EFFECT OF STAPHYLOCOCCAL ENTEROTOXIN A (SEA) ON CELL PROLIFERATION IN THE DENTATE GYRUS AND HIPPOCAMPAL-DEPENDENT LEARNING AND MEMORY

By

QING CHANG

A thesis submitted to the

Graduate School-New Brunswick

Rutgers, the State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Master of Science

Graduate Program in Psychology

Written under the direction of

Alexander W. Kusnecov

And approved by

_____

_____

_____

New Brunswick, New Jersey

October, 2015

ABSTRACT OF THE THESIS

EFFECT OF STAPHYLOCOCCAL ENTEROTOXIN A (SEA) ON CELL PROLIFERATION IN THE DENTATE GYRUS AND HIPPOCAMPAL-DEPENDENT LEARNING AND MEMORY

By QING CHANG

Thesis director:

Alexander W. Kusnecov

Staphylococcal enterotoxin A (SEA) is known as a T cell superantigen, with well-characterized neurobiological and endocrine effects. We have quantified the survival of new cells, labeled with BrdU, in the dentate gyrus of the hippocampus after SEA or Saline injection into male C57BL/6 mice. A reduction of BrdU+ cells was found on Day 11 after SEA treatment, but not on Day 4. This suggests that BrdU+ cell retention was reduced by T cell activation. To determine if this decline in BrdU+ cells might predict deficits in spatial learning, a second experiment was conducted in which the effect of SEA treatment on spatial navigation learning in the Morris water maze (MWM) and the water-based Radial Arm Maze (wRAM) was investigated. Male C57BL/6J mice were given 5 ug SEA (N=7) or Saline (N=7) and then subjected to MWM hidden platform (HP) training. Two weeks after the final MWM training, mice were subjected to 8 days of water-based radial arm maze (wRAM) training. The results showed no major SEA effect on initial MWM learning. Further, wRAM learning was not affected by SEA-treated

mice, but interfered with the interaction of SEA treatment and MWM pre-exposure. This may be due to a reduction in cognitive flexibility.

Acknowledgement

I would like to thank the members of my committee, Dr. Alexander Kusnecov, Dr. Tracey Shors and Dr. George Wagner for their guidance and support. I would also like to thank current and former members of the Kusnecov Lab, especially Elyse Mallimo, Dmitri Zorine, Shivangi Patel, Melissa Butler, Michael Steinfeld and Nizmi Ponery, without whom this project would not have been possible. I would like to thank my family and friends for all of their love, company and encouragement. I'm especially grateful to my parents. Thank you for the boundless love that raise me up through all difficulties. I'm very grateful to my fiancé, Yefei, for the infinite patience and understanding across the seas. Finally, I would like to express my deepest gratitude to my advisor, Dr. Alexander Kusnecov. Thank you for your unconditional support and guidance throughout this project. I learnt not only the facts, but also a broader perspective to understand research, and for that I am truly thankful.

Table of contents

List of Figures

Introduction

The central nervous system (CNS) can be influenced by a number of physiological changes, including immunological activity. Apart from the CNS functional changes that result from conventional sensory stimuli, such as visual and audio input, the immune system is found to have influence on CNS activation by regulating the immune activation and sensibility of the entire body toward external and internal antigen. The effect of cytokines on CNS is a classic example. Cytokines are proteins with a small molecular weight, mostly released by cells of the immune system, targeting at immune cells as well as cells involved in other functions, such as neurons. Pathogens and their toxins are considered as antigens and can strongly activate the immune system, causing immune cellular proliferation, antibody production, and the release of regulatory molecules called cytokines. Cytokine receptors are expressed on neurons, and the neuromodulatory effects of cytokines hence alter behavior and physiological function of the neurons. The present thesis will focus on this line of research, testing the effect of immune challenge with staphylococcal enterotoxin A (SEA) on neurogenesis in the dentate gyrus of hippocampus and the effect of SEA on spatial learning.

1. Overview of the immune system

The immune system consists of molecules, cells, tissues and organs that distinguish self from non-self and eliminate the latter to protect the organism. The non-self agents can vary from external pathogens, such as bacteria and viruses, to internal factors, such as tumor cells and apoptotic cells. Immune organs are comprised of bone marrow, thymus, spleen and lymph nodes. All types of leukocyte (including T cells) are differentiated from bone marrow multipotent hematopoietic stem cells (Kumar and Jack

2006). Different types of immune cells are all specialized in function throughout the sequence of immune activity. They are also mobile and either range from blood circulation system and lymph circulation system, or reside in tissue and organs to prevent possible infection.

The immune reaction can be classified into categories, namely innate immune immunity and adaptive immune immunity, with the latter divided into humoral immunity and cell-mediated immunity.

The innate immune system consists of cells and molecules that are able to defend against pathogen broadly and instantly. The innate immune system could be subdivided into 1) anatomical barriers, 2) phagocyte, neutrophil, dendritic cell and natural killer (NK) cell, and 3) the complement system. The innate immune system does not possess memory, and reacts to a broad range of antigens within hours.

Adaptive immunity reacts more precisely toward detected specific antigens. The adaptive immune cells are able to specifically recognize the individual antigens by their peptide sequence. These cells proliferate and exert potent mechanisms to neutralize and/or eliminate the antigen. The adaptive immune system could be subdivided into 1) humoral immunity mediated by B lymphocytes and the antibody derived from it, and 2) cell-mediated immunity mediated by T lymphocytes.

Leukocytes generate molecules such as antibodies, cytokines and chemokines that conduct different immune functions. Cytokines are generally released by cells of the immune system, in particular macrophages and T cells, targeting other immune cells as well as cells involved in other functions, such as neurons. For instance, activated T helper cells (Th cell) modulate the activity of other immune cells by releasing several type of

cytokines. The Th1 cell subset secrete tumor necrosis factor alpha (TNFα) and interleukin-2 (IL-2) and exert local (i.e., activation of macrophage) and systemic (i.e., maximize the proliferation of cytotoxic CD8+ T cells) effects that promote inflammation. The Th2 cell secretes interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-9 (IL-9) and interleukin-13 (IL-13) that stimulate B-cells into proliferation, induce antibody class switching and increase neutralizing antibody production (Mitsuyama and Liu, 2008). Besides proimflammatory effects, certain cytokines also exert anti-inflammatory effects. For instance, Th2 cells secrete IL-10 which down regulates pro-inflammatory cytokines such as IL-2, IL-3, and TNFα (Murphy and Reiner 2002). Cytokines enable leukocytes to communicate and exert effective and appropriate immune responses.

2. T cell antigen recognition and Superantigens (SAgs)

2.1 T cells and T cell receptor (TCR)

Generated from bone marrow, immature T cells migrate to the thymus where they undergo thymic selection mainly focusing on T cell receptor (Germain 2002). The T cell receptor (TCR) is a molecule located on the surface of T cells. It is one of the key molecules of antigen recognition. During the maturation in thymus, T cell co-receptors are expressed as well, dividing T cells into CD4+ and CD8+ subgroups and differentiate in their function (Jameson and Bevan 1998). This T cell maturation in the thymus finishes at an organism's early age, with fully mature T cells entrance in enter the periphery (in transport via the blood and lymph circulation).

