task (Brotto, Barr, & Gorzalka, 2000). It is important to note, however, that in the current study, these sex differences were only seen in the C57BL/6 mice. Given that BALB/c mice are a more “anxious” strain of mouse
strain at baseline, perhaps treatment-induced differences in anxiety would be harder to detect. In that case, SEA effects would be harder to see in Balb/c mice, and in fact, were not as prominent as in the C57BL/6 strain. In all, the current study provides added support for the notion that female C57BL/6 display reduced levels of anxiety as compared to males.

Interestingly, C57BL/6 animals exhibited higher levels of PPI compared to Balb/c animals across all three prepulse intensity trial types. Conflicting reports have been described in terms of the strain differences in PPI. A systematic review of 13 different inbred strains of mice found significantly greater PPI for Balb/c mice than C57BL/6 (Paylor & Crawley, 1997), whereas a similar study directly comparing the two strains found significantly greater levels of PPI in the C57BL/6 strain (Francis et al., 2003). A third study comparing eight different inbred strains of mice found similar levels of PPI between the two strains (Bullock, Slobe, Vázquez, & Collins, 1997). Though there is no established consensus in the literature on PPI in these strains, the current evidence is in keeping with Francis et al. (2003), in that greater PPI was observed for C57BL/6 mice.

With regard to the MWM, it was not surprising that significantly more Balb/c subjects showed a failure to learn, as compared to the C57BL/6 subjects. This strain difference in learning is well-documented. For the C57BL/6 mice, 19 out of 49 (~39%), animals failed to learn the task, whereas for the Balb/c mice 30 out of 46 (~65%) subjects failed to learn the task. Balb/c mice are known to be “poor learners” when it comes to the MWM task (Crawley et al., 1997; Owen, Logue, Rasmussen, & Wehner, 1997; Upchurch & Wehner, 1989), as opposed to C57BL/6 mice who are relatively good at
learning the MWM (Crawley et al., 1997; Owen et al., 1997; Upchurch & Wehner, 1989). This was confirmed in the current study.

In terms of maternal treatment effects on behaviors of the offspring, pups from SEA mothers exhibited decreased social behavior, increased anxiety-like behavior, showed deficits in MWM learning and displayed an increase in depressive-like behaviors (viz., floating). However, SEA offspring also exhibited increased locomotor activity, a greater interest in exploration of a novel object, and higher percent PPI. Therefore, it was not the case that offspring from SEA mothers were impaired for all behavioral parameters.

The effects of maternal SEA exposure were similar for both strains with regard to increased anxiety, heightened percent PPI and increased depressive-like behavior; whereas social deficits, increased locomotor activity, increased interest in a novel object, and interrupted learning of the MWM was only exhibited in C57BL/6 offspring. Furthermore, maternal SEA exposure promoted better short-term memory in the MWM 1-hour probe test for C57BL/6 mice, and not Balb/c mice. Possible reasons for these dissociations are discussed below.

It appears that from the current study, maternal SEA treatment generates increased anxiety-like behavior in the EPM and OR test, as stated above. Increased anxiety in MIA offspring is well documented for offspring from mothers treated with LPS (Babri, Doosti, & Salari, 2014; H. Golan et al., 2005; Lin, Lin, & Wang, 2012), influenza (Shi et al., 2003), and poly IC (U. Meyer et al., 2008; S. E. Smith et al., 2007). Most of these studies used the EPM and OF and found reduced entries into the open arms and center area,
respectively. The literature on this is conflicted, however, with some studies showing no changes in anxiety in LPS rat offspring (Bakos et al., 2004) or poly IC mouse offspring (Giovanoli et al., 2013), and one study displaying reduced anxiety-like behavior in LPS mouse offspring (Asiaei, Solati, & Salari, 2011). Where increased anxiety was observed, several investigators attribute this to elevated fetal IL-6 (U. Meyer et al., 2008; Shi et al., 2003; S. E. Smith et al., 2007). However, in the current study, while SEA-injected pregnant C57BL/6 mice did display extremely elevated levels of IL-6 (Figure 7), Balb/c pregnant mice did not produce very much IL-6 in response to SEA. This suggests that the high IL-6 response in C57BL/6 mice might explain the increased anxiety-like development in C57BL/6 offspring, but for Balb/c mice, the modest IL-6 changes are unlikely to adequately explain anxiety-development in Balb/c offspring.

In addition, there is also evidence that IFN-γ levels contribute to anxiety-like profiles in mice (Campos, Vaz, Saito, & Teixeira, 2014), and as shown in Chapter 3, SEA produced significantly elevated levels of IFN-γ in pregnant mice of both strains. Therefore, the increased production of IFN-γ may be sufficient to produce anxiety-like behaviors. Interestingly, there is recent evidence suggesting that IFN-γ is important in the development of social behavior (Filiano et al., 2016). Therefore, it is conceivable that maternal elevations in IFN-γ may be responsible for neurobehavioral changes in the offspring, due to prenatal SEA treatment.

Alternatively, others have pointed to elevated stress hormones, glucocorticoids, and changes in fetal hypothalamic-pituitary-adrenal (HPA) axis development as as possible mediators of increased anxiety in MIA offspring (Asiaei et al., 2011; Babri et al., 2014; Golan et al., 2006). The HPA axis is known to control fearful and anxiety-like
behavior in mice and humans, and therefore changes in that area of the brain could influence anxiety-like phenotypes (Giunta, 2008; Kronfol & Remick, 2000; Mallimo & Kusnecov, 2013). There is also evidence that increased proinflammatory cytokine production—as in the case of SEA injections for both C57BL/6 and Balb/c mice, as shown in Chapter 3—is accompanied by an increase in glucocorticoids (Babri et al., 2014; Zaharia, Kulczycki, Shanks, Meaney, & Anisman, 1996). Indeed, it has been demonstrated that the HPA axis response to SEA, as reflected by corticosterone elevations, is mediated by TNF (Rossi-George, Urbach, Colas, Goldfarb, & Kusnecov, 2005). Both C57BL/6 and Balb/c mothers displayed significantly elevated levels of TNF-α to SEA exposure (Figure 8), and so it is extremely conceivable that the production of these cytokines is accompanied by HPA axis response. The current study did not assess stress hormone production, but future studies would benefit from including that in their design. Though previous literature is conflicted on the outcome of anxiety due to MIA, in the current study, SEA produced consistently higher levels of anxiety in offspring from both strains than did controls.

Interestingly, SEA offspring from the Balb/c strain also displayed elevated levels of percent PPI compared to controls. Much of the literature on MIA offspring implies that the opposite usually occurs. Disruptions in PPI in MIA offspring is one of the most robust findings in the literature, having been displayed in both LPS offspring (Borrell et al., 2002; M.-È. Fortier et al., 2004; M. E. Fortier et al., 2007; Romero et al., 2007) and poly IC offspring (Makinodan et al., 2008; Urs Meyer, 2013; Urs Meyer et al., 2007; Ozawa et al., 2006; Vorhees et al., 2015). This effect, also, has been suggested to involve elevated IL-6 (Hsiao & Patterson, 2011; S. E. Smith et al., 2007), and that IL-6 is able to directly
cross the placenta in rats and enter the fetal circulation (Dahlgren, Samuelsson, Jansson, & Holmäng, 2006). However, as discussed in Chapter 3, both strains produced IL-6 and yet deficits were not exhibited. In light of this, there is the possibility that another product of SEA injections, aside from the elevated IL-6, is mediating increased sensorimotor gating abilities.

Prepulse inhibition is widely regarded to reflect sensorimotor gating, and is regulated by limbic circuitry such as the nucleus accumbens and hippocampus (Bakshi & Geyer, 1998; J. M. Petitto, Huang, Hartemink, & Beck, 2002). Dopamine agonists, and increasing dopamine concentrations in limbic areas, have consistently been shown to reduce PPI in both animals and humans (for review see Braff, Geyer, & Swerdlow, 2001). There is evidence to show that IL-2 has neuromodulatory effects on the dopamine system of the brain, including in the limbic areas (J. Petitto, McCarthy, Rinker, Huang, & Getty, 1997; Zalcman et al., 1994), and that at very high doses of exogenous IL-2 administration dopamine release was inhibited in the striatum (J. Petitto et al., 1997). Although the same study did not find changes in PPI due to IL-2 administration, the authors posited that the site of administration may influence the behavioral outcome (J. Petitto et al., 1997). Thus, increased IL-2 in the limbic system may be able to influence the behaviors controlled by that area, for instance PPI. These studies, however, involve IL-2 effects in adult animals. The critical question is whether uterine IL-2 is relevant to CNS development.

As discussed above, SEA administration induced increased levels of IL-2 in both the C57BL/6 and Balb/c pregnant mice (Figure 6). This may have influenced IL-2 production in the fetus, leading to dopamine and limbic system circuitry changes in the
fetus. Such a high production of IL-2 is unique to T cell superantigens as opposed to the conventional poly IC and LPS, which activate cells of the myeloid lineage (e.g., macrophages), and hence do not cause an elevation in T cell associated cytokines, such as IL-2 (K. A. Smith, 1988). This might explain why in the case of MIA by poly IC or LPS there is a decrease in PPI, but as in the case of SEA, there is an increase.

Both C57BL/6 and Balb/c offspring of SEA-exposed mothers displayed increased floating behavior during the 24-hour probe trial in the MWM. This is reminiscent of a behavioral test known as the forced swim test (FST), in which animals are placed in a cylinder of water and the time they spend not swimming (immobility time) is calculated (Cryan & Mombereau, 2004). The results of this test are used to measure depressive-like behaviors and learned helplessness (Cryan & Mombereau, 2004). There is much evidence that MIA–either through LPS or poly IC–can increase depressive behavior in mice, as measured by the FST (Abazyan et al., 2010; Babri et al., 2014; Khan et al., 2014; Mueller & Bale, 2008). It has been proposed that this is propagated by the increased production of proinflammatory cytokines (Abazyan et al., 2010; Khan et al., 2014), and in particular IL-2 (Mössner et al., 2007), IL-6 and TNF-α (Dowlati et al., 2010). Significant production of proinflammatory cytokines IFN-γ, IL-2 and TNF-α were exhibited in SEA-injected pregnant mice (section 3.3.), and could be propagating the production of these cytokines in fetal brains to influence the development of depressive-like behavior. Why this behavior is observed at 24 hours and not during the task itself or at the 1-hour probe could be due to learned helplessness. Animals are exposed to a stressful environment throughout Day 1, and when they are exposed to the exact same environment 24 hours
later, their motivation to escape might have diminished. Either way, both strains show marked increases in floating times in the SEA offspring at 24 hours.

Some of the behavioral effects of SEA-exposure on the mothers were seen only in C57BL/6 offspring, as we had expected. This is most probably due to the fact that SEA differentially affects the two strains based on their MHC II isotype (Taub et al., 1992; Watanabe et al., 2004), and as shown in Chapter 3, produced significantly higher concentrations of IFN-γ, IL-6, and TNF-α in the C57BL/6 strain. The first of these behavioral effects was that of decreased social interactions in terms of percent touch and percent time touching the target cup with a novel mouse. Social deficits have been reported before in MIA animals. Administration of Poly IC into pregnant females around the same time point as the current study (E12.5) has been shown to reduce both contacts and contact time of offspring with a novel mouse (Lipina et al., 2013; N. Malkova, 2010; Schwartz et al., 2013; S. E. Smith et al., 2007), and injections of LPS and influenza into pregnant mothers has shown similar results (Oskvig et al., 2012; Shi et al., 2003). Interleukin-6 has been shown to be necessary and sufficient to induce social deficits in MIA offspring (S. E. Smith et al., 2007), and SEA induced significant IL-6 production in the C57BL/6 mothers. Because much less IL-6 was produced in Balb/c mothers in response to SEA, offspring from this strain show no differences from control in their social behavior.

Although we discussed MWM above in the context of floating behavior, here it is important to comment on learning behavior in the MWM. The C57BL/6 offspring of SEA-treated mothers were less likely to learn the location of the hidden platform. Offspring of MIA mothers have been shown to be impaired in spatial learning on the
MWM as a consequence of LPS injection (M. E. Fortier et al., 2007) and poly IC (U. Meyer et al., 2009; U. Meyer et al., 2006; U. Meyer et al., 2008). This impairment has been attributed to changes in both hippocampal and HPA axis activity. Effective learning of the MWM is a hippocampal-dependent process, relying heavily on proper long-term potentiation and N-methyl-D-aspartate (NMDA) functioning in the region (Jeffery & Morris, 1993; Morris, 1983; Morris, Anderson, Lynch, & Baudry, 1986; Moser, Krobert, Moser, & Morris, 1998; Vorhees & Williams, 2006). These hippocampal processes can be affected by influences from the HPA axis, such that elevated production of glucocorticoids can lead to hippocampal cell loss and disturbances (Sapolsky, 1987; Stein Behrens et al., 1992). Maternal immune activation by LPS and poly IC has been shown to not only increase glucocorticoids, but has also been shown to directly affect neuronal activity in the hippocampus (Babri et al., 2014; Ito et al., 2010; Nyffeler, Meyer, Yee, Feldon, & Knuesel, 2006). As previously mentioned, these changes are most likely due to the elevated production of proinflammatory cytokines (Haddad, Saadé, & Safieh-Garabedian, 2002; U. Meyer et al., 2008; Webster & Sternberg, 2004). Because so many Balb/c animals failed to learn the task regardless of treatment, and because SEA did not produce as high concentrations of proinflammatory cytokines in mothers as the C57BL/6 strain, maternal SEA exposure did not seem to produce noticeable changes in the ability for Balb/c animals to learn the task.

Some unexpected results from SEA treatment were also observed in C57BL/6 mice, such as increased exploration of a novel object, increase locomotor activity, and increased memory for the location of the hidden MWM platform. The latter was
somewhat surprising, since C57BL/6 offspring of SEA mothers were slow in finding the hidden platform.

