EFFECT OF STAPHYLOCOCCAL ENTEROTOXIN A AND TUMOR NECROSIS FACTOR ALPHA ON EXPLORATORY BEHAVIORS TOWARD NOVEL STIMULI

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ABSTRACT OF THE DISSERTATION

Effect of Staphylococcal Enterotoxin A and Tumor Necrosis Factor Alpha on Exploratory Behaviors toward Novel Stimuli

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Staphylococcal enterotoxin A (SEA) is a superantigen that stimulates T cells. It has well-characterized neurobiological and endocrine effects, which have been shown to rely on the cytokine, tumor necrosis factor alpha (TNFα). We tested the effect of SEA and TNFα on mouse exploratory behavior toward a novel stimulus using multiple protocols. To test the effects of SEA challenge, novel object stimuli with and without a familiar object were presented to male C57BL/6 mice, and the mice were given one or more days to explore the familiar object before the test. When exposed to a novel object together with a familiar object, SEA-challenged mice displayed more immobile episodes and duration when observing the novel object. However, when the animals were given multiple trials to explore the novel object, the increased immobility was reduced. When the animals were tested in a different procedure involving a larger open field apparatus without the presence of the familiar object, mice showed less exploration to the novel object when treated with SEA. This was observed to be age-dependent, with older mice (10 months) showing a greater effect relative to younger mice (3 months). SEA stimulates T-cells and increases the level of tumor necrosis factor alpha (TNF-α).

Consequently, we conducted additional experiments in which the effects of central TNFα infusion was tested in a series of conditions looking at an object, food and social mate novelty. The results showed that TNF-α increased the exploratory behavior in the open field with the presence of a novel object, and decreased exploration and consumption of a novel diet. Overall, it was concluded that TNF-α, a major cytokine released in response to T-cell activation by SEA, affects the exploratory behavior of mice in multiple dimensions. Future studies should determine whether SEA effects involve TNF actions operating within the brain.

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CHAPTER 1
GENERAL INTRODUCTION

1.1. General Overview of the Project

As the chief executive of the central nervous system (CNS), the brain integrates and processes information that supports a variety of behavioral and physiological processes to be controlled and regulated. In particular, the brain coordinates the endocrine system, reproductive system and the immune system (Besedovsky and Sorkin, 1977; Fernald, R., 2017), thereby regulating hormonal and cellular processes that adjust feeding, mating, and host-defense (Besedovsky & del Rey, 1996; Pfaff 2002). The latter function is a primary role of the immune system, a collection of cells and molecular processes that defend the organism against external pathogens and internal threats. Although both the CNS and immune system have their specialized functions, their interaction is necessary for the survival and well-being of the organism. This has been demonstrated by the emergence of data that the CNS can alter immunological processes, and conversely, that immune responses can affect the brain and behavioral functions (Besedovsky & del Rey, 1996; Ader, 2000).

Research on the relationship between the CNS and the immune system, has been referred to as 'psychoneuroimmunology' (PNI), a term coined by Robert Ader (Ader, 2000). The term was suggested following his and others' demonstrations of behaviorally conditioned immunomodulation, in which immunosuppressive drugs or immune responses to antigen could serve as unconditioned stimuli (UCS) in Pavlovian conditioning paradigms (Ader & Cohen, 1975; Ader, 2000). Re-exposure of animals (and

humans) to conditioned stimuli associated with such UCSs, was found to reenlist the original immunomodulatory effects of the UCS. This suggested that changes at the level of the immune system could be registered by the brain. This is the fundamental notion underlying the meaning of PNI, which is that the brain and immune system are engaged in communication that produces consequences for both the CNS and the immune system. For example, conditioned responses (after CS-UCS pairings involving immunomodulation) demonstrated that a cognitive process can regulate certain immune functions by virtue of prior monitoring of immune events during CS-UCS trials. Since those original studies, it is now well established that immune products called 'cytokines,' fulfill signaling properties that modify brain and behavioral functions (Anisman& Merali, 2003).

Indeed, it is now clear that communication between the brain and the immune system is bidirectional. As mentioned earlier, the brain can influence immune function via the autonomic nervous system and pituitary-derived neuroendocrine activity. For example, psychological stress was found to alter immune function via the hypothalamus-pituitary-adrenal (HPA) axis, resulting in a decrease in the number of helper T cells and down-regulation of activity of natural killer cells and antigen presenting cells activity (Kiecolt-Glaser et al. 1986; Kiecolt-Glaser et al. 2005). Acute and chronic stress and the consequent alteration in HPA activity correlate with auto-immune diseases such as rheumatoid arthritis and delayed wound healing (Chida, Sudo, & Kubo, 2005; Stojanovich and Marisavljevich, 2008; Kiecolt-Glaser et al. 2005). Alternatively, the influence between the CNS and the immune system is not unidirectional, as the immune system activity can alter cognitive and endocrine functions. Besedovsky demonstrated the

exchange of signals among the multi-directional immune-neuro-endocrine interactions. The brain, immune response and neuroendocrine mechanisms share mediatory circuits involving immune cell products, the HPA axis and the sympathetic nervous system. The immune system has a similar function to a sensorial receptor that triggers the brain and associated neuroendocrine structures for hormonal and autonomic nerve responses, mediated by cytokines (Besedovsky & del Rey, 2007). And in humans, chronic inflammation, allergies and asthma, which are characterized by elevated cytokine production, are thought to contribute to increased anxiety and depression (Anisman & Merali, 2003; Bercik et al. 2010; Maes. 1999; Marshall and Colon 1993; Postolache et al., 2007; Caldera-Alvarado, Khan, Defina, Pieper & Brown, 2013).

Therefore, consistent with the foregoing, the overall goal of the current dissertation is to investigate the effect of SEA, a T-cell stimulator, and the downstream cytokine TNF-α, on cognitive and behavioral functions.

1.2. Overview of the immune system

The mammalian immune system is a physiologic homeostatic guardian system that defends the organism against disease and contributes to the constancy and integrity of the organism (Besedovsky & del Rey, 2007). It reacts quickly to both external and endogenous pathogens, identifies specific pathogens for a precise attack, and stores memories of the pathogen. The complexity of the immune function requires immune organs, cells and molecules to work cooperatively. The bone marrow, thymus, spleen and lymph nodes are the immune organs that produce, incubate and store the immune cells and molecules. The major immune cells fall into two main cell lineages: the lymphoid

cell lineage and the myeloid cell lineage. The lymphoid lineage includes T cells, B cells, and natural killer cells; while the myeloid cell lineage includes monocytes, granulocytes, macrophage, mast cells and myeloid dendritic cells. Immune cells either patrol through the circulation and lymph system or reside in specific tissues/organs to monitor the health status of nearby body regions.

The lymphocytes, including T cells and B cells, are white blood cells that flow in the blood, tissue fluid and lymph. B cells produce antibodies, large proteins that recognize and neutralize pathogens. T cells induce apoptosis to expose and eliminate the pathogens in the body's unhealthy cells that are non-functional or affected by pathogens. Macrophages are a group of cells that either reside in a specific tissue or circulate in the lymph system and assist the function of the lymphocytes. When an antigen is detected bearing no surface proteins marking healthy body cells, the macrophages perform their function of phagocytosis by engulfing and digesting to eliminate the threat (Kusnecov & Anisman, 2013). The immune cells can either communicate in a cell-to-cell manner or through secreted signaling molecules by the ligand-receptor pathways. The primary ligands that mediate intercellular communication are cytokines.

The immune system could be divided into innate immunity and adaptive immunity. These two types of immunity are complementary when facing immune challenges and cooperate with each other using various functions provided by specialized cells and molecules.

1.2.1 Cell-mediated immunity and cytokines

T cells play a critical role in cell-mediated immunity. Hematopoietic stem cells in the bone marrow are the origin of T cells. The hematopoietic progenitors migrate to the thymus and develop into mature T cells that can recognize self and non-self antigens. T cells can be sub-grouped into helper T cells (also known as CD4+ T cells), cytotoxic T cells (also known as CD8+ T cells), regulatory T cells, natural killer T cells and memory T cells. T cells are characterized by the T cell receptors (TCRs) that are expressed on the cell surface of T cells. Every T cell expresses a single type of monoclonal TCR. TCRs are heterodimer receptors mainly consisted of distinct polypeptides α and β, while some T-cells in the gut mucosa have TCRs consisted of γ and δ polypeptides (Holtmeier & Kabelitz., 2005). The structure of the TCR polypeptide chain distal domain is extremely variable in amino acid sequence, and is therefore called the V (variable) region. The variability is ensured by a particular combination of DNA encoded segments of the TCR genes (Bentley & Marriuzza 1996). The variance in this V region enables T cells to identify the difference between many different antigens and perform targeted immune responses. Each TCR recognizes a specific structure or sequence of a peptide and activates the T cells in a different situation to carry out the recognition of self and non-self-differentiation. Despite the variance of each TCR structure, the variable region of the β chain (V-β region) has specific motifs that are common across T cells with different antigen specificities. These are encoded by different Vβ genes, which have been numerically identified (eg., Vβ1, Vβ2, etc). The actions of staphylococcal enterotoxins are based on their recognition of these Vβ gene products, which will be described later.

Immune cells communicate together and coordinate the immune response mainly via cytokines. Cytokines are soluble cell signaling proteins around 5-20 kDa that binds to

receptors of the target cell and affect cellular behavior (Anisman, Hayley &Kusnecov. 2018). The ways that cytokines participate in cell regulation include autocrine, paracrine and endocrine signaling. The target cells of cytokines are not limited to immune cells only, but also include neurons, microglia, astrocytes and other cells in the CNS (Parnet, Kelley, Bluthé, & Dantzer, 2002; Konsman Parnet & Dantzer, 2002). Cytokine categories include chemokines, interferons, interleukins, hematopoietins, and tumor necrosis factors (TNFs). They are critical in regulating immune functions including hematopoiesis, innate immunity and acquired immunity.

Major proinflammatory cytokines include IL-1 and TNF-α, which play an essential role in mediating immune functions. IL-1 is a proinflammatory family that initiates and regulates inflammatory responses both at the receptor and the nuclear levels (Dinarello, 2009). It can be produced by a variety of cells including macrophages, monocytes, fibroblasts, dendritic cells, lymphocytes, microglia, and epithelial cells. The IL-1 family has 11 members. IL1-beta is one of the significant members that was first found. It is mainly produced by the monocytes and macrophage, causing a robust pro-inflammatory effect (Dinarello, 1994). IL-1 beta is critical for local and systemic inflammation. It can induce immune cell proliferation, differentiation and antigen presentation, as well as the production of other cytokines such as IL-6. IL-1 beta can elicit the reaction of fever and participates in many autoimmune diseases such as Crohn's disease (Sims & Smith, 2009).

TNF-α is a homotrimer protein of 17 kDa. It has two receptors TNFR1 and TNFR2. TNF-α is known to be involved in systemic inflammation. It is produced by

immune cells including macrophages, CD4+ lymphocytes, and also neurons and glial cells (Gahring, Carlson, Kulmer & Rogers, 1996). TNF-α was first found and named for its participation in tumor cell apoptosis and cachexia, and later was proved to be involved in host defense against pathogens as well as autoimmune disease (Feldmann & Maini, 2001). Both IL-1 and TNF-α are major pro-inflammatory cytokines that facilitate immune activation.

1.2.2 Innate immunity and the adaptive immunity

Innate immunity is the first line of defense that the immune system provides in a non-specific manner. The characteristics of innate immunity are rapidity, independence of antigen and can be found in a vast amount of species including both vertebrates and invertebrates. When a pathogen enters the skin and mucosa that serve as the first barrier of the innate immune system, they will be recognized as non-self immediately by sentinel cells carrying toll-like receptor or RIG-I-like receptors (Baum & Garcia-Sastre, 2010). Interferons will then be produced and they stimulate hundreds of related genes to be expressed. The gene expressions upregulate antiviral protein synthesis that carries functions to inhibit viral protein synthesis, degrade viral genetic material including DNA and RNA. This is a non-specific process that does not identify the specific antigen strain, but can detect it as an intruder by its molecule structure broadly shared by the pathogens.

The adaptive immune system reacts in a highly specialized manner with pathogen recognition and memory. It is present mainly in mammalian vertebrates and is also known as acquired immunity (Flajnik & Kasahara, 2010). Adaptive immunity identifies a diverse set of antigens both self and non-self. Lymphocytes mediate the elimination of a

specific kind of pathogen, and memory cells are created to be used for more efficient activation against future infections from a pathogen of a similar nature. The characteristics of adaptive immunity are specificity, memory, diversity, self-regulation, and discrimination of self vs. non-self (Moticka, 2015). The adaptive immune system takes time to initiate effector responses to eliminate the pathogen. In this process, the specificity of the pathogen is identified, targeted, eliminated and memorized. In an adaptive immune process against a typical bacterial infection, the pathogen will first go through the antigen presentation process, where bacteria will be phagocytosed by the antigen presenting cells (APCs), including macrophages and dendritic cells. After degradation, APC will expose the epitopes (i.e. antigens) by the major histocompatibility complex (MHC) molecules to appropriate CD4+ T cells (Th cells) and/or CD8+ T cells (cytotoxic T cells) (Abbas, Lichtman & Pillai, 1994). Antibodies will be selectively produced by B cells to neutralize antigen, and this will be accomplished with the assistance of Th cells. With regard to cytotoxic T cells, they will recognize the particular antigen, undergo clonal expansion, and induce apoptosis in target bacteria. Ultimately, the nature of the pathogen is stored by memory T cells and will allow for much faster responses should there be a subsequent invasion of the same type of pathogen.

The major histocompatibility complex (MHC) molecules that are part of the MHC I and II genes regulate immune responses by presenting antigens to T cells. This presentation to T cells is via a heterodimer on the MHC molecule that consists of an α and a β polypeptide chain and is categorized as class II, and is present on antigen presenting cells (APCs). The α and β polypeptides anchor on the cell membrane and form a peptide-binding cleft in its membrane-distal domain. This domain has a considerable

polymorphism in order to hold epitopes of different structures. The classical MHC class II molecules are of three types (DP, DQ and DR) that are highly polymorphic and linked to different immune diseases (van Lith et al., 2010).

The antigenic epitope is presented by the MHC to Th cells by specific recognition via T cell receptors (TCR) and its associated proteins. This process is required for almost all adaptive immune response. Additionally, the activation of T-cells calls for the function of a cluster of differentiation (CD) on the cell surface, for example, CD28 on T cell membrane and CD80 on APC (Bromley et al., 2001).

In summary, innate and adaptive immunity are not two systems that function separately. On the contrary, they cooperate closely to optimize immune activity. Many cells are involved, including macrophages that carry out phagocytosis functions in innate immunity to eliminate the pathogen. It can also serve as the APC that initiates and subsequently directs T cell responses. Additionally, the activated T cells will release cytokines to activate the natural killer cells that also belong to innate immune processes, but also overlap with adaptive immunity. Together, both innate and adaptive components effectively defend the organism from infection and disease.

1.3. The interaction between the CNS and the immune system

1.3.1 The blood-brain barrier and the CNS immunity

The brain was once thought to be exempt from the immune system by the separation of the blood-brain barrier (BBB) and thereby had privilege from any damage due to inflammation. The BBB refers to unique properties of endothelial cells in the CNS

vascular vessels that separates the peripheral circulation from direct contact with cells in the CNS. The differences of the endothelial cells include an absence of fenestrations, more extensive tight junctions and sparse pinocytic vesicular transport that limit the paracellular flux of hydrophilic molecules across the BBB while allowing entry of small lipophilic substances (Ballabh, Braun & Nedergaard, 2004). The BBB closely screens the material exchange and blocks most of the immune cells as well as pathogens from entering the CNS. This process is the basis for the fundamental idea that the brain is immune-privileged. However, as the interaction between the immune system and the CNS was further investigated, the immune system was found to have multiple ways to affect the brain.

Microglial cells are the resident immune cells that account for more than 10% of all cells in the brain (Lawsen et al., 1992). Serving as the local macrophage, microglia perform the function of innate immunity including monitoring, phagocytosis and signaling. Different from the peripheral immune cells, the microglial cell population is independent of bone marrow-derived progenitors. It is maintained by self-renewal after early development. The monitoring function of the microglial cells is more sensitive compared with the peripheral immune cells and is referred to as scavenging. Resting microglia ramify branches out and physically survey through the surrounding region for foreign material or non-healthy-self material and activate themselves to eliminate the antigens. When activated, the microglia cells shift into an amoeboid shape to perform phagocytosis (Torres-Platas et al., 2014). Apart from the immune functions, microglia also influence synapse transmission and plasticity (Paolicelli, et al., 2001).

The meninges are the membranes that shield the brain and spinal cord from the periphery of the body. In mammals, the meninges consist of the dura mater, the arachnoid mater, and the pia mater. The circulation system in the meninges supports the function of BBB. Cerebrospinal fluid (CSF) performs material exchange in the subarachnoid space between the arachnoid mater and the pia mater. The meninges protect the central nervous system as a physical barrier. T cells also reside in the meninges and patrol the CNS for immune surveillance (Derecki et al., 2010; Louveau et al. 2015). A recent discovery found CNS lymphatic vessels within the dura mater of the meninges that possibly enables T cells to enter and exit the brain via cerebrospinal fluid. This piece of evidence clarified that the immune surveillance was stronger than people used to believe, and helps to explain the T cell recruit and penetration in the brain immunity against infection.

The immune system can also influence the CNS indirectly through cytokines signaling at the vagus nerves (Hansen, O'Connor, Goehler, Watkins, & Maier, 2001). Moreover, the BBB itself can secret cytokines into the CNS. Finally, selected cytokines such as IL-1beta and TNF-α are able to cross BBB either circumventricular organs that lack the coverage of BBB, or by an inflammation leakage or a carrier-mediated transport system (Dunn, 2002., Banks 2005). Some cytokines such as IL-1 can be transported into the CNS, but the speed is slow, and the amount of transportation is minimal. The ventricular and subarachnoid CSF spaces where permeable endothelial cells reside do not exhibit the same immune privilege as the CNS. Many large molecules such as cytokines are able to enter the CNS in those regions.

As one of the most delicate organs of the body, the CNS requires minimum interruption from the immune system. However, the importance of the CNS requires sufficient immune surveillance to guarantee the defense from pathogens. Although traditional research regards the brain as an immune privileged, the studies mentioned above demonstrated that the immune system closely monitors the health of the CNS.

1.3.2 Stress, HPA axis, and the immune function.

The interaction between the immune system and the CNS is not unidirectional. A bidirectional communication exists; and cognitive processes are capable of influencing the immunological equilibrium as well. When the body is challenged physically or psychologically, stress serves as the cognitive reaction that regulates immune function. It has been shown that stress increases macrophage IL-1, lymphocyte IL-2, IL-6 and TNF-α level (Wilson, Finch & Cohen, 2002).

When the body undergoes physical or psychological stress, the HPA axis will be activated by releasing hypothalamic corticotrophin-releasing hormone (CRH), which causes elevations in ACTH and adrenal-derived glucocorticoids (i.e. corticosterone in rodents; cortisol in humans) will be upregulated. The HPA axis impacts a variety of immune cells including T cells, B cells, macrophages, and microglia as well as the synthesis and release of cytokines (Ashwell, Lu & Vacchio, 2000; Caramori et al.,2005; Sierra et al., 2008). Both IL-1 and TNF-α production could be inhibited by glucocorticoids, with TNF having a higher sensitivity (O'connor et al., 2000). It is widely used to control the inflammation response and some autoimmune diseases to protect the

host organism from the detrimental consequences of long-term hyperactivity of the immune system.

IL-1 is the most well-studied cytokine in the area of psychoneuroimmunology and plays a significant role in the immune modulation of the HPA axis (Goshen & Yirmiya, 2009). The HPA axis is regulated by IL-1 at the hypothalamic, pituitary, and adrenal level (Besedovsky & del Rey 1996): IL-1 can promote the secretion of CRH at the paraventricular nucleus of the hypothalamus; IL-1 also directly induces the downstream production of adrenocorticotropic hormone (ACTH) from the pituitary and then the terminal at the adrenal cortex where glucocorticoids were synthesized and released. Moreover, IL-1 receptors were found across all three levels of the HPA axis, potentially contributing to a cascade effect along the hypothalamic-pituitary-adrenal pathway involved in elevating glucocorticoids (Turnbull and Rivier 1999, Bornstein et al., 2004).

Another major cytokine, TNF-α, and its soluble receptors p55 (sTNF-R p55) and p75 (sTNF-R p75) regulate the HPA axis resulting in the release of corticosterone or cortisol (Arruda et al. 2010; Kobayashi 1997; Villar 2013). It has been found that TNF-α interplays the activity of the HPA axis (Elenkov and Chrousos, 1999; Tsigos and Chrousos, 2002). Plasma TNF-α up-regulates HPA axis, while elevated corticosterone suppresses the production of TNF-α. Peripheral TNFα has been found transported into the CNS by radioactive labeling TNFα from blood to brain in mice through a receptor-mediated mechanism (Gutierrez, Banks, Kastin, 1993).

1.3.3 Sickness behavior

Sickness behavior is one of the primary effects that the immune system elicits from the CNS. Sickness behavior refers to a coordinated set of behavioral changes that develop in individuals during the course of infection (Dantzer 2001). The classic model of sickness behavior can show one or more of the symptoms including weakness, malaise, decreased motor and social activity, anorexia, reduced water intake, altered sleep pattern and anhedonia. Sickness behavior is believed to sustain the natural homeostatic reaction the body uses to promote recovery from the illness (Hart, 1988). Pro-inflammatory cytokines in the CNS including IL-1 and TNF-α are believed to serve as the molecular cause of sickness behavior (Kelley et al., 2003).

Administration of IL-1 induces a series of behaviors including anorexia, social exploration, reduction of feeding and locomotion that are categorized similarly as sickness behavior (Bauer, Weingarten, Senn & Langhan, 1995; Hellerstein, Meydani, Meydani, Wu & Dinarello, 1989; Linthorst, Flachskamm, Muller-Preuss, Holsboer & Reul, 1995; Dantzer, 2001). Systemic administration of IL-1β and TNF-α disrupted consumption of a highly palatable food which suggested a possible anorexic and anhedonia effect (Brebner et al., 2000). Similarly, peripheral injection of IL-1 β i.p. and i.v. reduced novel food intake of saccharin diet immediately as well as chronically (Bauer et al., 1995; Hellerstein et al., 1989). The effect appears to be mediated by prostaglandins. Animals displayed a reduction of locomotion after central administration of IL-1 beta, and this effect was eliminated by IL-1ra pre-treatment. IL-ra also abrogated the decrease of social exploration in rats that was peripherally administered with IL-1 (Kent et al.,

1992). It has been reported that IL-1 disrupted operant conditioning and retention of spatial learning (Crestani, Seguy & Dantzer, 1991; Oitzl, Josephy & Spruijt, 1993).

Besides sickness behavior, cytokines such as IL-1 and TNF-α alter other CNS functions as well. IL-1 mediates the fever response during inflammation. Blocking the effect of IL-1 inhibits fever reaction during infection or inflammation (Kluger 1991; Dinarello 1991). In all species of human, rat, rabbit and mouse, administration of IL-1 induced fever by i.p., i.v. or i.c.v. LPS induced fever was attenuated by the blockage of the IL-1 receptor (Kluger 1991).