TCR is consisted of two different protein chains. In humans, 95% of T cells bear TCR that is composed of an alpha and beta chain, whereas 5% of T cells have TCR that

is composed of gamma and delta chains. All types of the chains can be divided into two extracellular regions: variable region and a constant region. The constant region is located proximal to the cell surface, connected by a transmembrane region and a short cytoplasmic tail for fixation (Bentley and Marriuzza 1996). The variable region (V region) is extracellular and binds to the peptide/MHC complex. It is the unique combination of DNA encoded segments that accounts most for the diversity in specificity of the T cell receptor for antigen recognition (Bentley and Marriuzza 1996). Every T cell expresses a single type of monoclonal TCR, and only T cells that express functional TCR that does not react to self-antigen (Ag) could pass though the selection(Jameson and Bevan 1998).

In the TCR, the variable region of the β chain (V-β region) has certain motifs that are common across various peptide specificities. These motifs are produced by V-β genes – of which there are over twenty different types in mouse: Vβ1, Vβ2, Vβ3, etc.

TCR are only able to recognize the peptides presented by major histocompatibility complex (MHC). MHC is a collection of glycoproteins that exist on the cell surface of all nucleated cells that controls a major part of the immune system and help differentiate self and non-self. MHC can be classified into two classes: MHC class I are expressed on all nucleated cell, while MHC class II only exists on antigen presenting cells, such as macrophage and dendritic cell (Mellman and Steinman 2001). Antigen presenting cells take antigen in through endocytosis, and the fragments of digested Ag are presented toward the cell surface to naïve T lymphocytes through MHC proteins (Mellman and Steinman 2001).

In traditional clonal antigen recognition, antigenic peptides are presented into the groove between the two major chains of the T cell receptor, and the association with various accessory molecules results in clonal activation of those T cells that have the appropriate complementary region in that groove.

A single TCR is only able to recognize a specific Ag that match exactly to its topological structure presented by MHC. In other words, not many naïve T cells will be activated by a single type of antigen without proliferation.

2.2 Bacterial Superantigens: Staphylococcal Enterotoxins

In order to maximize the effect of T-cell activation on CNS function during immunological challenge, previous studies have introduced the super antigen (SAg) models (Kusnecov, Liang et al. 1999; Kawashima and Kusnecov 2002; Rossi-George, Urbach et al. 2005). Superantigens (SAgs) (Kawashima and Kusnecov 2002) are toxins produced by bacteria (bacterial superantigens) and viruses (viral superantigens) that exert a strong capacity for in vivo immune stimulation. Unlike common antigens, SAgs activate the immune system by stimulating massive proliferation of T cells whose TCR non-specifically bind onto MHC (Figure). The best characterized SAgs are the staphylococcal enterotoxins (SEs) (Proft and Fraser, 2003) produced by staphylococcal aureus. SEs can be categorized into many subtypes, varying in the primary amino acid sequence (Proft and Fraser 2003).

Superantigens, apart from the ordinary pathogens, can omit the antigen presenting process and bind 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). In this way, SAgs can activate T cells and increase proinflammatory cytokine level. Since the antigen

recognition is not required in this process, many more T cells are activated non-specifically and result in stronger cytokine production (Proft and Fraser 2003). Compared with conventional antigens which would stimulate 1 percent of all T cells, SAgs can activate 20 percent of T cells (Proft and Fraser 2003).

The differences in the gene that encode the sequence motif of the V-β chain have an influence on the binding of SAgs and the MHC as well. The V-β region was translated from one of several different genes (i.e., Vβ1, Vβ2, Vβ3 etc). Each type of SAgs 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, Waterbury et al. 1996; Proft and Fraser 2003).

3. The effect of Superantigens on CNS and cognitive function

A vast amount of new cells are generated in the hippocampus area of the adult brain, and it is proved that those cells are involved in learning and memory (Gould, Beylin et al., 1999; Shors, Miesegaes et al., 2001; Shors, Anderson, et al., 2012). Many factors such as glucocorticoids, stress, aging, estrogen and environmental enrichment can influence the proliferation or survival of the neurogenesis in hippocampus (Gould et al., 1999).

Immune cells and immune activation are found to affect the neurogenesis in hippocampus and learning. Significantly reduction of hippocampal neurogenesis was found when CD4+ T cells were systemic depleted, impaired reversal learning in the Morris Water Maze (MWM), and showed no compensation effect by environment enrichment (Wolf, Steiner et al. 2009[1]; Ziv, Ron et al 2006). SEB injection was found to be associated with an increase of cell proliferation and neurogenesis in the hippocampus

of adult mouse (Wolf, Steiner et al. 2009[2]). A significant increase in precursor cells in the dentate gyrus was found on four days after the SEB intraperitoneal (i.p.) injection while BrdU was given three days after SEB injection as the marker of precursor cells(Wolf, Steiner et al. 2009[2]). These studies suggest that T cell activity contributes to the maintenance of neurogenesis in the adult hippocampus, and SEB stimulation promotes this effect.

It is well known that immune activation and behavior affect CNS function. Both cytokines and their receptors are widely found in the CNS, on neurons and glial cells (Vitkovic, Konsman et al. 2000; Dantzer, O'Connor et al. 2008; McAfoose and Baune 2009). A severe peripheral infection will induce massive release of cytokine. A small amount of cytokines can pass through the weak area on blood brain barrier and act on the CNS receptors, generating symptoms often referred to as sickness behavior (Bluthe, Crestani et al. 1992; Bluthe, Pawlowski et al. 1994; Kelley, Bluthe et al. 2003; Dantzer, O'Connor et al. 2008).

Sickness behavior refers to a coordinated set of behavioral changes that develop in sick individuals during the course of infection (Danzer 2001). Sickness behavior is believed to conserve energy and support the immune response driven by specific brain area. The classic model of sickness behavior can show one or more of the symptoms listed here: weakness, malaise and decreased motor activity, anorexia and decreased water intake, altered sleep pattern and anhedonia.

The physical changes, such as decreased motor activity, greatly reduce the validity of any animal study on the cognitive effect of cytokine release. The use of SAgs, however, circumvents this problem. For example, it was found that with a lower dose of

SAgs injection, animals showed a novel type of behavioral change that is very distinct from sickness behavior (Rossi-George, LeBlanc et al. 2004). Animals showed less anxiogenic behavior, but neophobic behavior was found in response to new food or a novel object (Rossi-George, LeBlanc et al. 2004). No decreased motor activity was shown in these studies, which make cognitive tests (i.e., MWM, object recognition) possible.

The SAgs model is utilized to study the effect of T cells activation and cytokines on cognitive function. Neither acute nor repeated i.p. injection (5ug) of SEA administrated were found to interfere with learning impairment or memory (Woodruff et al., 2010). However, in reconsolidation training 30 days after the primary training, the SEA group relearned better of the MWM with no additional treatment.

Specific Aim 1: Determine whether a behaviorally active dose of SEA affects neurogenesis in the hippocampus of adult animals.

We hypothesize that precursor cells in hippocampus is associated with SEA intraperitoneal injection in adult C57 mice.

Specific Aim 2: Determine whether the positive effect of SEA on reconsolidation after Morris water maze primary training can be generalized to different type of spatial memory test.