With regard to novel object contacts, C57BL/6 SEA offspring did not interact a greater number of times with the novel object, relative to the familiar object, but did show increased contact time with the novel object. Though some studies have found decreased interest in a novel object in MIA offspring (Ozawa et al., 2006; Shi et al., 2003), these injections were done at different time points than in the current study. One study that injected with poly IC at E12.5—as used in the current study—also found increased novel object preference in the offspring (Ito et al., 2010), as did one study injecting LPS at E17 (H. Golan et al., 2005). It was proposed that this behavior could be due to abnormal processing of non-spatial information in the hippocampus, leading to increased preservation behavior and an enhanced expectation for the familiar object, which would increase sensitivity to the novel object (Ito et al., 2010). Whatever the explanation, it appears that the current findings, in showing increased contact time, suggest increased exploration of the new object due to maternal SEA exposure. The cognitive explanation for this, is not immediately evident and needs to be explored further.

The C57BL/6 SEA offspring also traveled greater distances in the OF, exhibiting higher levels of locomotion and activity than controls. This has been recorded before for LPS offspring (Zaodung Ling et al., 2009), and has been attributed to changes in dopamine activity in the offspring (Zuckerman & Weiner, 2003). It is important to note, however, that the increased locomotor activity was only found in young mice (P90), whereas in older mice (P240), locomotor activity decreased (Zaodung Ling et al., 2009).
Offspring in the current study were around seven weeks old (P47) when they were tested on the OR, corresponding well with the younger-aged mice in the LPS studies.

Lastly, SEA offspring from the C57BL/6 strain displayed enhanced memory in the MWM 1-hour probe, while Balb/c offspring exhibited worse memory. At 1 hour, C57BL/6 SEA offspring spent significantly more time in the platform quadrant than did controls, a measure usually used to indicate memory for where the platform had been hidden. This enhanced memory behavior has been seen in one other study looking at MIA effects in offspring using LPS, and the authors of that study were also surprised by the unexpected result (H. Golan et al., 2005), as most MIA research has found the opposite (U. Meyer et al., 2006; U. Meyer et al., 2008; Samuelsson et al., 2006; S. E. Smith et al., 2007). Though the difference between Balb/c SEA offspring and controls at the 1-hour probe does not reach significance, there is a strong trend for SEA offspring to spend less time in the platform quadrant. This result has support in the literature, as many have found impairments in learning and memory in the MWM in MIA offspring (U. Meyer et al., 2006; U. Meyer et al., 2008; Samuelsson et al., 2006; S. E. Smith et al., 2007). The difference between the strains’ reactions to maternal SEA may be due to the differing responses of the mothers to the injections. While increased proinflammatory cytokines like IL-6 have been shown to impair learning and memory processes (Samuelsson et al., 2006; S. E. Smith et al., 2007), there is also evidence that T-cells and their cytokine products, like IFN-γ, are necessary for hippocampal neurogenesis, learning and memory, and proper hippocampal functioning (Shaked et al., 2005; Ziv et al., 2006). While IFN-γ was produced in both strains during pregnancy, only the C57BL/6 mice had extremely
high concentrations in response to SEA. Though learning abilities in the MWM were still impaired in the C57BL/6 mice, perhaps this concentration of IFN-γ was able to differentially affect the two cognitive processes of acquisition and storage, and instead of producing deleterious effects on short-term memory, it was able to influence heightened memory abilities. The effects on MWM memory was only seen in the short-term, 1-hour probe but not in the long-term 24-hour probe. In all, these results are surprising and are in need of further elucidation.

To summarize, offspring from both Balb/c and C57BL/6 SEA-injected mothers displayed heightened anxiety, increased percent PPI levels, and depressive symptoms. In addition, C57BL/6 offspring displayed decreased social behaviors, increased locomotion and novel object exploration. Interestingly, even though C57BL/6 mice exhibited decreased learning in the MWM, they showed increased memory at 1-hour. However, Balb/c mice trended towards the opposite effect, and displayed decreased memory in the MWM at 1-hour. These results are most likely due to a combination of increased production of T-cell-derived proinflammatory cytokines and their effects on neural development during the embryonic and/or early postnatal period.
CHAPTER 5: BEHAVIORAL PHENOTYPE OF MALE AND FEMALE OFFSPRING FROM MOTHERS TREATED WITH STAPHYLOCOCCAL ENTEROTOXIN B

Relevant Background and Rationale

Staphylococcal enterotoxin B is both extremely similar and crucially distinct from its SEA counterpart. Though both SEs bind to MHC II molecules, SEB only binds to the $\beta$ chain, and also specifically recognizes region 3.2 on the $\gamma$ chain (Pinchuk et al., 2010). In addition, SEB binds to human $\text{V}\beta 3$, $\text{V}\beta 12$, $\text{V}\beta 14$, $\text{V}\beta 15$, $\text{V}\beta 17$ and $\text{V}\beta 20$ chains (Papageorgiou, Tranter, & Acharya, 1998). In mice, SEB administration can cause weight loss, thymus depletion and immunosuppression (Marrack, Blackman, Kushnir, & Kappler, 1990). In humans, it has been implicated in food poisoning and food-borne illnesses, and non-menstrual toxic shock syndrome. Because it is able to be aerosolized, it is very stable even at high temperatures, and it can cause extensive systemic damage, SEB is the only SE that has been studied as a biological weapon (Williams, 2001). At low doses, SEB can cause persistent and incapacitating symptoms and at a dose of 0.02 mcg/kg death can occur (Papageorgiou et al., 1998). Injections of SEB into mice produces vast amounts of IL-2, TNF-$\alpha$, IL-1 and IFN-$\gamma$, with each cytokine peaking at a different time point (Miethke et al., 1992b; Miethke et al., 1993). In addition, T-cell-derived IL-4 also increases to detectable levels following SEB administration (Miethke et al., 1993). All in all, SEB is a potent superantigen that can cause widespread biological symptoms through increased levels of T-cell activation and cytokine production.

The rationale for conducting the behavioral tasks on pups from SEB-injected mothers is the same as described in Chapter 4 for the SEA offspring. In addition, injection at E12.5 is also justified above.
As mentioned above, MHC II molecules exist in varied allele polymorphisms such that different animals contain differing isotypes of the molecules (Taub et al., 1992). Staphylococcal enterotoxins differentially bind to different isotypes of the MHC II molecule, such that one SE will produce a more robust response in one form of the molecule over another. Staphylococcal enterotoxin B is more potent in animals expressing the H-2d isotype, such as the Balb/c mouse strain (B. Stiles et al., 1993; Taub et al., 1992). As shown above in Chapter 3, SEB produced higher levels of circulating pro-inflammatory cytokines IL-2 and IFN-γ in Balb/c mothers as opposed to their C57BL/6 counterparts. Because of this, we anticipate differing behavioral results between the two strains of offspring. We expect Balb/c pups to display a more severe behavioral phenotype associated with maternal immune activation with SEB than the C57BL/6 pups. In particular, we anticipate lower levels of social interaction, novel object exploration and prepulse inhibition, higher levels of anxiety, and increased cognitive impairment in regards to the MWM. While we may see some of the same behavioral patterns in the C57BL/6 mice—as SEB did cause significant production of IL-2, IFN-γ, and IL-6—we do not expect as strong a result.

Material and Methods

Experimental groups and procedure

Similarly-aged female C57BL/6 and Balb/c mice were injected on embryonic day 12.5 (E12.5) of pregnancy. Animals from each strain were randomly assigned to be injected with either 5μg (approximately 250 μg/kg) of SEB (Toxin Technology, Sarasota, FL) or 0.9% saline. The number of mothers per group, with average litter size in parentheses, can be seen in Table 8. There were no significant differences in litter size (F_{3,13}=0.7466,
p=0.5434. Pups were weaned three weeks after birth, and were separated based on sex at five weeks after birth. The numbers of male and female pups per behavioral group are listed in Table 9.

A detailed description of the behavioral tests can be seen in Chapter 2, and a brief description of the schedule can be seen in Chapter 4.

Data Analysis

Behavioral data was analyzed using 2x2x2 (strain x treatment x sex) ANOVAs, repeated measure ANOVAs, chi-square tables of frequency, and correlation tests. All outliers were removed from the data a priori to analysis and were determined as being more than two standard deviations away from the mean of each group (characterized by strain, treatment and sex). In instances where there was a main effect of strain differences, planned one-way ANOVAs for each strain were conducted to parse out differences within one strain that may have been affecting overall treatment effects. There was a large amount of variability within and between the groups resulting in differing sample sizes for many analyses. In no instance were more than 2 animals excluded from any group. However, due to technical errors incurred for the computer running the tracking software, the data for twenty animals for days 1, 3 and 4 of the Object Recognition Task are missing. Table 12 below has the number of animals missing for each group and the new Ns for those groups. Data analyzed using only Day 5 data points were made of the usual group sizes listed in Table 9. Whether this missing data influenced the outcome of the analyses will be discussed below. Behavioral data for the EPM, OR and MWM was recorded using the Any-Maze software program (San Diego Instruments). Social interaction data was recorded through a customized program that
registered the number of contacts with the target and control cups. Finally, PPI data was collected through the SR-Lab startle response system (San Diego Instruments). All data was analyzed using JMP software (SAS).

**Results**

**Social Interaction**

**Number of Contacts.** Three to five data points were missing for the social interaction analyses, and two points were deemed outliers *a priori* to analysis. The number of contacts with each cup was recorded, and the percent of total touches made with the target cup \( \frac{\# \text{ touches target cup}}{\# \text{ touches target cup} + \# \text{ touches to control cup}} \) were analyzed using a 2x2x2 (strain x treatment x sex) ANOVA. There was a significant main effect of strain \( F_{(1,81)}= 4.8774, p=0.0300 \), since overall the C57BL/6 mice had higher percentages of touching the target cup than Balb/C mice (Figure 23a). In addition, there was a significant strain x sex interaction \( F_{(1,81)}=4.15, p=0.048 \), reflecting increased target cup touches by C57BL/6 females, as compared to C57BL/6 males. Importantly, there was a significant strain x treatment interaction, \( F_{(1,81)}=10.83, p=0.0015 \), which appeared to be due to a higher percentage of target cup touches by Balb/c SEB offspring as compared to Balb/c control offspring (Figure 23a). This interaction also reveals that the main effect of strain is due to a difference in the control offspring between the strains but not the SEB offspring. These results indicate increased social interactions in C57BL/6 saline mice compared to Balb/c saline mice, but in terms of SEB exposure during pregnancy, the main difference was for the Balb/c SEB offspring, when compared to BALB/c saline offspring.

**Total Time Touching Cups.** The total time each animal spent touching the cups was recorded and a percentage of time spent touching the target cup
was calculated and analyzed with a 2x2x2 (strain x treatment x sex) ANOVA. There was a main effect of strain, $F_{(1,85)}=9.64$, $p=0.0026$, such that Balb/c mice had higher percentages of time touching the target cup than did C57BL/6 mice. However, there was a significant strain x treatment interaction, $F_{(1,85)}=13.80$, $p=0.0004$, indicating that this strain difference was only apparent in SEB offspring, and, importantly, that Balb/c SEB offspring had a higher percentage of time touching the target cup compared to Balb/c control offspring (Figure 23b).

Though C57BL/6 saline mice had an overall greater percentage of interactions with the target cup as compared to the Balb/c mice, the Balb/c SEB mice spent a larger percentage of their interaction time touching the target cup. This implies that the C57BL/6 control mice would come and go from the target cup more often, while the Balb/c SEB mice would interact with the cup for longer stretches of time. In addition, for both percent touch and percent time, Balb/c SEB offspring interacted with the target cup significantly more and for a longer duration than Balb/c saline offspring.

*Elevated Plus Maze (EPM)*

Animals were recorded in each part of the EPM apparatus, and the amount of time they spent in the closed arms, open arms, and center area was collected. There were five total outliers determined *a priori* to data analysis. The Balb/c mice spent a significantly greater percentage of the total time in the closed arms of the apparatus than C57BL/6 mice, $F_{(1,84)}=16.03$, $p=0.0001$. However, of those Balb/c mice, SEB offspring spent significantly more time in the closed arms than did the saline offspring, $F_{(1,84)}=7.46$, $p=0.0076$. For both the percent time spent in the center area and open arms, there was a significant main effect of strain ($F_{(1,84)}=16.09$, $p=0.0001$; $F_{(1,84)}=18.50$, $p<0.0001$,
respectively), such that C57BL/6 mice spent more time in the center and open arms of the EPM apparatus (Figure 24a). Likewise, there was a main effect of treatment for both percent time in center and percent time in open arms, indicating that offspring of saline-injected mothers spent more time in the open areas than did the SEB offspring ($F_{(1,84)}=4.41, p=0.0387; F_{(1,84)}=6.57, p=0.0121$, respectively). However, these differences were mainly due to the difference in Balb/c offspring, in which the SEB offspring spent significantly less time in the center area than the saline offspring, $F_{(1,84)}=5.35, p=0.0232$. Furthermore, this was confirmed by a planned one-way ANOVA, which revealed less time in the open arms for the SEB offspring, $F_{(1,39)}=4.40, p=0.0424$ (Figure 24a). In addition, the percent time in the open arms was positively correlated with the percent time in the center area, $r=0.4198, p<0.0001$, but negatively correlated with percent time in the closed arms, $r=-0.7957, p<0.0001$. These results indicate that not only were Balb/c animals spending less time in the open areas than the C57BL/6 animals, but Balb SEB offspring spent significantly less time in the open areas than the Balb saline pups.