Lipopolysaccharides (LPS) are large molecules that serve as the major component of the outer membrane of Gram-negative bacteria that consist of a lipid and a polysaccharide composed of O-antigen. Peripheral administration of LPS, a key ligand for toll-like receptors, induces the release of IL-1beta, which then drives the behavior effects of sickness behavior (Dantzer and O'Connor, 2008). This process can be eliminated by administering the antagonists of the cytokine, proving the necessity of IL-1 in this process (Layé et al., 2000). In this study, the peripheral administration of LPS successfully reduced food intake after the treatment for up to ten hours. The LPS effect was significantly attenuated by pretreatment with IL-1 receptor antagonist (IL-1ra) centrally, whereas central administration of IL-1ra did not alter peripheral increases of IL-1 beta in response to LPS. Central administration of IL-1 by itself was found to elicit sickness behavior as well, and the effect can be similarly blocked by local treatment of IL-1ra (Dantzer and O'Connor 2008). The studies demonstrated that central but not

peripheral IL-1 plays a pivotal role in mediating the cognitive response to innate immune activation.

1.3.4 The impact of IL-1 and TNF-α on cognitive functions.

Subsumed under the general term of "sickness behavior", are behavioral changes that imply alterations in cognitive processes, the most common being learning and memory. IL-1β was shown to impair the contextual fear learning, and IL-1 receptor antagonist rescued the impairment (Pugh, Fleshner, Watkins, Maier, & Rudy, 2001; Goshen et at., 2009). Another study showed that IL-1 overexpression impaired memory acquisition and retention in the Morris water maze (MWM) (Moore, Wu, Shaftel, Graham, & O'Banion, 2009; Yirmiya, Winocur & Goshen, 2002). However, besides the cognitive impairments, IL-1 is also known to facilitate hippocampal-dependent learning and memory. IL-1 was also found necessary as the IL-1 receptor knock out mice showed deficits in fear conditioning and MWM learning and memory (Avital et al., 2003). The effect of IL-1 on learning and memory may be relevant to the HPA axis. IL-1 receptor knock-out mice showed increased exploratory behavior in the open field test, which indicated a decrease of anxiety level. As the HPA axis has a dose effect on learning and memory, the different impact of IL-1 on cognitive ability could be due to a dose effect.

Recent research showed that a local increase of TNF-α in the hippocampal dentate gyrus was found to impair memory via astrocytes (Habbas et al., 2015). This is consistent with TNF-α being linked to regulation of neuronal morphological development in the hippocampus, affecting synaptic plasticity and inhibition of long-term potentiation (LTP) (Cunningham, Murray, O'neill, Lynch & O'connor, 2004; Butler, O'connor & Moynagh,

2004). In addition, TNF-α in the CNS also works as a facilitator for glutamate excitotoxicity and affects glutamate transmission (Ye et al., 2013; Bruce et al. 1996; Gary et al.1998).

Peripheral administration of TNF-α was also found to induce sickness behavior including reduced locomotor activity, decreased fluid intake, and body weight loss (Biesmans et al., 2015). Additionally, it should be noted that repeated TNF-α was found to induce a time-dependent sensitization effect of brain monoamine activity, plasma corticosterone activity and sickness behavior (Hayley, Brebner, Lacosta, Merali & Anisman, 1999). The pronounced effect occurs days after the initial treatment. However, the carrier effect of bovine serum albumin may be relevant to the sensitization effect (Anisman& Merali, 2003). Carrier-free TNF-α infusion elicited monoamine activity changes only, with no change in plasma corticosterone activity and sickness behavior.

Clinical evidence suggests that TNF-α is linked to depression, as TNF-α has also been shown to contribute to depression-like behaviors after chronic stress, and TNF-α inhibitor decreased depression and anxiety-like behavior in a rat model of chronic mild stress (Diniz et al., 2010; Karson, Demirtaş, Bayramgürler, Balcı & Utkan, 2013). A higher TNF-α level has been suggested to associate with depression (Dowlati et al., 2010). The meta-analysis using data from 13 studies showed a significantly higher concentration of TNF-α calculated by the weighted mean difference in depressed subjects compared with control subjects.

Cytokines including IL-1, IL-2, TNF-α, and IFN were observed to accompany depression, yet correlational relationships were observed rather than causation (Anisman

& Merali, 2003). In clinical studies, an increase in serum levels of TNF-α takes place in major depression and during manic and depressive episodes; persistently elevated TNFα was associated with prospectively determined treatment resistance in depression (Schiepers, Wichers, Maes, 2005; Simon et al., 2008; Brietzke and Kapczinski, 2008; Strawbridge et al., 2015). TNF-α inhibitor therapy reduces symptoms of depression, mood or anxiety disorders in patients with chronic disease such as rheumatoid arthritis and Crohn's Disease (Abbot et al., 2015; Uguz, Akman, Kucuksarac, & Tufekci., 2009). Additionally, elevated peripheral levels of TNF-α and its receptors may serve as a trait marker for bipolar status in bipolar depression (Soczynska et al., 2009). However, no conclusion has been made about the relationship between TNF-α and depression. The linkage between them could be the psychological stressors affecting immunity and cognition together. Cytokines may serve as trait markers of depression but not an etiological role in depression (Anisman et al. 1999). A better understanding of the effect TNF-α exerts on the CNS will help to reveal the mechanism of depression.

TNF-α was found to impair learning and memory including passive avoidance memory, classical conditioning and hippocampal-dependent learning (Fiore et al., 2000; Paredes, Acosta, Gemma, & Bickford, 2010, Belarbi et al., 2012). Genetically modified mice overexpressing TNF-α showed reduced learning and memory capacity in the Morris water maze due to impairment of the hippocampal function (Fiore et al., 2000). However, deletion of TNF receptor 1 and 2 (TNFR1 and TNFR2) was found to impair learning and memory (Baune et al., 2008; Camara et al., 2013). TNF has been proved to have many neuroprotective effects including regulation of neuronal activity and maintenance of myelin (Probert, 2015). The effect of TNF-α on cognitive function still needs to be

further investigated, since few studies have examined the behavioral effects of TNF after central infusion.

1.4. Superantigens and Cognition.

1.4.1 Bacterial Superantigens and staphylococcal enterotoxins

Unlike the traditional antigens that require specific recognition of the antigenic epitope, superantigens (SAgs) activate the immune system without the TCR specific pairing with MHC. SAgs are toxins from bacteria (bacterial superantigens) and viruses (viral superantigens) that induce a strong in vivo immune stimulation and massive cytokine release (Kawashima and Kusnecov, 2002). Their protein size varies between 22 to 30kD, and they are highly resistant to proteases and denaturation (Krakauer, 2013). SAg stimulation causes massive proliferation of T cells (5~30%) at picomolar concentrations compared with the traditional specific antigen recognition that activates 0.0001-0.001% of T cells (Kotzin et al., 1993). The massive inflammation response of SAgs includes a cascade of the activation of macrophages and high level of IL-1 and TNF-α release. This effect can result in severe and life-threatening symptoms such as toxic shock syndrome and multiple organ failure.

The best-characterized SAgs are the staphylococcal enterotoxins (SEs) (Proft and Fraser, 2003) produced by *Staphylococcus Aureus (S. aureus)*. Staphylococcus aureus is a Gram-positive, round-shaped bacterium found on mucosa and the skin of 20% to 30% of the human population. It causes minor skin infections such as pimples and severe diseases such as food poisoning, pneumonia and meningitis. The enterotoxins are protein exotoxins released by *S. aureus* microorganism, and that target the digestive system. The

structure of the SEs consists of N and C-terminal domains with a long alpha-helix in the middle of the molecule and a characteristic beta-barrel at the N-terminal domain and a beta-grasp motif at the C-terminal domain. SEs can be categorized into many subtypes including SEA - SEJ. The SEs have similar function but vary in the primary amino acid sequence forming different binding modes and activate different groups of T cells (Proft and Fraser 2003). Among all the SEs, SEA and SEB are the most well-studied toxins.

The traditional specific binding requires the TCR to present the epitope to a group of specific T cells that could match the structure of the epitope and dock with the MHC. The non-specific binding process of the SAgs omits the antigen presenting process and binds specifically to the V-β region of TCR and the α-region of the MHC II molecule (Proft and Fraser, 2003; Hong, Waterbury and Janeway, 1996). The binding was outside of the MHC and TCR pocket. Since the MHC presents no epitope to the T cell for recognition, the cross-link was referred to as non-specific binding (Proft and Fraser 2003). However, this process does require the match of the SAgs and the Vβ subsets of TCRs, as well as the complex of the SAg and the MHC molecule. Each type of SAg has a different affinity to different V-β genotype. For example, SEA shows higher affinity to Vβ3+ and Vβ11+ T cells, while SEB preferentially binds to and activates Vβ8+ T cells (Hong et al. 1996; Proft and Fraser 2003).

1.5. The effect of superantigens on CNS and cognitive function

As previously mentioned, the immune system consists of innate immune and adaptive immune components, which are equally essential to maintain a healthy organism from diseases. As a traditional model for immune activation, LPS is widely accepted to

study the interaction between the immune system and the CNS. This model well addresses the activity of the innate immune system. However, most of the studies using the LPS model involve very little about the adaptive immune response, and the interaction between CNS and the adaptive immune system is still unresolved. Additionally, the LPS model generates sickness behavior that significantly decreases the locomotion of animals. Consequently, measuring cognitive function using many standard tests such as MWM and object exploration can be difficult in the LPS model.

The model using superantigen staphylococcal enterotoxin A (SEA) is one of the few models that address the interaction between adaptive T cell immune response and CNS. Unlike the traditional LPS model, SEA model induces no change in an animal's locomotion ability, granting the cognitive test result apart from the influence of any physical discomfort. This model benefits the cognitive tests that rely on locomotion measurements to be more reliable, such as open field test, MWM test, plus maze test, and rotarod test. The SEA model facilitates the limited research on the interaction between T-cell and CNS explicitly. The vast activation of T-cells produces complex cognitive alternations that have been studied by our lab. Without changing the traveling speed of mice, SEA injected animals showed an elevated level of anxiety in open field test and reduced consumption of a novel diet, which was interpreted as increased anxiety level (Kawashima & Kusnecov, 2002; Rossi-George Urbach, Colas, Goldfarb & Kusnecov, 2005). The exploratory behaviors were not affected by the SEA treatment in the first session of the open field test. However, during the second phase, a tall novel object was introduced and animals treated with 5ug of SEA showed decreased exploration. The animals treated with 1µg SEA was not affected. Animals also displayed taste neophobia

in both novel and familiar environments when treated by SEA. The taste neophobic effect of the SEA was later found absent in TNF $^{-/-}$ mice, which suggested the effect being mediated by TNF-α, whose level was elevated after SEA injection (Rossi-George et al., 2005). However, in contrast to the evidence of increased general anxiety level, SEA injected animals showed increased entrance into the distal portions of the open arms in elevated plus maze (EPM) test (Rossi-George, LeBlanc, Kaneta, Urbach & Kusnecov 2004), which could be interpreted as increased motivation to explore an unfamiliar context. This phenomenon argues against the altered anxiety hypothesis neophobic effect created by novel food. Additionally, the learning and memory ability of SEA injected animals in MWM was found not disturbed, and an enhanced memory was observed in SEA treated mice during reconsolidation of the same test (Woodruff, Schorpp, Lawrenczyk, Chakraborty & Kusnecov, 2011).

AS the SEA model has been found to affect the HPA axis, the behavior effect may be mediated through this pathway. Results from our lab showed that challenge with SEA up-regulated corticosterone and adrenocorticotropic hormone (ACTH) levels (Kawashima and Kusnecov, 2002). Blocking the upstream CRH in the CNS attenuated the anorexia effect that SEA induced in control group (Kaneta and Kusnecov, 2005). Those conclusions suggest that SEA may induce the anorexic behavior by mediating the HPA axis. Whether TNF-α mediates the SEA treatment influence HPA axis is yet to be revealed.

Unlike the LPS model that induce IL-1 production, SEA and SEB treatment induces high plasma levels of TNF-α (Kawashima and Kusnecov, 2002). However, there

is limited knowledge about the effect of SEs induced TNF-α on cognitive function. Exploratory behavior, learning and memory.

Exploratory Behavior and the Immune System

Exploratory behavior is the gathering of information about the environment driven by the tendency to explore or investigate a novel environment (Mettke-Hofmann, Winkler & Leisler, 2002). This self-driven tendency is believed to be related to novelty and curiosity (Berlyne 1950). The curiosity here was explained as a perceptual curiosity, the driving force that motivates organisms to seek out novel stimuli and diminishes with continued exposure (Kidd & Hayden, 2015). Exploratory behavior benefits the animals by gaining information about feeding and foraging, competitors and predators as well as potential mates (Renner, 1988). It was first proposed that animals approach novel stimulus by exploratory motivation in 1955 (Montgomery 1955). However, in a particular circumstance or certain species of animals, there can be a delay or reluctance to explore a novel stimulus, and researchers related this to fear as opposed to motivated exploratory behavior (Halliday 1966; Russell 1973). Halliday believed that exploration is negatively associated with the single fear factor rather than novelty. A high level of fear leads to less exploratory behavior, and mild fear predicts more exploration. The modern two-motivational system of exploratory behavior proposed an approach-avoidance conflict in which novelty induces both exploratory and fearful motivations (Russell 1973, Murphy 1978, Wood-Gush & Vestergaard 1991, 1993). Exploratory stimuli could be either "absolute novel", or relatively novel on a temporal base (Murphy 1978). Novelty induces exploratory behavior including locomotor response and an investigatory response. The

fear response includes avoidance or withdraw actions, freezing or inhibition of movement.

It should be noted that the fear of novelty could be masked by hunger, while fear could inhibit feeding. Anxiety was shown to be related to exploration and have an adverse effect on the preference for a novel object (Griebel et al., 1993). This finding suggested that anxiety shifts the balance of exploration toward the avoidance side.

Exploratory behavior is closely related to learning. It controls what information is learned and helps to optimize subsequent memory performance (Voss, Gonsalves, Federmeier, Tranel, & Cohen, 2011). It has been demonstrated that animals acquire useful information by using exploratory behaviors in an enriched environment and performed better in an escape test in both a learned and an unfamiliar maze (Renner 1988). The result was interpreted that the animals in an enriched environment learned better exploration strategy. A novel environment induces acetylcholine release but not GABA nor glutamate (Giovannini et al. 2001). Giovannini interpreted that the cholinergic effect was explained as both related to motor activity and related to attention, anxiety and fear. It is well-accepted that the cholinergic system is involved in memory and attention processes (Hasselmo 2006). ACh enhances the activity of many cortical neurons and facilitates fear conditioning behaviors (Jiang et al. 2016). Additionally, the degeneration of cholinergic neurons in the basal forebrain leads to memory loss (Muir et al., 1993).

Many factors could alter animals' behavior between exploration and avoidance. One example that shifts the balance to the neophobic side is the sickness behavior

mentioned in the previous section. Animals going through sickness behavior display reduced exploration under specific situations, such as the presence of a new social mate (Dantzer 2001; Larson & Dunn, 2001).

Immune challenge such as enterotoxin and mycoplasma fermentans decreased animals' locomotor and exploratory activity as well as social behaviors (Yirmiya., 1996., Yirmiya et al., 1997, Salarzar., 2013). Primarily derived from the immune cells, cytokines have been found to play a role in the exploratory behavioral changes following immune activation (Kelley et al. 2003). Cytokines can affect the brain in two ways: cytokines are produced in the central nervous system (CNS) by glial cells and astrocytes (Beveniste, 1992); additionally, peripheral cytokines can affect the nervous system via the vagus nerves and humoral volume transmission into the brain parenchyma (Dantzer, 2001).

The effect of IL-1 on exploratory behavior is well-demonstrated. The behavior effect of sickness behavior can be induced by IL-1, including reduced exploration toward a novel environment and stimulus as well as neophobia. IL-1 decreased exploration in an open field test and was associated with impairment in long-term memory for novel object recognition (Barichello et al., 2015; Swiergiel & Dunn 2007). Intracerebroventricular infusion of IL-1beta induced anorexic effect, which is mediated by prostaglandins, and pretreatment with IL-1 receptor antagonist attenuated LPS-induced depression of food intake (Hellerstein et al., 1989; Layé et al., 2000). Both peripheral and central administration of IL-1 beta decreased social exploratory behavior in rats, and the peripheral effect can be attenuated by vagotomy (Bluthé et al., 1996).

However, comparing with other cytokines that are well-studied, such as IL-1, not much literature focuses on the direct effect of central TNF- α and its effect on exploration toward other novel stimuli. Neither was the effect of central TNF-α on memory-related exploration investigated.

In the current dissertation, to determine the behavioral effect of central TNF- α administration on novel stimuli, the first experiment tested animals' exploratory behavior regarding a novel environment, food, social mate, and objects. To examine the effect of central TNF-α administration, animals were fitted with an indwelling intracerebroventricular cannula, and tested in an open field test, novel food (prosobee) test, social interaction test, and object exploration test. We hypothesized that TNF-α will alter exploratory behavior in novel context toward various stimuli. The animals will display food-neophobic behavior but could become risk takers when exposed to other stimuli.

CHAPTER 2
GENERAL METHODOLOGY

Subjects

Adult male C57BL/6J mice were group-housed (2-4 per cage) upon arrival and maintained on a constant 12:12 h light: dark cycle with lights on at 0700 h. Animals were given access to food and water ad libitum. Mice in chapter 3 were purchased from Jackson Laboratories (Bar Harbor, Maine) and allowed at least one-week acclimation to our facilities prior to the start of surgery. Animals were single-housed after surgery to protect the surgical wound and control for infection. Mice in chapter 4 were either purchased from Jackson Laboratories or inbred from breeding pairs of the same origin. All experiments were conducted following 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.

Behavioral Testing

All animals were pre-handled at least five times before subjected to any behavioral test. Experimenters wore gloves and lab coats, pick up animals and placed them on hand for free exploration of 1-1.5 minutes. Animals were allowed to move on experimenters' hand as well as the forearm with coverage of a lab coat. Animals were handled by the experimenter r multiple times. For the animals in chapter 3, animals were restrained by scruff for 30 seconds after the handling. Because animals needed to be immobilized to insert the internal cannula, they were adapted several times prior to the infusion days to handling by being firmly restrained by hand and then picked up to feign

infusion. . Animals were placed back to home cages after being handled. The animals were handled both prior and post-surgery.

All behavior tests were done with experimenters blind to the treatment. Animals were transferred to the test room using transfer cages. Animals were picked up and placed onto experimenter's palm and placed into a test apparatus with care. The test room was controlled for minimum noise during the test session.

Behavioral testing apparatus:

Open field apparatus: The apparatus was made of black Plexiglas sheets (56 × 62 × 28 cm, LxWxH) as an open box form (Figure 1). For the purpose of zone mapping, the bottom of the apparatus was painted in blue color and evenly-divided by 5x6 red lines into thirty identical cells. The large object used in the apparatus used was a metal cylinder (diameter: 6.25 cm; height: 15.25 cm). The smaller objects used for object recognition were: Object G, a white golf ball fixed onto a plastic base derived from the cap of a 50 ml Falcon tube; object M, a piece of metal tube that was of the similar size as object G. The objects were fixed onto the base of the apparatus during experiment.

Novel diet consumption apparatus: The apparatus was a metal open-topped box divided into two compartments measured 25.5 ×19 ×38cm (LxWxH) by a black Plexiglas (Figure 2). The floor was made of stainless wire mesh. A 50ml falcon tube contains the novel liquid diet and was capped by a rubber stopper with a stainless steel nozzle. The container was fixed upside down at the end of one chamber and was filled with prosobee solution before the start of the experiment.

Social interaction apparatus: The apparatus was made of white Plexiglas and was divided into a three-chamber system measured 26.5 x 46 x 30cm (LxWxH) (Figure 3). Two small opening (4cm x 4cm) at the bottom center of the wall between the center chamber and test/control chamber allowed the test animal to travel among the three compartments. A round cage measured 10 x 10cm (RxH) made of metal wire was placed at one of the distant corners of the test chambers to restrain the stranger mouse (Figure 3). An identical cage was placed in the diagonal corner in the control chamber.

Novel object exploration apparatus: The apparatus was a circular arena made of Plexiglas sheets measured 14 x 23cm (RXH). The wall was purple-colored and the floor was blue colored. The floor was covered by 1cm alpha-dry bedding before the test, and beddings from different test animal cages were sampled and mixed into the apparatus bedding to fast-forward the habituation process. The same small objects as in the open field test were placed into the apparatus approximately at the thirding points of the diameter.

Behavioral scoring

All training and testing sessions were recorded using an overhead video camera that instantly processed by behavioral tracking software ANY-maze. Protocols were set-up prior to the tests and sample trials were run to confirm the program will successfully complete the designed trials. In the behavior tracking set-up, virtual zones were drawn according to the apparatus, and the placement of the apparatus went through micro-adjusted before each trial to re-fit the map and the apparatus and the objects.

The contacts to the stimulus (an object, a novel food container or a cage of a test animal) were manually inputted into ANY-maze by the experimenter blind to the treatment during every trial. The contacts to a stimulus include sniff and touch. Sniff was defined as the test animal's snout approaching the stimulus with a distance shorter than 0.5 cm. Touch was defined as the animal's front paw(s) making physical contact with the objects. The sniff and touch was input into ANY-maze using specifically assigned keys. The duration of contact and the duration animals consuming novel food were recorded manually by experimenters holding an assigned key on the keyboard. As the resolution of the camera and software are limited, the experimenters used their best judgment to input the numbers, and the result was later sampled and verified by another experimenter to make sure the quality of the manual input.

Data collection and analysis

The raw data of behavior tests were extracted from ANY-maze into MS Excel. Data collected include the animal ID, test ID, distance traveled in the trial, mean speed during the trial (similar to the distance traveled as it equals distance divided by test-time), freezing episodes (interpreted as immobile episodes in the data analysis, as the software detects animal not moving), time freezing, number of entrances, time spent and distance traveled in the designated zones, mean distance from the object zone(s) or center zone and the manual input of experimenters. The manual input included number of sniff and touch to objects/cups, and duration of the contacts. Raw data were unblinded and input into various software for analysis. Data were cleaned and organized using MS Excel and R. Data visualization was performed in R software. Statistical tests including independent

sample t-test, repeated measures ANOVA and Post-Hoc test were performed using SPSS software for consistency of algorism.

CHAPTER 3

THE EFFECT OF SEA ON OBJECT RECOGNITION AND A "ODD-BALL" NOVEL STIMULUS

3.1 Relevant Background and Rationale

There is increasing evidence that T-cells influence the CNS, possibly by providing neuroprotective and cognitive-enhancing effects (Moalem et al., 1999; Ziv et al., 2006). A major finding was that T cells travel through lymphatic vessels in the meninges and may execute immune surveillance (Louveau et al, 2015). Furthermore, activated T cells secrete neurotrophins, which can promote neuronal survival and maintenance of neuronal health (Moalem et al., 2000). Conversely, in severe combined immune deficient (SCID) mice, which lack T cells, and in adult wild-type mice that went through acute depletion of lymphocytes, evidence was provided of impaired acquisition of cognitive tasks including MWM, water-free Barnes maze and six-radial arm water maze (Brynskikh. et al., 2008). This suggests that when T cells are absent, the behavioral function is impaired.

Superantigens are known to produce complex cognitive alternations via activation of T-cells. SEA injected animals showed altered exploratory patterns in an open field test with a novel object and showed reduced consumption of a novel diet (Kawashima and Kusnecov 2002). The result was not likely due to a change in locomotion ability as the traveling speed of the animals was unaffected. The results were interpreted as an increased anxiety level. However, in contrast to the evidence of increased general anxiety level found in the OF test, the elevated plus maze test did not find the SEA treated animals experienced higher anxiety level. In fact, SEA treated animals made more

entrances into the distal portions of the open arms in the elevated plus maze (EPM) test (Rossi-George et al., 2004), suggesting either an increased motivation to explore an unfamiliar context or search to escape. Additionally, results from the light-dark box test found no significant change of the time spent in the light area after treatment with another SAg, SEB (Rossi-George et al., 2004). Both the EPM and the light-dark box test suggested no increase of anxiety level from the analysis of the exploratory behaviors. The increased "anxiety" level in the OF test is a case that includes an introduction of a novel stimulus only. The conclusions from the tests are uncertain, but also implies that SEA may not have a main effect on the level of anxiety, but could have an interaction effect with the presentation of novel stimuli only.