We hypothesize that intraperitoneal injection in adult C57 mice together with Morris water maze training will affect the probe test after water-based radio arm maze test training.

Method

1. Experiment 1. Effect of T cell activation on cell proliferation in the central nervous system.

1.1 Animals

The experiments were conducted with C57BL/6J mice from Jackson Laboratory (Bar Harbor, ME) which, at the start of the injection stage, were 12 weeks of age. Subjects were housed four per cage in a 12:12 hour light:dark illumination (lights on 0600h). Subjects were allowed four weeks acclimation with food and water at libitum prior to the start of treatment. Disposable cages with food and bottled water ad lib were used to house animals after injection for possible hazardous contamination.

1.2 Experimental Procedures

Immune stimulation and cell labelling

Two time points (day4 and day 11 after SEA injection) were selected to study how SEA treatment affects the baseline of neurogenesis and the survival of the new cells. The Day4 experiment utilized C57BL/6J mice (N=10) that originated from breeding pairs obtained from Jackson Laboratory and were bred in our colony room. Animals were assigned randomly to SEA or saline group: 0.2 ml of saline (N=5), 5 μg of Staphylococcal Enterotoxin A (SEA; Toxin Technology) diluted in 0.2 ml of saline (N=5). Day11 experiment utilized adult C57BL/6J mice (N=14) that were obtained from Jackson Laboratory. The animals were acclimated for two weeks before being subjected to experimentation. Animals were assigned randomly to two treatment groups: 0.2 ml of saline (N=7); 5 μg of Staphylococcal Enterotoxin A (SEA; Toxin Technology) diluted in 0.2 ml of saline, 7 day sacrifice (N=7). All injections were given between 1000 and 1200

h. 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. All experiments were approved by the Rutgers Institutional Animal Care and Use Committee.

BrdU is a synthetic nucleoside that is utilized as analog of thymidine. It could be detected in the DNA of proliferating cells by immunohistochemical or immunofluorescent (IF) staining procedure (see below).

On Day 3 after SEA or saline injection, all animals received 50 mg/Kg injection of bromodeoxyuridine (BrdU, Sigma-Aldrich B5002, stored at -20ºC) IP. All injections were given between 1000 and 1200 h.

Tissue Collection and Fixation

On day 4 and day 11, animals were anesthetized with 0.23ml/10g intraperitoneal injections of 1.2% Avertin and were perfused transcardially with 4% paraformaldehyde (PFA;). The brains were subsequently harvested and post-fixed in PFA at 4ºC overnight before being switched to 30% sucrose solution to dehydrate for sectioning purpose.

Dorsal hippocampus performs cognitive functions such as spatial memory while ventral hippocampus is related to emotion distinction (Fanselow and Dong, 2010). A freezing microtome was used to collect the dorsal hippocampus for analysis of the rostra-caudal extent of the dentate gyrus. One-in-Six series (A-F) of dorsal hippocampus tissue (35µ m) was collected in order using a freezing microtome. Series A was mounted with phosphate-buffered saline (PBS) on Fisherbrand Superfrost Plus precleaned glass slides (25x75x10mm) directly after sectioning, and series B-F were kept in cryoprotectant for

future analysis. Tissue mounted on slides were left to air-dry before they went through fluorescent immunochemistry staining.

BrdU immunofluorescent staining

Brain tissue sections were pretreated with citrate buffer, trypsin solution and HCL solution for antigen retrieval and DNA denaturation. Tissue sections were incubated in 1.5 percent normal donkey serum (Abcam, ab7475) blocking buffer followed by 1:500 monoclonal rat-anti-BrdU primary antibody (AbD Serotec, OBT0030) overnight at 4 degree Celsius. Tissue sections were then incubated in 1:500 donkey -anti-rat IgG secondary anitibody (Abcam, AlexaFluor 488) and coverslipped with DAPI (Life technology, P36935). PBS was used for washes between each incubation period.

Quantification of Double-Labeled Cells

Neuroanatomically distinct subregions (interaural 2.86mm, bregma -0.94mm to interaural 1.34mm, bregma -2.46mm) of the dorsal hippocampus were identified by the mouse atlas of Franklin and Paxinos (1997). Seven to eight sections were processed per animal for both hemispheres. Quantification of BrdU positive cells was performed blind with Nikon Eclipse E400 light and fluorescent microscope at 100X magnification located. All qualified fluorescent cells in the subgranular and granular zone were quantified in the entire sampled rostro-caudal extent of the dentate gyrus of both hippocampi.

Data Analysis

Cell counts were analyzed by two separate independent sample T tests since the two experiment were performed seperately.

2. Experiment 2. Effect of SEA on water radial arm maze after pre-experience of Morris water maze.

2.1 Animals

The experiments were conducted with 36 C57BL/6J mice from Jackson Laboratory (Bar Harbor, ME) which, at the start of the MWM training, were 12 weeks of age. Subjects were housed four per cage in a 12:12 hour light:dark illumination (lights on 0600h). Subjects were allowed four weeks acclimation prior to the start of treatment with food and water ad libitum. Disposable cages with food and bottled water ad lib were utilized after injection for possible hazardous contamination.

2.2. Immune Stimulation:

Animals were treated with SEA (N=16) or Saline (N=16), with 8 animals in each group randomly selected to a home caged condition, while the other 8 in each treatment were subjected to MWM learning (see figure3). All animals were subjected to water-based radial arm maze (wRAM) learning 21 days after the first SEA treatment (Figure 1).

All animals were injected intraperitoneally (IP) with either 5ug of staphylococcal enterotoxin A (Toxin Technology, stored at -80ºC, diluted in 0.2 ml of physiological saline) or physiological saline in a volume of 0.2 mL on the first day and second day of hidden platform training mice. This is considered as repeated treatment of SEA. All injections were given between 1000 and 1200 h.

2.3 Apparatus

Morris water maze

The first phase of spatial learning was assessed by Morris water maze (MWM) test, a hippocampal-dependent visuo-spatial learning task. A galvanized steel tub

(diameter 110 cm, height 59 cm) was utilized as the apparatus in this experiment. Tub was filled with water to a height of 49 cm and tinted with white Crayola nontoxic paint. Water temperature was maintained at 21-23º Celsius during everyday experiment. The platform was a circular (9 cm diameter) piece of clear Plexiglas with perforations to provide mice with traction. The platform was maintained 1cm below the water surface affixing to a steel rod with a height of 48 cm. During testing, white and black cotton curtains were suspended between the experimenter and the pool to create contrast. Different spatial cues were located around side of the tub, and consisted of two horizontally aligned light bulbs near the 'north' quadrant, a constellation of holiday lights aligned horizontally near the 'east' quadrant, the black and white curtains with 2 pieces of star shaped white cardboard near the 'south' quadrant, and a Jamaican Flag and a constellation of holiday lights aligned vertically near the 'west' quadrant.

Radial-arm water maze (wRAM)

The wRAM was introduced as the second spatial learning test. It was a swimming based a radial arm maze, which took advantage of eliminating food odors in the traditional radial arm maze. This maze used black Plexiglas and has an octagonal shape with eight identical swim alleys (arms) radiating out from an open central area. Visible and hidden escape platforms could be submerged at the end of one goal arm. The top view and dimension of wRAM were shown in figure 2. The platform was a square (8 cm x 5 cm) piece of clear Plexiglas with perforations to provide mice with traction.