The number of entries into the open arms and center area were significantly correlated with the percent time spent in the open arms, $r=0.5499, p<0.0001; r=0.4107, p<0.0001$, respectively, and were also found to have significant main effects of strain, $F_{(1,86)}=8.62, p=0.0107; F_{(1,86)}=6.87, p=0.0025$, in that C57BL/6 mice had more entries into the open arms and center area. In addition, a planned one-way ANOVA revealed that there was a trend towards a main effect of treatment for the number of entries into the open arms, indicating significantly higher amounts for Balb/c saline offspring compared to SEB offspring, $F_{(1,39)}=3.74, p=0.0602$ (Figure 24b).
The distance traveled in each area of the EPM apparatus was also recorded and analyzed. For the total distance traveled throughout the five minutes, there was a main effect of treatment, \((F_{(1,89)}=14.49, p=0.0003)\), such that SEB offspring moved more than saline offspring (Figure 24c). This difference may be due to the significant difference in the distance traveled in the closed arms of the EPM, where the SEB pups also traveled greater total distances than the saline pups, \(F_{(1,86)}=12.97, p=0.0005\) (Figure 24c). In fact, total distance traveled in the EPM was significantly correlated with distance traveled in the closed arms, \(r=0.9838, p<0.001\). Taken together, this data suggests that animals did not travel much in the open arms, but preferred to stay within a small circumscribed area, whereas animals in the closed arms tended to move greater distances. Therefore, because SEB pups spent more time in the closed arms than in the open areas, they traveled greater distances.

As expected, there were main effects of strain for both latency measures, such that C57BL/6 mice had lower latencies to enter the open arms and center areas, \(F_{(1,84)}=10.05, p=0.0021; F_{(1,87)}=7.76, p=0.0066\). In addition, both the latency to enter the open arms and the latency to enter the center area had significant strain x sex x treatment interactions, indicating significantly longer latencies for female Balb/c SEB offspring than female Balb/c saline offspring, \(F_{(1,84)}=4.55, p=0.038; F_{(1,87)}=6.89, p=0.012\).

Taken together, Balb/c mice showed a significant delay in leaving the closed arms, made less entries into the open areas, and spent less time outside the closed arms than C57BL/6 mice. Most importantly, Balb/c SEB offspring spent significantly less time outside the closed arms than their Balb/c saline counterparts, and SEB offspring in general entered the open arms less often than saline offspring. Additionally, Balb/c
female SEB mice had a higher latency for leaving the closed arms than Balb/c female controls; and SEB offspring travelled longer distances than saline offspring, but primarily inside the closed arms.

Object Recognition (OR)

The OR task was a 5-day test, with data collected on each day. For days 1, 3 and 4, twenty data points were lost due to technical error, and the Ns used for those days can be found in Table 12. The OR task was a 5-day test, with data collected on each day (Figure 2). For each day, an animal was considered “inactive” if it spent more than 50% of the 5-minute trial in a stationary spot. Most of the time this spot was the point at which the animal was placed in the OF, in the middle of the center area. Because of an experimenter error, most of the animals on Day 1 were placed in the corner of the OF instead of the center, so very few animals were considered inactive on Day 1. Though 20 pieces of data were missing for days 1-4, we analyzed the inactivity of the subjects for which there was data. Consistent with the SEA results in chapter 4, a chi-square test revealed that significantly more Balb/c animals than C57BL/6 animals were inactive every day from Day 2 to Day 5, \[X^2=15.58, p<0.0001 \text{ (Day 2, Figure 25a)}; X^2=13.09, p=0.0003 \text{ (Day 3)}; \]
\[X^2=11.61, p=0.0007 \text{ (Day 4)}; X^2=18.85, p<0.0001 \text{ (Day 5)}]. There were no significant main effects of strain and/or treatment on inactivity.

The amount of time the animals spent in the perimeter area of the OR apparatus (also known as thigmotaxis) and the time spent in the center area were collected and transformed into percent of total time in the apparatus (300s). Days 1, 2, and 5 were the main days of interest, as those were days that the animals were presented with a novel situation. Inactive subjects were removed from the data analysis. The percent time the
mice spent in the center on the first day (with no objects present) was not significantly different between groups, all p’s > 0.2559 (Figure 25b). However, the total distance traveled on Day 1 was greater for C57BL/6 mice than Balb/c mice, having traveled significantly more both in the perimeter area and the open area, $F_{(1,69)}=68.65$, $p<0.0001$. For Days 2 and 5, the inactive subjects were excluded from the data. There were no significant main effects or interactions for the percent time spent in the center for Day 2, all p’s > 0.1950, but there was a main effect of strain on distance traveled, such that C57BL/6 mice traveled more than Balb/c mice, $F_{(7,58)}=32.53$, $p<0.0001$. For Day 5, there was a significant main effect of strain on percent time in the center area, with C57BL/6 animals spending more time in the center, $F_{(1,76)}=5.29$, $p=0.0242$, and a main effect of sex on distance, indicating increased distances for females as compared to males, $F_{(1,76)}=10.88$, $p=0.0015$. Because of the missing data for the Days 1, 3 and 4, we could not run a repeated-measures ANOVA comparing percent time in the center area from Days 1-5.

Lastly, interactions with the “new” and “old” objects on Day 5 were recorded. A total of 19 animals (6 C57BL/6, 13 Balb/c) did not interact with either object on Day 5. There was also a slight trend for SEB offspring to show no interaction with the objects on Day 5 compared to saline-offspring controls, but this difference did reach significant levels, $X^2=2.375$, $p=0.0988$. Excluding the 19 animals that did not interact with either object and one outlier, the percent of total interactions with the novel object and percent time interacting with the novel object on Day 5 was analyzed, but there were no differences found between groups, all p’s > 0.1901 (Figure 25c); all p’s > 0.1852, respectively. In addition, it is possible that animals may have increased their interactions
from the old object on Day 4 located in the position that the new object would be placed on Day 5 (which was counterbalanced between subjects) to the interactions with the new object; this could imply increased curiosity in the new object as opposed to a place preference. No individual group significantly increased their interactions or time of interaction between the days, and so there were no significant differences between groups, all p’s > 0.1315 (Figure 25d); all p’s > 0.1675.

In all, for the Object Recognition task there were significant strain differences for much of the task, but no major maternal treatment difference. In general, C57BL/6 mice were more active, traveling more and interacting with the objects more, whereas the Balb/c mice remained more stationary and/or did not interact with either of the objects. Despite the missing data and experimenter error, this result is consistent with the results above in chapter 4. There was, however, a significant treatment x strain effect on Day 4, in which Balb/c SEB offspring did significantly reduce their interactions with the objects compared with Balb Saline offspring. Unfortunately, between the missing data of the 20 animals on Days 1-3, and the 19 animals that did not interact with either object on Day 5, much of the data could not be confidently analyzed for the object recognition test and our results might reflect a low N. Future studies are needed to confirm these findings.

Prepulse Inhibition (PPI)

Refer to chapter 4 for trial types and determination of percent inhibition. A total of ten outliers were removed from the analysis.

For startle responses to the pulse alone, there was a significant main effect of strain, such that Balb/c mice reacted with significantly greater startle, $F_{(1,74)}=37.62$, $p<0.0001$ (Figure 26a). In addition, there was also a treatment effect for elevated startle
responses in the SEB offspring regardless of strain, $F(1,74)=12.19, p=0.0008$ (Figure 26a). For percent PPI across all three types of prepulse trials, there was a main effect of strain, with C57BL/6 mice exhibiting higher percentages of inhibition of startle compared to Balb/c mice, $F(1,77)=28.15, p<0.0001$ (+2dB); $F_{1,77}=12.36, p=0.0007$ (+4dB); $F_{1,77}=11.91, p=0.0009$ (+8dB). Interestingly, for the highest prepulse stimulus, namely the +8dB prepulse trials, there was a main effect of treatment, which was due to higher inhibition percentages for SEB offspring compared to saline offspring, $F(1,77)=5.36, p=0.0233$ (Figure 26b). In the +4dB prepulse trials, there was a trend for Balb/c SEB offspring to have increased levels of percent PPI compared to Balb/c controls, $F(1,77)=3.06, p=0.0844$, and this level reaches significance with a one-way ANOVA, $F_{(1,35)}=4.29, p=0.0458$ (Figure 25c). Similarly, in the +2dB prepulse trials, SEB offspring display greater percent PPI than saline offspring, $F(1,77)=8.09, p=0.0057$, and one-way ANOVAs revealed that this difference was only true for the males of each strain, $F(1,25)=6.13, p=0.0204$ (C57BL/6); $F_{(1,10)}=3.29, p=0.04898$ (Figure 25d).

There were strong significant correlations between percent inhibition during every type of prepulse intensity trial; in addition, startle responses are significantly correlated with percent inhibition during the +4dB and +8dB prepulse trial (Table 13). A repeated-measures ANOVA was used to analyze differences in percent PPI across the prepulse trial types for each group of subjects. There was a main effect of strain, $F_{2,76}=5.20, p=0.0077$, such that Balb/c mice increased in percent PPI across prepulse intensities, while C57BL/6 mice only increased percent PPI at the +8dB prepulse (Figure 26e).

To summarize, Balb/c mice and SEB offspring regardless of strain and sex display higher levels of startle responses than their C57/BL6 mice and saline offspring
counterparts. For all three prepulse intensity levels, C57BL/6 mice exhibited higher PPI than Balb/c offspring, but generally did not vary in their %PPI across prepulse trial types. In the +4dB prepulse trials, there was a slight trend for Balb/c SEB offspring to have higher PPI levels than Balb/c control offspring, and in the +2dB trials both strains showed this affect, with males displaying this difference more clearly. In the +8dB trials, SEB offspring had significantly higher levels of %PPI than saline offspring regardless of strain. Percent PPI was correlated at every prepulse intensity, and startle responses correlated with %PPI in +4dB and +8dB trials. C57BL/6 mice did not vary in their PPI responses in different prepulse trials, except for C57BL/6 SEB male offspring whose %PPI increased from +4dB trials to +8dB trials. Overall, Balb/c mice tended to increase in their %PPI responses as the prepulse intensity increased.

Morris Water Maze (MWM)

The MWM was run as described in Chapter 4 (see 4.3). As in chapter 4 animals varied in their learning rates. Sample learning curves for “Learned” and “Not Learned” can be seen in Figure 21. A chi-square analysis was run comparing all groups on the frequency of “Learned” vs “Not Learned,” and there was a significant main effect of treatment such that significantly more saline offspring learned the task than did SEB offspring, $X^2=3.49$, $p=0.0482$. Upon further analysis, this difference was found to only be significant for the Balb/c offspring, $X^2=3.885$, $p=0.0487$, and not for the C57BL/6 animals, $X^2=0.531$, $p=0.4664$ (Figure 27a).

All subjects were run through a probe trial both 1 hour and 24 hours after the final learning trial, with the probe data analyzed using only the 61 animals that learned the task (Table 14). One hour after the task, there were no significant differences between the
groups in the percent of total time they spent in the platform quadrant, all p’s > 0.1251, with most groups averaging about 40% of the total time spent in the platform quadrant. At 24 hours after the learning stage, there were significant interactions of strain x sex, \(F_{1,51}=4.95, \ p=0.0305\) and strain x treatment, \(F_{(1,51)}=4.95, \ p=0.0339\), indicating significantly more percent time was spent in the platform quadrant by Balb/c females as compared to males, and Balb/c control offspring as compared to SEB offspring. These interactions were inherent in a strain x sex x treatment interaction—which was likely due to Balb/c male saline offspring spending significantly more time in the platform quadrant than did Balb/c male SEB offspring. This was confirmed by a closer examination of this interaction using a one-way ANOVA \(F_{(1,14)}=8.11, \ p=0.0129\) (Figure 27b).

A repeated measures ANOVA was used to analyze if there were any significant changes in the percent time spent in the platform quadrant between the 1-hour probe and the 24-hour probe. There was a significant strain x treatment effect, such that Balb/c SEB offspring decreased percent total time in the platform quadrant compared to Balb/c controls, \(F_{(1,51)}=11.84, \ p=0.0012\) (Figure 27c). There was a trend for C57BL/6 saline offspring to reduce the percent time in the platform quadrant from the 1-hour probe to the 24-hour probe, but this was not statistically significant, \(t(29)=1.33, \ p=0.0975\). Despite these changes between the probe tests, the percent time spent in the platform quadrant during the 1-hour probe was significantly correlated with the percent time during the 24-hour probe (\(r=0.3463, \ p=0.0005\)).

The distance traveled during the probe tests were also collected and analyzed. There was a significant interaction of strain x sex x treatment such that Balb/c male SEB offspring traveled significantly less in the platform quadrant than Balb/c male saline
offspring during the 24-hour probe, $F_{(1,45)}=4.1865, p=.0466$. There were no significant differences between groups for the 1 hour probe.

In summary, offspring of saline-injected mothers were significantly better at learning the MWM task than were offspring of SEB-injected mothers, and Balb/c SEB offspring—particularly males—exhibited impaired memory for the test 24 hours after the learning phase.

**Discussion**

Through the five behavioral and cognitive tasks discussed above, it is apparent that the offspring of mothers injected with SEB display a distinct behavioral phenotype to those offspring of saline-injected mothers; however, strain plays a crucial role in the development of the behavioral phenotype (Table 11). Many, but not all, of the results found in the SEB offspring are similar to the results found in SEA offspring, and are discussed in length in Chapter 4.

First, in every test except the MWM, there is a clear distinction between the C57BL/6 strain and the Balb/c strain, regardless of treatment and/or sex. C57BL/6 mice appear to be more social, less anxious, more active, and have greater PPI than Balb/c mice. Differences in strain anxiety, activity and sensorimotor gating were discussed in Chapter 4.