Studies also have focused on the effect of SEA treatment regarding learning and memory. SEA did not impair learning and memory ability in the MWM, but SEA promoted the reconsolidation of the initial learning effect (Woodruff et al. 2011). However, a related study demonstrated that animals that went through an initial MWM training performed worse in a water Radial Arm Maze weeks after the MWM acquisition (Qing Chang, Masters Thesis). The mechanism by which SEA affect the long-term memory process is not readily apparent. This may be more easily investigated using simpler forms of learning-related behavior.

In this chapter, we applied different protocols to test the effect of SEA on novel object exploration and object recognition. The protocols all attribute whether SEA has an effect on the long-term memory retrieval of a familiar object, and whether SEA affects the exploratory behavior of a novel object with and without the presence of a familiar object.

Many studies on novelty do not investigate discrete changes in stimulus change, but simply expose animals to a new environment. For example, Kalueff studied the exploration to various novel apparatus and compared their exploratory behavior in novel and familiar environment. While this may be novel, it is also a stressor. Therefore, the design in the present study was an approach similar to human studies in which oddball stimuli are presented after repeated exposure to the same stimulus (Debener, Makeig, Delorme & Engel, 2005). Using this approach, animals can first be habituated to a specific stimulus, and then "surprised" by the introduction of a new stimulus similar in size as the habituated stimulus.

3.2 Method:

3.2.1 Subjects

All animals used were male C57bl/6J mice. Animals in experiment 3a, 3b, 3d-3f were adult male mice (average age of 6 months old) bred in-house from breeding pairs purchased from Jackson Laboratory (Bar Harbor, ME USA), and were group-housed (2-4 per cage). Animals in experiment 3c were male mice aged seven-week-old, purchased from Jackson Laboratories ((Bar Harbor, ME USA)). Food and water were provided ad libitum. The colony room was maintained on a constant 12:12 h light: dark cycle with lights on at 0700 h. All animals were pre-handled by the experimenters for five days, 1 minute each day, before the initiation of experimental treatments and/or behavioral testing. All experiments were conducted following the Guide for the Care and Use of Laboratory Animals, as promoted by the National Institutes of Health, and was approved by the Rutgers Institutional Animal Care and Guidance Committee.

3.2.2 Experimental design

Three protocols were utilized to investigate the exploratory behavior of animals under different contextual conditions. Table 1 provides a summary of the protocols that were used. Experiments were differentiated conceptually as "Object Recognition" experiment or "Odd-Ball" novel object experiments, as will be explained. Each experimental protocol varies with regard to the amount of familiarization that animals receive to the different stimulus conditions of the testing environment. In experiment 3a, animals went through an eight-day protocol including i) habituation to the apparatus (Day1 – Day3), ii) familiarization with the identical object (Day4 – Day6) and iii) test with one familiar and one novel object (Day7). For experiment 3b, animals went through a seven-day protocol including i) habituation to the apparatus (Day1 – Day6), ii) familiarization with two identical objects (Day7) and iii) test with one familiar and one novel object (Day8). In experiment 3c, a novel protocol was set up with i) familiarization with one object (Day1 – Day9) and ii) test with another novel object (Day10). This protocol was utilized for experiment 3d-3f with minor modifications.

3.2.2.1 Object Recognition Experiments

Experiment 3a: Effect of multiple-day object familiarization

Experiment 3a is schematized in Figure 5. This experiment investigated the effect of SEA on object recognition in a small circular arena using a seven-day procedure modified from Vogel-Ciernia and Wood, 2014. For details see Chapter 3. Animals (N = 12) were allowed to habituate to the apparatus for three days (Days 1-3), five minutes each day, and then on Days 4-6, allowed to explore two identical simultaneously-presented objects (referred to as the familiar [F] objects) for 10 minutes each day. As

described in Chapter 3, the two objects were either golf balls (objects G) or two short metal pipes (objects M) close in size to the golf balls. Familiarization to G or M objects was counterbalanced. On the test day (Day 7), animals were treated intraperitoneally with 5ug (approximately 200µg/Kg) SEA (n = 6) or a similar volume (0.2ml) of Saline (n = 6) two hours prior to exposure to the novel object. Injections occurred between 1000-1300 h. Animals were returned to the home cage after injection. The order of injection was staggered based on the treatment group to ensure that any influence of circadian rhythm was equally applied to saline and SEA-treated mice. That is, animal injections were separated by twenty-minute intervals, alternating between saline and SEA injections. The experimenter was blind to the treatments since vials were coded. For each animal, behavioral testing began 2 hours after injection and lasted 10 minutes.

A Microsoft video camera was used to capture the test video. Live recording and analysis of animal movements and patterns of exploration were performed by ANY-Maze tracking software (Stoelting Co., Wood Dale, IL). For behavioral analysis, the software was used to create a variety of zones in the apparatus: object zone A, object zone B, observation zone A, observation zone B, border zone and a neutral zone (See figure 4). The distance traveled, number of entries and time spent in each zone were all measured. Also, the software possessed the capability to monitor episodes when the animals were briefly immobile. By default, ANY-Maze records an immobile episode when no movement is detected for 250 msec. However, while these episodes were recorded, additional immobility that lasted ≥1 second was also recorded.

Experiment 3b: Effect of one day of object familiarization

Experiment 3b is similar to Experiment 3a, with the exception that familiarization to two identical objects occurred for one day only (Schematized in Figure 5). Experiment 3b modified the protocol and used the same design as Vogel-Ciernia and Wood in 2014. Two groups (n = 7 for SEA group, n = 6 for the saline group) of animals were tested using the same object recognition arena as experiment 3a and followed a modified eight-day protocol. Animals were allowed to habituate in the apparatus for six times, five minutes each, and then exposed to two identical objects (referred to as the familiar objects) for 10 minutes on only one day, Day7. On the following test day (Day8), animals were treated with either SEA or saline, and two hours later exposed to a novel object and a familiar object. Exposure lasted 10 minutes.

The two objects (object G and M) used in experiment 3a were again used in this experiment for the animals to explore. The same behavioral parameters were collected as in experiment 3a using ANY-maze.

3.2.2.2 "Odd-Ball" Experiments

Experiment 3c: Establishing "odd-ball" protocol

We investigated the exploratory behaviors of animals during a nine-day training phase toward a constant familiar object and a novel object on the tenth day. Experiment 3c is schematized in Figure 5. The animals were familiarized with the presence of a single object (object G or M) as the exploration target in the center of an open field apparatus that was the same apparatus as the one used in the OF test in chapter 3. Once the exploration behavior of animals had reached a plateau for three consecutive days, the familiarized object was replaced with an object different in shape, color and texture (object M or G) while other experiment conditions remain unchanged. Exploratory

behavior toward the objects, including the number of sniff and touch, exploration duration and distance traveled during exploration were recorded every day. The number of contacts with the objects, the distance traveled, number of entries into each zone, and time spent in each zone were all measured using ANY-maze. Distance traveled during the ten-minute session was interpreted as the parameter indicating general mobile ability. Time and distance traveled in the border of the OF were also recorded.

Various experimental conditions were assigned to different groups to control for factors that could contribute to the novelty detection process. The assignment is shown in table 1. Animals in group A were exposed to object M on day 1-9 and tested with object G on day 10. Group C animals were exposed to object M throughout day 1-10 and served as the control for the novelty from a different object. To counterbalance the possible difference of interest between the two objects, Group B and group D were added using opposite object order to that of groups A and C. To study the ability to detect the appearance of a novel object, group E was added with no object in the training phase. Group F remains home-caged during the training phase, which serves as a control group experiencing maximum novel stimulus including the object and context. This experiment does not involve SEA treatment.

Experiment 3d: Effect of SEA on response to "odd-ball" novel stimulus

This experiment continues to determine the two-hour-short-term effect of SEA injection on exploratory behaviors toward a novel object using the protocol developed in experiment 3c (Schematized in Figure 5) in the open field box (OF). Mice (N = 12) received training of eleven consecutive days in the OF with the familiar objects and one-day test phase with a novel object.

On the test day, animals were treated intraperitoneally with 5ug SEA (n = 7) or 0.2ml Saline (n = 6) two hours before exposure to the novel object, between 1000-1300 h. Animals were returned to the home cage after injections. Similar animal order assignment was used as experiment 3a. The same exploratory behavior parameters were monitored during the testing as experiment 3c.

Experiment 3e: Delayed effect of SEA on response to "odd-ball" novel stimulus

This experiment focuses on the short-term effect of SEA on familiar object exploration, and the delayed effect of SEA treatment on novel object exploration 24 hours after injection (Schematized in Figure 5). 12 animals were tested to the novel object twenty-four hours after the SEA injection.

A ten-day training phase as in the previous experiment was given toward the familiar object used in experiment 3d. On day 11, animals were given SEA or Saline, and was later exposed to the familiar object as in previous sessions. Two hours after injection, animals were tested in the OF with the same familiar object as training phase.

On the next day (Day 12), animals in both groups (SEA or Saline) were exposed to the novel object.

The same parameters measured in experiment 3d were recorded in experiment 3e.

Experiment 3f: Effect of SEA on exploratory behaviors to an "odd-ball" novel object in younger adult mice

Experiment 3d was tested using animals with an average age of 10 month-old. According to Vogel-Ciernia and Wood (2014), age affects their exploratory behavior. In this experiment, we tested the exploratory behaviors of young adult mice with an average

age of 3-months to confirm the effect of SEA on exploratory behaviors toward a novel object.

3.2.3 Statistical analyses:

Behavioral data: Parameters looked at were distance traveled and time spent in the various object and non-object zones of the particular apparatus, as defined by ANY-maze protocol. For experiment 3c, the number of contacts to object M and G was compared to check for the inherent difference of interest with object M and G from Day 1 to Day 9 for this purpose. As no difference was detected between the counterbalance use of object M and G, the data were pooled together for analysis.

For the object recognition experiments, to determine the percentage of contacts made with the novel object, the following formula was used:

$$\textit{Percent Novel object contacts} = \frac{Novel\ object\ contacts}{(\ novel\ object\ contacts + familiar\ object\ contacts)} * 100$$

This formula was also applied for other parameters (eg., percent entrance into object zone). It should also be noted that the object pairs were always placed in the same locations in the apparatus, during training and test trials. In subsequent analyses, where "novel object" is referred to on training trials, this is simply referring to the familiar object in the location where a novel object will be placed on the test day. This is done to control for the possibility that changes in object exploration are driven by location preference, and not actual detection of object novelty.

The behavioral data collected were analyzed using independent sample t-test and repeated measures ANOVA when necessary. Comparison was also made between the test phase and the last day of the training phase. Post hoc test used the Tukey method was done for pairwise comparison.

3.3 Results - Part I: Effect of SEA on Object Recognition:

The object recognition test is commonly used to test the object memory for mice. It adopts the idea that recognition could be inferred from preferential looking to the novel target, similarly as the 'visual paired comparison paradigm' in primates' tests (Leger et al., 2013). In this test, two identical objects (designated 'familiar') were present in our study during the first session, and then one of the two objects is replaced by a novel object in the test session. The exploration of the new object provides an index of recognition memory. In our study, we adopted two protocols, containing a long (3Day, experiment 3a) and a short (1Day, experiment 3b) training phase. The object pairs were placed in the same spots of the apparatus in the training trials and the test trial. The habituation curve to the novel object is shown in Figure 6. For data collection purpose, the "novel object" in the training trials referred to the object that stayed on the spot of the test day's novel object.

3.3.1 Experiment 3a

Comparisons of exploratory behavior on the test day were made with the last training day of the familiar objects. Irrespective of treatment, no change was found in the overall total distance traveled in the apparatus across the two days. However, the within-subject day factor was found to have a significant influence when analysis of exploratory behaviors was confined to the observation zones and the neutral zone. The entrance ($F_{(1,9)}$

= 9.89, p = .01), time ($F_{(1,9)}$ = 7.15, p = .02) and distance traveled ($F_{(1,9)}$ = 5.00, p = .05)in the novel object zone decreased on the test day, when compared to exploration in the same location on the previous day (see Figure 7). The day effect was also significant for the number of contacts to both the novel object ($F_{(1,9)}$ = 12.62, p < .01) and the familiar object($F_{(1,9)}$ = 6.11. P= 0.03) (see Figure 7). The contacts increased on the test day. The within-subject day effect was not significant for all other parameters investigated including immobile time and episodes and exploration in other zones.

While changes in exploratory behavior were noted upon presentation of a novel object, there was no interaction effect found regarding the treatments. The contacts toward the objects were indifferent (p > 0.05) between the novel object and the familiar object. The *Novel object contacts percentage* was derived (Figure 8), and similar percentages were calculated for parameters including the number of entrances, distance traveled and time spent using the same formula. No treatment interaction effect was found. SEA and Saline treated groups were similar in other exploratory behavior parameters as well.

We performed independent sample t-test comparing the treatment effect on the data for the test day as well. However, the SEA and Saline-treated animals did not show a major difference in the parameters representing exploratory behaviors.

3.3.2 Experiment 3b

This experiment tested animals' exploratory behavior to a novel object substitution with the presence of a familiar object previously presented only once. Animals went through six habituation trials in the empty round arena (Figure 4), and one training trial with two identical objects (referred to as "familiar objects") in the arena

before exposed to the novel object in the test trial (Figure 5). Parameters including total distance traveled in the apparatus, total immobile time, the number of contacts to each object as well as distance traveled, time spent and entrance into all defined zones were analyzed using repeated measures ANOVA comparing two levels of the within-subject variable: the training day and the test day. The training day serves as the baseline of exploratory activity and balances the possible difference of animals' interest in the object positions of the apparatus. The data analysis process was similar to experiment 3a.

As the animals were exposed to the familiar objects for one trial only, the habituation curve to the objects would not reach plateau. The day factor was found to significantly affect the parameters including traveling speed ($F_{(1,12)} = 92.17$, $p < 0.01$), immobile time ($F_{(1,12)} = 7.63$, $p = .02$), contacts to the novel object ($F_{(1,12)} = 60.90$, $P<0.01$) and familiar object ($F_{(1,12)} = 20.92$, $p < 0.01$). The mean speed and contacts decreased while immobile time increased. Additionally, the percentage of contact to the novel object ($F_{(1,12)} = 5.54$, $p = 0.04$) (Figure 9) and novel object contact duration ($F_{(1,12)} = 6.91$. $p = 0.03$) also significantly decreased on the test day. The effect of day on immobile time, travelling speed, the numbers of contacts to the novel and familiar objects was shown in Figure 10.

The SEA treatment was found to have an interaction effect on the immobile time ($F_{(1,12)} = 6.18$, $p = 0.03$). The number of immobile episodes also showed a borderline effect ($F_{(1,12)} = 4.602$, $p = 0.055$). The treatment effect was found not to be significant for all other parameters investigated when comparing the SEA and Saline treated groups ($p > 0.05$), including the distance traveled during the trial.

To better study the immobile behaviors in this test, the length of the immobile behavior (originally quantified as no freezing behavior for >1s) was re-calculated using a minimum duration of 250msec, 2secs and 3secs. Immobile duration longer than 4secs was rare in the test and not analyzed consequently. Additionally, the 5-minute test session was binned by each minute and analyzed for the change of the immobile behaviors across the test trial. Finally, the zones that immobile behaviors took place were also studied.

Animals treated with SEA showed a significant interaction effect both using the 250msec ($F_{(1,12)} = 6.10$, $p = 0.03$) criteria and the 3sec criteria ($F_{(1,12)} = 6.17$, $p = .03$) in total immobile time. No effect was observed when using the 2sec criteria ($p > 0.05$). Animals treated with SEA also showed an interaction effect both using the 3sec criteria ($F_{(1,12)} = 5.58$, $p = .04$) in the immobile episodes. No effect was observed when using the 250msec and 2sec criteria ($p > 0.05$).

The bin analysis revealed that animals' immobile behavior (>1s) changes across the 5 bins in both time ($F_{(4,3.187)} = 3.43$, $p < .05$) and episodes ($F_{(4,4)} = 5.48$, $p < .01$). The immobile time and episodes both increased. No significant interaction effect of the treatment was found.

The zone analysis of the immobile behavior was done dividing the apparatus into the novel observation zone, the familiar observation zone and the neutral zone. A major day effect was found in the immobile episodes ($F_{(1,12)} = 9.01$, $p = .01$) and time ($F_{(1,12)} = 5.93$, $p = .03$) in the familiar observation zone. An interaction effect of the treatment was also found. The immobile episodes ($F_{(1,12)} = 10.26$, $p = .01$) and time ($F_{(1,12)} = 5.70$, $p = .04$) in the familiar observation zone was found increased in SEA treated animals on the test day comparing with the training day. Although no interaction effect was found in the

distance traveled over minutes in each zone, SEA treated animals had interaction effects in time spent in the neutral zone and familiar object observation zone.

Additional analysis using t-test on the test day data comparing the treatment effect was performed. However, no treatment effect was found between the SEA and Saline-treated animals in the parameters representing exploratory behaviors.

3.3 Discussion - Part I: Effect of SEA on Object Recognition:

In this part, we used two different protocols to test the effect of SEA on exploratory. In experiment 3a, the animals successfully detected the novel stimulus, reflected by the changes in the number of contact to the novel object. The number of contact to the novel was decreasing during the training phase, and then increased on the test day. The experiments also demonstrated that SEA treatment did not affect the memory retrieval of the familiar object. However, no major treatment effect was observed on exploratory behaviors in experiment 3a. SEA treated animals was not different from saline-treated animals in the exploration of the novel object. In experiment 3b, SEA treatment had a strong interaction effect on immobile time and episodes.

Experiment 3a consisted of three days of habituation, three days of training with two familiar objects, and one day of test. On the test day, animals received i.p. injections and novel object exposure together with one familiar object exposure. The travel distance during the test trial was not different between the treatment groups, verifying that 5 ug SEA treatment did not affect animals' motor ability or exploratory motivation. This conclusion has been demonstrated by various behavior tests addressing the effect of SEA by our lab (Kawashima & Kusnecov, 2002; Rossi-George et al., 2004; Woodruff et al., 2011).

Additionally, it is unlikely that SEA treatment induced anxiety-like behavior, as the time spent in the border zone was not different between the groups. Animals under stress would avoid investigating the central region of the apparatus and spend more time hiding in the corner or border of the apparatus with little movement (Bailey, Crawley, & Buccafusco. 2009).

In experiment 3a, the increase in the number of contact to both the novel object and the familiar object suggested that the presence of the novel object promotes general exploratory behaviors not only to the novel object, but also to the familiar object. The observer summarized the common exploration pattern, and at the beginning of the test trial animals looped between contacting one object and then contact the other object in turns. As shown in Figure 8, although the saline injected animals had an increase of the novel object contact percentage, the percentage stayed not far from 0.5. The duration of the contacts was not different between the two objects as well. The exploratory parameters did not show a significant preference to the novel object, but reflected an equal interest of exploration between the objects.

The conclusion was different in experiment 3b. In this experiment, the training of the familiar objects was shortened from three days to one day. There was a decrease in the number of contacts to both the novel object and the familiar object in the test trial. This is an opposite change direction comparing with experiment 3a. The novel object contact percentage further decreased significantly, showing a preference to the familiar object. The test animals made less voluntary exploration in experiment 3b, especially to the novel object, which could be interpreted as a neophobic behavior pattern. The interpretation is further supported by a decrease in travel distance, which could be related

to an elevated anxiety level (Bailey, Crawley, & Buccafusco. 2009). The difference between the protocols of experiment 3a and 3b lies in the number of exposures to the familiar objects. Animals did not collect enough information from the single exposure to the familiar object in experiment 3b, and the presence of the novel object induced greater stress. Exploratory behavior on the test day declined as a consequence. This phenomenon contradicts to the statements of the protocol established by Vogel-Ciernia and Wood (2014). In this protocol, a single exposure to the familiar object would be sufficient to habituate the animals. When a novel object was introduced, there was an increase of exploration to the novel object. Experiment 3b intended to replicate the paper by strictly following the protocol. The difference of the experiment result could be due to the difference in the physical setting of the laboratories as well as the general condition of the subjects.

Additionally, a significant interaction effect was found in the immobile time in the test trial of experiment 3b. SEA injected animals displayed longer immobile time (the sum of the length of immobile episodes that lasted more than 1s) in the test trial when compared with the saline animals using the training trial as the baseline. Additionally, analysis for immobile episodes that lasted more than 3s also showed that SEA treated animals have longer immobile time and episodes compared with the saline-treated animals. The results implied that SEA treated animals showed altered exploratory pattern and were more likely to pause during their exploration in the presence of a novel object. However, it is not sufficient to explain whether the increase of immobility was due to attentiveness to stimuli, freezing response from fear or a lack of motivation to move.

A bin analysis that divided the five-minute test session into five equal segments showed that the immobile time and episodes increased as the test time went by. If fear was driving the increase of immobility, the immobility could be considered as related to freezing behavior. In this case, the animals should be freezing more at the beginning of the test when first introduced to the object, and gradually display less immobility as the test time prolonged. However, the data showed that immobility increased as the test prolonged, ruling out it being freezing behavior and suggesting the immobility was driven by other mechanisms.

The zone analysis was done to examine the animals' interests in the neutral zone and the novel and familiar object observation zones. The analysis showed that the immobile episodes and time increased in the familiar object zone, with a significant interaction of SEA that driven the main effect. It is possible that SEA decrease the exploratory motivation to a familiar object and caused the increase in immobility in the familiar zone. However, it is too early to claim that SEA decreased the motivation to explore novel stimuli, as the immobile episodes and time did not differ between the treatment in the novel object observation zone. It could be an effect on attention that follows the recognition of the novel object, yet the mechanism is not immediately clear.

We found that the immobility increased as time went by, while the time spent around the objects was not affected. It is possible that animals' immobility was due to attentiveness to the objects. Animals could be anxious at the beginning of the test and made less close observation that required them to stop moving and investigate the object. As time went by, animals were more comfortable to make more pauses in the observation zone of the objects. However, there is no evidence to support that animals paid more

attention to the objects during the pauses, although cognitive process comparing the novel object's information with the familiar object's information could take place during this pause. To confirm this hypothesis, further experiments should compare the immobile episodes of animals with one and three exposures to the familiar object in the same test trial to compare their immobile episodes and time. The hypothesis would be better supported if animals with fewer experience with the familiar object show altered immobility at the beginning compare with the ones with more experience, and gradually equals as time goes by. A control group treated with SEA and tested with the two familiar objects is also suggested as a future study.

The results suggested that when animals were not sufficiently habituated, they were less exploratory, and SEA has a more significant effect to alter the exploratory behavior. This implies that animals were more attentive to the environment when sick. SEA could have increased the attentiveness to novel stimulus when animals are uncomfortable. As previously suggested, SEA treatment activates the HPA axis. It is possible that with the effect of SEA alone in experiment 3a, the activation of HPA axis did not reach the threshold to induce neophobic behaviors. However, the SEA treatment induces corticosterone release into the circulation. When the animals experienced external stress together with SEA treatment, the HPA axis was further activated to induce a stronger stress effect, and animals displayed altered exploratory behavior pattern as a consequence.

3.4 Results - Part II: Effect of SEA on "Oddball" Novel Object Recognition

3.4.1 Experiment 3c

All mice (N = 24) exposed to the familiar objects habituated to the open field and object showing a decrease in traveled distance over Day 1 to Day 9 (Figure 11). Their exploratory behavior reached a plateau status on Day 4.