The wRAM apparatus was filled with water to a height of 10 cm and tinted with white Crayola nontoxic paint. Water temperature was maintained at 21-23º Celsius during everyday experiment. The platform was maintained 1cm below the water surface

affixing to a steel rod stand of 9 cm in height. A white cotton curtain was suspended between the experimenter and the maze. Different spatial cues were located around the maze, and consisted of a constellation of holiday lights aligned vertically near the “east” side, one light bulb near the ‘north’ side, a black triangle-shaped cardboard and a black laboratory glove near the ‘west’ side, and a star-shaped, a circle-shaped black cardboard near the ‘south’ side. Arms were labeled one through eight on the outside for experimenter’s convenience.

2.4 Morris Water Maze Testing:

Visible Platform Training:

Visible platform training was performed on Day1 and Day2 to familiarize animals with the escape task. This session serves to exclude animals with possible visual or motor deficits that could interfere with performance. Daily sessions consisted of 4 trials. Before individual trials the escape platform was semi-randomly placed in the center of one of the four quadrants with a cylindrical striped flag indicating its location. In each trial, test animal was semi-randomly placed in the maze close to the edge of one of the other three quadrants, facing the tub edge. Mice were run in squads of four and were picked up immediately after they arrive at the platform with four limbs, or after 60 seconds of swimming. After each trial, animals were placed in heated cages with dry bedding during the inter-trial interval (ITI). For each mouse, all locations were tested on a given day.

Hidden Platform Training:

Hidden platform trials began 2 days after the completion of visible platform trials and consisted of four trials per day per animal with a 4 minute ITI. Hidden platform trials

were conducted for five consecutive days (Day4-8). Platform location was semi-randomized in the first trial of the first day for individual animal, and remained at the same location during hidden platform testing. In each trial of a day, individual animal starts from a novel location. Start locations were designated semi-randomly in one of the other three quadrants. Mice were run in squads of four and were picked up immediately after they arrive at the platform, or after 60 seconds of swimming. After each trial, animals were placed in heated cages during the inter-trial interval.

Probe Test:

2 and 48 hours after the last trial of hidden platform training on Day8, the platform was removed and mice were placed in the maze for 60 seconds free swim.

2.5 Radial-arm water maze training

WRAM test was performed 14 days after the last probe test for the MWM.

Visible platform training:

Visible platform training was performed on Day23 and Day24. Daily sessions consisted of 4 trials. The escape platform was placed in the end of one of the eight arms with a cylindrical striped flag indicating its location. In each trial, the test animal was semi-randomly placed in the end of one of the free seven arms, facing the end of the arm. Mice were run in squads of four and were picked up immediately after they arrive at the platform with four limbs, or after 60 seconds of swimming. After each trial, animals were placed in heated cages during inter-trial interval. For each mouse, all odd numbered start locations were tested on Day 23, and all even numbered locations were tested on Day 24.

Hidden platform trials began 2 days after the completion of visible platform trials and consisted of four trials per day with a 4 minute ITI. Hidden platform trials were

conducted for five consecutive days (Day25-29). The hidden platform located in the end of arm number 7 for every animal during all trials across the entire session, while animals started from a novel start location for each trial. Mice were run in squads of four and were picked up immediately after they arrive at the platform with four limbs, or after 60 seconds of swimming. After each trial, animals were placed in heated cages during inter-trial interval. Four arms were selected as the starting arm among the rest of the seven arms. For each mouse, all odd numbered arms were tested on one day, and all even numbered locations were tested on the consecutive day, and this cycle repeats.

Probe Tests:

2 and 24 hours after the 5th day (Day28) of wRAM hidden platform training, the platform was removed and mice were placed in the maze for 60 seconds free swim.

2.5 Statistical Analysis:

All MWM and wRAM sessions were timed by stopwatch, as well as videotaped and analyzed using SMART software (Spontaneous Motor Activity Recording & Tracking; San Diego Instruments, San Diego). In visible and hidden platform testing, the latency and distance traveled to locate the escape platform were calculated. In both probe test, the time and distance traveled in the destination quadrant (quadrant formerly containing the escape platform in hidden platform training) and other quadrants were calculated.

Results

Effects of behaviorally active dose of SEA on generation and survival of the precursor cells in the hippocampus of adult animals.

5ug SEA injection suppressed the survival of neural precursor cells in the hippocampus seven days after generation but not the baseline level (one day after BrdU injection).

The Day1 and Day7 BrdU experiments were conducted separately, and therefore two independent sample t-tests were conducted on the data.

BrdU existence was measured in the dorsal hippocampal regions. Figure 3 shows examples of BrdU positive staining in DG area. Statistical analyses of manual cell counts revealed no difference between the two treatment groups for Day1 (CI =-160.6617, 105.3617, p=.6381, one animal in SEA group was excluded due to unsuccessful staining), and a significant difference of treatment condition for Day 7 (CI =-112.2640, -20.3074, p =.0085) (Figure 4).

SEA treatment have a negative effect on probe test of wRAM learning after Morris water maze primary training.

2.1 Morris water maze visible platform training

Sixteen mice in experiment 1 learned to locate a visible escape platform in Morris water maze across two days. Repeated measures of analysis of variance (ANOVA) factor was utilized to analyze the data. Decreasing latency was observed on day 2 compared to day1 for all 16 animals ($F(1,14)= 45.37$, $p < .0001$) (data not shown). No significant difference was observed between the two treatment groups by day ($F(1,14)= 1.964$, $p = .183$). However, a significant difference was observed between the two treatment groups

when analyzing the treatment factor alone ($F(1,14)= 8.337$, $p = .012$). Prior to any injection or treatment, the saline group located the escape platform faster than the SEA group. The learning curve of visible platform training was shown in Figure 5.

2.2 Morris water maze hidden platform training

All mice in experiment 1 learned to locate a hidden escape platform in Morris water maze. Decreasing latency was observed over five consecutive days of hidden platform training. Data was analyzed using repeated measure ANOVA. The assumption of sphericity was not met ($p<0.001$) in Mauchly test. Huynh-Feldt test was utilized to calculate an appropriate adjustment to the degrees of freedom of the F-test. A significant difference was found over day factor for all 16 animals ($F(2.257,31.595)= 10.377$, $p=.036$). No interaction was found between day*treatment factor ($F(2.257,31.595)=.368$, $p = .720$). No significant difference was observed by treatment factor itself ($F(1,14)=.610$, $p = .448$). The learning curve of hidden platform training was shown in Figure 6.

2.3 Morris water maze probe test

Two-way ANOVA was utilized to analyze the overall velocity, travelling distance and time spent in the target zone, percentage of time and distance in the target zone, the latency before animal enter the target zone and number of entrance into the target zone. No significant difference was found by either the treatment factor or the time interval between last training and probe test for all the dependent variables above (data not provided).