In tests of social interaction, C57BL/6 control animals make more direct contacts, spend more time in the area of a novel mouse, and sniff a novel mouse more times than Balb/c animals do (Sankoorikal, Kaercher, Boon, Lee, & Brodkin, 2006). In the present study, although Balb/c control mice touched the cup less than the C57BL/6 control mice, the overall percent of time SEB offspring spent touching the cup was greater. This
implies that Balb/c control offspring would approach the target cup less number of times but the SEB offspring would stay there for longer. This could be due to the tendency for Balb/c SEB mice to be more anxious (Crabbe, 1986; Crawley et al., 1997; Francis et al., 2003), and thus they spend more time in one spot compared to the more ambulatory C57BL/6 mice.

The tendency for higher levels of anxiety in Balb/c mice mentioned above could also explain the difference in the distance traveled on Day 1 in the OR task. Though there was no difference between the strains in the percent time spent in the center of the OF, C57BL/6 animals traveled further in the perimeter center areas, generating an overall greater distance traveled. The fact that the Balb/c mice did not travel as much but spent comparable amounts of time in both areas implies that they tend to remain in one place for extended periods of time before migrating to another location. This is illustrated in Figure 9, which shows sample movement maps of C57BL/6 and Balb/c mice: the C57BL/6 animal moves around quite often in all areas of the OF, while the Balb/c animals travel much less and relocate less often, staying in the same area. Anecdotally, Balb/c mice generally sat in the location in which they were placed in the middle of the OF for an extended period of time before moving to the perimeter. They then more often than not remained in the perimeter until the end of the task. On the other hand, C57BL/6 mice were usually in constant motion exploring the OF, passing through the center area on multiple occasions. It is possible that this is why the percent time in the center is similar for the two strains, a measure that does not accurately capture their behavioral differences.
It is also interesting to note that there was no strain difference in the MWM learning task, and that each strain learned the task equally as well. This is different to what we saw in Chapter 4, with Balb/c mice displaying deficiencies in learning the MWM task as compared to C57BL/6 mice. Balb/c mice are known to be “poor learners” when it comes to the MWM task, never learning where the platform is hidden (Crawley et al., 1997; Owen et al., 1997; Upchurch & Wehner, 1989). One reason we may not have detected any strain differences in learning is because of the difference in the number of animals in each group. While almost equal numbers of each strain did not learn the task [C57BL/6 = 19 (34%); Balb/c=17 (40%)] there were over ten more C57BL/6 subjects than Balb/c subjects. Adding more Balb/c subjects to match the amount of C57BL/6 might increase the percentage of non-learners in the Balb/c offspring. Were there equal numbers of each strain, perhaps this difference would have been made greater or at least have come out significant.

In sum, most of the strain differences reported above reflect already-established differences between Balb/c and C57BL/6 mice. C57BL/6 mice are more social than Balb/c mice and take greater interest in a novel mouse, as described in the current study. Balb/c mice are known to be more anxious and less active than C57BL/6 mice, which is reflected in their hesitation to leave the closed arms of the EPM and to remain inactive in the OR task. Their increased startle responses are also indicative of higher levels of anxiety. Though contradicting some of the literature, C57BL/6 mice had increased PPI at all three prepulse intensities, and there was no difference between the strains in their ability to learn the MWM task. Future studies should ensure that there are equal, or close to equal, numbers of animals in each group to validate these findings.
The most important aspect of this experiment is the influence of maternal SEB treatment on offspring behavior. There were many significant maternal treatment effects on the behaviors of the offspring. These were observed mainly for the Balb/c offspring. This is most probably due to the fact that SEB differentially affects the two strains based on their MHC II isotype (Taub et al., 1992; Watanabe et al., 2004), and as shown in chapter 3, produced significantly higher concentrations of IL-2, IFN-\(\gamma\), and IL-4 in the Balb/c strain. In only two instances were there differences between C57BL/6 SEB offspring and saline offspring (+8dB prepulse trials, and learning in the MWM), which will be discussed below. However, for the purposes of this discussion, all SEB/saline differences discussed are referring to the Balb/c subjects and not the C57BL/6 subjects, unless otherwise specified.

Offspring of SEB-injected mothers, as compared to saline controls, displayed overall increases in social interaction behavior, increased anxiety, increased levels of sensorimotor gating as measured by PPI, reduced spatial learning ability, and reduced memory 24 hours after a learned task. Though sex did play a role in some of these phenotypes, for the most part, these differences were seen in animals regardless of sex.

It is surprising that SEB offspring displayed increased rates of both social interaction with a novel mouse and PPI of acoustic startle at all three prepulse intensities. Much of the literature on MIA offspring implies that the opposite usually occurs. Administration of Poly IC into pregnant females around the same time point as the current study has been shown to reduce both contacts and contact time of offspring with a novel mouse (Lipina
et al., 2013; N. Malkova, 2010; Schwartzer et al., 2013; S. E. Smith et al., 2007), and injections of LPS and influenza into pregnant mothers has shown similar results (Oskvig et al., 2012; Shi et al., 2003). Disruptions of PPI in MIA offspring is one of the most robust findings in the literature, having been displayed in both LPS offspring (Borrell et al., 2002; M.-È. Fortier et al., 2004; M. E. Fortier et al., 2007; Romero et al., 2007) and poly IC offspring (Makinodan et al., 2008; Urs Meyer, 2013; Urs Meyer et al., 2007; Ozawa et al., 2006; Vorhees et al., 2015). As raised in the discussion of chapter 4, it has been suggested that elevated IL-6 is the essential mediator of MIA effects on offspring, necessary and sufficient to producing deficits in social behavior and PPI (Hsiao & Patterson, 2011; S. E. Smith et al., 2007), and that IL-6 is able to directly cross the placenta in rats and enter fetal circulation (Dahlgren et al., 2006). It is interesting that in the current study that maternal concentrations of SEB-induced IL-6 did not differ between the two strains, and yet there was a difference in social and sensorimotor behavioral outcomes. These differences, however, were not deficits but enhancements. It is possible that because a significant production of IL-6 was not generated, deficits in social behavior and PPI were not produced, and the changes in concentrations of the other cytokines affected these behaviors. Support for this idea comes from another recent study that found elevated levels of IL-6 in LPS-injected mice from both the C57BL/6 strain and the NMRI strain but only saw behavioral changes in the NMRI offspring (Babri et al., 2014). The authors of this study posited multiple reasons for this, one of them being that it was not the IL-6 mediating the change, but rather other biological differences produced by the LPS.
Assuming that elevated levels of IL-6 are unlikely to be the basis of the increases in social interaction and PPI in the Balb/c mice, one or more of the other three maternal cytokines analyzed could be the source of these changes. A recent study showed a strong link between adaptive immunity, IFN-γ and social behavior (Filiano et al., 2016). Meningeal T-cells release IFN-γ into the brain, and mice deficient in T-cells showed decreased levels of social interaction (Filiano et al., 2016). Injecting IFN-γ in the cerebrospinal fluid activated layer I neocortical neurons, GABAergic neurons most probably driving regional inhibition of neural circuits. This inhibition was proposed to reduce hyperconnectivity in the prefrontal cortex, a condition indicated in the brains of those diagnosed with social disorders such as Autism (Filiano et al., 2016). Staphylococcal enterotoxin B is not only a potent T-cell activator, but as the current study showed, produces an extremely high production of maternal IFN-γ. Though increased levels of IFN-γ have been known to be lethal to pregnancies (Krishnan et al., 1996), it is also a crucially important cytokine in the placental environment, necessary for successful pregnancy (Ashkar & Croy, 2001), suggesting that the right balance of its concentration could help rather than hurt fetal development. Perhaps the SEB-induced activation of the maternal adaptive immune system - T-cells and subsequent IFN-γ production - in Balb/c mice led to IFN-γ release in the fetal environment and developing CNS, leading to an enhanced phenotype of social behavior. Increased percent PPI in SEB offspring was surprising, but was also seen in the SEA offspring (for a detailed discussion for why this could be, see Chapter 4.).

The limbic area may represent a uniquely sensitive site to the presence of excessive proinflammatory cytokines (Nyffeler et al., 2006), and thus even though the
elevations of IFN-γ, IL-6 and IL-2 were relatively low compared to Balb/c animals, they may have been enough to cause changes in the limbic areas. This might also explain why C57BL/6 SEB offspring also displayed increases in PPI in the +8dB prepulse trials. As mentioned above, PPI is also considered to be controlled by the limbic system (Bakshi & Geyer, 1998; J. M. Petitto et al., 2002), and though C57BL/6 did not display increases in PPI at the lower prepulse intensity levels, at the loudest, most demanding prepulse level, changes in the limbic system might have been detected.

To summarize the unexpected results of social behavior and PPI, the findings of enhanced phenotypes of both of these behaviors seems to contradict the previous literature on MIA. Though IL-6 is usually deemed the main influence, behind MIA-induced deficits in social behavior and PPI, it does not seem to play a role in SEB-induced MIA. Increased IFNγ has been implicated in higher levels of social behavior, while increased IL-2 decreases dopamine release in the limbic system, which controls sensorimotor gating abilities. However, because no studies have yet looked at the fetal cytokine effects of MIA by bacterial T cell superantigens, it is not possible to speculate on the fetal milieu in response to maternal cytokine production. To gain a better understanding of how SEB injections in the pregnant mother affect the fetal environment, studies must be done to assess cytokine production in the placenta and fetus.

Offspring of Balb/c SEB-injected mothers displayed increased anxiety in the EPM and the OR task. Moreover, regardless of strain, SEB offspring exhibited higher startle responses during the PPI task, which may also be indicative of increased levels of anxiety. The increase in anxiety was most apparent in the EPM, in which Balb/c SEB
offspring spent significantly more time in the closed arms and less time in the open arms and center area than the saline offspring. Though SEB offspring traveled significantly more than controls, this travel was mainly in the closed arms. This difference is not likely to be indicative of changes in locomotor activity, but rather a function of the protected nature of the closed arms - “safe” areas to move in. Alternatively, the open arms–where saline animals spend more time–is likely perceived as less “safe.” The behavior of the BALB/c mice reflected that, as they were more immobile in the open arms, but more active in the closed arms. We would have expected to see similar results to the EPM in the OR task on Day 1, when there were no objects in the open area and the animals were being exposed to the OF for the first time, but because a technical error and missing data for 20 animals, we believe that the power of the statistical tests was not sufficient to see any significant differences. There was, however, a trend for Balb/c SEB offspring to be more inactive on Day 5 in the OR, when one of the familiar objects was replaced with a new object. The inactive behavior could also be seen as an increase in anxiety levels in SEB offspring, as the addition of a strange object can be seen as stressful, but this difference did not reach significance. Increased anxiety was also displayed in offspring from SEA-injected mothers, and a detailed discussion of this can be found in Chapter 4.

In addition to strain and treatment affects in the EPM and PPI tasks, there were also significant sex x treatment interactions on some of the test measures. In the EPM, only female Balb/c SEB offspring displayed longer latencies to enter the open arms and only male Balb/c SEB offspring showed increased PPI in +2dB prepulse trials. This implies that SEB might be differentially affecting the sexes in terms of anxiety levels and PPI at low prepulse intensities. Similar sex-related increased latencies in the EPM was
also shown for female offspring born to mothers who had been stressed during mid-to-late gestation (Bowman et al., 2004). The immune system, sex steroids and the HPA axis all interact with one another to respond to stress in a sex-dependent manner (Rohleder, Schommer, Hellhammer, Engel, & Kirschbaum, 2001). It has been shown that circulating gonadal hormones affect male and female rats in different ways, such that reducing levels of male hormones does not affect anxiety levels in males, but reducing female hormones increases anxiety in females (Zimmerberg & Farley, 1993). This could be because estrogen and progesterone interact with the GABA<sub>A</sub> receptor (Belelli, Lan, & Gee, 1990), producing anxiolytic properties, and the tampering with this communication–either through reducing the hormones or by changing the efficacy of the receptor–could counteract these effects. Progesterone can reduce the production of proinflammatory cytokines after trauma (He, Evans, Hoffman, Oyesiku, & Stein, 2004; Kipp et al., 2007), and it is possible that they may have an inverse relationship: the more progesterone the less proinflammatory cytokines, and the more proinflammatory cytokines, the less progesterone. In this way, increases in proinflammatory cytokines due to SEB-injections could reduce progesterone interactions with the GABA<sub>A</sub> receptor, preventing its anxiolytic affects. One study using poly IC did, in fact, show changes in GABA<sub>A</sub> receptor neurochemistry in the limbic areas (Nyffeler et al., 2006), however, sex differences were not analyzed in this study. Though maternal SEB injections increased anxiety in Balb/c offspring regardless of sex for most measures in the EPM, perhaps females were slightly more affected. Once an SEB offspring did experience the open arms it tended to spend its time in the closed arms, but females were so inhibited it took them significantly more time than controls to even try to venture out.
There was also a slight sex x treatment interaction in the +2dB prepulse trials. While all SEB offspring had overall increased PPI compared to controls, only the Balb/c males displayed this difference at the lowest prepulse intensity trials. There is evidence that males may be more susceptible to the effects of MIA compared to females (Bale et al., 2010; Mueller & Bale, 2008). In fact, many studies mainly examine male offspring based on this assumption (Abazyan et al., 2010; Golan et al., 2006; Xuan & Hampson, 2014; Yee et al., 2011). The effects of maternal SEB injections on the offspring—namely, increased PPI—appeared to increase sensorimotor gating of males at the lowest prepulse intensity.

Lastly, significant treatment effects were seen during the MWM task, both in the learning phase and the long-term memory phase of the task. Similar effects were seen in C57BL/6 SEA offspring, and a discussion of how SAg exposure could produce such behavior is discussed in Chapter 4, section 4.4. It is interesting that in the case of SEB, this effect was seen in both Balb/c mice and also C57BL/6, as most of the behavioral effects of SEB have only been displayed in the Balb/c strain and the MWM deficit in SEA offspring was only seen in C57BL/6 mice.