The homogeneity was tested between the counterbalanced groups first. The counterbalanced groups are groups using opposite object orders for the training and test sessions. No difference was found between either group A and B or group C and D (See Table 1) by a one-way ANOVA test in all exploratory behavior parameters on the last day of the training session except the time spent in the object zone. The post-hoc Tukey test suggested that one group (group B, G-M) had significantly longer time spent in the object zone compared with all other groups. However, the result may not reflect a true difference in the animals' interest to explore the two objects. The result could be a type 1 error: The other group of the counterbalanced pair, using an M-G order, did not show any difference of time spent in the object zone compared with all other groups. Additionally, other parameters including the distance traveled in the zone, entrance into the zone and latency before entering the zone were not different regarding the counterbalanced groups (G-M and M-G). Finally, the groups did not display any difference between the two objects on day 1 in time spent in the object zone. However, the results for time spent in the object zone was analyzed using the design of six groups. No difference was found using Tukey's HSD test between other pairs except group B. Other exploration parameters that were found to have no different counterbalance effect were analyzed combining group A and B (M-G and G-M) as Fam/Novel and group C and D (M-M and G-G) as Fam/Fam.

Analysis using one-way ANOVA revealed a significant difference among the groups on parameters including the distance travelled in the apparatus ($F_{(3,32)} = 6.41$, $p < 0.01$), number of contacts toward the test object ($F_{(3,32)} = 5.40$, $p < 0.01$), number of entrances into the object zone ($F_{(3,32)} = 5.10$, $p = 0.01$), distance travelled in the object zone ($F_{(3,32)} = 5.63$, $p < 0.01$) as well as the time spent in the object zone ($F_{(5,30)} = 9.117$, $p < 0.01$). Post-Hoc tests were done for all pairwise comparisons. Selected results are reported as following:

For distance traveled in the apparatus, the home-caged animals made significantly less traveling compared with group Fam/Fam and Fam/Novel ($p < 0.05$), while no difference was found among the rest of the groups.

The Fam/Fam group and Fam/Novel group are significantly different in the number of contacts toward the test object ($P = 0.013$). The animals exposed to novel objects (Fam/Novel) made significantly more contact toward the test object compared with the Fam/Fam group. The complete result of the Post-Hoc test is presented in the appendix.

This model suggests that animals exposed to a new object on test day showed significantly more contact compared with the animals exposed to the same familiar object that was habituated during training. This indicates that animals were able to differentiate different test objects in this specific experimental design. Thus we established a successful protocol for experiments 3d, 3e and 3f involving SEA treatment.

3.4.2 Experiment 3d

In this experiment, animals were subjected to a single object exploration using the protocol established in experiment 3c. On Day 12, animals were treated with either SEA or saline and placed into the open field apparatus with the presence of the novel object two-hour after the treatment (See Figure 5). All mice habituated to the open field with the familiar objects and showed a reduction in travel distance and number of contacts from day 1 to day 11. Their exploratory behavior reached plateau on day 4. The habituation curve reflected by the number of contacts by day is shown in figure 12.

Data of the last training day (Day 11) revealed no difference between the baseline of the treatment groups in exploratory behavior parameters including distance traveled, number of entrance and time spent in all zones. The number of contacts toward the objects between groups was not different ($p > 0.05$). On test day, independent sample t-test showed no treatment effect between the test groups in the distance travelled ($t_{(11)} = 1.80$, $p > 0.05$) or the entrance into the object zone ($t_{(11)} = 1.66$, $p > 0.05$). However, animals treated with SEA made less contacts toward the object, measured by the number of sniff ($t_{(11)} = -2.18$, $p = 0.05$) and touch ($t_{(5.707)} = -2.64$, $p = 0.02$) on the test day compared with the saline treated animals. SEA treated animals also spent less time ($t_{(5.614)} = -2.90$, $p = 0.03$) and traveled shorted distance ($t_{(11)} = -3.29$, $p = 0.01$) in the object zone. The time spent in the observation zone was also shorter in the SEA treated group ($t_{(11)} = -2.94$, $p = 0.01$) when compared with the Saline treated group.

The data was also analyzed using repeated measures ANOVA taking the test day and the previous training day as the two levels of the within-subject variable. The day factor had a significant effect on the exploration, together with an interaction of treatment effect on the number of contacts and time spent in the object zone. The conclusion did

not differ from the independent sample t-test (data not presented due to similarity with the t-test results).

3.4.3 Experiment 3e

This experiment studied animals' exploratory behavior toward a familiar object after SEA treatment. Additionally, animals were tested for the long-term (24hr) effect of SEA treatment on the exploration of a novel object. In this experiment, animals were subjected to a single object exploration test using the protocol established in experiment 3c. On Day 11, animals were treated with either SEA or saline and exposed to a familiar object to explore in the open field apparatus. After 24 hrs, the animals were exposed to a novel object to explore in the same apparatus. All mice (N = 12) habituated to the open field apparatus with a familiar object and showed a decrease in travel distance and number of contact over day 1 to day 10. Their exploratory behavior reached plateau on day 4. The habituation curve addressing the number of contacts by day is shown in Figure 13.

Data analysis of the last training day (day 11) with independent sample t-test revealed no difference of the baseline between the treatment groups in exploratory behavior parameters including distance traveled, time spent and entrance in all zones ($p > 0.05$). The number of contact toward the object of the two treatment groups was not different either ($p > 0.05$).

Data collected from Day 11 (test day1) when i.p. injections were given was analyzed by independent sample t-test. No difference in the number of contacts toward

the familiar object ($t_{(11)} = 0.345$, $p > 0.05$) was found between the treatment groups. Other exploratory behavior parameters including distance traveled, time spent and entrance in all zones did not differ ($p > 0.05$) between the treatment groups by independent sample t-test either. On Day 12 (test day 2), animals treated with SEA 24hr before showed no difference compared with the saline-treated animals in the number of contacts to the novel object ($t_{(11)} = -0.393$, $p > 0.05$). No difference was found in exploratory behavior parameters including distance traveled, time spent and entrance in all zones, defined by independent sample t-test ($p > 0.05$). Selected t-test results are shown in Table 5 and Table 6.

The data was also analyzed using repeated measures ANOVA taking the last training day, test day1 and test day2 as the three-level within-subject variable. The day factor had a significant effect on the exploration. However, no treatment effect was observed. The conclusion did not differ from the independent sample t-test.

3.4.4 Experiment 3f

As experiment 3d was done using mice of older age, experiment 3f was conducted to verify the SEA treatment effect in younger adult mice. All mice habituated to the open field apparatus with the familiar objects and showed a decrease in travel distance and number of contacts over day 1 to day 14. Their exploratory behavior reached plateau on day 3. The exploratory behavior is shown in Figure 14 represented by the number of contacts to the object. Data of the last training day (Day 14) revealed no difference between the treatment groups in exploratory behavior parameters including distance traveled, number of entrance and time spent in all zones ($p > 0.05$). The number of

contact toward the object was not tested different between treatment groups either ($p > 0.05$).

On test day, no main effect was found in the exploratory behaviors to the novel object. SEA treated animals showed no difference in the number of contacts to the novel object ($t_{(9)} = -.24$, $p > 0.05$). No difference was found in exploratory behavior parameters including distance traveled, time spent and entrance in all zones by independent sample t-test ($p > 0.05$).

The data was also analyzed using repeated measures ANOVA taking the test day and the previous training day as the two levels of a within-subject variable. The day factor had a significant effect on the exploration, supported by a significant difference in the immobile episodes and time spent in the center zone. However, no interaction of treatment effect was found. The conclusion did not differ from the above (see appendix for detailed results).

3.5 Discussion Part II

In part II, three experiments utilized the protocol established in experiment 3c and tested the effect of SEA on exploratory behaviors toward a novel object. The animals all successfully identified the novel object, reflected by one or more parameter changes including contact toward the object(s), immobile time, total travel distance/average speed, as well as travel distance, entrance and time spent in various zones. The identification of familiarity and novelty demonstrated that SEA treatment did not affect the memory retrieval of the familiar object. However, no major treatment effect was observed on exploratory behaviors except experiment 3d. SEA treatment decreased exploration to the novel object in experiment 3d. In experiment 3e and 3f, SEA treated

animals was not different from saline-treated animals in the exploration of the novel object.

The result from experiment 3d - 3f suggested that there was no difference in the locomotion ability of the SEA injected animals, which had been proved by experiment 3a and 3b as well. Nor did we find that SEA affected the anxiety level of animals compared with saline treated animals in experiment 3d - 3f: no treatment effect was found when analyzing the travel distance or time spent in the corner zone.

In experiment 3d, SEA injected animals showed decreased contact toward the novel object, but the number of entries into the object zone did not differ from the saline-injected group. The interpretation of this phenomenon is that the SEA injected animal are able to identify the difference between objects, yet they are more cautious and reluctant to directly contact the novel object than the saline injected animals.

SEA injected animals showed no change in contact toward familiar object 2hr after SEA administration, and no change in novel object exploration 24hr after administration, indicating the neophobic effect of SEA lasted less than 24hrs. The object neophobia could be attributed to the effect of TNF-α. The difference between the effect of SEA and TNF will be further discussed in the general discussion.

Experiment 3d and 3f utilized the same experimental design. Experiment 3d concluded that the SEA treatment reduces the exploratory behavior toward a novel object. However, the treatment effect of SEA was not consistent with the other two experiments. The treatment effect of experiment 3d is not likely a type I error for the single parameter, as the SEA treated animals spent less time in the object zone as well.

It should be noted that the subjects in experiment 3d were on average older compared with other groups. These animals were an average age of 10-months. As has been shown in other rodent studies, age is an independent variable that affects animals' exploratory behavior (Shukitt-Hale et al., 2001; Vogel-Ciernia and Wood, 2014). Aged animals were overall less exploratory and showed a reduction of exploration toward novel stimulus (Goodrick 1971, Shukitt-Hale et al., 2001). Since animals in experiment 3f were younger by 6 months, these data suggest that for this particular behavioral test age may be an important variable affecting SEA effects on novel object exploration.

The decreased contact toward the novel object reflected a more cautious status in the older animals. When sick, animals of older age were physically more venerable, and it is more dangerous to contact a novel object that the animals are not sure whether it is safe or not. As animals aging, movements and reflexes slow and their hearing and vision weaken, and the fluid intelligence decreases as well. It is rational to make less risk behavior for older sick animals as they may face more challenge in novel situations. Moreover, aged animals were less motivated to explore novel stimuli. Rat's exploratory behavior was found decreased from 4-month old to 12-month old in an open field apparatus (Goodrick 1971). A similar phenomenon may exist in mice and it could be an interaction effect between age and the SEA treatment that they do not prefer the novel object to explore (Shukitt-Hale et al., 2001).

Another factor that could contribute to the inconsistency issue of experiment 3d is the inconsistent baseline. On day 10, the baseline of the two groups had an insignificant difference in the number of contacts to the object. The difference could be enlarged on the test day and result in a false positive t-test result. Two Saline treated showed more

contact to the novel object on the test day, which could be outliers that driven the difference of the two groups (Figure 14). However, the SEA treatment effect was also proved significant by a repeated measures ANOVA including data from both the last training day (Day 11) and the test day. The SEA affected the exploratory behavior regardless of the previous-day baseline. The data from the previous training, especially from Day 9-Day 11 did not have any outliers in either group, and indicate that animals are well-habituated to the experiment setting. The change on the test day should be an effect of the novel stimulus together with an interaction of treatment effect.

The process of novel stimulus detection in this chapter requires long-term memory retrieval. From the results above, we concluded that 5ug i,p, treatment of SEA did not affect the object memory retrieval process. Similar to the MWM test, the hippocampus is required to complete this cognitive task. The experiments were organized in the order of increasing cognitive load. Among all the experiments in this chapter, experiment 3a is the least challenging test: the animals had multiple exposures to the familiar object; the familiar object was present in the test and could serve as a comparison when the novel object was introduced. The cognitive load of this task is the minimum and the task has the least sensitivity of testing the possible cognitive function impairment associated with SEA treatment. Experiment 3b reduced the training to a single trial. The memory consolidation was not repeated before the test. However, the presence of the familiar object provided easier memory retrieval to identify the difference between the novel and familiar objects. The following experiment 3d, 3e and 3f removed the presence of the familiar object, and isolated the long-term memory retrieval process

independent from the acquisition of the same object. The experiments demonstrated that the object memory retrieval process was not affected by 5ug SEA i.p. injection.

The experiments in this chapter demonstrated that 5ug SEA i.p. treatment does not affect the locomotion ability of the animals nor the memory retrieval of the familiar object. The treatment can influence animals' exploratory behavior in some conditions. In the object recognition test, SEA treatment affected the immobile time and immobile episodes of the animals with one-day exposure to the familiar object. In the open-field apparatus, older animals treated with SEA showed decreased exploration to the object. Both effects were detected 2hrs after the SEA treatment.

CHAPTER 4

THE EFFECT OF TNF-A ON EXPLORATORY BEHAVIOR TO NOVEL STIMULI

4.1 Background

The term ‘novelty' refers to any stimulus or set of stimuli, that differ in form or quality from previous knowledge of a given object or set of circumstances. For the organism, a behavioral or cognitive response to novelty is essential, as it demonstrates differentiation between stimuli as familiar and unfamiliar (i.e., novel). When responses to novelty are absent, as has been argued to occur in certain psychiatric conditions (eg., latent inhibition test in schizophrenia), this is thought to reflect general cognitive dysfunction and impaired memory (Schmajuk. Christiansen. & Cox., 2000). Indeed, defining the range of novel situations is extensive, and this has been argued to contribute to learning and memory. Moreover, recognizing novelty, or detecting unfamiliar signals from the norm, is a critical element of attention, one with potential adaptive significance. Biological states that increase attention to novelty, may represent modifications to neurobiological functioning that ensure sharper detection of contrast between the familiar and unfamiliar.

Information acquired from the exploration of a novel stimulus may not serve current needs, but forms latent learning instead and serves as a future reference (Renner, 1988). The information is stored in long term memory and facilitates foraging, escaping and mating, and dopamine modulates novelty-seeking behavior during decision making (Costa, Tran, Turchi & Averbeck, 2014). For example, it has been shown that animals that have more exploration experience used a shorter time to escape from chasing in an unfamiliar maze (Renner, 1988).

Compared with familiar stimuli, novel stimuli can elicit stronger exploratory behavior. Berlyne concluded that the novelty exploration was driven by curiosity (Berlyne, 1950), and Montgomery proposed that novel stimuli actually evoke exploratory drive (Montgomery 1955). Additionally, novel stimuli induce fear-like behaviors and fear was hypothesized to participate in the decision-making process under patterns of exploration (Montgomery 1955; Russel, 1973). Animals can display neophobic behavior such as freezing, hiding and avoiding contacts with novel stimuli (White and Weingarten, 1976; Mason et al. 1978). By integrating the exploratory drive and fear into one theory, the two-motivation system theory suggested that novelty created a competition between neophobia and exploratory drive, resulting in an approach-avoidance conflict (Murphy, 1978, Wood-Gush & Vestergaard 1991, 1993). The balance of the scale will shift according to the strength of the two motivations, determining the exploratory pattern of animals. As a result, an animal will choose to either bravely explore the stimuli or hide in a safe place and make no approach to the stimuli. Later research linked the exploratory motivation toward novel stimuli to the dopamine-driven reward system, while the amygdala drives the neophobic behaviors (Dulawas, Grandy, Low, Paulus & Geyer, 1999).

The immune system, like the brain, evolved the capacity to differentiate between the familiar and novel, and has been likened to a cognitive system, in which foreign material (such as bacteria and viruses) are responded to on the basis of a discrepancy between their molecular makeup and that of the host. While this operates at the cellular level within the immune system, there is now increased knowledge of neural-immune

interactions, and strong evidence that immune-derived cytokines can affect a variety of behavioral functions.

When an animal is immunologically challenged, it will show a set of adaptive behavior changes that orient the organism's priorities to cope with infectious pathogens. Those behaviors are subsumed under the general sickness behavior. Sickness behavior consists of weakness, malaise, anxiety, lethargy, anorexia, decreased water intake, and withdrawal from the physical and social environment (Dantzer, 2009). Sickness behavior is believed to be driven by specific brain areas to conserve energy and support the immune response. Specific cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) are sufficient to induce sickness behavior (Bluthé et al., 1994; Kelley et al., 2003). Sickness behavior driven by cytokine treatments is found with reduced exploration under certain situations, such as the presence of an unfamiliar social mate (Dantzer 2001; Larson & Dunn 2001).

Primarily derived from the immune cells, cytokines have been found to play a role in the exploratory behavioral changes following immune activation (Kelley et al. 2003). Cytokines can influence the brain in three ways: cytokines are produced in the central nervous system (CNS) by glial cells and astrocytes and make a direct effect on the CNS (Beveniste, 1992); additionally, the peripheral cytokines can affect the nervous system via the vagus nerve in the periphery; and finally, cytokine can act via the humoral system (eg. Blood) and act on the CNS (Dantzer., 2001).

Tumor necrosis factor (TNF) -α is a multifunctional proinflammatory cytokine released by macrophages and monocytes. It plays a crucial role in the inflammatory

process and interacts with multiple levels of the hypothalamic–pituitary–adrenal (HPA) axis (Turnbull & Rivier., 1999). TNF-α exists in the CNS and regulates novelty related behaviors such as anorexia and social exploration (Fiore et al., 1998; Bernstein et al. 1991; Palin et al., 2009). Transgenic mice with overexpressed TNF-α in the CNS displayed impairments in exploratory performances in a mild stress-inducing hole-board test (Fiore et al., 1998). TNF-α induced anorexia has been observed in both rat studies, and patients undergoing chemotherapy (Bornar et al. 1989; Michie et al. 1989; Bernstein et al. 1991). TNF-α reduced social exploration when administered into cerebral ventricles, and the process was mediated by TNF-R1 and a TNF-R1 adapter protein FAN (a factor associated with neutral sphingomyelinase activation) (Palin et al., 2009). However, compared with other cytokines that are well-studied, such as IL-1, not much literature has focused on the direct effect of central TNF- α on exploration toward novel stimuli, such as novel environment and novel objects. Neither has the effect of central TNF-α on memory-related exploration been accessed.

In order to further investigate the behavioral effect of central TNF- α administration on the CNS, this experiment tested animals' exploratory behavior in a novel environment, exposure to novel food, social exploration, and object recognition. To test the effect of central TNF-α administration, animals were fitted with an indwelling intracerebroventricular (i.c.v.) cannula and tested using the open field test, novel food (prosobee) test, social interaction test, and object exploration test. We hypothesize that TNF-α will alter exploratory behavior in a novel context toward various stimuli. The animals will display food-neophobic behavior but could become risk takers when exposed to other stimuli.

4.2 Materials and Methods

4.2.1 Animals

Male C57 mice aged seven-week-old were purchased from Jackson Laboratories ((Bar Harbor, ME USA)) and colonized at the psychology building animal facility. Animals were housed 4 per cage and maintained on a constant 12:12 h light: dark cycle with lights on at 0700 h. Food and water were available ad libitum. Animals were allowed two-week acclimation before the start of the surgery. All animals were pre-handled by the experimenters for five days, 1.5 minutes each day before any behavior tests to minimize handling stress. All experiments were conducted following 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.

4.2.2 Experimental Procedures

Stereotaxic surgery and recovery

Animals were examined for health conditions before the surgery and mounted onto a stereotaxic device (Model 900, David Kopf instrument, Tujunga, CA) with anesthesia mouthpiece to maintain animal under isofluorane throughout the surgery. A permanent, indwelling guide cannula, projecting 2mm below the pedestal, (Plastics One Inc., Roanoke, VA) was aimed toward the left lateral ventricle area (relative to Bregma: anterior-posterior: -0.34mm, medial-lateral: 1.1mm) using cyanoacrylate gel (Fisher Scientific) and dental cement (Co-oral-ite, Dental MFG. Co., Diamond Springs, CA). Immediately after surgery, mice were given heating pad underneath home cage single-housed and monitored till wakefulness. Mice were allowed three days to recover.

Animals were monitored for general health and possible infection. A topical antibiotic ointment was applied when necessary. Animals were handled again once a day starting from the second day of the surgery.

Drugs and infusions

Recombinant murine TNF-α (R&D Systems Inc., Minneapolis, MN, USA) was dissolved in artificial cerebrospinal fluid (aCSF). TNF-α was administered i.c.v. at 200ng/mouse. All infusions were given between 1000 and 1300 h with lights on at 0600. The i.c.v. infusion apparatus consists of an internal cannula (projection length; 0.15mm below the guide), tygon tubing (0.020 ID) and a 10µl Hamilton syringe. On the test days, animals were infused with the assigned solution (TNF-α solution, or aCSF) at a rate of 0.5µl/min. When infusion was complete, the internal cannula remained in place for an additional three minutes to avoid loss of solution due to the low diffusion rate. Animals were closely monitored for 5 minutes after the infusion.

Verification of cannula placement

After all tests were completed, animals were anesthetized with isoflurane and infused with 0.5% methyl green. Two minutes after the infusion, animals were sacrificed by CO_2 exposure. Brains were removed and immediately sliced coronally at the pinhole of the cannula to verify the spread of the dye in the ventricles.

4.2.2.1 Experiment 1

Behavioral Testing

The same groups of animals were used for all behavioral testing with the following order: (i) the open field/novel object test (OF/NO), (ii) the novel food

consumption test (NF), (iii) the social interaction test (SI) and (iv) the object recognition test (OR). The OF was conducted on the fourth day following the completion of the surgery. Each subsequent test was run 48 hrs after the previous test. A Microsoft video camera was used to capture the test video. Live recording and analyzation of animals' behavior was performed by the ANY-Maze tracking software (Stoelting Co., Wood Dale, IL).

Open field and novel object testing

Animals received the first i.c.v. infusion and were tested 1.5hrs afterward for the OF. The open field/novel object test was conducted with a protocol established in our lab (Cooper and Kusnecov, 2007). The test was divided into two phases, five minutes each. In phase 1, animals were placed into a rectangular open field apparatus that measured 56 × 62.25 × 28 cm (LxWxH) to start the test and allowed 5 minutes to explore the apparatus. In phase 2, a cylindrical metallic object measured 6.25 ×15.25 (R × H) was introduced into the open field serving as a stress-inducing stimulus. Exploratory behaviors were measured in both phases.

For the behavior analysis of the software, the apparatus map in ANY-maze was divided into four zones, named center zone, corner zone, border zone, and observation zone (See Figure 1). Specific behavioral parameters, including exploration time, distance traveled, zone entrance and the mean distance from a specific zone were computed both phases.

Novel Food Consumption

Two days after the OF/NO test, animals received the second i.c.v. infusion and were tested 1.5hrs afterward for the consumption of a novel liquid diet (Prosobee baby formula). This test was conducted with a protocol established in our lab (Kusnecov et al., 1999). The protocol does not require food or water deprivation in advance, thus minimizing the stress. The test animal was placed into a metal chamber divided into two compartments measured 25.5 ×19 ×38cm (LxWxH) by a Plexiglas wall for 20 minutes (Figure 2). A small opening (5cm x 5cm) at the bottom center of the wall serves as a door for the test animal to travel between the two compartments. A prosobee solution bottle was present and fixed to one end of the chamber. The other compartment was kept empty and measures the baseline effect in a chamber. Animals were placed in the control chamber to start the twenty-minute test. The prosobee bottles were weighed immediately before and after to calculate the amount of solution consumed by the test animal. Behavior parameters including exploration time, distance traveled, zone entrance and mean distance toward a specific zone were computed for each compartment. The experimenter recorded the number of contacts and contact duration toward the prosobee bottle. All observations were scored blind to the experimental treatment.