2.4 Water-based radial arm maze visible platform training

All mice in experiment 1 learned to locate a visible escape platform in water-based radial arm maze. Decreasing latency was observed on day2 comparing to day1 for all 32 animals participated in the test ($F(1,28)= 28.000$, $p = .006$). No two-way interaction was found between day*treatment ($F(1,28)= 0.94$, $p = .761$) and day*pretraining ($F(1,28)=.459$, $p=0.504$). Additionally, no main effect was observed on treatment ($F=0.060$, $p=0.808$) or pretraining ($F= 0.137$, $p=0.715$). No two-way interaction was found between day*treatment ($F(1,28)<0.001$, $p = .998$). The learning curve of visible platform training is shown in Figure 7.

2.5 Water-based radial arm maze hidden platform training

All mice in experiment 1 learned to locate a hidden escape platform in the water-based radial arm maze. Data was analyzed using repeated measures ANOVA. No adjustment was needed on the degree of freedom ($p=0.08$ in Mauchly test). Decreasing latency was observed over five consecutive days of hidden platform training for all animals ($F(4,25)= 6.039$, $p=.002$). No interaction was found between day factor and treatment factor ($F(4,28)=.626$, $p = .648$), or day factor and pretrain factor ($F(4,28)=1.894$, $p = .143$). The SEA+wRAM group generally took the longest time to reach the platform although no significant difference was found between the four groups over the five day learning.

For main factors, a significant effect was found on the MWM pretraining effect ($F(1,28)=5.849$, $p = .022$). No significant difference was observed on treatment effect ($F(1,28)=.992$, $p = .345$). No interaction was found between pretrain factor and treatment factor ($F(4,28)=2.135$, $p = .155$). The learning curve of hidden platform training is shown in Figure 8.

2.6 Water-based radial arm maze probe test

Repeated measures ANOVA was utilized to analyze the average velocity, travelling distance and time spent in the target zone, percentage of time and distance in the target zone, the latency before animal enter the target zone and number of entrance into the target zone. No significant difference was found by either the treatment factor or the time interval between last training and probe test for all the dependent variables above (data not provided).

2.5 Water-based radial arm maze probe test

Average velocity in Water-based radial arm maze probe test.

Average velocity indicates the ability to move in the spatial maze. It reflects the animal's motivation and ability to escape from the maze. No significant difference was found between the four treatment groups in both 2hr probe and 24 hr probe test by repeated meansures ANOVA (p_{SEA}=0.832, $p_{pretrain}$=0.931, $P_{SEA*pretrain}$=0.919).

The percentage of travelling distance in the end of the target arm over travelling distance of the 60 second probe test.

The percentage of travelling distance in the end of the target arm reflects the confidence of the animal of the long-term memory formed in hidden platform training. No main effect was found for treatment in both 2hr probe and 24 hr probe test by 3-way ANOVA (p_{SEA}=0.533). A significant difference was found for the pretrain factor ($p<0.003$). An interaction was found between treatment and pretrain (p=0.049).

Latency from start to reach the end half of target arm.

Latency from start to reach the end half of target arm reflects the long term memory of the hidden platform training. No main effect was found in both 2hr probe and 24 hr probe test by 3-way ANOVA (p_{SEA}=0.626, $p_{pretrain}$=0.089). However, an interaction was found between treatment (SEA or saline) and pretrain (MWM or HC) (p=0.051). Latency from start to reach the end half target arm was shown in figure 11.

Number of entries to the end of the target arm.

Total entries to the end of the target arm may reflects the persistence of the animal of the long-term memory for the location of the hidden platform. No main effect was found for treatment in both 2hr probe and 24 hr probe test by repeated measures ANOVA (p_{SEA}=0.534). A significant difference was found for the pretrain factor (p=0.004). No interaction was found between treatment and pretrain (p=0.126).

Time spent in the end of the target arm.

No main effect was found for treatment in both 2hr probe and 24 hr probe test by repeated measures ANOVA (p_{SEA}=0.463). A significant difference was found for the pretrain factor (p=0.006). No interaction was found between treatment and pretrain (p=0.328).

Discussion

The results from Experiment 1 showed that SEA treatment was not found to have a significant effect on cell proliferation in dentate gyrus three days after its introduction (24 hrs after the cells generation), yet it is associated with a reduction in the survival of the BrdU+ cells 11days after SEA introduction compared with the control group. Previous studies has revealed that SEB, a different T cell activator, can cause an increase in the numbers of BrdU+ cells in the dentate gyrus of C57 mouse four days after the i.p. injection (Wolf et al. 2009[2]). However, in the current study, SEA treatment did not affect the number of BrdU+ cells measured 4 days after immune activation. The difference between the two studies can be a result of the difference between the subgroups of T-cell stimulated by SEA and SEB. SEB are recognized by TCR that carries Vβ8 region (Kawabe and Ochi 1990, Vidlak, Mariani et al., 2011), while SEA is recognized TCR that possess Vβ3 and Vβ11 regions and consequently activates more CD4+ T cells comparing to SEB (Omoe, Nunomura et al., 2010). There is a distinct difference in the hypothalamus-pituitary-adrenal axis and proliferative response of T cells toward SEA and SEB in C57BL/6 mice. Additionally, the dose effect could also contribute to the difference between the current study and the one study using SEB (Wolf, Steiner et al, 2009[2]). In the previous study, 0.1μg per 1g body weight of SEB (2.5ug for a mouse weighing 25g) was given, while 5ug SEA was given to each animal in the current study.

Activated CD4+ T cells will migrate and relocate onto meninges (Dereck, Cardani et al., 2010). Without infiltration, into the brain, CD4+ T cells are able to release cytokines such as IL-2 and IL-4 into the CNS. It has been suggested that CD4+ T cells

have a neuroprotective role and neurogenesis was severely reduced in CD4+ T cell depleted animal (Wolf, Steiner et at., 2009; cl, Ron et al., 2006). However, not many studies have been conducted on CD4+ over-activated animals, as carried out in the present study using SEA. Interleukin-4 has been proved to promote microglial cell activation (Butovsky, Talpalar et al., 2005), and the role of IL-4 as well as its effect through microglial activation is still under study. The effect of microglial cells on neurogenesis has been a controversial topic for many studies. It is possible that the CD4+ T cell act as a neuro-proliferator within a certain range of concentrating. Yet a higher level of expression can possibly have a negative effect on neuro-proliferation.

Additionally, no other cell markers were stained together with BrdU. The actual phenotype of the BrdU+ cells is unclear. The suppression on cell proliferation might not only be neurons, but also astrocytes and microglia cells. A double stain for doublecortin (neural progenitors), GFAP (astrocytes) and/or CD11b (microglia) is necessary to determine the cell type of the BrdU+ cells. Furthermore, a dose-response study with SEA might reveal greater BrdU+ cells at lower SEA doses.

Experiment 2 investigated the effects of SEA on learning and memory in the MWM and wRAM. A subset of these animals were treated with SEA or Saline and remained in the home cage, prior to wRAM testing (the HC/SEA and HC/Saline groups). Since these animals are naïve to behavioral testing prior to wRAM, but immunologically challenged, it might be predicted that low BrdU+ cells in the hippocampus might influence wRAM learning and memory.