The difference in male Balb/c SEB offspring and their control counterparts during the 24-hour probe test is not necessarily indicative of impaired long-term memory, but may actually reflect a difference in motivation and depressed behavior between the two groups. Anecdotally, when the SEB offspring were placed in the MWM 24 hours after the test day, they showed a tendency to immediately float in the water as opposed to actively searching for the platform. As discussed in chapter 4, this is reminiscent of the forced swim test (FST), The results of this test are used to measure depressive-like behaviors
and learned helplessness (Cryan & Mombereau, 2004). There is much evidence that MIA—either through LPS or poly IC—can increase depressive behavior in mice, as measured by the FST (Abazyan et al., 2010; Babri et al., 2014; Khan et al., 2014; Mueller & Bale, 2008). It has been proposed that this is propagated by the increased production of proinflammatory cytokines (Abazyan et al., 2010; Khan et al., 2014). In the current study, only males showed this behavior, but another study found a similar sex difference in depressive behavior (Mueller & Bale, 2008), and this may be due to the sex differences discussed above. In addition, this depressed-like phenotype might also explain why male Balb/c SEB offspring were significantly more inactive on Day 4 of the OR. It was the third day that animals were exposed to a specific environment, and thus their motivation to move around may have diminished and resulted in immobile behavior just as in a FST.

In summary, the strain differences detected in the behavioral tests align with previous literature on the differences between Balb/c mice and C57BL/6 mice. Increases in social interaction and PPI in the Balb/c SEB offspring contradict most of the findings in the MIA literature, but could be due to the extremely high concentrations of IFN-γ and IL-2 produced in response to SEB, which may influence social behavior and prepulse inhibition, respectively. An increase in anxiety levels is a robust finding in the MIA literature and is most probably due to the enlarged production of proinflammatory cytokines and possibly glucocorticoids. There were some sex-by-treatment interactions, with females displaying elevated anxiety and males exhibiting depressive behavior, which most likely reflect differing neurobiological responses to increased production of proinflammatory cytokines. Finally, SEB offspring from both C57BL/6 and Balb/c mice
displayed impaired learning on the MWM and increased PPI at high prepulse intensities, hippocampal- and limbic-area-dependent tasks—thus indicating that even small rises in cytokine concentrations may cause visible changes in those highly-sensitive areas. Thus, it is apparent that offspring of SEB-injected mothers exhibit behaviorally unique phenotypes relative to saline controls.
CHAPTER 6: GENERAL DISCUSSION

The current study proposed and tested a new model for maternal immune activation, using the T-cell-stimulating superantigens staphylococcal enterotoxins A and B (SEA; SEB). The results showed that while C57BL/6 mice responded more strongly to SEA, and Balb/c mice responded more strongly to SEB, exposure to either superantigen caused increased cytokine production of IL-4, IFNγ, IL-2, IL-6, TNF-α, and IL-17A, when compared to saline controls (Table 7). In addition, distinct behavioral phenotypes, largely dependent on strain and maternal treatment, emerged in offspring from superantigen-injected mothers. The C57BL/6 offspring from SEA-injected mothers displayed decreases in social behavior and spatial learning, and increases in anxiety, locomotion, interest in a novel object, short-term spatial memory, and depressive-like behaviors (Table 11). Similarly, Balb/c offspring from SEB-injected mothers displayed behavioral alterations. This included decreases in spatial learning, and increases in social behavior, anxiety, sensorimotor gating abilities, and depressive like-behaviors (Table 11). Overall, through the novel use of SEA and SEB as prenatal immune challenges, we were able to elicit significant cytokine concentrations in the mothers and altered behavioral profiles in their offspring. As discussed further, this both mirrors and diverges from previous models of maternal immune activation in important ways.

The phenomenon of maternal or prenatal immune activation posits that stimulation of a mother’s immune system during pregnancy can cause long-lasting neurobiological and behavioral changes in the offspring. Immune-challenging events such as bacteria, viruses, or stress, can cause a robust immune response, including a significant production of cytokines. These cytokines, and particularly proinflammatory
cytokines such as IL-6, IFN-γ, and TNF-α, which upregulate an immune response, may have the ability to cross the placenta connecting mother and fetus and infiltrate the developing fetal brain (Dammann & Levi, 1997). Alternatively, the maternal immune response may cause the fetus itself to upregulate its own production of proinflammatory cytokines (U. Meyer et al., 2006). Since cytokines play a natural role in brain development – contributing to cortical migration and neuronal growth, regeneration, development and survival (Bauer et al., 2007; Burns et al., 1993; Mehler & Kessler, 1997; Skundric & Lisak, 2003; Stolp, 2013) – the influx of proinflammatory cytokines can disrupt the delicate balance needed for normal neuronal development. This phenomenon has been implicated in the etiology of developmental psychiatric disorders, most prominently autism and schizophrenia (Boksa, 2010; Alan S Brown & Derkits, 2010; A. S. Brown & Patterson, 2011; Urs Meyer, 2013; U. Meyer et al., 2011; Patterson, 2011).

To date, the animal models used to model MIA utilize pathogens that mainly elicit an immune response via the innate immune system. Exposure to LPS leads to the activation of the innate immune response by binding to toll-like receptor-4 (TLR-4) on macrophages and other innate immune cells, which triggers a cascade of intracellular signals that ultimately leads to the production of pro-inflammmatory cytokines like IL-6 and TNF-α (Aderem & Ulevitch, 2000). Another innate stimulus, Poly IC, mimics the actions of a viral infection by binding to TLR-3 in the innate immune system and causing an induction of pro-inflammmatory cytokines (Doughty et al., 2006; Koga et al., 2009). The influenza virus can also activate the innate immune system, although, it will also induce an adaptative immune response that elicits B and T cell effector responses (eg,
antibody against the virus and cytotoxic T cell responses). As the three main models for MIA, these immune challenges mainly activate an immune response via the innate immune system. In most cases, due to the difficulty of maintaining facilities for viral infection research, most studies have opted for the use of LPS and Poly I:C, and this is where much of the evidence has been gathered.

Though the above-mentioned pathogen models ultimately produce increased levels of proinflammatory cytokines, their main method of action is through the activation of innate immune cells like neutrophils, and macrophages, and not through direct activation of T-cells. Therefore, LPS and poly IC fail to capture the full extent of immunological involvement in a wide range of possible immune challenges. The use of superantigens circumvents this problem, since this provides the opportunity to elucidate the role of T-cells and T-cell derived cytokines in MIA and the development of neurobiological and behavioral changes in affected offspring.

Superantigens, like SEA and SEB, are a class of pathogens that uniquely interacts with the adaptive immune system to directly activate T-cells (Fraser & Proft, 2008; B. Torres et al., 2000; B. A. Torres et al., 2001). Because SAgs bind outside the antigen-binding groove (Dellabona et al., 1990) to the external portion of the variable region of the beta chain (vβ) on TCRs (Fraser & Proft, 2008; McCormick et al., 2001; B. A. Torres et al., 2001), T-cells can be activated without specific recognition of antigen, thus activating ten-fold greater numbers of T-cells than would normally be activated in response to conventional peptide antigens. When specific antigen recognition is needed to activate T-cells, only about .01% of the body’s total T cell pool is activated (Abbas et al., 2011); however, when superantigens are present, about 20% of the body’s T-cells are
stimulated (Fraser & Proft, 2008). The robust stimulation of CD4+ T-cells causes an extreme production of T-cell-derived cytokines such as, IL-2, IFN-γ and TNF-α (Fraser & Proft, 2008; B. Torres et al., 2000).

The use of superantigens as a means of producing MIA is additionally important because there is a significant chance that a pregnant mother could be infected with a superantigen-like pathogen during her pregnancy. Staphylococcal aureus (S. aureus), the gram-positive bacterium that produces SEA and SEB, can regularly colonize up to 30% of the general population (Kluytmans et al., 1997; Krakauer & Stiles, 2003), while 60% of the general population are recurrent carriers of the bacterium (Kluytmans et al., 1997). Crucially, S. aureus has been shown to be present in the vaginal-rectal area in 17% of pregnant women, with 2% of those strains showing methicillin-resistance (K. T. Chen, Huard, Della-Latta, & Saiman, 2006). The presence of these resistant bacteria in pregnant women is not only growing, but the most common time of initial presentation of S. aureus in pregnancy is during the second trimester (Laibl et al., 2005)–a time point shown to be a particularly vulnerable one for MIA effects on the fetus (as discussed above in the introduction to Chapter 4). Moreover, there is evidence that active human endogenous retroviruses (HERVs), virus genomes incorporated into human DNA, can produce superantigen-like effects on the immune system when activated (Morandi, Tarlinton, Tanasescu, & Gran, 2017). Since all humans have elements of HERVs in their genomes, pregnant women are at just as much risk of these effects during their pregnancy.
Much of the behavioral results reported in the current study in response to SEA and/or SEB are consistent with previous literature using LPS, poly IC, and influenza virus to produce MIA in pregnant mice and rats. These include: changes in social behavior, increased anxiety in the elevated plus maze (EPM), increased locomotion and exploration of a novel object in the object recognition (OR) task, and decreased Morris water maze (MWM) learning. These behavioral alterations are of particular interest in that they have all been implicated in autism and schizophrenia disorders. Decreased social behavior—seen in C57BL/6 SEA offspring—is similar to the inhibition of social behavior seen in autism, whereas increased anxiety and locomotion reflects common symptoms associated with both autism and schizophrenia (Cheung et al., 2010; U. Meyer et al., 2011; Skokauskas & Gallagher, 2010). The mechanism behind these changes, and why some of them only appear in one strain versus the other has been discussed in detail above (see chapters 4 and 5), and does not need to be expounded on further. Though deficits in sensorimotor gating abilities are usually associated with both neuropsychological diseases (Cheung et al., 2010; U. Meyer et al., 2011), the current study found an increase in sensorimotor gating in MIA offspring. This difference, in addition to other behavioral results that differ from the current MIA models, such as increased social interactions in Balb/c SEB offspring, and increased short-term memory in the MWM task, have also been discussed in detail above.

Although much of that discussion was speculative since fetal cytokine concentrations were not measured in the current study, there is evidence that maternal immune challenge can affect cytokine concentrations in fetal brains (Tables 1, 2). Importantly, SEA and SEB elicit different patterns of cytokine concentrations as
compared to LPS, poly IC, and influenza virus, in that exponentially more T-cell-derived IL-2 and IFN-\(\gamma\) are produced, in addition to high levels of TNF-\(\alpha\). There were also increased concentrations of IL-6 measured, but only in C57BL/6 mice in response to SEA. What exact cytokines differences this might have elicited in fetal brains is unknown—and should be explored further in the future—but it is safe to say based on previous research that these maternal cytokine productions are impacting fetal development.

The exact mechanism of maternal immune activation is currently unknown, yet all the research agrees that the heavy production of proinflammatory cytokines in the mother somehow leads to a similar production in the fetus, and ultimately causes a cascade of events that generates neurobiological and behavioral deficits in the offspring. Different mechanisms of how the proinflammatory cytokines exert their influence have been proposed, including via genetic manipulation, neurotransmitter system changes, and metabolite dysfunctions. First, how the increased cytokine production in the mother influences fetal cytokine production is reviewed, after which the effects on central nervous system development are discussed.

Whether maternal cytokines have the ability to cross the placenta and enter the fetus is still debated, yet all theories agree that the end result is an increased presence of proinflammatory cytokines in the fetal environment. Evidence for transplacental cytokine transfer (cytokines passing directly from mother to baby) has been seen with IL-6 (Dahlgren et al., 2006; Kent, Sullivan, & Elder, 1994; Zaretsky, Alexander, Byrd, & Bawdon, 2004) and TNF-\(\alpha\), IL-1\(\alpha\), and IL-1\(\beta\) (Kent et al., 1994). However, there is also
evidence that the latter three cytokines may not to be able to cross the placenta (Zaretsky et al., 2004). For IL-6, optimal transfer through the placenta and fetal access is thought to occur during mid-gestation in comparison to late gestation (Dahlgren et al., 2006). Once the proinflammatory cytokines traverse the placenta, the fetus itself responds by endogenously producing more cytokines, further amplifying an inflammatory presence (Urs Meyer et al., 2007). Alternatively, even if maternal cytokines are not able to cross the placenta, they are able to cause a cascade of events at the maternal-fetal interface, initiating proinflammatory cytokine production in the lining of the uterus known as the decidua (Hsiao & Patterson, 2011), placenta, the umbilical veins or fetus itself (Dammann & Leviton, 1997). Evidence of this has been seen with an increase in both cytokine mRNA and cytokine protein in the embryonic serum following maternal immune challenge using LPS and poly IC (Ashdown et al., 2006; Hsiao & Patterson, 2011; U. Meyer et al., 2006; Urakubo et al., 2001). Whether maternal cytokines can or cannot cross the placenta, the same result is observed: after MIA, elevated levels of proinflammatory cytokines such as IL-6, TNF-α, and IL-1β are present in maternal serum, the uterus, amniotic fluid, fetal circulation, and the fetal brain (Dammann & Leviton, 1997; U. Meyer et al., 2006; Urs Meyer et al., 2007; Oskvig et al., 2012). It is yet to be determined what specific effects T-cell-mediated MIA has on the production of proinflammatory cytokines in the maternal-fetal interface and fetal brain. Though SAg exposure produces proinflammatory cytokines like the current MIA models, these cytokines are mainly produced by T-cells and are made up of a majority of IFN-γ. However, it is likely safe to assume that increased production of proinflammatory cytokines in the maternal-fetal interface and fetal brain occurs, yet which cytokines and
their patterns of production are in need of clarification. Regardless of exposure type, it is clear that accumulation of maternal cytokines in response to an immune challenge can impact the environment in which the fetus is growing.