Social Interaction Test

Animals received the third i.c.v. infusion and were tested 1.5hrs afterward for social interaction with a stranger mouse for 10 minutes. The test animal was placed into a white Plexiglas three-chamber system that measured 26.5 x 46 x 30cm (LxWxH) (Figure 3). The apparatus was separated into three chambers: a test chamber and a control chamber each measured 26.5 x 18 x 30cm (LxWxH); the center chamber 10 x 15.25 x 30cm (LxWxH) connected the two chambers measured). Two small openings (4cm x

4cm) located centrally and at the bottom of the wall dividing the center chamber and test/control chamber allowed the test animal to enter each compartment. A round cage measured 10 x 10cm (RxH) made of metal wire was placed at one of the distant corners of the test chambers to contain the stranger mouse (Figure 3). An identical cage was placed in the diagonal corner in the control chamber. The test and control chambers were counter-balanced.

A test animal was first introduced into the center chamber and allowed 5 minutes for habituation toward the apparatus. The test animal was temporarily removed from the chamber, and an adult stranger animal was then placed in the cage presenting the test chamber. The test animal was then placed back into the center chamber and allowed to explore for an additional 10 minutes. During the test, the experimenter recorded the number of times the animals made contact with the cage in the test chamber and cage the control chamber which was empty.

Object recognition test

Animals received the fourth i.c.v. infusion and were tested 1.5hrs afterward for object recognition. The test was conducted using an eight-day protocol modified from Vogel-Ciernia and Wood (2014). Animals were exposed to the apparatus with no object (apparatus only) for 5 minutes in four consecutive days (D1-D4). Then animals were introduced to two identical objects (referred to as the familiar objects) from D5 to D7 for 10 minutes and tested on D8 with a novel object replacing one familiar object. A circular arena made of purple-colored Plexiglas measured 14 x 23cm (RXH) was used in this test.

The floor of the apparatus was covered with bedding taken and mixed from each cage, and the same bedding material was used to test every animal on the same day.

The familiar and novel objects were of similar size, but were different in shape, texture and color. Object G was a white golf ball firmly glued to a round base made of a 50ml falcon tube cap, and object M was a silver-colored metal cylinder with a rough surface. Previous research of our lab showed that animals have no preference between the two objects (data not shown).

For behavior analysis using the tracking software, the apparatus map was divided into four zones: object zone A, object zone B, observation zone 1, observation zone 2 and neutral zone (See figure 4), to analyze the exploration pattern of the animal. Specific behavioral parameters including exploration time, distance traveled, zone entrance and mean distance toward a specific zone were computed for each zone for both phases.

Experiment 2

Experiment Design

A second experiment was run using the same procedure of surgery and testing in the object recognition test followed by the open filed/novel object. This was done to determine whether the order of the two tests would make a difference in the results since the OF/NO test preceded the object recognition test in Experiment 1. Two days after completion of all surgeries, animals were tested in the object recognition test. 48 hrs after the completion of the first test, animals were introduced into the open field and tested without and with the big novel object.

Data Analysis

Data included traveled distance, travel time and entrance to each zone was collected. In addition, the number of contacts and contact duration were entered by an observer blind to the treatment of the test animals. Data from the open field/novel object test, novel food test, and social interaction test were analyzed by t-test, and data from the object recognition test were analyzed by repeated measures ANOVA. The test sessions were further broken down into 1 minute, 2 minutes or 2.5 minutes intervals (i.e. bins) to study the change of exploration pattern in each test.

4.3 Results

4.3.1 Experiment 1

Open field/novel object test

This test consisted of two five-minute phases. 12 animals (TNF-α = 7, aCSF = 5) were included in the analysis (one aCSF treated animal was excluded from the data analysis due to an operation error). The first phase (from 0 s to 300 s) was an open field test without the object. Parameters including total distance traveled in the apparatus, total freezing time, time and entrance into the center, observation, border, and corner zones were analyzed using independent sample t-test. Overall, the two groups were not different in the open field phase (see Figure 15).

For the novel object phase, TNF-α infused animals showed increased exploration when compared with the aCSF group were more exploratory when the big novel object was introduced.

Total freezing time was measured using the sum of the duration longer than 1s when animals were not detected to be moving. The time that test animals froze in the apparatus is shown in Figure 15A. A significant difference in freezing time was found between the two groups: TNF-α infused animals displayed significantly less freezing time ($t_{(10)}$ = -2.91, p = .015) when compared with aCSF animals.

The average distance between the test animal and the center zone of the apparatus is shown in Figure 15B. A significant difference was found between the two groups: TNF-α infused animals displayed significantly shorter distance ($t_{(10)}$ = - 3.02, p = .013) when compared with aCSF animals (Figure 15B). The animals treated with TNF- α were on average closer to the center of the apparatus.

The time spent in the four corner zones of the apparatus is shown in Figure. A significant difference was found between the two groups: TNF-α infused animals spent significantly less time in the corner zone ($t_{(10)}$ = - 3.93, p = .003) when compared with aCSF animals. In addition, the number of entrance into the border zone of the apparatus was higher for the TNF-α infused animals ($t_{(10)}$ = 5.40, p < .001) when compared with aCSF animals (Figure 15D). Conversely, time spent in the border zones of the apparatus (Figure 15E) was for the TNF-α-infused mice ($t_{(10)}$ = -2.36, p = .040).

The entrance into the observation zone of the apparatus where the novel object is located is shown in Figure 15F. A significant difference was found between the two groups: TNF-α infused animals made significantly more entrance ($t_{(10)}$ = - 3.26, p = .008) when compared with aCSF animals. Similarly, time spent in the observation zones (Figure 15G) was increased TNF-α infused animals ($t_{(10)}$ = 2.81, p = .018). And

consistent with this, the distance traveled in the observation zone (Figure 15H) was greater in the TNF-α infused animals ($t_{(10)} = 2.584$, $p = .027$) when compared with aCSF animals.

Novel food consumption test

For this test, parameters included total distance traveled in the apparatus, total freezing time, the number of contacts toward the prosobee bottle, latency before the first contact toward the prosobee bottle, the total amount of prosobee consumed as well as distance traveled, time spent and entrance into both chambers. Overall, TNF-α infused animals consumed less prosobee and made less contact with the prosobee container.

The amount of prosobee consumed during the test session is shown in Figure 16. It was found that TNF-α infused animals consumed significantly less prosebee ($t_{(11)} = -2.65$, $p = .028$) when compared with aCSF animals (Figure 16A).

Similar results were found when examining the number of contacts toward the prosobee sipper ($t_{(11)} = -2.74$, $p = .019$) (Figure 16B) and the duration of contact with the prosobee sipper in (Figure 16C) ($t_{(11)} = -2.76$, $p = .019$).

No difference was found between the treatment groups for latency to make first contact with the bottle, and the total distance traveled in both chambers of the apparatus. There was also no difference in time spent in both zones, nor the number of entries into the prosobee and control chambers ($p > .05$).

Social interaction test

Similar exploratory and contact parameters were tested as in previous tests. The percentage of contacts with the target animal is shown in Figure 17, and was not affected by TNF-α infusion.

Overall, animals in both treatment groups made more exploration in the social chamber (containing the target animal) compared with the control chamber. Animals made significantly more contact and longer total contact duration toward the target compared with the control cage ($p < 0.001$). Parameters including distance, entrance and time spent were found to be significantly different between the animal chamber and the control chamber ($p < 0.05$), but were equal for TNF-α and aCSF animals.

Object recognition test

One animal (animal number 20) treated with TNF-α was sick and removed from the analysis. Parameters tested were total distance traveled in the apparatus, total freezing time, number of contacts with each object, latency before the first contact with each object, as well as distance traveled, time spent and entrance into all defined zones were analyzed using repeated measures ANOVA comparing two levels of the within-subject variable, the last training day and the test day. The last training day serves as the baseline of exploration activity and balances the possible difference of interest in objects' position in the apparatus. To directly compare animals' interest in each object, percentages were calculated for entrance into the novel zone by the addition of entrance into both object zones:

Novel entrance % = entrance into novel object zone/(entrance into novel object zone + entrance into familiar object zone) *%

The same method was used to calculate the percentage of distance traveled in the novel object zone and time spent in the novel object zone.

No change was found in the total distance traveled between the two days. However, the day factor was found to affect the results significantly for parameters including entrance into the novel object zone ($F_{(1,10)} = 19.60$, $p = 0.047$) while time spent in the novel object zone was not different ($p>0.05$). The test day effect was also significant for distance traveled in the border zone ($F_{(1,10)} = 19.92$, $p = 0.047$) and a border effect for time spent in the border zone ($F_{(1,10)} = 15.33$, $p = 0.059$. Longer and more visits to the border zone were observed when a novel object was introduced.

The treatment effect was significant when comparing the time that animals spent in the novel object observation zone (Figure 18A) and travel distance ($F_{(1,10)} = 11.422$, $p = 0.007$) in the novel object observation zone (Figure 18B). Animals spent less time in the novel object observation zone when treated with TNF-α.

No significant difference was found between the treatment groups regarding the number of contacts made to each object, distance traveled during the test session, nor distance traveled and time spent in the remaining zones ($F_{(1,15)}<1$, $p>0.05$).

4.3.2 Experiment 2

In experiment 1, object recognition was tested by the fourth TNF infusion. To better study the repeat treatment effect of the TNF-α on long-term memory, animals were

tested on object recognition after the first infusion of TNF. The same analysis was done as the experiments in the original order.

In the object recognition test, the day factor had a significant effect over the entrance into the familiar ($F_{(1,10)} = 11.46$, $p = 0.04$) and novel ($F_{(1,12)} = 7.32$, $p = 0.02$) object zones. The number of entrances decreased for both zones. The time spent in the border zone also decreased on the test day ($F_{(1,12)} = 14.83$, $p = 0.002$). No effect of the test day was found regarding distance traveled during the test session, the entrance to the familiar object zone, and other parameters ($F_{(1,10)}<1$, $p>0.05$).

However, in both the OF and the NO phase, the animals showed a similar but weaker treatment effect. In the NO phase of the OF/NO test of experiment 1, t-test showed the treatment made a significant difference for parameters including time freezing, the mean distance from the center, time spent in the corner, entrance and time in the border zone, entrance, time and distance in the observation zone. In the NO phase of the OF/NO test of experiment 2, fewer parameters were found significant for the treatment effect, including time freezing, time spent in the center zone, entrance and distance in the corner zone, entrance and distance in the border zone, distance traveled in the observation zone as well as distance traveled in the phase. The T-test result was presented in Table 2 for selected parameters.

4.4 Discussion

The current study proposed and tested the effect of central administered TNF-α on novel stimulus exploration, using a battery of tests including open field test, novel food

test, social exploration test and object recognition test. The aspect of anxiety level was also monitored in the study. The study did not observe a change in the exploratory pattern by central administered TNF-α in the open field phase. However, the TNF-α promoted exploration to a big-size novel object in the same open field apparatus.

On the contrary, in the OR test, TNF-α decreased the observation time and travel instance. Yet this effect in OR was only observed in animals treated with repeated TNF-α infusion, but not in the naïve animals. Additionally, the study confirmed that central TNF-α infusion induces a reduction of novel food intake. No behavior changes of the treatment were observed in the social interaction test.

TNF-α has been suggested necessary for inducing anxiety-like behavior in specific situations, such as persistent inflammatory pain and viral infection measured by OF (Chen et al., 2013; Karson et al., 2013). However, the direct effect of TNF-α on exploratory behavior in the OF has not been studied before. In this study, the animals were tested both in the empty open field apparatus and followed by a second phase that has a large object inducing anxiety in the same apparatus (van Gaalen & Steckler., 2000). No effect of the TNF-α i.c.v. infusion was observed in the first phase of the current experiment. However, TNF-α infused animals displayed more exploratory behavior in the NO phase, reflected by the increased exploration time and distance in the observation zone. As the TNF-α infusion has been verified as effective in the NO phase of the study, the absence of a TNF effect in the OF phase draws a different conclusion from the observation of increased anxiety level in the study of Chen and Karson (Chen et al., 2013; Karson et al., 2013). In the two studies, TNF-α inhibition was found to reduced

depression and anxiety-like behavior in an inflammation model and a chronic mild stress model of depression in rats, demonstrating that TNF-α is related to anxiety-like behavior.

On explanation for the discrepancy from our results is that TNF-α does not induce anxiety alone, but is necessary for the display of anxiety-like behavior in an altered physical condition such as illness. A similar interpretation was found for the working memory. Systemic TNF-α treatment (50 μg/kg) had no impact on the performance of normal mice when tested for working memory in a T-maze, but produced acute impairments in progressive neurodegeneration animals (Hennessy et al., 2017). Alternatively, the apparatus used in this study was larger compared with the two studies mentioned above and could cause a difference in exploratory behavior.

In the current study, in the NO phase of the test, TNF-α treated animals displayed more exploration in the observation zone. The appearance of a large novel object appeared to induced this effect, such as TNF-α treated animals may have been more motived or less fearful in object approach. Since TNF-α is associated with sickness behavior, which should increase neophobic behavior, it is surprising that the effect of TNF-α was to increase exploration motivation. This may have been due to increased risk-taking behavior, which is at odds with previous conclusions regarding TNF effects (Dantzer, 2001). Since many of the effects of TNF are studied after systemic treatment, further studies should focus more on descriptions of central RNF influences.

The novel food experiment tested animals' exploration and consumption of a novel liquid diet stimulus. Rat studies have long established that central administration of TNF-α induces anorexic behavior using a solid diet (Bornar et al. 1989; Michie et al.

1989). A study using Staphylococcal Enterotoxin A demonstrated that systemic TNF-α is necessary for the downstream anorexic effect (Rossi-George et al., 2005). The novel food test in this study confirmed the anorexic effect of TNF-α using a liquid diet in mice, and verified the efficacy of the TNF-α infusion protocol. However, it should be noted that there is a possibility that the animals could experience a loss of appetite, which is related to a level of malaise that inhibited locomotion. The decrease in food consumption may not only affect the consumption of a novel diet, but to a familiar diet as well. However, we did not find changes in the distance traveled and entrance into the test chamber, which indicated that the animals were willing to explore and not displaying sickness behavior. A further study that tests the animals using a familiar diet may well address this question.

In the social exploration test, all animals showed a preference toward the animal chamber over the control chamber. However, no effect of TNF-α treatment was observed. TNF-α was found to reduce social exploration using a protocol that placees a juvenile social mate into the home cage and measured the social interaction in the dark phase of the day (Palin et al. 2009). The difference in social investigation protocols could result in different observation. The protocol utilized in this study may induce more stress to the test animal as the apparatus was not habituated as much as the home cage, and an adult animal was more threatening than a juvenile. However, the protocol used in this study designed a control chamber that can better address the preference toward the social mate and has demonstrated that the stress mentioned above did not affect the preference toward the social mate. Another possibility is that by the third infusion, the behavior reaction to TNF may not be as effective as the first infusion, as we found that the effect

of central TNF on OF/NO exploration was stronger in experiment 1 than the same test in experiment 2.

The first test used a familiar object and a novel object to access the long-term memory of the animals. No literature has addressed the effect of TNF-α on long-term-memory-related exploration. The significant effect of the test day factor demonstrated that both treatment groups successfully identified the novel object, reflected by the difference of the two trials using repeated measures ANOVA. However, both groups displayed neophobic behavior on the test day, and the TNF-α treated animals made less exploration around the novel object. This effect contradicts what we concluded in the open field with a large object presence: TNF-α did not alter the anxiety level, as the novel object introduced elicited greater approach in TNF animals. It may be, however, that the different effect of TNF-α in the object recognition test is due to changes in memory. TNF-α has been known to impair passive avoidance memory (Fiore et al., 2000) and cerebellar learning (Paredes, Acosta, Gemma, & Bickford, 2010). In addition, TNF-α mediates memory deficits after chronic LPS administration (Belarbi et al., 2012). How TNF-α altered memory is not clear yet and need further investigation.

Another possibility for reduced novel object exploration is the sensitization effect of TNF-α. When the object recognition test was scheduled as the first test (experiment 2), no significant change was observed to the first TNF-α infusion. However, repeated administration of TNF-α was reported to cause increased severity of the sickness behavior (Anisman et al., 2013). Although it should be noted that these experiments involved i.p. TNF-α treatment. Nonetheless, if the sensitization effect does occur, later

i.c.v. treatment effect on the NO phase of the OF test should be more salient when animals received a second infusion of TNF-α (experiment 2). We should either see a sickness-behavior-like exploration pattern in the first OF phase, or a stronger increase in the exploration in the NO phase. However, this result was similar but weaker compared to that seen in experiment 1. With this being said, it is unlikely that the result is mainly because of a sensitization effect.

In an ideal case, each behavioral test should follow a primary TNF-α infusion (i.e., no prior TNF exposures). However, the reverse order of testing in experiment 1 and 2 has addressed the major questions regarding order effect. Moreover, the anorexic effect has been well-documented in other literature in multiple species, and observing this after a second TNF-α infusion suggests that TNF-α continues to be biologically active.

The methyl green staining confirmed all the infusion was successful. Unfortunately, the study did not have the chance to measure the distribution of TNF-α in each brain region, such as the amygdala and hippocampus. It would be helpful to quantify the effect of TNF-α in order to confirm the critical brain regions that were affected by the infusion. Further analysis of the dose-effect could be addressed accordingly.

Overall, we do see an altered exploratory behavior pattern after the TNF-α central administration. The animals did not show an anxiety-like behavior in the OF test, but did display anorexic and altered exploratory behavior toward novel objects in this study.

CHAPTER 5
GENERAL DISCUSSION

The interaction between the CNS and immunity is now well accepted, and cytokines are recognized as members of the mediators that communicate between the two systems. As a fundamental part of the immune system, the adaptive immunity is not well-studied in terms of its effect on CNS. The current series of experiments pursued to expand on the understanding of the connection of the two systems by characterizing the cognitive changes of SEA and those of the downstream cytokine TNF-alpha. To that end, experiments in chapter 3 measured the effect of SEA systemic treatment on various conditions of novel object exploration whereas experiments in chapter 4 quantified changes following TNF-alpha central administration on novel stimuli exploration.

Collectively, we found that SEA did not impair memory retrieval of a familiar object, and it affected the exploratory behavior toward novel stimuli under given conditions. Animals treated with SEA were found to have more immobile episodes and time when exposed to a novel object together with an object that they explored on the previous day. Increasing the training trials to the familiar object removed the effect of SEA on immobility. Additionally, older mice were more sensitive to SEA treatment and had a reduction in the exploration of a novel object in a familiar environment, but the effect was not observed in younger mice. The conclusion contributes to unraveling the impact of T cells on learning and memory, and it is to the best of our knowledge the first demonstration of adaptive immune activation influenced novelty exploration. In addition,

the current study developed a new protocol to test the effect of long-term object memory in mice.

We also found that the administration of TNF-α created a complex behavioral effect toward novel stimuli including reduced novel food intake and altered exploration pattern to novel stimuli. When exposed to an empty open field apparatus and then tested with a large novel object in the center of the apparatus (OF/NO test), TNF-α infusion increased exploratory behavior to the object. However, when the same animals were tested using both a familiar apparatus and a familiar object together with a novel object, the animals' exploratory behavior toward the novel object showed a reduction in the observation zone. Changing the order of the experiments resulted in a weaker effect, but did not make a dramatic difference in the conclusion. The results from the current study added to the understanding of TNF-α modifying cognitive functions, and it shed lights on the mechanism that SEA affects the decision-making process of the brain.

The current study found the effect of TNF-α on novel object exploration does not fully overlap with the SEA effect in the OF/NO test. A previous study of our lab observed SEA had an effect on the NO phase of the OF/NO test (Kawashima & Kusnecov, 2002). SEA treated group was not different in the OF phase. However, in the NO phase of the test, animals received 5 ug SEA treatment were less exploratory and made less line-crossing, which is in an opposite change direction compared with the TNF-α effect we observe in this study.

It should be noted that besides TNF-α, several other cytokines are released following systemic SEA treatment. Although TNF-α is necessary for inducing neophobic

effect by SEA treatment (Rossi-George et al., 2005), SEA treatment induces cellular immune changes that shift the homeostasis of the immune system with a complex molecular level adjustment. Besides TNF-α, IL-2, IL-6 and IFN-γ levels were also found elevated after SEA administration (Pichereau et al., 2011, Carlsson & Sjögren, 1985). All of the cytokines can act on the CNS and induce behavioral impacts. IL-2 modulates dopamine activity that induces climbing behavior and motor activity (Zalcman, 2002; Zalcman et al., 1998). IL-6 was found to modulate ambulation, rearing, digging and grooming in BALB/c mice moved to a new shoe box (Zalcman et al., 1998). IFN-γ derived from meningeal T cells was associated with increased tonic GABAergic inhibition in projection neurons and influence social behaviors (Filiano et al., 2016). The behavioral changes following SEA treatment is a combination of the effect of the mentioned cytokines, activating various pathways. TNF-α may be the key factor in inducing anorexic behavior, but it could be other cytokines that mediate the effect of SEA in the OF/NO test. Further studies should address the roles of other cytokines in mediating the behavioral effect of SEA. And an interaction effect among the cytokines should be considered as well.

Meanwhile, the process that SEA affects the CNS function could go through other indirect mechanisms besides cytokines directly impacting HPA axis. The vagus nerve could affect the HPA axis from the periphery (Dantzer., 2001), and the microglial cells resident in the CNS can be activated by SEA treatment in vitro, and they upregulate IL-1 and TNF-α production in the CNS (Dantzer., 2001, Vidlak et al., 2011). Systemic treatment of SEA would induce molecular and cellular changes affecting both the periphery and the brain. And the periphery changes could end up-regulating multiple loci

of the brain. Vagotomy experiments could be performed to study the SEA treatment and the vagus nerve, in which the vagus nerves were sectioned under the diaphragm so as not to compromise cardiac and pulmonary function (Dantzler, 2009). The periphery cytokine effect, on the other hand, could be addressed either by marking the periphery cytokine with an isotope such as Deuterium and track their concentration in the brain, and control the activation of microglia cells.

With all that being said, it does not necessarily mean that the effect of SEA in the NO phase is not related to TNF-α. It should be noted that SEA effect in the OF/NO test is dose-dependent: the reduction of exploration was observed in animals treated with 5ug of SEA, but not in the 1ug treatment groups. The difference in the dose of SEA may result in a difference in the amount of TNF-α released by T cells. A difference in the concentration of TNF-α may induce different behavior response. The concentration of TNF-α in the CNS 2hrs after 1ug and 5ug SEA i.p. treatment hasn't been quantified, but it will be very helpful to resolve the difference of the behavioral result found in this experiment. It should be noted that the TNF-α concentration in human CSF is 8.62 ± 3.05 pg/mL (Bromander et al., 2012). The highest TNF-α level detected in rat brain after meningitis induced by Streptococcus pneumoniae was around 200pg/100mg of tissue in the hippocampus and frontal cortex (Barichello et al., 2009). We adopted the amount of 200ng TNF-α per mouse for infusion. To better estimate the dose difference, it is suggested that we measure the concentration of TNF-alpha after the infusion was completed. It is also suggested to test the dose effect and use multiple doses of TNF infusion to testify which dose get close to 200pg/100mg, the natural infection response mentioned above.

Nonetheless, TNF-α i.c.v. infusion altered animal's exploratory behavior in the OF and novel food consumption test. Additionally, multiple studies have demonstrated the increase of TNF-α induced by SEA both in vitro and in vivo (Yan, Yang, Neill & Jett, 1999; Huang, Lin & Won, 1997), however, the distribution of TNF-α level change in the brain after SEA treatment was not clear yet. A systemic investigation of the concentration changes of TNF-α in the periphery and critical brain regions following SEA treatment is beyond the scope of the present study, but should be examined in future experiments. To better address the influence of SEA on the brain, the concentration of TNF-α and possibly the TNF-α receptor should be quantified in the brain regions including hippocampus, amygdala, hypothalamus and prefrontal cortex. With that information, we would generate a hypothesis on the likelihood of SEA effect being carried out via TNF-pathway.