Many studies have revealed that neurogenesis is highly interacted with learning and memory (Deng et al., 2010; Leuner and Gould, 2010). Neurogenesis is positively

correlated with spatial learning, and the reduction of neurogenesis caused by CD4+ T cell deficiency interferes with spatial learning in the C57 mouse (Ziv et al., 2006). It is possible that the reduction of BrdU+ cells in the current study was not due to a decrease in neural precursor cells, but other cell types. It was found that SEA-induced reductions in BrdU+ cells was associated with better memory formation in the wRAM 3 weeks after the first injection of SEA without MWM pre-training. On the other hand, improved wRAM memory was not seen in the MWM/SEA pre-trained group. The reasons for this are not clear. In a previous study (Woodruff et al., 2011), animals that were MWM-trained and treated with SEA had better reconsolidation of MWM a month later. It can be hypothesized that the precursor cells born during the immune activation will benefit spatial learning when the neurons reach maturation, unless a new but different spatial task is introduced. It is supported by the suggestion that the neural progenitor cells born and mature at certain periods of time may make distinct contributions to events that occur in the same time period (Nokia et al., 2012).

Additionally, MWM by itself (eg., Saline/MWM group) also reduced memory formation in the wRAM. It could be that the neurons that were born during MWM training interfered with the computational model in the hippocampal circuit of neurons born during wRAM training, and thus diminished the effect of SEA on later learning. A double labeling experiment tracing BrdU and doublecortin positive cells generated from the MWM learning would help answer this question by correlating the number of double labeled cell with the learning and memory in later wRAM test.

If the reduction of BrdU+ cell was a result of reduced neural precursor cells, it could be the ability of cognitive flexibility that was affected. Figure 10 indicates that

there is no difference in the percent distance travelled in the target arm in the saline groups that were pretrained (MWM exposed) or not pretrained (Saline/HC). But for SEA animals, the ones that were pre-trained had worse memory compared with the SEA/HC (i.e. not pretrained) group. This phenomenon can also be seen as a trend in the acquisition phase, although statistical significance was not found (Figure 9). These differences occurred on both probe test days, and could not be accounted for by travelling speed. Therefore, these data (using latency and distance travelled measures) indicate that the animals that were introduced into MWM before wRAM had a worse memory retrieval in the wRAM, compared with animals that were not trained in the MWM.

These observations could be interpreted as interactions with cognitive flexibility, which is the ability to flexibly use and ignore familiar associations when contingencies change (Burghardt et al 2012). Animals in which adult neurogenesis was blocked had a higher error rate when the shock zone and safe zone were reversed in a spatial fear conditioning task. However, blocking neurogenesis did not affect learning of the original learning conditions (Burghardt et al 2012). Future studies should determine whether theory is justified in the SEA model.

Conclusion

This study is the first to directly assess the effect of staphylococcal enterotoxin A (SEA) on neurogenesis and to examine the effect of SEA on the generalization of spatial cognition. It was found that injection of SEA did not affect the number of new cells in the dentate gyrus of hippocampus three days after SEA injection, but had a negative effect on it eleven days after the SEA injection. The SEA injection was found to have no influence on the Morris water maze acquisition and memory. However, home caged animals that were naïve to Morris water maze (MWM) showed better memory in water-based radial arm maze (wRAM) three weeks after the injection of SEA compared with animals that were previously trained in MWM. This could be a result of changes in hippocampal circuits caused by neurogenesis, or changes in cognitive flexibility.

List of Figures

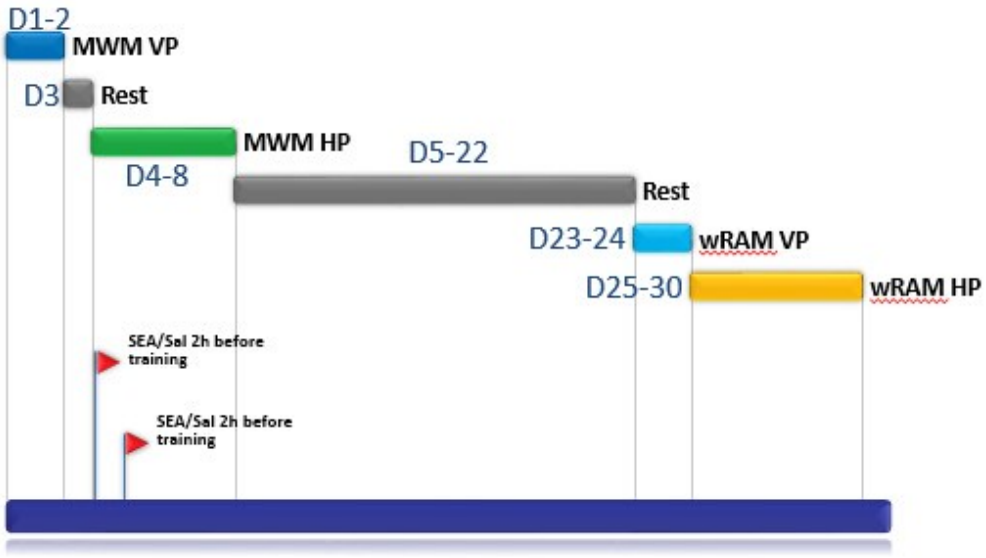

Figure 1. The timeline of experiment 2. Conditions are stated in bold at the end of each bar. Injections with SEA or Saline are indicated by flags on the bottom bar. There were two injections, each given on consecutive days.

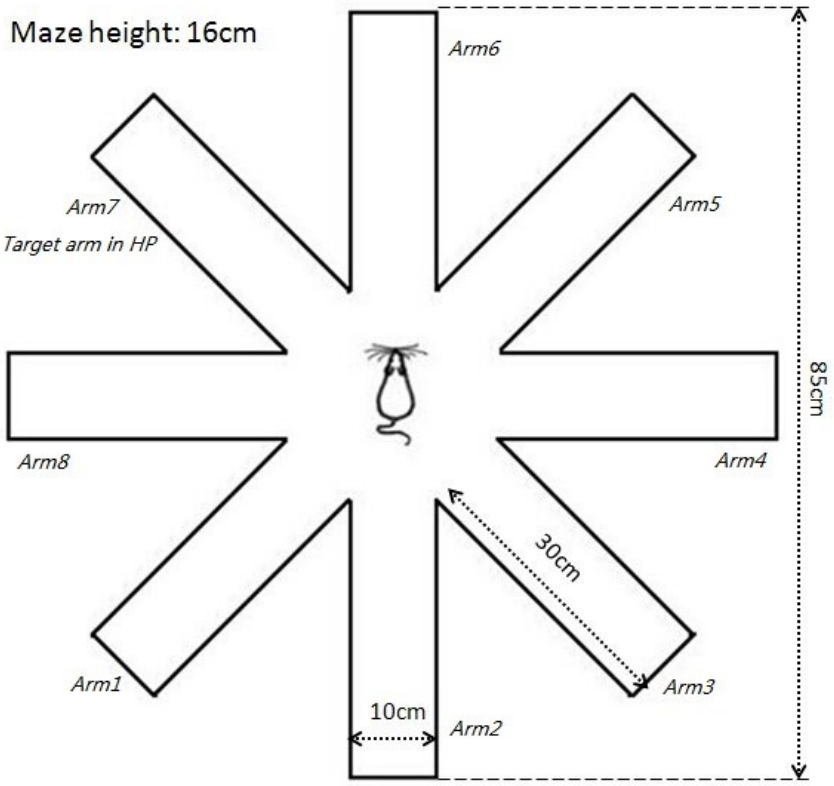

Figure 2. Diagram of the water-based radial arm maze. Arm measurements were (L x W x H) 30cm x 10cm x 16cm. The angle between two arms is 45°.