The buildup of proinflammatory cytokines in the fetal environment is potentially dangerous because these immune products can readily access the nervous system of the developing mouse embryo, since the blood brain barrier (BBB) has not fully developed. Therefore, excess amounts may be disruptive. On the other hand, these cytokines may also be critical mediators in the correct development of the brain during the prenatal period, and if present at optimal amounts, may promote improved development. For instance, in the developing brain, there are neurotrophic cytokines that act through specific receptors as intercellular mediators to regulate neuronal growth, regeneration, survival, and differentiation (Bauer et al., 2007; Skundric & Lisak, 2003; Stolp, 2013). Such cytokines have also been implicated as crucial factors in synaptic plasticity and in modifying certain behaviors (Bauer et al., 2007). These cytokines, among them IL-1, IL-6 and TNF-α, are necessary for appropriate growth and neural connectivity of the developing brain. Interferon-γ, in particular, has been shown to promote neuronal differentiation in developing brains (Zhao & Schwartz, 1998).

In contrast to these positive neuronal effects of cytokines, when the optimal balance of neurotrophic cytokines is altered through peripheral cytokines coming from extra-CNS regions in the embryo, or from the mother, widespread and permanent damage to the developing brain may occur. The proinflammatory cytokines might directly injure neurons, oligodendrocytes and microglia, all supporting the normal growth and development of the brain. Additionally, in experiments where fetal neuronal cultures
taken from rat fetuses were mixed with high doses of IL-1β, IL-6 and TNF-α, there was a significant reduction in dendritic nodes, dendritic length and primary dendrites overall (Gilmore, Fredrik Jarskog, Vadlamudi, & Lauder, 2004). Not only do these results suggest that proinflammatory cytokines can significantly reduce dendrite development and complexity of developing cortical neurons, but these findings also strongly indicate that cytokines play a key mechanistic role in the link between prenatal exposure to infection and risk for diseases characterized by these dysfunctions.

In addition, microglia are crucial to the number of neurons that can be formed in the developing brain. When microglia are activated in utero through immune challenge, there is a significant decrease in the amount of neural precursor cells found in the brain of the rat embryo (Cunningham et al., 2014). Because the human BBB is not fully developed during the early gestational period (Goasdoué, Miller, Colditz, & Bjorkman, 2016), and may not have its protective tight junctions in early fetal development (Goasdoué et al., 2016; Kjeld Møllgård, Balslev, Lauritzen, & Saunders, 1987; K Møllgård & Saunders, 1986), any of the above implicated cytokines might have access to the fetal brain, and may wreak its havoc through direct contact with it. Therefore, during prenatal brain development normal stability of the immune system is needed in order to form a well-functioning brain, and an influx of external cytokines may be harmful for that stability.

A number of other theories proposed about the mechanism of action of MIA include targeting of specific neurotransmitter or metabolite systems that ultimately impact fetal neurodevelopment. First, MIA has been suggested to affect the kynurenine pathway of tryptophan degradation, a metabolic pathway, the products of which can cross
through the mature BBB and interact with the brain (Fukui, Schwarcz, Rapoport, Takada, & Smith, 1991). The neuroprotective product of the kynurenine pathway is kynurenic acid, which was shown to be decreased in the offspring of MIA-injected mothers, while the neurotoxic product, quinolinic acid, was increased (Zavitsanou et al., 2014). This increase could interfere with proper brain maturation.

Another mechanism proposed identifies impairments in the GABAergic system as being a direct consequence of MIA (Labouesse, Dong, Grayson, Guidotti, & Meyer, 2015; Oskvig et al., 2012). Promoter regions for critical enzymes in GABA synthesis were hyper-methylated in offspring from MIA mothers, leading to a decrease in GABA expression which was correlated with deficits in cognitive behaviors (Labouesse et al., 2015). Another study found large disruptions in genes for GABAergic interneuron migrations in developing brains in offspring of exposed mothers (Oskvig et al., 2012). It has been proposed that MIA-induced deficiencies in GABAergic transmissions result in a disturbance of cortical inhibitory activity, leading to cognitive deficits in the offspring (Nyffeler et al., 2006). The GABA theory of MIA helps to elucidate the hypothesis proposed previously (see chapter 5)–based on literature that found a relationship between IFN-γ and GABA cortical neurons (Nyffeler et al., 2006)–that IFN-γ production in response to SAg exposure is mediating the effects on social behavior seen in the current study. While both Balb/c and C57BL/6 animals showed increased production of IFN-γ in response to SEB and SEA, respectively, Balb/c SEB offspring displayed increased social behavior while C57BL/6 SEA offspring displayed decreased social behavior. The concentration of IFN-γ in C57BL/6 mothers in response to SEA was almost two-fold greater than in Balb/c mothers administered SEB, in addition to having greater
concentrations of IL-6 as well. These greater maternal responses may be producing detrimental actions on the fetal GABA system, whereas the concentration of IFN-γ produced in Balb/c mothers might be producing socially beneficial actions. Cytokine production to SAg-mediated MIA may be interacting with the GABA system in differential ways depending on strain and T-cell response.

Aside from GABA, other neurotransmitter systems may be affected by MIA. Previous research has demonstrated increased sensitivity of prefrontal serotonin receptors and decreased glutamate receptor functioning in the offspring of mothers injected with LPS (Wischhof, Irrsack, Dietz, & Koch, 2015). Similarly, maternal poly IC and stress increased the serotonin 5-HT2A receptor but decreased the metabotropic glutamate 2 (mGlu2) receptors in the prefrontal cortex of mice (Holloway et al., 2013). There is also experimental in vivo evidence that maternal poly IC exposure increases the number of dopamine neurons (U Meyer, Engler, Weber, Schedlowski, & Feldon, 2008) and dopamine function (Ozawa et al., 2006) in the midbrain and hippocampus. Both serotonin and dopamine has been shown to be crucial in central nervous system development (Mazer et al., 1997; Money & Stanwood, 2013) and in postnatal hippocampal neurogenesis (Borta & Höglinger, 2007; Gould, 1999). Proper execution of both of these processes is crucial for healthy cognitive functioning, and hippocampal processes are responsible for many learning and memory behaviors, including the MWM (Jeffery & Morris, 1993; Vorhees & Williams, 2006), and has also been implicated in proper sensorimotor gating (Bakshi & Geyer, 1998; J. Petitto et al., 1997). In addition, dopamine plays a central role in locomotor activity with increased overall dopamine activity levels producing increased locomotion (Beninger, 1983). Interestingly, our results showed that
memory for the MWM at 1-hour and locomotor activity in the OR was increased in C57BL/6 SEA offspring, as was percent PPI in offspring from both strains. These results diverge from previous MIA studies using LPS and poly IC, which have shown decreases in these measures. As MWM memory, locomotion and PPI are mediated by hippocampal processes, which are in turn influenced by brain dopamine and serotonin, future studies would benefit from looking at the effects of T-cell-mediated MIA on dopamine and serotonin production in the brain.

Another means by which MIA might interfere in fetal development involves its impact on gene expression. Researchers injected LPS into pregnant rats mid-gestation, and after measuring significantly elevated levels of proinflammatory cytokines in amniotic fluid and fetal rat brains, whole genome microanalysis displayed gene dysregulation in 3,285 genes (Oskvig et al., 2012). Of the 3,285 genes, 2,192 were abnormally upregulated and 1,093 were abnormally downregulated. When these genes were categorized into restricted functional categories genes related to the cell cycle—including genes for oxidative stress, hypoxia, and cell death—were the most enriched for the upregulated genes, and genes related to nervous system development and function—including genes for neuronal migration—were the most enriched for the downregulated genes (Oskvig et al., 2012). These genes, and the natural balance of their activity, are critical for the development of the central nervous system; the changes caused by the increase in proinflammatory cytokines could be detrimental to proper brain development. This proposed molecular mechanism of MIA requires further analysis and inclusion of SAg-induced MIA, and is likely to involve a complex pattern of gene interactions.
Nonetheless, it is a promising approach to unravelling some of the key molecular pathways affected by MIA in the developing brain.

Lastly, different cytokines have been pinpointed as major culprits behind the disruptive effects of MIA on brain and behavioral development. First, as discussed in previous chapters, IL-6 has long been thought as both necessary and sufficient for the developmental effects of MIA. Reinforcing this idea, injections of IL-6 alone, with no other immune challenge, has led to the development of neurobehavioral deficits in offspring of the injected mother (Samuelsson et al., 2006; S. E. Smith et al., 2007). In addition, IL-6 antagonists can prevent some of the behavioral abnormalities usually seen in the offspring of Poly IC-injected mice (S. E. Smith et al., 2007). Further, IL-6 has been shown to activate the JAK/STAT3 pathway, ultimately mediating harmful gene expression in the spongiotrophoblast layer of the placenta (Hsiao & Patterson, 2011). Part of the IL-6 family of cytokines, leukemia inhibitory factor (LIF) has recently been indicated as crucial in the biological consequences of maternal immune challenge. This cytokine is required for fetal brain development (Hatta et al., 2006; Hatta, Moriyama, Nakashima, Taga, & Otani, 2002), and more specifically, for neurogenesis (Simamura et al., 2010). Researchers have shown that a mid-gestational injection of poly IC disrupts the production of LIF, ultimately reducing levels of it in fetal cerebrospinal fluid and promoting decreased neurogenesis in the cerebrum (Tsukada et al., 2015).

Interestingly, IL-6 was not a major cytokine produced by either strain in response to SEB (Figure 14), and was also very low in Balb/c SEA offspring (Figure 7), and yet behavioral deficits were displayed in these animals. There are a number of possible explanations for this. First, as discussed at length in Chapters 4 and 5, IL-6 may not be
necessary or sufficient to producing behavioral effects in MIA offspring. Though this may contradict some previous literature, SEA and SEB produce significantly greater levels of other proinflammatory cytokines such as IL-2 and IFN-γ that were not considered in previous models of MIA. Second, IL-6 has been shown to peak at 4 hours post-injection of SEB (Miethke et al., 1993), while the current study measured cytokine concentrations at 2 hours post-injection; it is possible (but unlikely) the concentrations measured above do not reflect peak IL-6 production in response to SAg injections. Third, although IL-6 production was low, perhaps large amounts of IL-6 are not necessary for MIA-induced changes in offspring. Exposed mothers did, on average, still produce significantly more IL-6 than controls, and even at low concentrations it could have been enough to induce neurobiological and behavioral effects in the offspring. Whatever the correct explanation is, further study is needed on the role IL-6 plays in T-cell-mediated MIA.

Recently, IL-17 has become a focus of MIA literature. Interleukin-17 is predominantly produced by Th17 cells, and has also been shown to significantly increase in a poly IC MIA model (Choi et al., 2016). With that increase, offspring also show behavioral dysfunctions and cortical development abnormalities, and direct administration of IL-17 into fetal brains promotes those changes as well (Choi et al., 2016). Further, the blocking of IL-17 during maternal immune challenge with poly IC prevents these neurological and behavioral dysfunctions from taking place (Choi et al., 2016). This might suggest that IL-17 could be necessary and sufficient to producing MIA effects in offspring. However, much like IL-6, SEA and SEB produced extremely low concentrations of IL-17 in both strains. This is not surprising since IL-6 is a key factor in
Th17 differentiation, and without IL-6 production, there is no IL-17 production (Choi et al., 2016). Therefore, the same possible explanations for the behavioral results in light of low IL-6 production can also apply for low IL-17 production.

The foregoing discussed a range of plausible mechanisms for disruption of neural and behavioral development due to MIA. However, the exact mechanism of how MIA works is still unknown and highly debated. Whether maternal cytokines themselves cross into the developing fetus, or whether they initiate fetal production of proinflammatory cytokines—or, possibly, whether both occur—a buildup of cytokines in the fetal environment can lead to a number of harmful neuroanatomical and behavioral consequences for the offspring (discussed in Chapter 1). Because MIA may not work through mutually exclusive neurotransmitter and metabolite systems, nor may it be the presence of a singular cytokine that is causing the damage, each of the above proposed mechanisms and cytokines represent pieces in a growing puzzle of how a mother’s immune system may impact fetal brain development. In addition, studying the effects of T-cell-mediated MIA through the use of superantigens can further elucidate the role specific proinflammatory cytokines play in the development of neurobiological and behavioral deficits.

Of course, it is important to note that all of the LPS and poly IC literature, as well as the research presented in the current study, are performed using animal models. These models are the only ways to perform experimental-type studies to examine the effects of MIA on offspring biology and behavior. It is important to keep in mind that while these are the best approximations one can perform preclinically in laboratory animals, they
may still not be perfectly correlated with human outcomes. However, recent studies have been performed in humans looking at specific markers of maternal inflammation and immunological insult in pregnant mothers and relating them to the psychological and cognitive outcomes of the offspring. Specifically, increased levels of IL-8 during the second and third trimesters of pregnancy not only predicted the development of diagnosable schizophrenia in the offspring (Alan S Brown, Hooton, et al., 2004), but they were also significantly correlated with structural neuroanatomic changes in adult children that have been implicated in schizophrenia spectrum disorders (Ellman et al., 2010). In addition, low maternal hemoglobin was related to poorer neuromotor functioning and general intellectual abilities in offspring diagnosed with schizophrenia (Ellman et al., 2012), while maternal iron deficiencies were found to be a risk factor for the offspring to develop a schizophrenia spectrum disorder (Insel, Schaefer, McKeague, Susser, & Brown, 2008). For autism spectrum disorder (ASD), increases in C-reactive protein—a marker of inflammation—during the first and early second trimester elevated the risk of the offspring to be diagnosed with ASD by 43% (Alan S Brown et al., 2014). By continuing to find specific immunological risk factors in human pregnancies for the development of neuropsychiatric diseases, the animal models used in the laboratory can become increasingly applicable to human health and disease.