Another way also exists to explore whether TNF-α participates in the SEA effect in the OF test (Kawashima & Kusnecov, 2002). It has been proved that the anorexic effect of TNF-α was not observed in TNF-α knock-out mice, which demonstrate the necessity of TNF-α in conducting neophobia to novel food (Rossi-George et al., 2005). Further research can address this question by blocking TNF-α synthesis centrally, or using similar TNF -/- mice and test the animals in experiments including OF and OR.

Future studies could focus on the effect of TNF in the OF/NO test. The OF/NO test has been used for measuring the level of anxiety. The result of the test showed that animals were not different in the anxiety level and the reason that animals showed increased exploration to the novel object needs further investigation. In experiment 3c, animals in group E habituated to an empty OF apparatus and then test with a novel object

should decrease exploration to the novel object compared with animals in group Fam/Nov, which habituated to an OF apparatus with a familiar object. This indicated that the appearance of an object may be stressful to the animals in experiment 3c group E. Similarly, the appearance of the object in the OF/NO test of chapter 4 should be stress-inducing as well. Yet animals treated with TNF-alpha showed increased exploration to the object. The effectiveness of our OF/NO protocol can be tested by using animals treated with drugs for anxiety disorder, and check on their exploratory behavior pattern to the novel object. Animals under acute stress should also be tested as well. If a similar behavior pattern were observed in the animals received anxiety treatment and animals under acute stress showed less exploration, then we should reconsider the role that HPA axis played in this experiment.

Another future experiment addressing the role of the HPA axis in the OF/NO test could involve blocking the effect of CRH receptors. In previous studies (Kaneta and Kusnecov, 2005), CRH receptor blockage abrogated anorexia induced by SEA. It is also known that IL-1 can promote the secretion of CRH from neurons in the paraventricular nucleus of the hypothalamus (Besedovsky, 1996). In addition, a similar study using the same OF/NO protocol with IL-1 i.c.v. infusion, showed a similar exploration pattern as TNF in this test (data unpublished from our lab). As the phenomenon appeared to be similar, and both IL-1 and TNF can activate the HPA axis, downstream elements of the HPA axis (eg., glucocorticoids) may play an essential role in affecting behavior. Therefore, the involvement of CRH in the TNF induced behavior change in the OF/NO test should be examined: CRH may be necessary for the effect of TNF in the OF/NO test.

For such an experimental approach, the same surgery and habituation protocol can be adopted as in the OF/NO test in Chapter 4. On the test day, a test group will receive non-selective receptor antagonist, αhCRH9–41, or Astressin, i.c.v. prior to the infusion of TNF. Control groups will be treated with aCSF before the TNF infusion. Animals will be tested in the OF/NO test, and the location of the cannula, as well as the diffusion of the antagonist, will be checked post-mortem. By comparing the exploratory behavior of the control and test groups, the effect of CRH in mediating TNF effect will be verified.

The neophobic effect in the novel food test should be verified in future experiments as well. Animals should be tested for food preference using a familiar diet to compare with the novel diet. This experiment will ascertain whether the reduction of prosobee intake was due to avoidance of novel food, or loss of appetite. Animals in control groups will be subjected to prosobee for three days, and the test animals will have water in the prosobee container to control for the novelty of the experimental apparatus. The same protocol will be used for surgery, habituation, infusion of TNF and behavioral test. Data of exploration, as well as the amount of prosobee consumption, will be compared. If both the control group and the test group experienced the same level of reduction in food intake, the result in the novel food test of chapter 4 is due to loss of appetite. If animals that were habituated to prosobee consumed more food compared with the animals that received water in the habituation phase, the reduction of prosobee intake is likely due to the novelty of prosobee.

The anorexic effect induced by TNF-α and SEA could share the same pathway including the amygdala and hypothalamus. Previous research found that SEB increases

hypothalamic and amygdaloid expression of CRH mRNA and emotional reactivity to novelty (Kusnecov, Liang and Shurin, 1999). The activation of the HPA axis could induce the behavioral changes in chapter 3 studies as well. Experiment examine the level of TNF, CRH, corticosterone in the amygdala and hypothalamus could be done by measuring the concentration of the molecules in each brain region first, and then closely quantified with immunohistochemistry. The importance of CRH and TNF-α could also be addressed by blocking the receptors i.c.v.. To better address the molecular changes and brain regions involved in the novel food neophobia, the corticosterone concentration is suggested to be measured as well.

The current study added to the understanding of the interaction between immune system and the brain: SEA being a model of T cell activation affects the exploration pattern toward a novel object stimulus, and TNF-α, the cytokine that released as a messenger can affect the cognitive reaction to novel stimuli. Although the whole picture of how the two systems work together is still covered, we could interpret the results as the immune system reports to the CNS with the body's health condition. Cytokine receptors enable the brain to take immune activity as a factor in the cognitive process, and adapt the decision of exploratory behavior accordingly. The effect of immune activation on cognition may not simply be evaluated as promoted or impaired learning and memory, but it could rather take a detour in the cognitive process to generate the behavior pattern that best suit the sickness situation. For example, in the object recognition test, the SEA treated animals may take more time to reflect on the information just collected before making the next move. Similarly, in the previous study of our lab, we did not find a difference between the learning ability of SEA treated mice

and the control group in Morris water maze. However, the strategy they used to accomplish spatial learning may be different, and they may locate the hidden platform using a different route. This was not examined due to a technical limitations at the time of the study, but should be considered.

In a natural situation, the course of Staphylococcal aureus infection usually lasts for several days, and the synthesis of TNF-alpha is not an instant large dose, but rather a constant release that lasts for longer time. In the case of Legionella pneumophila, the elevation of TNF-alpha was detected in mice lung lavage fluid up to 48 hours after the bacteria was introduced into the lung (Blanchard et al., 1987). It is beyond the current study to predict how a constant increase of TNF-alpha would alter animals' cognition and behavior. To better mimic this process, it is suggested to test the long-lasting effect of SEA and TNF-alpha. Long-term infusion or multiple treatments could be given for consecutive days, and both the acquisition and memory retrieval performance can be examined in separate experiments. Additionally, the behavioral change should not simply be quantified as an increase or decrease, but considered a multi-dimensional exploration pattern.

The current study focused on the T-cell-mediated adaptive immune response induced cognitive change. It should be noted that in this model of SEA treatment, a larger group of T cell is activated comparing with it of specific-recognition. Consequently, the impair of the immune activation on the brain could be more influential than in immune activity of specific antigen recognition. The observation in the cognitive changes of the

current SEA studies may not be observed or weaker in other pathogen related immune activity.

Among the four studies in chapter 4, the novel food consumption test showed the most salient effect of TNF-treatment. Its effect in the open field/novel object test was more powerful when running as the first experiment but was weaker when run as the second one. The novel food consumption test was run as the second test in experiment 1 but still showed a strong effect of TNF. The reason could be the nature of the novel food test more sensitive to the TNF treatment, just as the biological preparedness in classical conditioning. Food is digested with limited ways to fully reverse the process, and poisonous food can cause death. Thus animals may be more cautious and reluctant to explore and try novel food. On the other hand, a novel object or social mate that could be abandoned or escaped anytime during the exploration is less dangerous. For an animal that is sick and weaker than their normal status, it is better to be more alarmed to a novel food that could cause death even for a healthy animal.

A previous experiment of our lab has demonstrated that TNF-alpha is necessary for the SEA induced anorexic behavior with TNF -/- animals (Rossi-George et. al., 2006). The current study aimed to demonstrate the role that central TNF plays in inducing a similar anorexic effect of SEA. Besides the question of loss of appetite mentioned in the discussion in the previous chapter, there is not enough evidence to claim that TNF-alpha is sufficient to induce the anorexic effect of SEA. There may be different mechanisms and pathways that mediated similar behavior effect. For example, IL-1 could induce a

reduction of novel food consumption, yet no elevation of IL-1 level was detected following SEA treatment in the previous study of our lab.

In conclusion, T-cell-mediated immune activation and TNF-α treatment are valid and valuable models for studying the effects of immune challenge on exploratory behavioral alterations. The current research contributed to unraveling the interaction between the brain and the immune system. We did not observe acute systemic delivered SEA or central TNF-α treatment affected the motor ability of mice. However, both treatment altered the exploratory pattern of the animals.

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Tables

Table 1: Experimental conditions and group assignments for Experiment 3c

Group	Number of animals (N)	Treatment Day1-Day9	Treatment Day 10
A	6	Exposure to object M	Exposure to object G
B	6	Exposure to object G	Exposure to object M
C	6	Exposure to object M	Exposure to object M
D	6	Exposure to object G	Exposure to object G
E	6	Exposure to experimental apparatus only	Exposure to object G
F	6	No Stimuli: Home-caged in the colony room	Exposure to object G

Table 2. Parameters and t-test results in the novel object (NO) phase of the open field test in Chapter 3, Experiment 2 (reverse order open field test)

Parameter	Treatment	Mean	t value	P value	significance
NO; Distance	TNF	12.115625	**-1.837**	**.087**	**n.s.**
	CSF	15.519625			
NO; Center: time	TNF	36.187500	.526	.607	n.s.
	CSF	26.287500			
NO; Center : distance	TNF	1.991375	.721	.483	n.s.
	CSF	1.317125			
NO; Corner: time	TNF	125.050000	0	1	n.s.
	CSF	125.062500			
NO; Corner : entries	TNF	16.25	**-2.838**	**.013**	**Sig.**
	CSF	24.00			
NO; Corner : distance	TNF	1.963375	**-2.242**	**.042**	**Sig.**
	CSF	2.750125			
NO; Border : time	TNF	114.425000	1.421	.177	n.s.
	CSF	121.987500			
NO; Border : entries	TNF	27.63	**-2.171**	**.048**	**Sig.**
	CSF	36.75			
NO; Border : distance	TNF	6.011375	**-2.481**	**.026**	**Sig.**
	CSF	8.682500			
NO; Observation : distance	TNF	2.149625	-1.311	.211	n.s.
	CSF	2.769750			
NO; Observation : time	TNF	23.312500	-.504	.622	n.s.
	CSF	25.675000			
NO; Observation : entrance	TNF	20.00	-.456	.655	n.s.
	CSF	21.75			

The TNF-α treated animals showed less exploration in the corner and border zone, but did not differ in the center and observation zone compared with the aCSF infused group, reflected by the distance and entries into the four zones ($p < 0.05$).

Figures

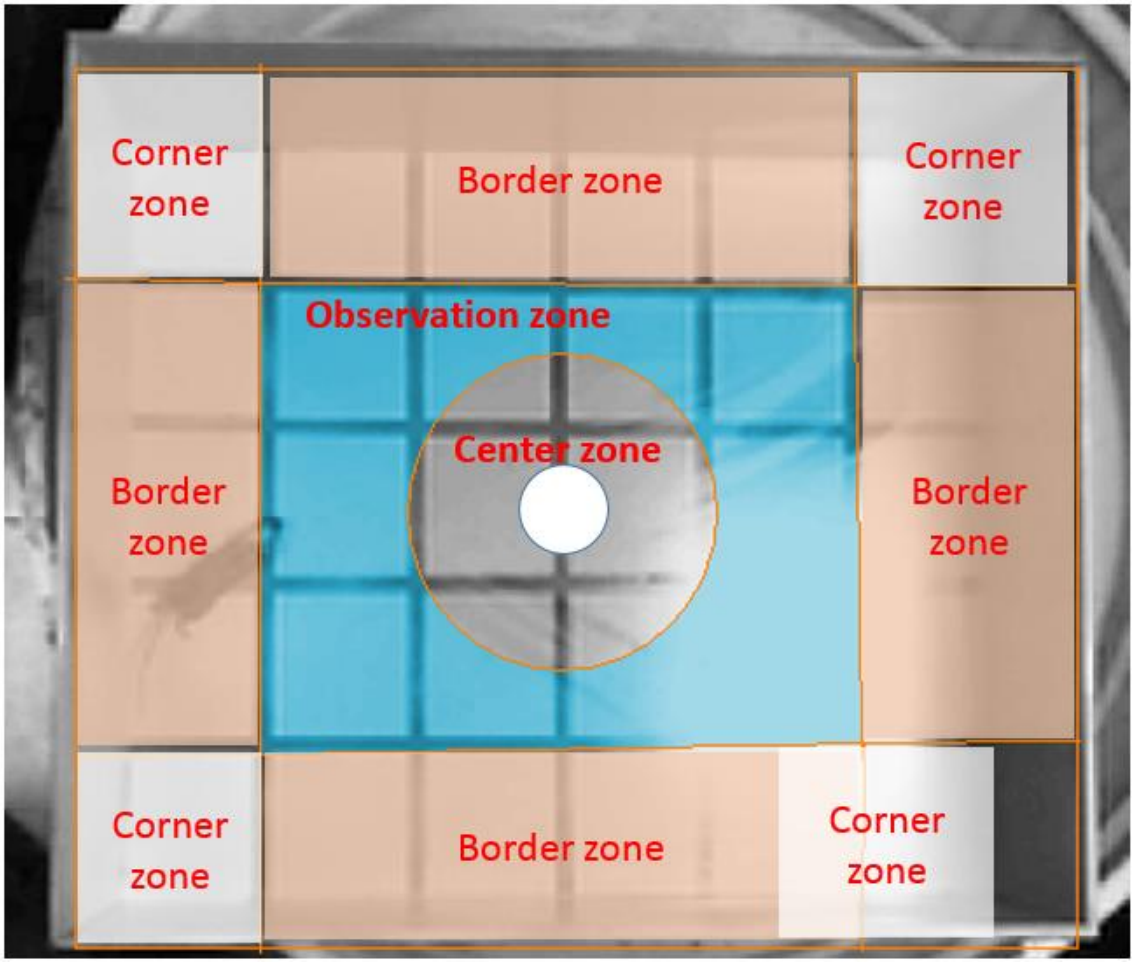


Figure 1. The division of the open field apparatus using ANY-maze for tracking animals' behavior. The apparatus map was divided into four zones, named center zone (in clear background of the figure), corner zone (white background), border zone (pink background) and observation zone (blue background), to analyze the exploration pattern of the animal. Specific behavior parameters including exploration time, distance traveled, zone entrance and mean distance toward a specific zone were computed for each zone for both phases.

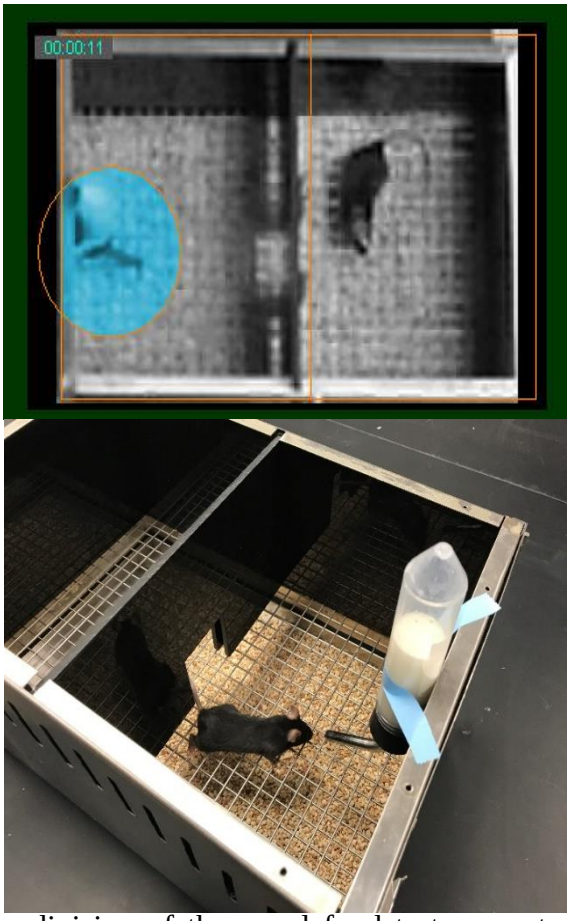


Figure 2. The division of the novel food test apparatus using ANY-maze for tracking animals' behavior. The apparatus map was divided according to the two test chambers as exploration zone and control zone, with a bottle zone on top of the exploration zone to measure the approach and time spent around the bottle. The number of contacts and the time consuming prosobee was separately quantified manually using the test video. The ANY-maze software computed specific behavior parameters including exploration time, distance traveled, zone entrance and the mean distance toward a specific zone.

Figure 1. The division of the open field apparatus using ANY-maze for tracking animals' behavior. The apparatus map was divided into four zones, named center zone (in clear background of the figure), corner zone (white background), border zone (pink background) and observation zone (blue background), to analyze the exploration pattern of the animal. Specific behavior parameters including exploration time, distance traveled, zone entrance and mean distance toward a specific zone were computed for each zone for both phases.

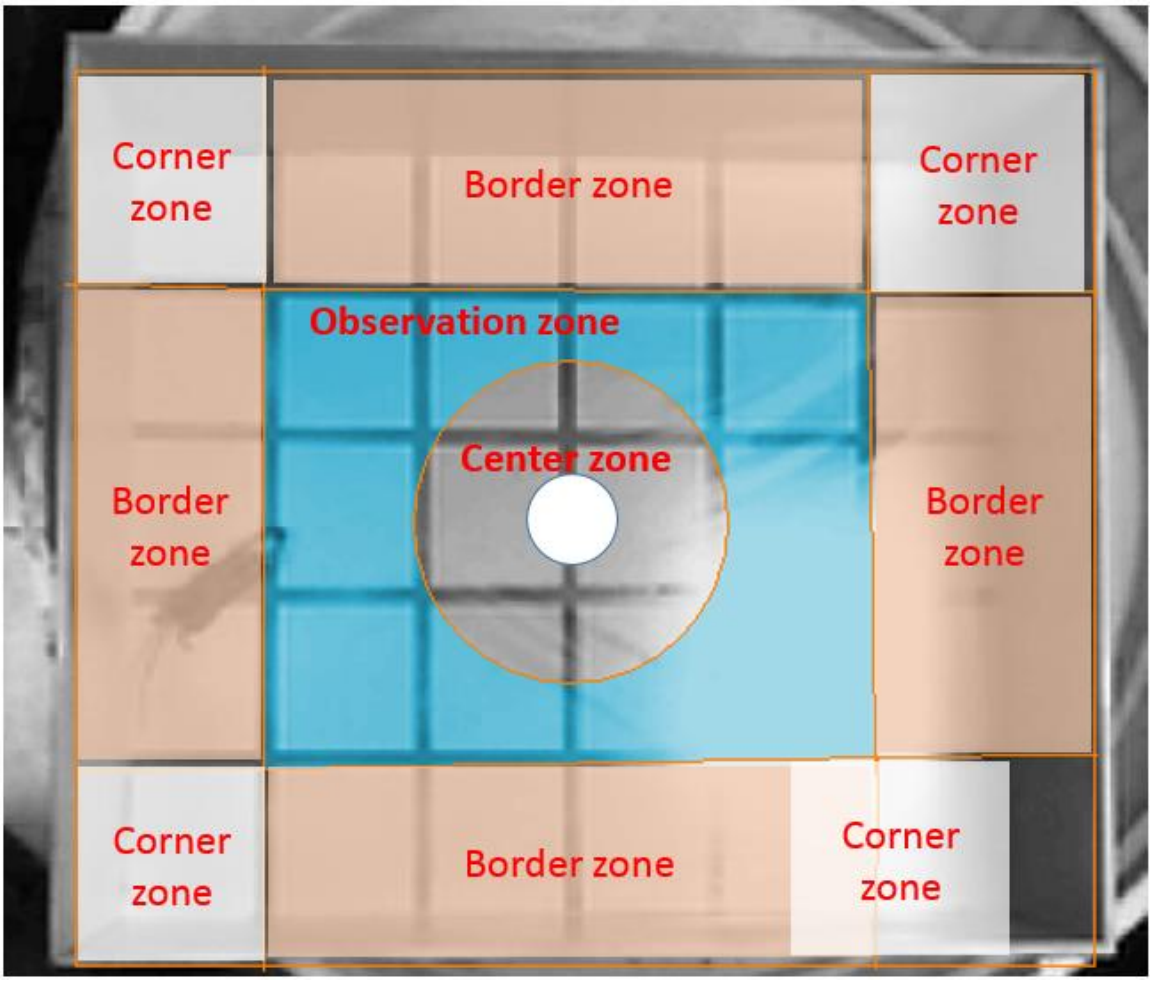

Corner zone
Border zone
Corner zone
Observation zone
Center zone
Border zone
Border zone
Corner zone
Border zone
Corner zone

Figure 3. The division of the social interaction map using ANY-maze for tracking animals' behavior. The apparatus map was divided into four zones, named center zone (the starting chamber that separates the test and control chambers), animal chamber (the chamber that contains the social target), control chamber (the chamber that has the empty cup). Two zones named test and blank were on top of the animal chamber zone and control chamber zone to quantify the behavior of the test animal around the cages (zones shown in blue). Specific behavior parameters included exploration time, distance traveled, zone entrance and mean distance toward a specific zone.

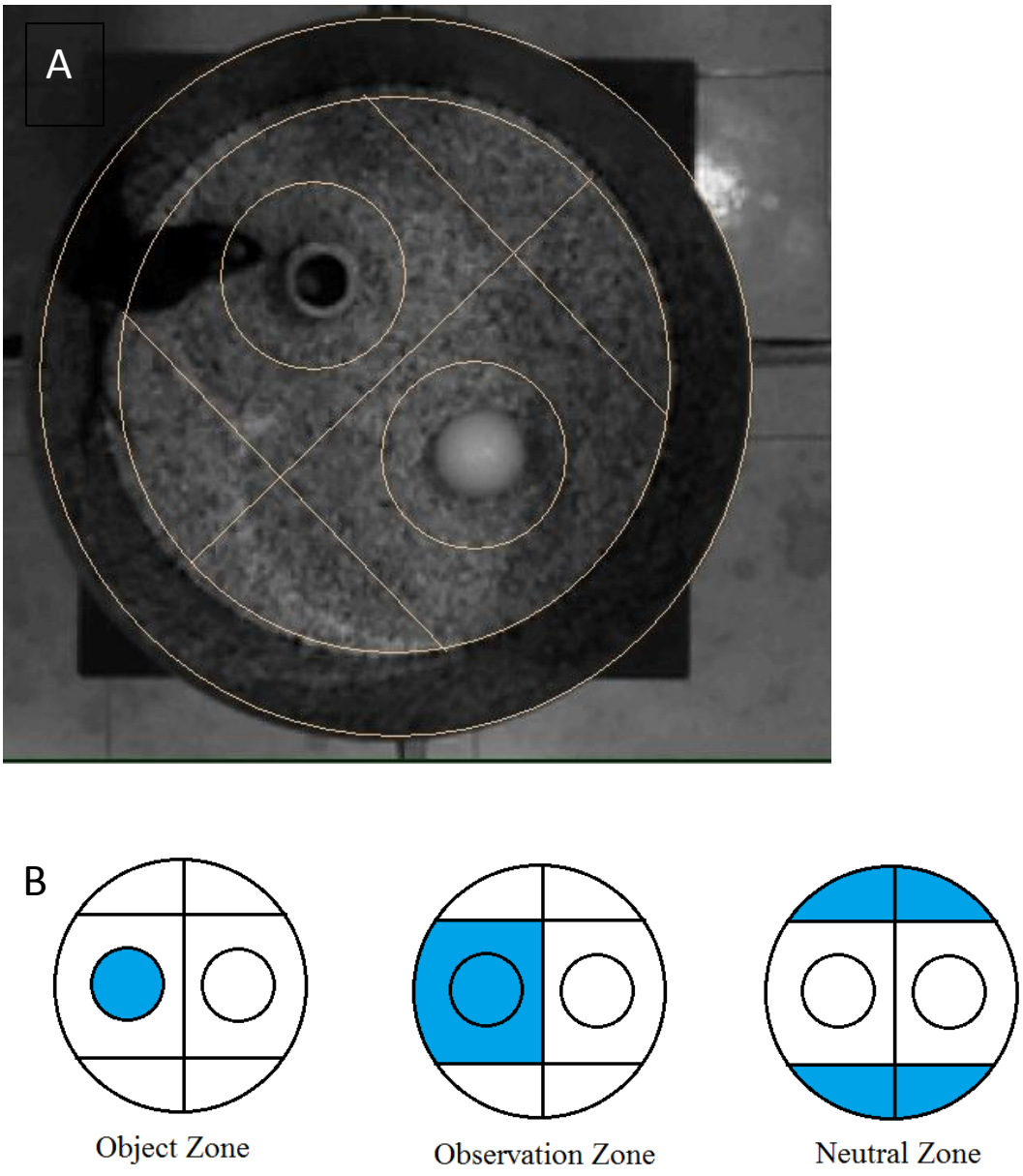
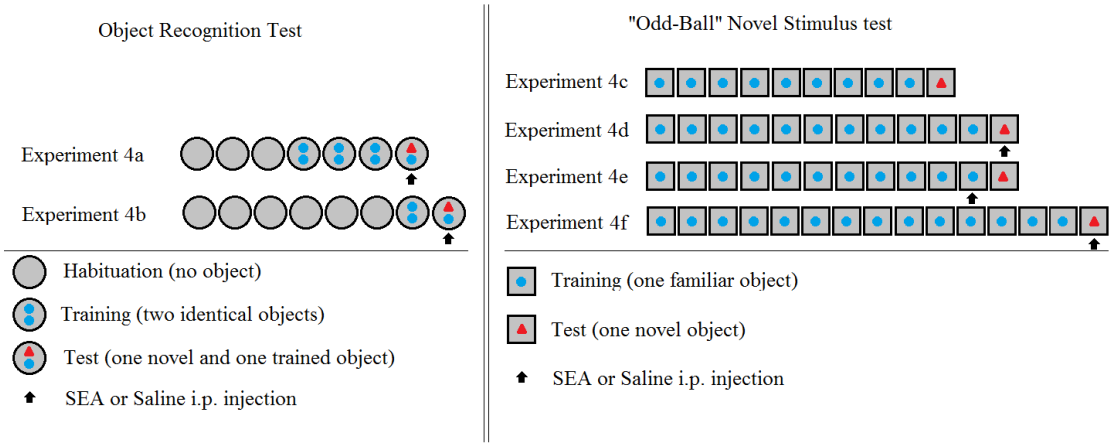


Figure 4. The division of the object recognition apparatus map using ANY-maze for tracking animals' behavior. The apparatus map was divided into three zones, named neutral zone (the two crescent zones next to the border zone), observation zone 1 (include object zone A), observation zone 2 (include object zone B). 4a. The actual size of the zones relative to the animal. 4b. The identification of the object zone, the observation zone and the neutral zone. The two object zones A and B contain the object at the center of the zones. The location of the novel object is counterbalanced. Specific behavior parameters including exploration time, distance traveled, zone entrance and mean distance toward a certain zone were computed for each zone for both phases.