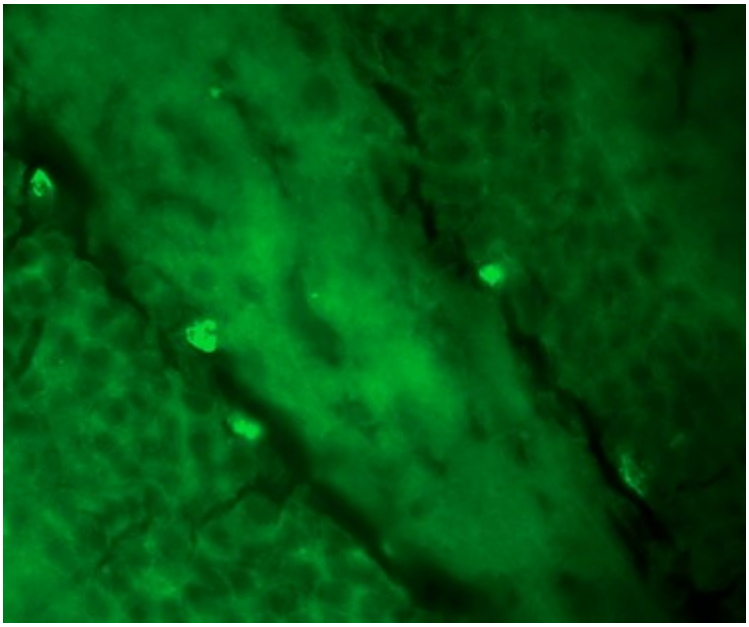

Figure 3. Experiment 1: BrdU+ staining in dentate gyrus area. The green fluorescent neurons are BrdU positive. Overlap cells are identified by focus adjustment.

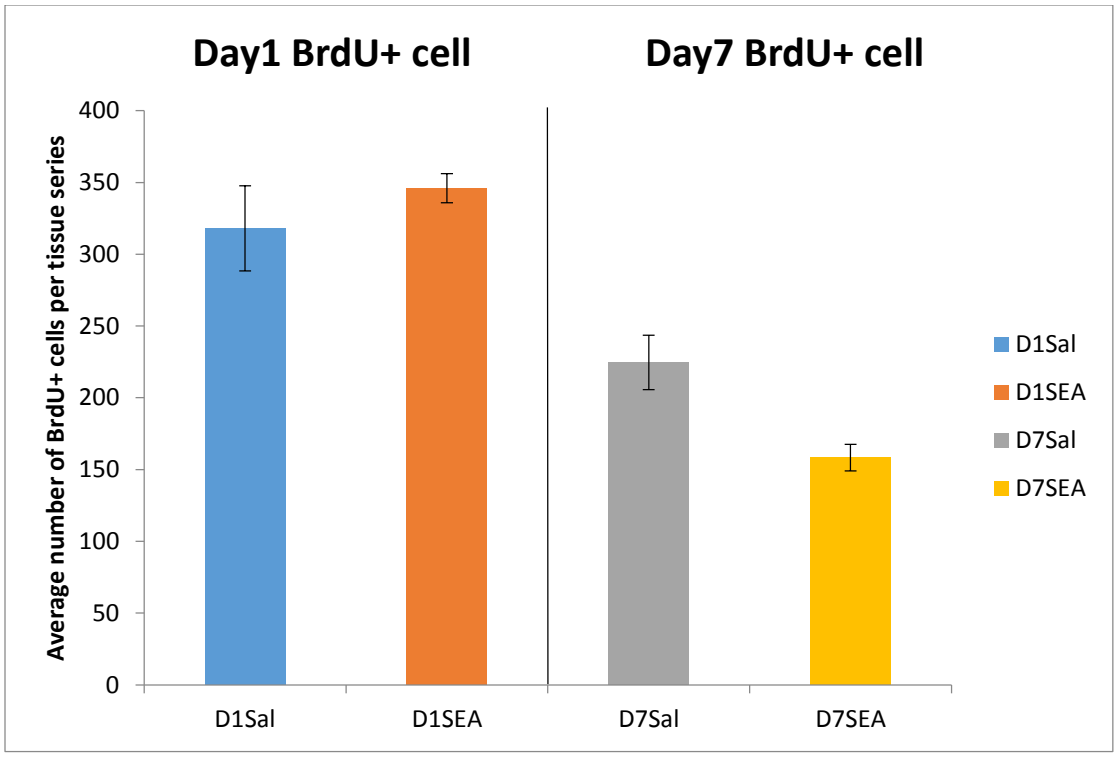

Figure 4. Experiment 1: Counting of BrdU positive cells. Mean number of BrdU+ cells in the dorsal hippocampus for each treatment group. The average per animal was calculated across 7-8 sections spanning the rostro-caudal extent of the dorsal dentate gyrus. N = 5-7/gp.

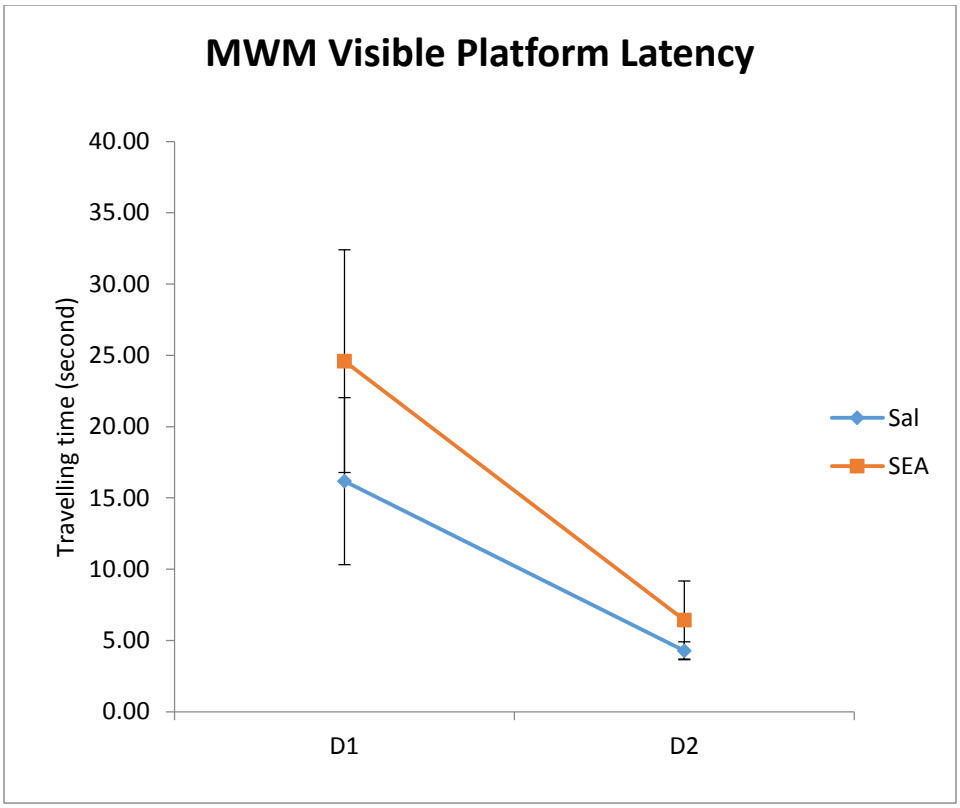

Figure 5. Experiment 2: MWM visible platform training. Mean latency to reach escape platform in visible platform training of MWM showed no difference between the two groups.