There were a number of limitations in the current study. First, because so many different groups of animals were needed for testing, some of the sample sizes were not as optimal as they could have been. Moreover, many litters were skewed in the sex of the pups, causing greater differences in sample sizes when grouped by sex. In addition to small
sample sizes, technical errors prevented us from analyzing data from a large amount of subjects, further decreasing sample sizes and/or forcing us to completely disregard certain groups in the analyses. This was specified above when it occurred, but measures in the Object Recognition task for the SEB experiments were specifically affected. In terms of the data analysis itself, it would be useful to analyze the data in terms of each litter as opposed to each individual animal. Since variation exists in immune responses and cytokine productions to infection between individuals, grouping the data by litter might be a more effective way of viewing the data. For instance, instead of sample size reflecting the total number of animals in a specific group, it would reflect the number of mothers injected to produce each group. The means used in the data analysis would be made up of the averages of the pups from each mother then averaged together to get the mean for each litter. However, the goal of the current study was to establish that T-cell-activating SAgs are a useful model for MIA, and not necessarily to determine a specific immune mechanism for their effects.

Much can be done to further the findings of the current experiment and elucidate the role T-cells and T-cell-induced proinflammatory cytokines play in the effects of MIA. Crucially, future studies should assess changes in cytokine concentrations in the maternal-fetal interface and in fetal brains in response to T-cell-activating pathogens. Though LPS and poly IC have been shown to induced significant productions of cytokines in these critical areas (discussed above and shown in Table 7), a different pattern of cytokines is produced in mothers in response to SEA and SEB, and their downstream effects on the developing fetus are currently unknown. This is the essential
and logical next step in further understanding what role activated T-cells and their products play in influencing biological and behavioral changes in the offspring. While the current study found many matching behavioral results to previous MIA literature, there were also stark differences, which may be a result of the differences in cytokine production between the models. Understanding what is happening in between the mothers’ immune response and the offspring’s behaviors can further clarify the differences between the previous MIA models and the one in the current study.

In addition to cytokine changes, assessing glucocorticoid changes and productions in mothers and also fetal and postnatal brains would be extremely useful, as well. As mentioned above, glucocorticoids and their influences on the hypothalamus-pituitary-adrenal (HPA) axis have been shown to play a large role in the effects of MIA (Asiaei et al., 2011; Babri et al., 2014; Golan et al., 2006), with increases in proinflammatory cytokines, and specifically TNF-α after SEA exposure, leading to increases of glucocorticoids and changes in the HPA axis (Babri et al., 2014; Rossi-George et al., 2005; Zaharia et al., 1996). Because the HPA axis plays such a crucial role in many cognitive behaviors, it is important to understand how this system and the production of glucocorticoids are affected by MIA.

Lastly, there is the possibility that behavioral abnormalities in MIA offspring could be partly due to their rearing by MIA-exposed mothers. In this case, it is not necessarily the biological changes induced by maternal exposure to immune challenges that is causing behavioral alterations in the offspring, but rather the behaviors of the mothers rearing the offspring that is contributing to these changes. Evidence for this has been shown in two similar studies using poly IC, indicating that control offspring raised
by poly IC-injected mothers exhibited behavioral abnormalities on latent inhibition tasks and in the potentiation of dopamine agonist sensitivity on locomotion (Urs Meyer et al., 2008; Urs Meyer, Schwendener, Feldon, & Yee, 2006). Offspring of MIA mothers raised by control mothers also exhibited these defects, indicating that the prenatal immune challenge was enough to affect these behaviors, but may not be necessary. Further studies of this kind are important ways of parsing out prenatal influences on neurobiological behaviors versus postnatal impacts.

In conclusion, T-cell-mediated maternal immune activation is a valid and valuable model for studying the effects of prenatal immune challenge on neurodevelopmental and behavioral alterations in offspring relevant to psychological diseases. Infection by bacterial T-cell superantigens is not only a significant risk factor for the development of these diseases, but the ability to utilize its effects on T-cells and production of T-cell-derived cytokines provides researchers with the opportunity to better understand the role these factors play in the current paradigm of maternal immune activation. Including this model in the MIA research and literature will benefit our understanding of how to devise means of prevention, intervention, and treatment of serious psychiatric diseases.


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Meyer, U., Nyffeler, M., Schwendener, S., Knuesel, I., Yee, B. K., & Feldon, J. (2008). Relative prenatal and postnatal maternal contributions to schizophrenia-related
neurochemical dysfunction after in utero immune challenge. *Neuropsychopharmacology, 33*(2), 441-456.


and cognitive impairment in the offspring: a neurodevelopmental animal model of schizophrenia. *Biological psychiatry, 59*(6), 546-554.


glutamate without evoking inflammatory mediators. *Journal of Neurochemistry, 92*(5), 997-1009.


Williams, J. (2001). CBRNE—Staphylococcal enterotoxin B.


### Tables

#### Fetal brains

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<tr>
<th>Source</th>
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<th>Animal, Strain</th>
<th>Day</th>
<th>Cytokine</th>
<th>Response</th>
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<td>Cai et al., 2000</td>
<td>LPS, 4mg/kg</td>
<td>Rat, Sprague-Dawley</td>
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<td>IL-1B, TNF-α</td>
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<td>IL-1B, TNF-α</td>
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</tr>
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<td>Ning et al., 2008</td>
<td>LPS, 500ug/kg</td>
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<td>TNF-α</td>
<td>Increase</td>
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<td>Meyer et al., 2006</td>
<td>poly IC, 5mg/kg</td>
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<td>Increase at 3 hour, no change at 6 hours</td>
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<td>IL-10</td>
<td>Increase</td>
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<td>TNF-α</td>
<td>Increased at 3 hours, no change at 6 hours</td>
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Table 1. Changes in fetal brain cytokine concentrations in response to maternal immune activation in the mother.
### Table 2. Changes in postnatal brain cytokine concentrations in response to maternal immune activation in the mother.

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<tr>
<th>Source</th>
<th>Injection, dose</th>
<th>Animal, Strain</th>
<th>Day of brain injection</th>
<th>Day of brain extraction</th>
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<th>Response</th>
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<td>Yesimilark et al., 2007</td>
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<td>P7</td>
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<td>Ling et al., 2002</td>
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<td>IL-1β, IL-6, IL-4, IL-2</td>
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<td>IL-2</td>
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<td>Borrell et al. (2002)</td>
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<td>Romero et al. (2007, 2010)</td>
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<td>Rat, Wistar</td>
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<td>Fortier et al. (2007)</td>
<td>LPS 50ug/kg</td>
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<td>E18 and E19</td>
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<td>Mouse, C57BL/6</td>
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<td>P70, P200, P400, P600</td>
<td>Learning in radial arm maze</td>
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<td>P28-56</td>
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<td>Reduced</td>
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<td>Rats, Sprague-Dawley</td>
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<td>P56</td>
<td>PPI</td>
<td>Reduced</td>
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<td>Wolff and Bilkey (2010)</td>
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<td>P35, P90–P120</td>
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### Behavioral Results, cont’d

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<th>Day of Testing</th>
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<th>Result</th>
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<td></td>
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<td>P98-112</td>
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<td>Smith et al. (2007)</td>
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<td>Adult, age unspecified</td>
<td>PPI</td>
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Table 3. Behavioral alterations in offspring of MIA mothers.
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<td>Saline (vs SEA)</td>
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<td>6</td>
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<tr>
<td>SEB</td>
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<td>5</td>
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<tr>
<td>Saline (vs SEB)</td>
<td>4</td>
<td>6</td>
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</table>

Table 4. ELISA data: final n’s for each group in cytokine immunoassays.

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<th>IL-4</th>
<th>IFN-γ</th>
<th>IL-2</th>
<th>IL-6</th>
<th>TNF-α</th>
<th>IL-17A</th>
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<td>IL-4</td>
<td>–</td>
<td>0.59***</td>
<td>0.67***</td>
<td>0.59***</td>
<td>0.66***</td>
<td>0.56***</td>
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<tr>
<td>IFN-γ</td>
<td>0.59***</td>
<td>–</td>
<td>0.79***</td>
<td>0.90***</td>
<td>0.90***</td>
<td>0.51*</td>
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<tr>
<td>IL-2</td>
<td>0.67***</td>
<td>0.79***</td>
<td>–</td>
<td>0.78***</td>
<td>0.87***</td>
<td>0.74***</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.59***</td>
<td>0.90***</td>
<td>0.78***</td>
<td>–</td>
<td>0.88***</td>
<td>0.51**</td>
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<tr>
<td>TNF-α</td>
<td>0.66***</td>
<td>0.90***</td>
<td>0.87***</td>
<td>0.88***</td>
<td>–</td>
<td>0.71***</td>
</tr>
<tr>
<td>IL-17A</td>
<td>0.56***</td>
<td>0.51*</td>
<td>0.74***</td>
<td>0.51**</td>
<td>0.71***</td>
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Table 5. Correlations of cytokine concentrations in response to SEA-exposure.
*p=0.0005; **p=0.0004; ***p<0.0001

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<tr>
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<th>IFN-γ</th>
<th>IL-2</th>
<th>IL-6</th>
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<td>IL-4</td>
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<tr>
<td>IFN-γ</td>
<td>0.81***</td>
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<td>0.78***</td>
<td>0.36*</td>
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<td>0.53**</td>
<td>0.78***</td>
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<td>0.63***</td>
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<tr>
<td>IL-6</td>
<td>0.11</td>
<td>0.36*</td>
<td>0.63***</td>
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Table 6. Correlations of cytokine concentrations in response to SEB-exposure.
*p=0.0268; **p=0.0007; ***p<0.0001
### Table 7. Summary of the ELISA results.

All animals responded with significantly higher concentrations of all cytokines measured in response to SEA and SEB injections compared to saline. Certain cytokine concentrations were increased in one strain as compared to the other, or in one group of animals based on pregnancy status over the other group.

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<tr>
<td>IFN-γ</td>
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<td>C57BL/6 &gt; Balb/c</td>
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<tr>
<td>IL-2</td>
<td>Not pregnant &gt; Pregnant</td>
<td>–</td>
</tr>
<tr>
<td>IL-6</td>
<td>Not Pregnant &gt; Pregnant</td>
<td>C57BL/6 &gt; Balb/c</td>
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<tr>
<td>TNF-α</td>
<td>Not Pregnant &gt; Pregnant</td>
<td>C57BL/6 &gt; Balb/c</td>
</tr>
<tr>
<td>IL-17A</td>
<td>Not Pregnant &gt; Pregnant</td>
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</tbody>
</table>

Table 8. Behavioral data: final n’s and average litter size for the number of mothers injected with either SEA, SEB or Saline whose pups were used for behavioral tests. Because the SEA and SEB behavioral tests were run at separate times, each had separate corresponding control subjects. The average litter size of each group appears in parentheses next to the number of mothers injected. There were no differences in average litter sizes between groups.

<table>
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<td>C57</td>
<td>5 (5.2)</td>
<td>4 (5.5)</td>
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<tr>
<td>Balb</td>
<td>4 (6.0)</td>
<td>5 (5.6)</td>
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</table>
Table 9. Behavioral data: final n’s for the number of offspring in each group tested on the behavioral tasks. Because the SEA and SEB behavioral tests were run at separate times, each had separate corresponding control subjects.

<table>
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<tr>
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<th>SEB (vs SEB)</th>
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</tr>
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<td>Balb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
<td>13</td>
</tr>
</tbody>
</table>

Table 10. Learned Morris Water Maze: final n’s of SEA and Saline offspring that successfully learned the MWM task and were used in the analyses for the probe tests.

<table>
<thead>
<tr>
<th></th>
<th>SEA</th>
<th>Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Balb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Female</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 11. Summary of the behavioral results.
*There is a lot of missing data for this particular test.
Table 12. Data missing for Days 1, 3, and 4 on the Object Recognition task for the SEB offspring and the final n’s for these days.

<table>
<thead>
<tr>
<th></th>
<th>SEB</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Missing</td>
<td>New N</td>
</tr>
<tr>
<td>C57</td>
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<td></td>
</tr>
<tr>
<td>Male</td>
<td>-4</td>
<td>5</td>
</tr>
<tr>
<td>Female</td>
<td>-2</td>
<td>11</td>
</tr>
<tr>
<td>Balb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>-3</td>
<td>7</td>
</tr>
<tr>
<td>Female</td>
<td>-4</td>
<td>14</td>
</tr>
</tbody>
</table>

Table 13. Learned Morris Water Maze: final n’s of SEB and Saline offspring that successfully learned the MWM task and were used in the analyses for the probe tests.