Summary of SEA experiment design in chapter 3 (need a clearer form)

Experiment name	Apparatus	# of object In each trial	Habituation (No object)	Familiarization (Same object)	Test	Number of mice
Experiment 3a	Small round arena	2	3d	3d	1d	12
Experiment 3b	Small round arena	2	6d	1d	1d	13
Experiment 3d-3f	Open field	1	NA	6-14d	1d	13+12++12 +10

Figure 5. The summary of SEA experiment design in chapter 3. No preference was found between the two objects.

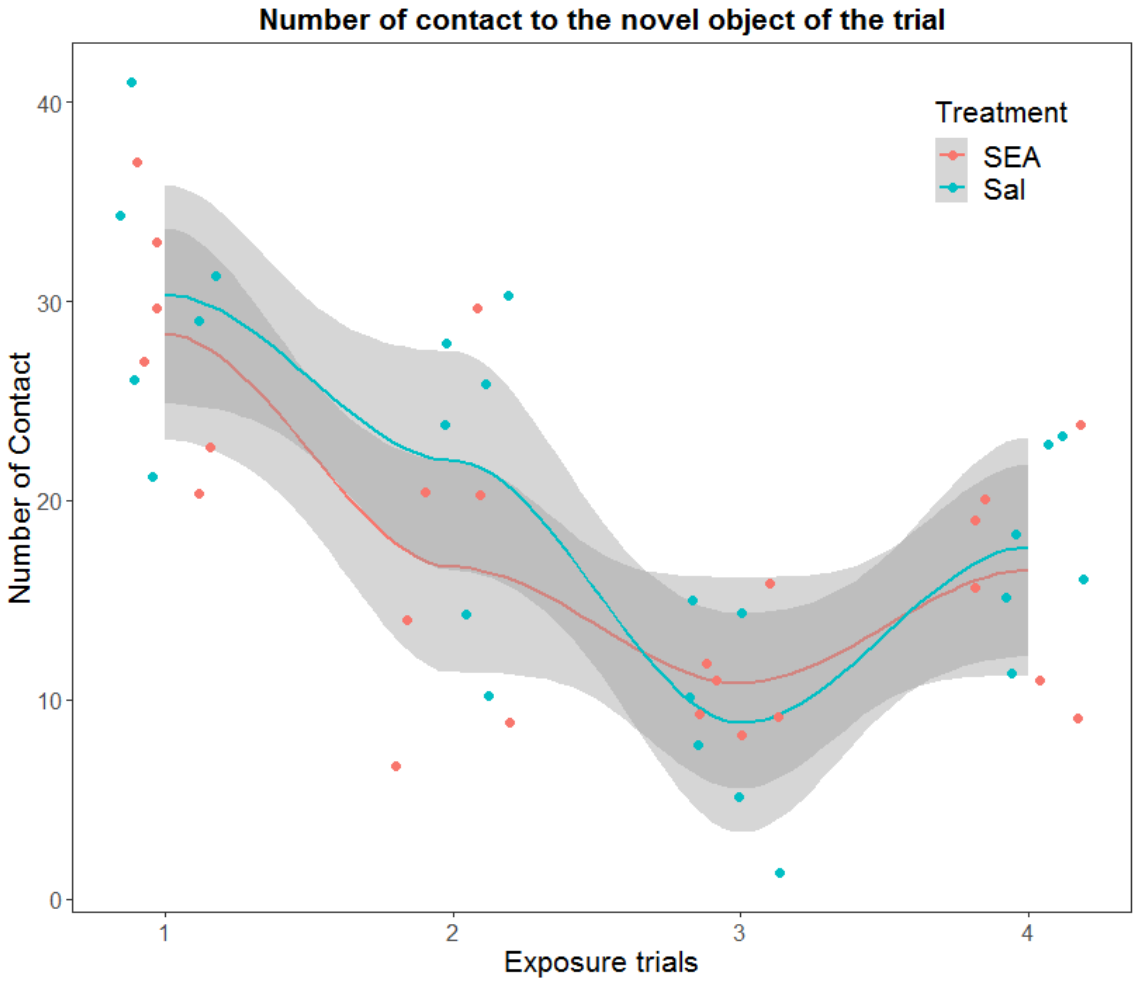


Figure 6. The number of contact to the novel object by the two treatment groups on each day in experiment 3a. The error band was calculated together with the means of both treatment groups. All data points were shown in the figure.

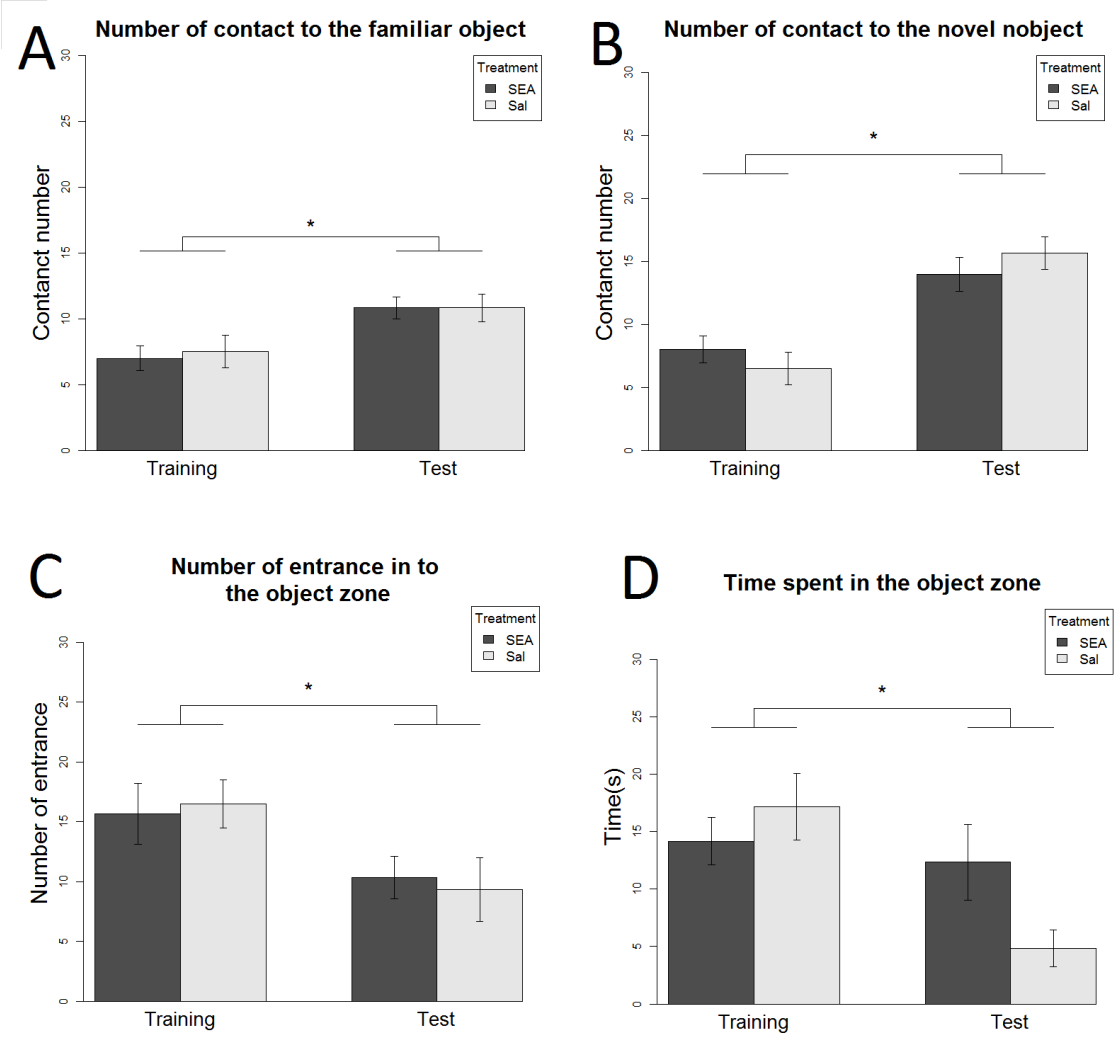


Figure 7. Summary for the last training trial and the test trial in experiment 3a for selected behavior parameters relative to exploration comparing the SEA treated animals (N=6), and the saline-treated animals (N=6). **Data was collected by ANY-maze tracking software. Bars represent mean ±1SEM. * = significantly different from the training day and the test day by comparing the within-subject variable.** (A)The number of contact toward the familiar object; (B) Number of contact to the novel object; (C) The average number of entrances test animals made into the novel object regions; (D) Time spent in the novel object regions (border effect, p = 0.076).

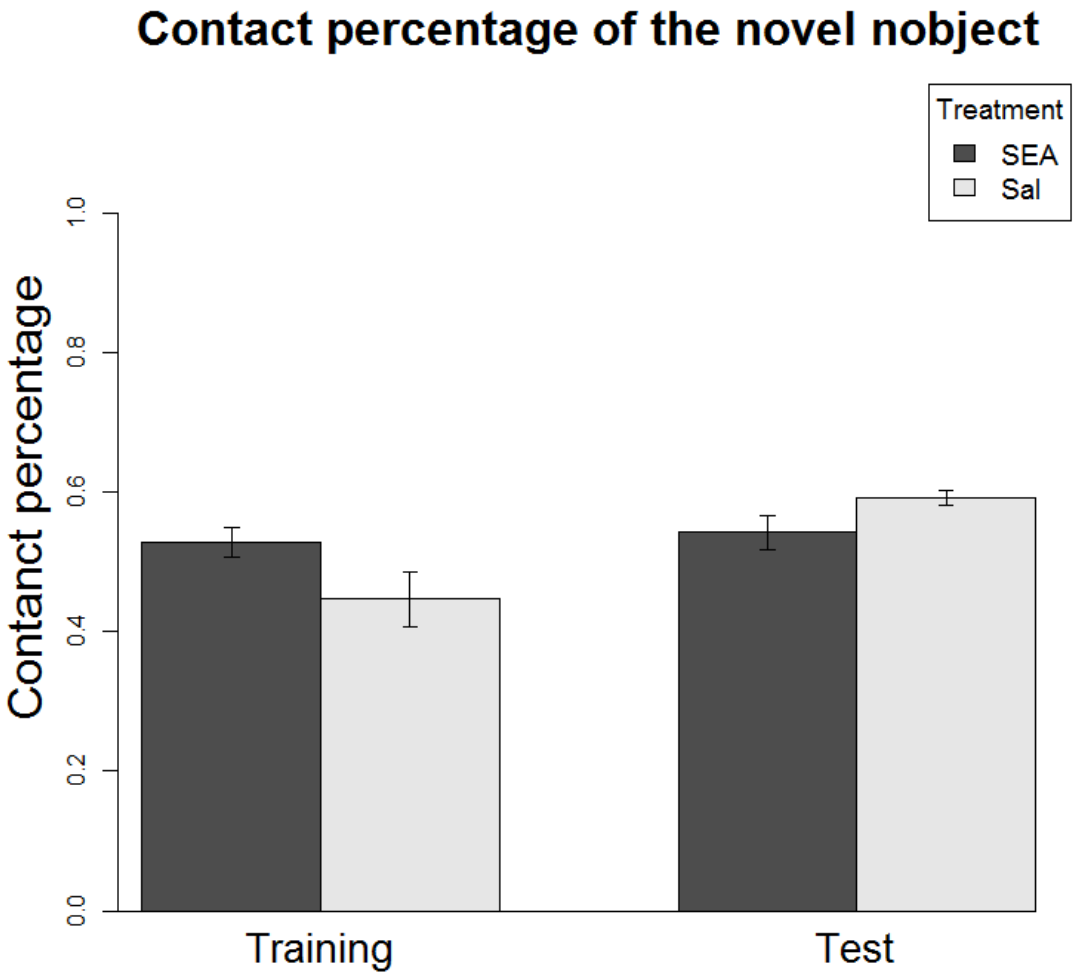


Figure 8. The calculated *novel object contacts percentage* in experiment 3a. The novel object contact percentage on the last training day and test day. Note that contacts on 'Training' day is for the object in the location where a novel object is placed on the 'Test' day. The percentage is calculated to show the preference to the novel object. No treatment effect was found ($p = 0.07$). Bars represent mean ±1SEM.

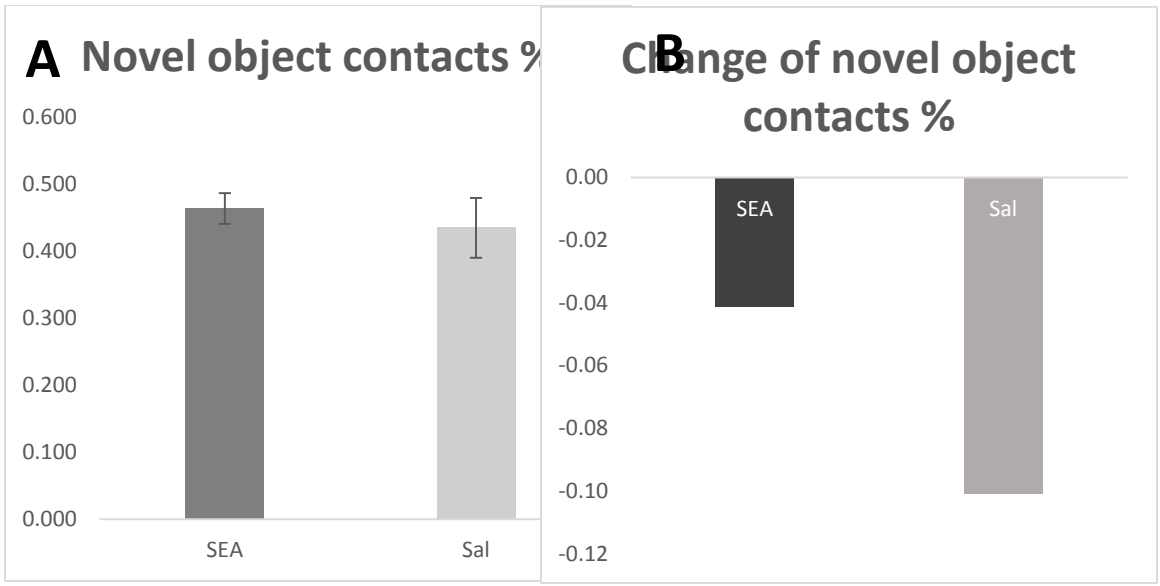


Figure 9. The calculated *novel object contacts percentage* in experiment 3b. A. The novel object contact percentage on the test day. B. The change of the test day comparing with the previous training day. The day factor significantly decreased contacts to the novel object ($p < 0.05$). An interaction treatment effect was found ($p < 0.05$). Data manually entered into ANY-maze tracking software by experimenter blind to treatments. Bars represent mean ±1SEM.

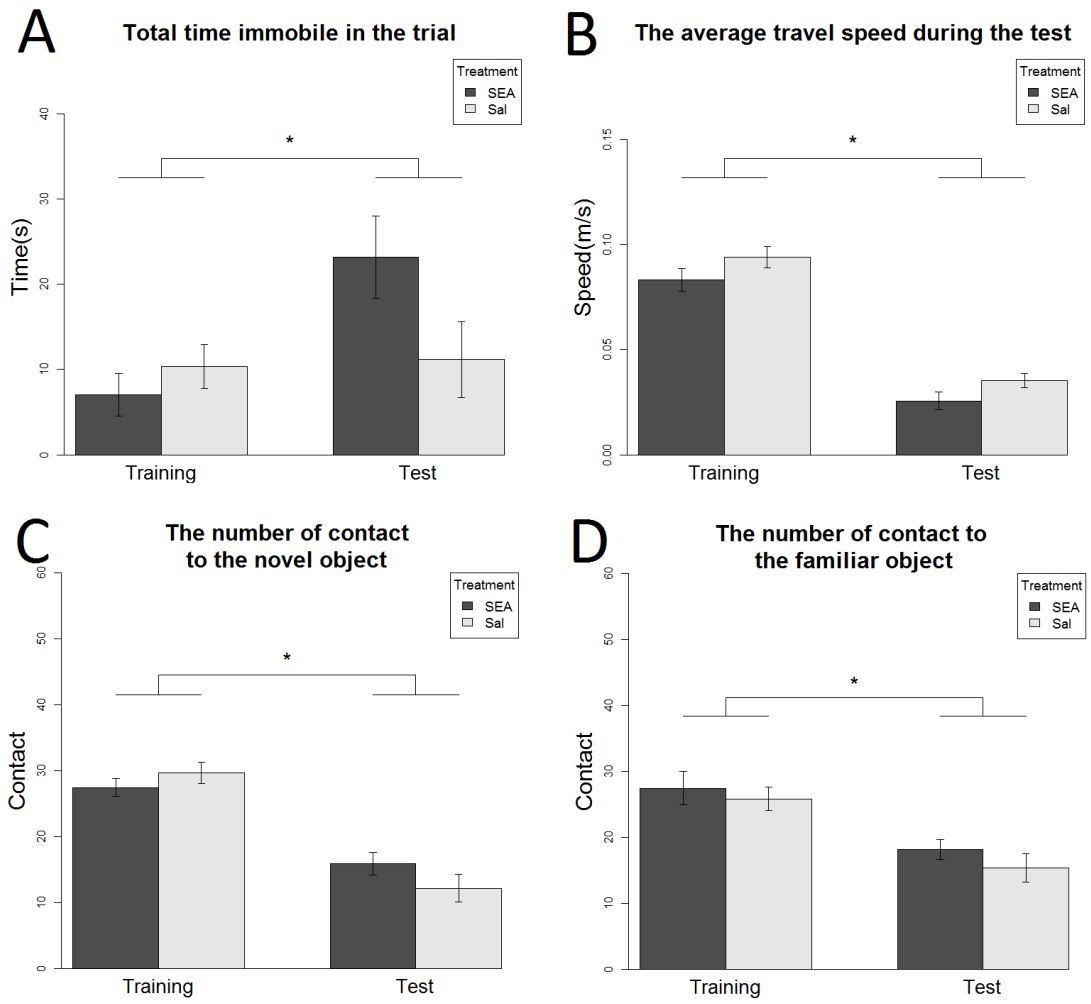


Figure 10. Summary for the training trial and the test trial in experiment 3b for selected behavior parameters relative to exploration comparing the SEA treatment animals (N=6) and the saline-treated animals (N=6). **Data was collected by ANY-maze tracking software. Bars represent mean ±1SEM. * = significantly different from the training day and the test day** (A)Total immobile time during the trial. The immobile time is calculated by adding the time periods that animals were detected immobile over 1s; (B) The average traveling speed of the test animal; (C) The average number of contacts made to the novel object regions; (D) The average number of contacts made to the novel object regions.

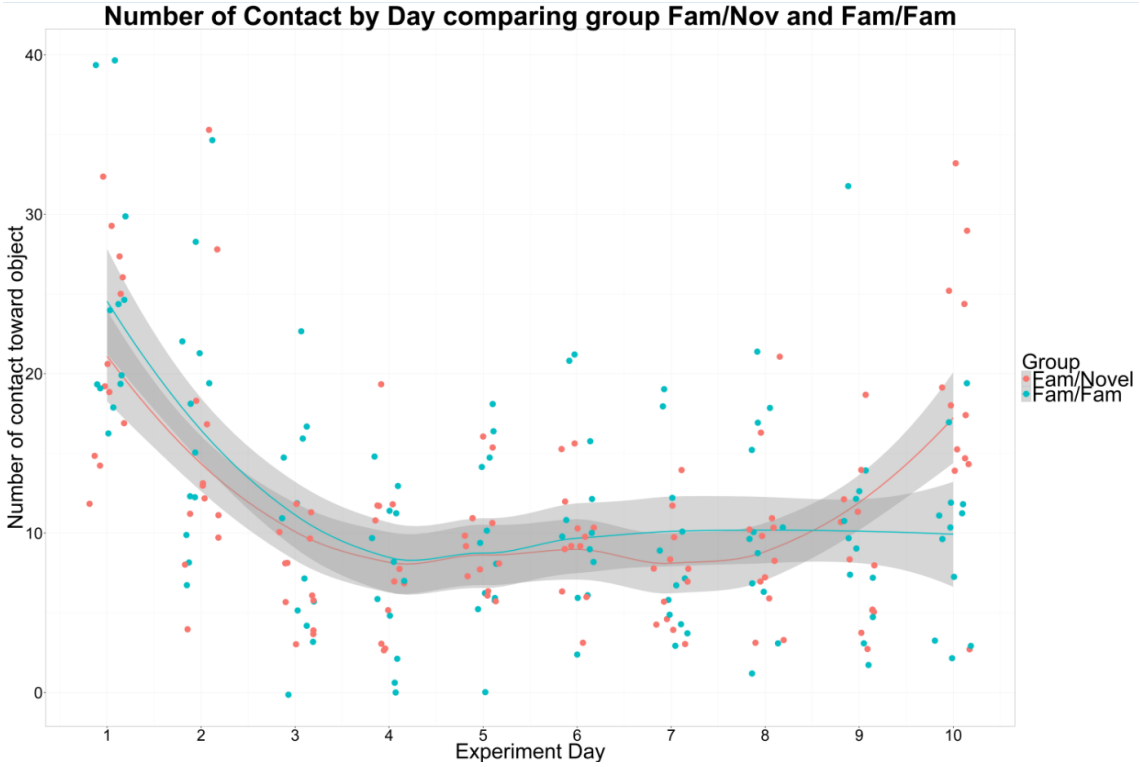


Figure 11. The habituation curve addressing the number of contacts toward the object during the training and test sessions of experiment 3c that was designed to establish the "odd ball" protocol. Exploratory behavior reached plateau on day 4. All data points were shown as well as the standard error band. No difference was found among the four groups during the training phase. Group A and B (M-G and G-M) were combined and shown as group Fam/Novel, and group C and D (M-M and G-G) was combined and shown as group Fam/Fam. It is evident that on the substitution day (Day 10), when a new object is introduced (the "odd ball"), animals showed greater interaction with the object.