<table>
<thead>
<tr>
<th></th>
<th>SEB</th>
<th>Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Balb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
<td>7</td>
</tr>
</tbody>
</table>
Figure 1: Method of action of superantigens versus normal peptide antigen presentation. T-cells specifically recognize peptide antigens presented by an antigen-presenting cell (APC), binding only antigens (containing a short peptide sequence) that fit into the antigen-binding grooves in the variable regions of the T-cell receptor (TCR). Superantigens – in their unprocessed and holotoxin (full protein) state - bind outside the \( \alpha \beta \) region of the TCR to the MHCII molecule on the APC, initiating a T-cell response without the need for specific antigen recognition. For this reason, the number of proliferating T cells to superantigens is quite pronounced, since many T cells with different peptide antigen specificities would be affected by a stimulus that bypasses clonal specificity. Why T cells evolved to retain this capacity is not fully understood.
Figure 2. Timeline of the behavioral tests. Animals were weaned and separated by sex at 3 weeks (P21), and began behavioral testing at 6 weeks (P42). In order to reduce testing effects and fatigue, animals were given one resting day between the EPM and the first day of OR, two resting days between the last day of the OR and PPI, and three weeks between the PPI and MWM. In total, testing spanned almost 5 weeks. Abbreviations used: EPM = elevated plus maze; OR = object recognition task; MWM = Morris water maze; PPI = prepulse inhibition
Figure 3

a. Social interaction test

b. Elevated plus maze
c. Open field and Object Recognition (OR) Task

Day 1

Day 2–4

Day 5
d. Startle chamber for PPI

![Startle chamber](image)

e. Morris Water Maze

![Morris Water Maze](image)

**Figure 3. The five behavioral tasks.** (a) The social interaction task (animal in cylindrical cage serves as a social target); (b) Elevated plus maze (animal is shown in one of the “open” arms); (c) Object Recognition task day 1 (empty open field), days 2-4 (two similar objects), day 5 (one familiar object and one novel object); (d) Startle chamber for testing prepulse inhibition (animal is placed inside the cylinder); (e) Morris water maze showing the platform location.
Figure 4. Mean IL-4 concentrations (per µg of total protein) in response to SEA and saline exposure. Bars represent mean ±SEM. # = main effect of SEA treatment, $p < 0.05$. N=4-8/gp.
Figure 5. Mean IFN-γ concentrations (per µg of total protein) in response to SEA exposure. No saline-injected animals produced detectable concentrations of IFN-γ. Bars represent mean ± SEM. * = main effect of Strain, $p < 0.05$. N=4-8/gp.
Figure 6. Mean IL-2 concentrations (per µg of total protein) in response to SEA exposure. No saline-injected animals produced detectable concentrations of IL-2. Bars represent mean ±SEM. + = main effect of pregnancy status, \( p < 0.05 \). N=4-8/gp.
Figure 7. Mean IL-6 concentrations (per µg of total protein) in response to SEA exposure. No saline-injected animals produced detectable concentrations of IL-6. Bars represent mean ±SEM. + = main effect of pregnancy status; * = main effect of strain, p < 0.05. N=4-8/gp.
Figure 8. Mean TNF-α concentrations (per µg of total protein) in response to SEA exposure. No saline-injected animals produced detectable concentrations of TNF-α. Bars represent mean ±SEM. + = main effect of pregnancy status; *= main effect of strain, $p < 0.05$. N=4-8/gp.
Figure 9. Mean IL-17A concentrations (per µg of total protein) in response to SEA and saline exposure. Bars represent mean ±SEM. # = main effect of treatment; + = main effect of pregnancy status, $p < 0.05$. N=4-8/gp.
Figure 10. Mean whole spleen protein concentrations in response to SEA and saline exposure. Bars represent mean ±SEM. + = main effect of pregnancy status, $p < 0.05$. N=4-8/gp.
Figure 11. Mean IL-4 concentrations (per µg of total protein) in response to SEB exposure. No saline-injected animals produced detectable concentrations of IL-4. Bars represent mean ±SEM. + = main effect of pregnancy status; * = main effect of strain, $p < 0.05$. N=4-8/gp.
Figure 12. Mean IFN-γ concentrations (per µg of total protein) in response to SEB exposure. No saline-injected animals produced detectable concentrations of IFN-γ. Bars represent mean ±SEM. + = main effect of pregnancy status; * = main effect of strain, p < 0.05. N=4-8/gp.
Figure 13. Mean IL-2 concentrations (per µg of total protein) in response to SEB exposure. No saline-injected animals produced detectable concentrations of IL-2. Bars represent mean ±1SEM. + = main effect of pregnancy status; *= main effect of strain, $p < 0.05$. N=4-8/gp.
Figure 14. Mean IL-6 concentrations (per µg of total protein) in response to SEB exposure (including outliers). No saline-injected animals produced detectable concentrations of IL-6. Bars represent mean ±SEM. There were no significant main effects for IL-6 production. N=4-8/gp.
Figure 15. Mean whole spleen protein concentrations in response to SEB and saline exposure. Bars represent mean ±SEM. + = main effect of pregnancy status, \( p < 0.05 \). \( N=4-8/gp. \)
Figure 16. Social Interaction Results for SEA and Saline male and female (combined) offspring. (a) Mean proportion of the total touches made to the target cup; (b) Mean proportion of the total time touching both cups spent touching the target cup. There were no significant sex differences. Bars represent mean ±SEM. # = main effect of treatment, \( p < 0.05 \). N=22-26/gp.
Figure 17. Elevated Plus Maze Results for SEA and Saline offspring. (a) Mean proportion of total time in the open arms in the EPM; (b) Mean total numbers of entries into the open arms. There were no significant sex differences. Bars represent mean ±SEM. # = main effect of treatment; *= main effect of strain, $p < 0.05$. N=22-26/gp.
18a.

OR, Inactive on Day 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Inactive Day 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>Balb</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
</tr>
</tbody>
</table>

b.

OR, Balb/c Inactive on Day 2?

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inactive Day 2?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>Male</td>
</tr>
<tr>
<td>SEA</td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td>Female</td>
</tr>
</tbody>
</table>

* p < 0.05

# p < 0.01
c. OR, Proportion of Total Time Spent in Center Area Across all Five Days

![Graph showing proportion of total time spent in center area over 5 days for different treatments.]

d. OR, C57BL/6 Total Distance Day 1, 2 and 5

![Graph showing total distance for different treatments across days 1-5.]

Responses

OR, Proportion of Total Time Spent in Center Area Across all Five Days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEA</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Proportion of Total Time

<table>
<thead>
<tr>
<th>Day</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
<td>1.0</td>
</tr>
</tbody>
</table>

# # # #
OR, Proportion of Total Time Interacting Spent Interacting with Novel Object

- Proportion of Total Time of Interacting

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>SEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57 Strain</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Balb Strain</td>
<td>0.4</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Treatments:
- Saline
- SEA

Legend:
- #
Figure 18. Object Recognition results for SEA and Saline offspring. (a) Inactivity on Day 1. There were no main effects of sex or treatment. N=49, 46 (b) Inactivity on Day 2 for the Balb/c mice. No C57BL/6 were inactive for this day. N=9-15/gp. (c) Mean proportion of the total time spent in the open arena by C57BL/6 mice across all five days of the task. Because too many Balb/c mice spent too much time being inactive most days, this data could not be analyzed. There was a similar pattern of proportion of time spent in the open arena across the days regardless of strain, sex, and treatment. N=23, 26/gp. (d) Total distance traveled by C57BL/6 mice on days 1, 2, and 5, the main days of interest. Because too many Balb/c mice spent too much time being inactive most days, this data could not be analyzed. N=23, 26/gp. (e) Mean proportion of the total time interacting with both objects spent touching the novel object on day 5. There was no main effect of sex, so the data were collapsed. N=22-26/gp. (f) Comparison between days 4 and 5, for mean proportion of total time interacting with the (familiar) object on day 4, located in the same location (or spot) where the replacement with a new object was made on day 5. There was no main effect of strain. N=22-26/gp. Bars represent mean ±SEM. # = main effect of treatment; * = main effect of strain or day, ∞ = main effect of sex, p < 0.05.
Figure 19. Movement maps in the OR for representative individual BALB/c mice showing restricted movement when compared with a typical highly active C57BL/6 mouse. The blue dot represents the animal’s starting location, while the red dot represents the animal’s end location once 5 minutes had elapsed. (a) Representative female Balb/c SEA offspring on Day 2. (b) Representative female Balb/c SEB offspring on Day 2. (c) Representative female Balb/c saline offspring on Day 2. (d) Representative female C57/BL6 SEB offspring on Day 2. The blue dot is located in the corner because the animal was in that position by the time the video began to record – an indication that C57 animals initiate movement very quickly once placed in the field.
20a. PPI, Mean Startle

- C57: Saline (blue) vs. SEA (red)
- Balb: Saline (blue) vs. SEA (red)

Statistical significance:
- p=0.0640

20b. PPI, %PPI at +8dB Prepulse

- C57: Saline (blue) vs. SEA (red)
- Balb: Saline (blue) vs. SEA (red)

Statistical significance:
- p=0.0640
Figure 20. Prepulse inhibition results for SEA and Saline offspring. (a) Mean startle responses to the pulse alone. There was no main effect of sex or treatment. N=22-26/gp. (b) Mean percent prepulse inhibition in the +8dB prepulse trials There was no main effect of sex. N=22-26/gp. (c) Mean percent inhibition for each strain across prepulse intensity types. N=44/gp. Bars represent mean ±SEM. * = main effect of strain, + = significant difference between prepulse intensity trials, p < 0.05.
21a. Figure 21. Sample learning curves for the Morris Water Maze. (a) A sample curve for a male C57BL/6 saline offspring considered to have learned the task. (b) A sample curve for a male C57BL/6 saline offspring considered to not have learned the task.
22a.

**MWM, Learned the Task?**

- **Learned**
  - C57: 
  - Balb: 
  - *p = 0.086*

- **Didn't Learn**
  - C57: 
  - Balb: 

**Strain**
- C57
- Balb

**Treatment**
- Saline
- SEA

b.

**MWM, Proportion of Total Time Spent in Target Quadrant for the 1-hour Probe**

- **Strain**
  - C57
  - Balb

- **Proportion of Total Time**
  - C57: 
  - Balb: 
  - *p = 0.088*

**Treatment**
- Saline
- SEA
Figure 22. Morris Water Maze results for SEA and saline offspring. (a) The mean number of animals that learned or did not learn the MWM task. There was no main effect of sex. N=22-26/gp. (b) The mean proportion of the total time the animals spent in the platform quadrant during the 1-hour probe test. There was no main effect of sex. N=7-12/gp. (c) The mean amount of time animals floated during the 24-hour probe test. There was no main effect of sex. N=22-26/gp. Bars represent mean ±SEM. * = main effect of strain, # = main effect of treatment, p < 0.05.
Figure 23. Social Interaction Results for SEB and Saline offspring. (a) Mean proportion of the total touches made to the target cup. The strain difference was only observed for control offspring. There was a main effect of sex, indicating increased touches for C57BL/6 females versus males (not shown). (b) Mean time spent touching the target cup expressed as proportion of the total time spent touching both cups. The strain difference was only observed for SEB offspring. Bars represent mean ±1SEM. * = main effect of strain; # = main effect of treatment, p < 0.05. N=13-31/gp.
24a. EPM, Proportion of Total Time Spent in Open Arms

![Graph showing proportion of total time spent in open arms by C57 and Balb strains under Saline and SEB treatments.]

- C57: Saline, SEB, p=0.06 *
- Balb: Saline, SEB

24b. EPM, Mean # Entries into Open Arms

![Graph showing mean number of entries into open arms by C57 and Balb strains under Saline and SEB treatments.]

- C57: Saline, SEB, p=0.06 *
- Balb: Saline, SEB
Figure 24. Elevated Plus Maze Results for SEB and Saline offspring. (a) Mean proportion of the total time spent in the open arms. There was no main effect of sex. (b) Mean numbers of entries into the open arms. There was no main effect of sex. (c) Mean total distance traveled. There were no main effects of strain or sex. Bars represent mean ±SEM. * = main effect of strain; # = main effect of treatment, p < 0.05. N=15-32/gp.
25a. OR, Inactive on Day 2

25b. OR, Proportion of Total Time Spent in Open Arena on Day 1
Figure 25. Object Recognition results for SEB and Saline offspring. (a) Inactivity on Day 2. There were no main effects of sex or treatment. N=54, 43/gp. (b) Mean proportion of the total time spent in open arena on day 1. There were no significant main effects or interactions. N=14-32/gp. (c) Mean proportion of the total time interacting with both objects spent touching the novel object on day 5. There were no significant main effects or interactions. N=15-32/gp. (d) Mean proportion of the total time on day 4 interacting with the object that is located in the same spot that the new object was located on day 5 compared to the percent total time on day 5 interacting with the new object. There were no significant main effects nor interactions. N=15-32/gp. Bars represent mean ±SEM. # = main effect of treatment; * = main effect of strain, p < 0.05.
PPI, Mean Startle

- **C57**
- **Balb**

PPI, %PPI at +8dB Prepulse

- **C57**
- **Balb**
Figure 26. Prepulse inhibition results for SEB and Saline offspring. (a) Mean startle responses. There was no main effect of sex. N=12-30/gp. (b) Mean percent prepulse inhibition in the +8dB prepulse trials. There was no main effect of sex. N=12-30/gp. (c) Mean percent prepulse inhibition in the +4dB prepulse trials. There was no main effect of sex. N=12-30/gp. (d) Mean percent prepulse inhibition for +2dB prepulse trials. There was a significant interaction of sex x treatment, such that only the male SEB offspring of each strain displayed increased percent PPI. N=6-21/gp. (e) Mean percent inhibition for each strain across prepulse intensity types. N=47, 39/gp. Bars represent mean ±SEM. * = main effect of strain, # = main effect of treatment; + = significant difference between prepulse intensity trials, p < 0.05.
b. **MWM, Proportion of Total Time Spent in Target Quadrant in 24-hour Probe**

- **Strain by Sex**
  - C57 Male: 0.45, Female: 0.35
  - Balb Male: 0.50, Female: 0.45

- **Treatment**
  - Saline: S
  - SEB: #
Figure 27. Morris Water Maze results for SEB and saline offspring. (a) The mean number of animals that learned or did not learn the MWM task. There was no main effect of sex. N=15-32/gp. (b) The mean proportion of the total time the animals spent in the platform quadrant during the 24-hour probe test. There was a significant strain x sex x treatment interaction, such that only male Balb/c offspring displayed significant differences. N=5-12/gp. (c) The mean proportion of time animals spent in the platform quadrant during the 1-hour and 24-hour probe. There was no main effect of sex. N=12-21/gp. Bars represent mean ±SEM. * = main effect of strain, # = main effect of treatment; + = significant difference between probe trials, p < 0.05.