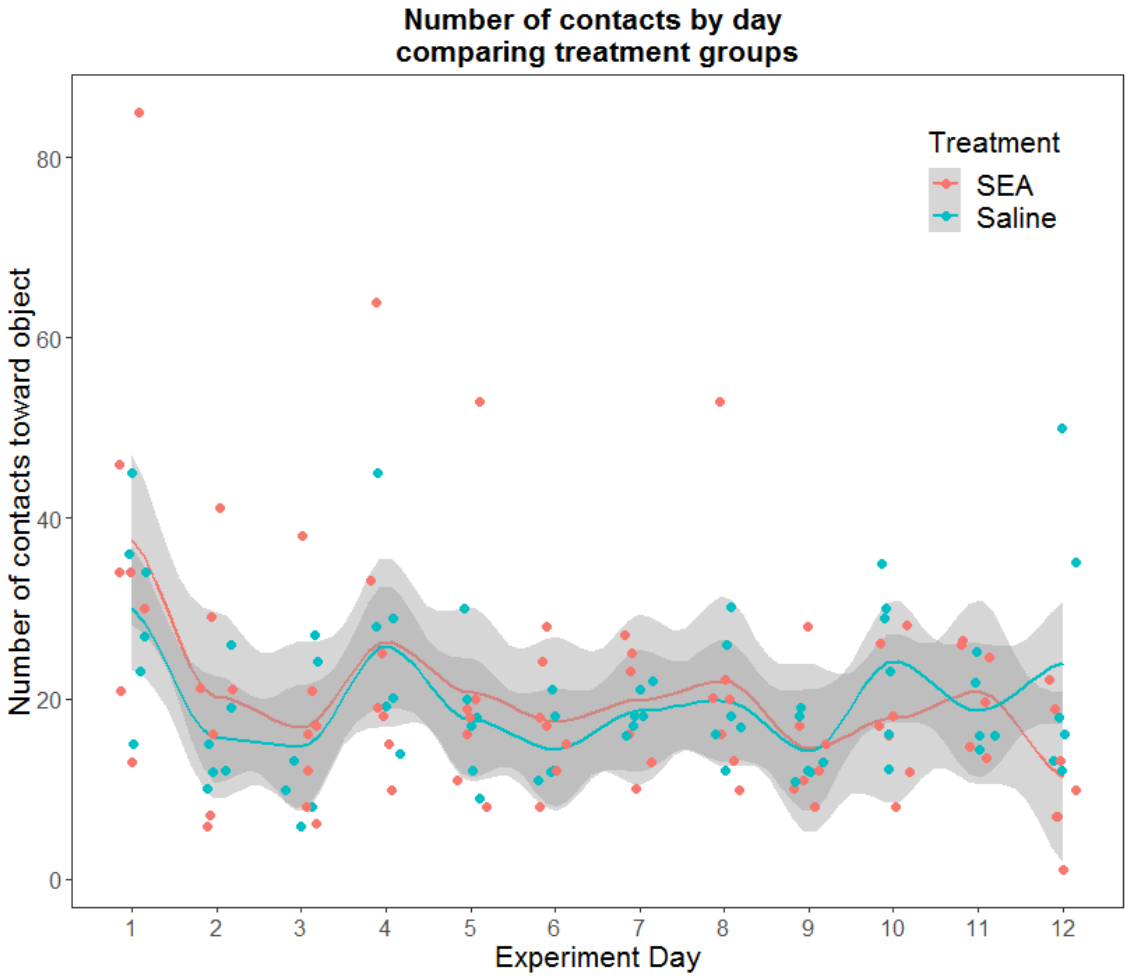


Figure 12. The number of contacts over the experiment days by the two treatments (saline and SEA) in experiment 3d. The error band was calculated together with the means±1SEM of both treatment groups. All data points were shown in the figure. The outlying data points are not the reading of the same animal. No outlier is identified. On Day 12, randomly selected animals were treated with either saline or SEA and presented with the novel object. Saline animals showed greater exploration of the object than SEA-treated animals.

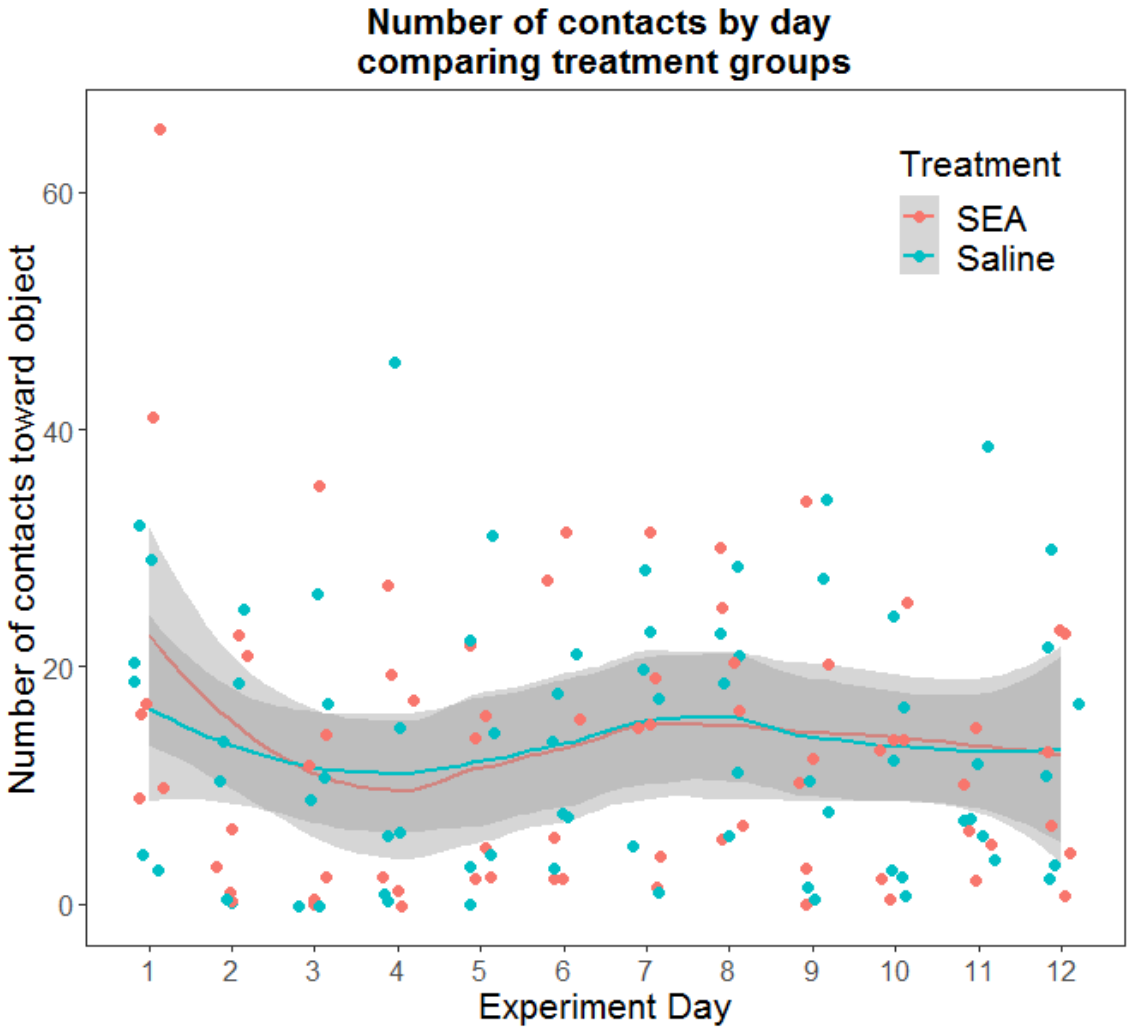


Figure 13. The number of contacts by the two treatment groups on each day in experiment 3e. The error band was calculated together with the means of both treatment groups. All data points were shown in the figure. On Day 11, animals were randomly chosen for Saline or SEA treatment and exposed to the same object (familiar) as on the previous days. There was no change interaction with the familiar object. On Day 12, a novel object was introduced, in place of the previously familiar object. There were no treatment effects exploration with the novel object, suggesting that 24 hours after SEA treatment, there is no reduction in novel object exploration.

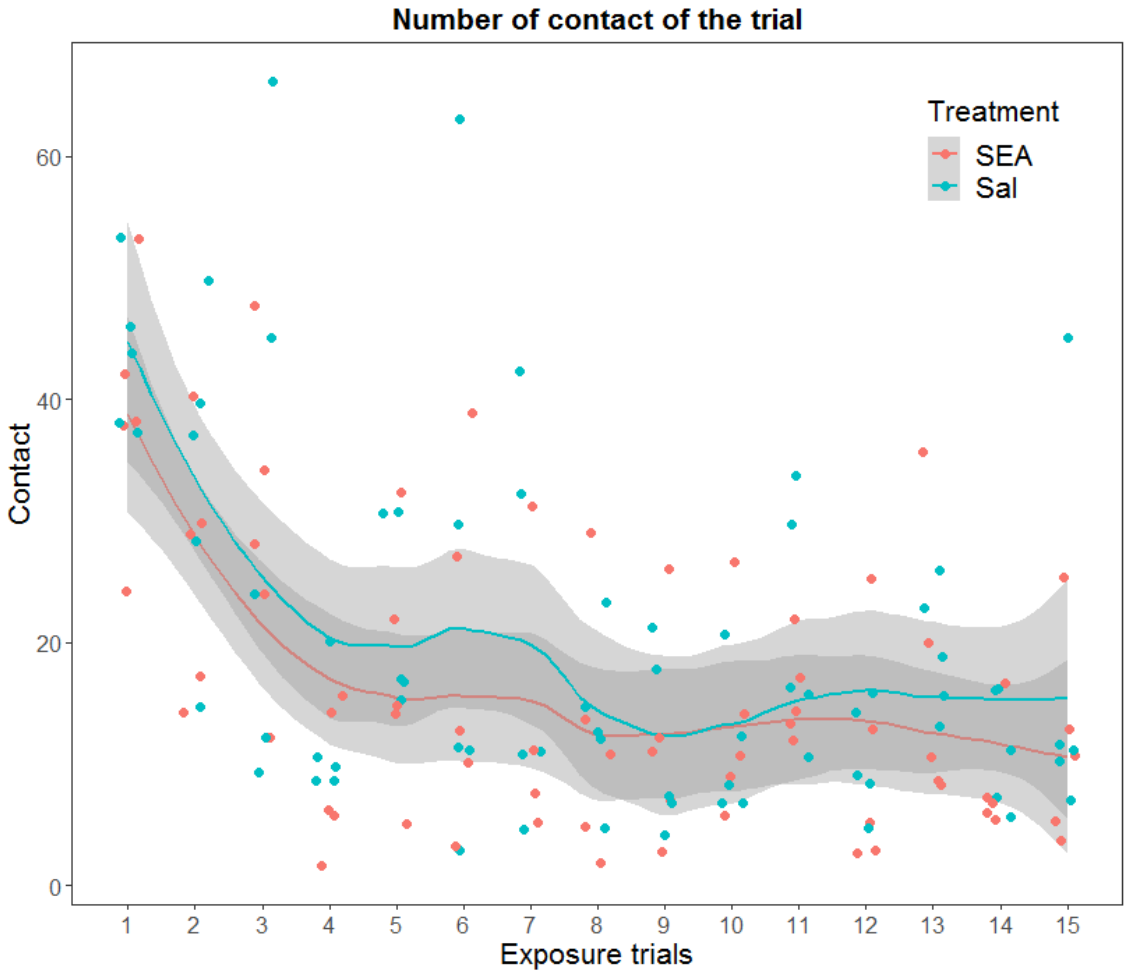


Figure 14. The number of contact by the two treatment groups on each day in experiment 3f. The error band was calculated together with the means of both treatment groups. All data points were shown in the figure. On Day 15, animals were randomly selected for SEA or Saline treatment, and then presented with the novel object. Although SEA-treated animals showed a decline in contacts, this was not significant.

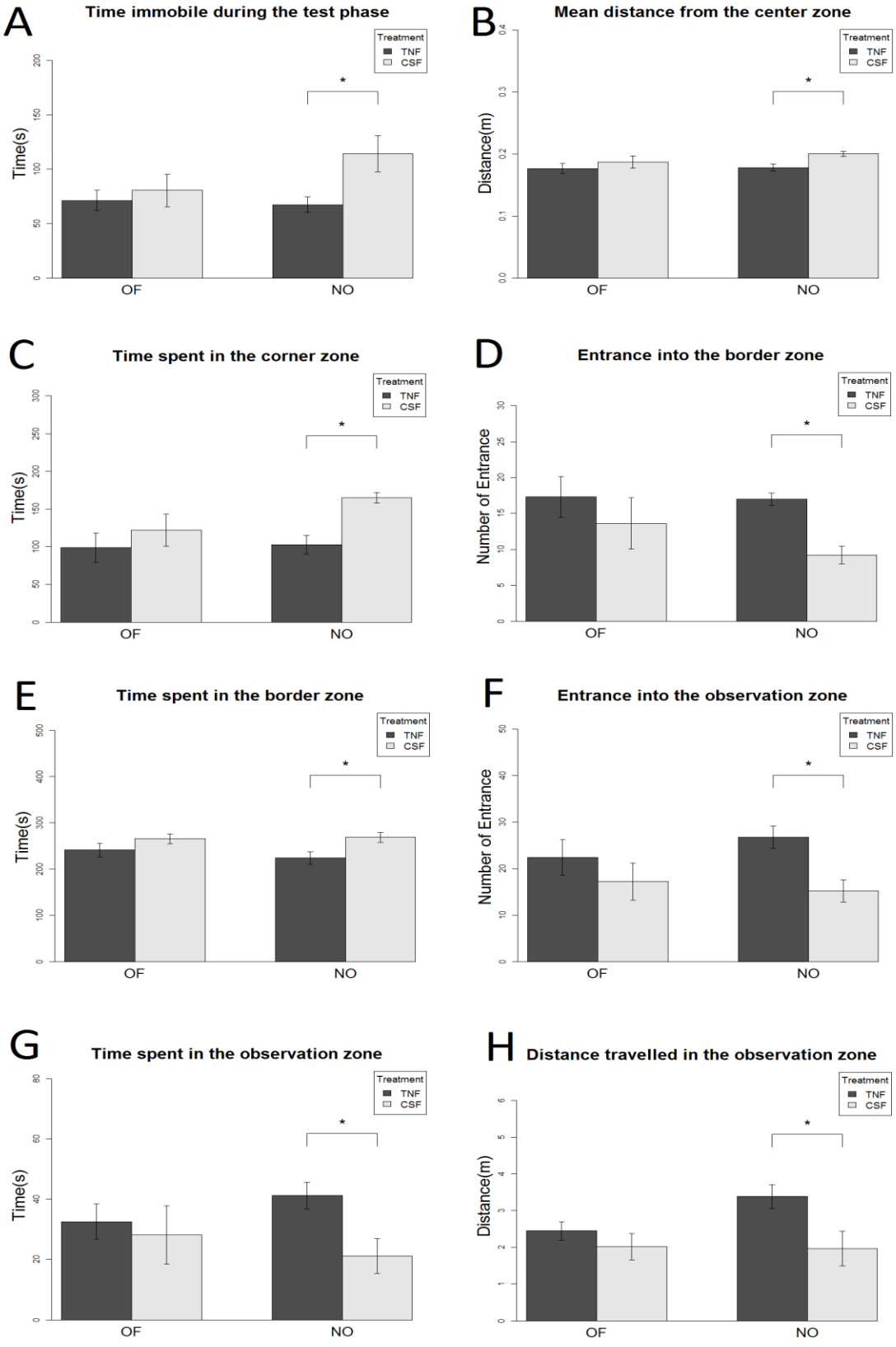

A
Time immobile during the test phase
Time(s)
Treatment
TNF
CSF
*
OF
NO
B
Mean distance from the center zone
Distance(m)
C
Time spent in the corner zone
D
Entrance into the border zone
Number of Entrance
E
Time spent in the border zone
F
Entrance into the observation zone
G
Time spent in the observation zone
H
Distance travelled in the observation zone

Figure 15. Summary during the open field (OF) and novel object (NO) phases (300s each) for different behavior parameters relative to exploration comparing the TNF-α infused animals (N=7), and the aCSF infused animals (N=5). **Bars represent mean ±1SEM. * = significantly different from infusion from CSF, $p < 0.05$.** (A)Time immobile during OF and NO phase (with minimum no-movement time >=1s); (B) Average distance from the center of the apparatus; (C) Time spent in the four corner regions; (D) The average number of entrances animals made into the four border regions; (E) Time spent in the four border regions; (F) The average number of entrances test animals made into the observation zone; (G)Time spent in the observation zone; (H) Travelling distance in the observation zone.

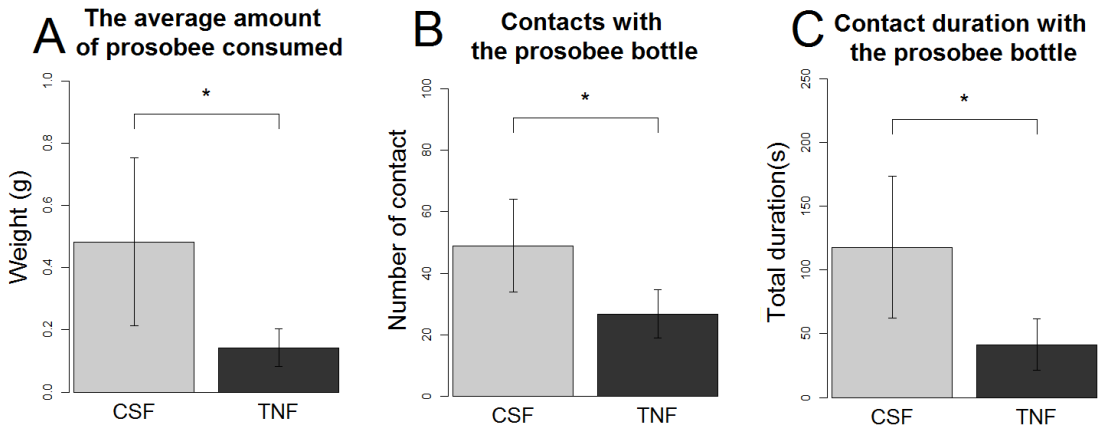


Figure 16. Summary for different behavior parameters relative to the novel food (prosobee) test (20 min) comparing the TNF-α infused animals (N=7) and the aCSF infused animals (N=6). **Bars represent mean ±1SEM. * = significantly different, $p < 0.05$.** A. The amount of prosobee consumed (calculated by the change of the weight of the prosobee bottles before and after the test. B. The average number of contacts toward the prosobee container. C. The average of total time the test animals consuming prosobee.

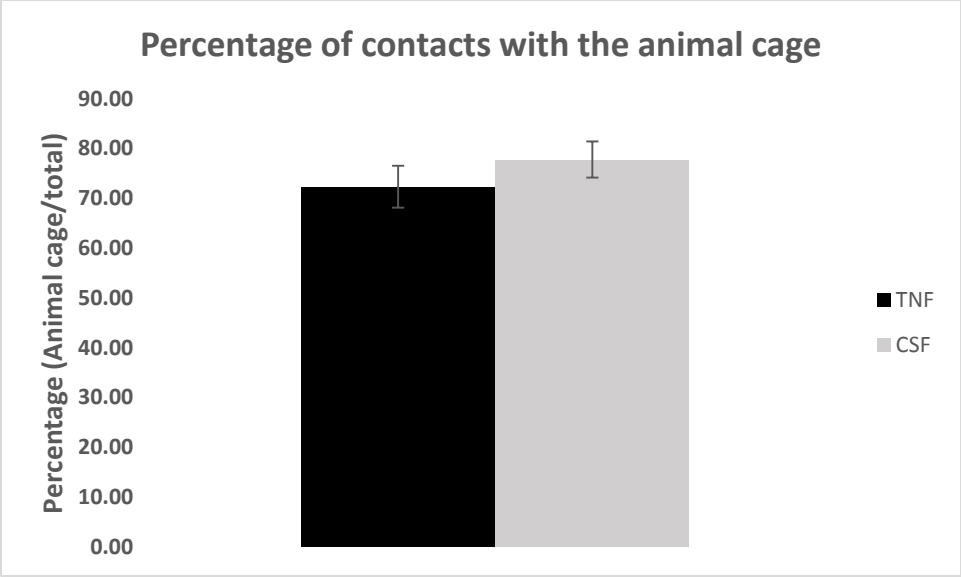


Figure 17. The rate of the number of contacts that test animal made with the social mate chamber divided by the total contacts. TNF-α infused animals (N=7) did not differ in the rate comparing with aCSF infused animals (N=6). All animals showed a preference toward the social chamber. Bars represent mean ±1SEM.

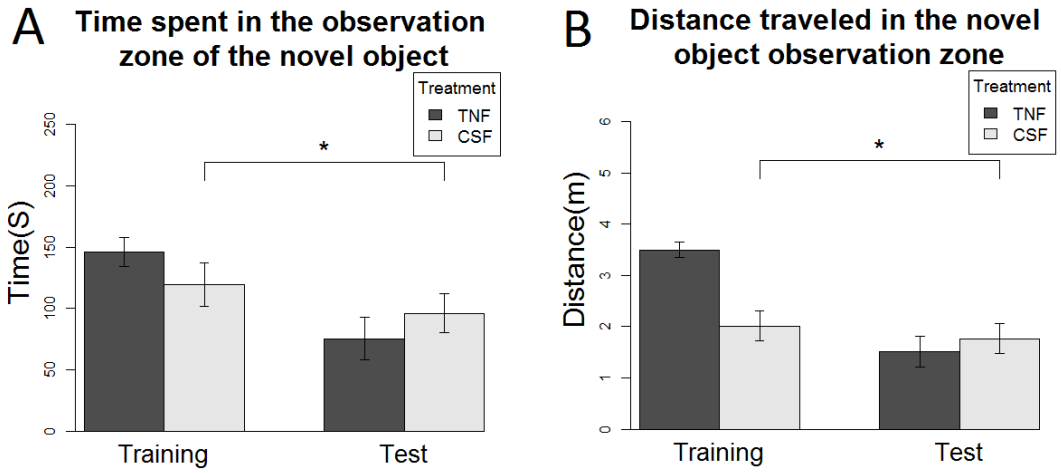


Figure 18. Summary time spent and distance traveled on the last training day and test day related to the object recognition test (10 min) comparing the TNF-α infused animals (N=6) and the aCSF infused animals (N=6). TNF-α infused animals (N=6) was significantly different from aCSF infused animals (N=6) in the change of the two days. **Bars represent mean ±1SEM. * = significantly different from infusion of TNF-α or CSF, $p < 0.05$.** A. Average time spent in the observation zone of the novel object. B. Average distance traveled in the observation zone of the novel object.