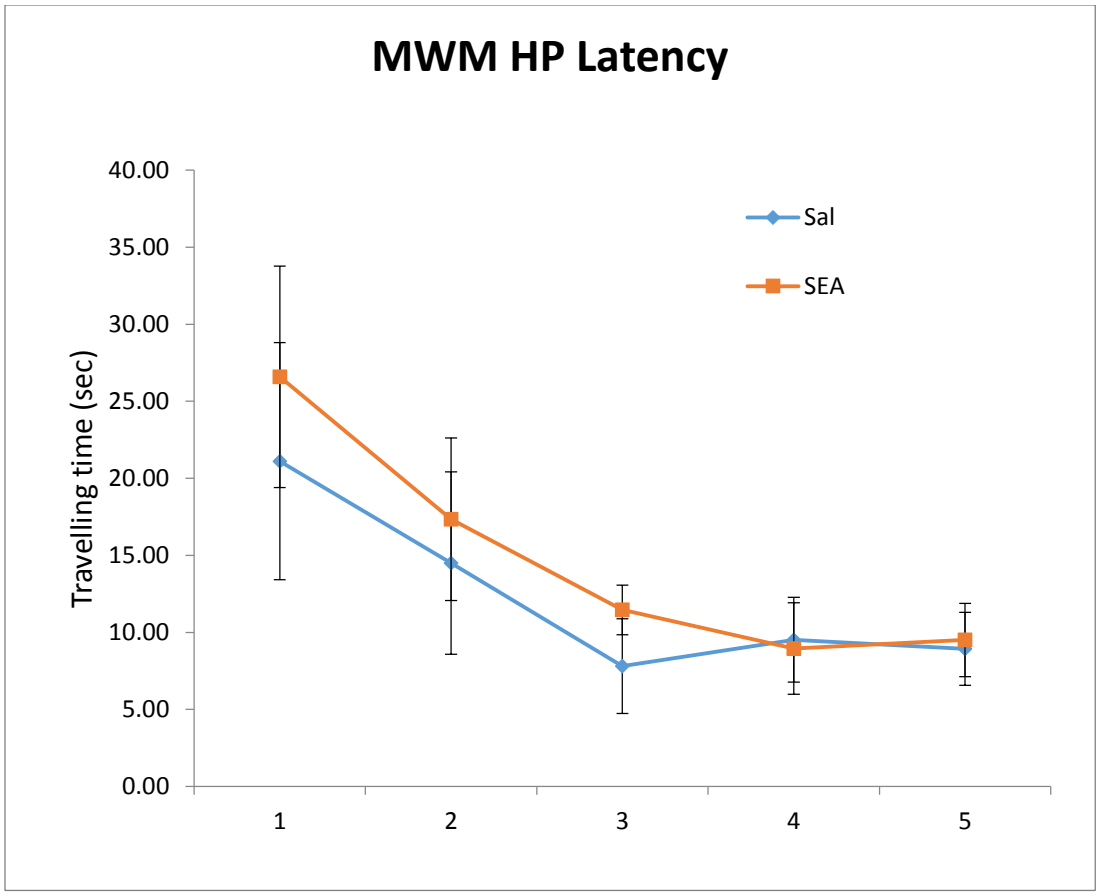

Figure 6. Experiment 2: Latency to reach platform in HP training of MWM. No difference was found between groups.

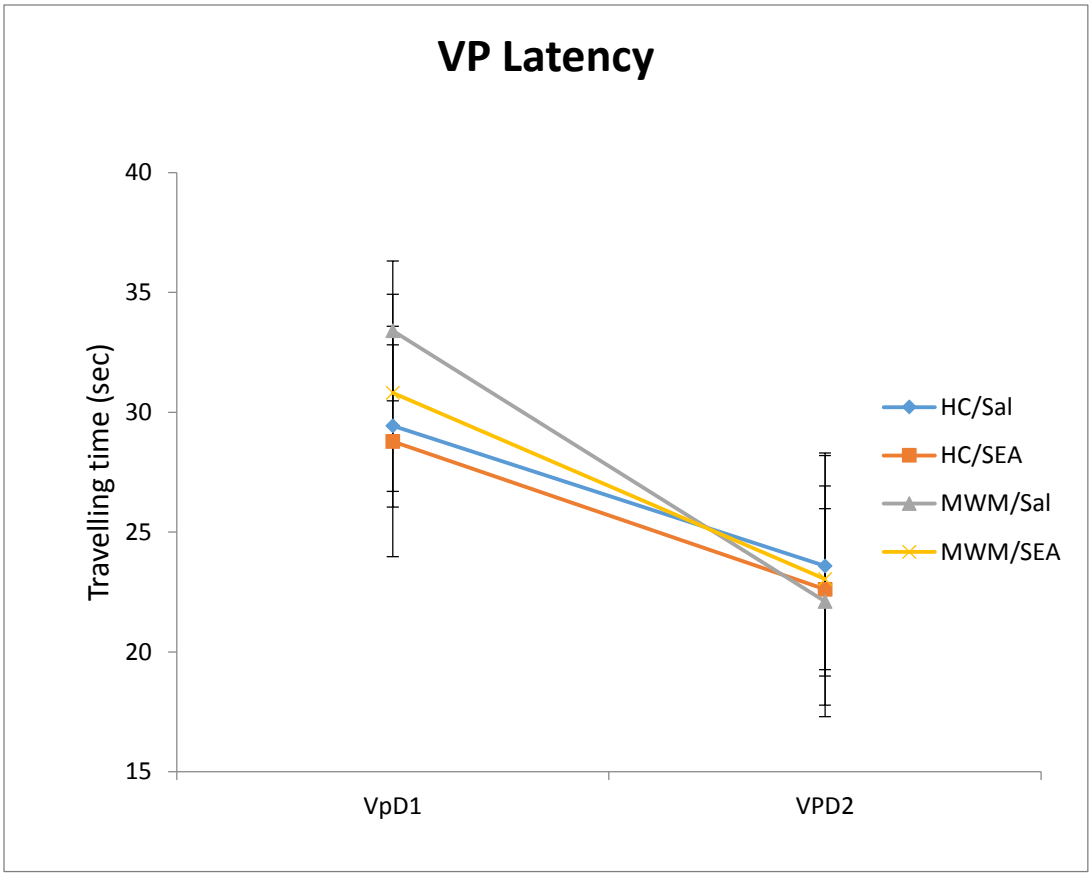

Figure 7. Experiment 2: Latency to reach escape platform in VP training of wRAM. No difference was found between groups.

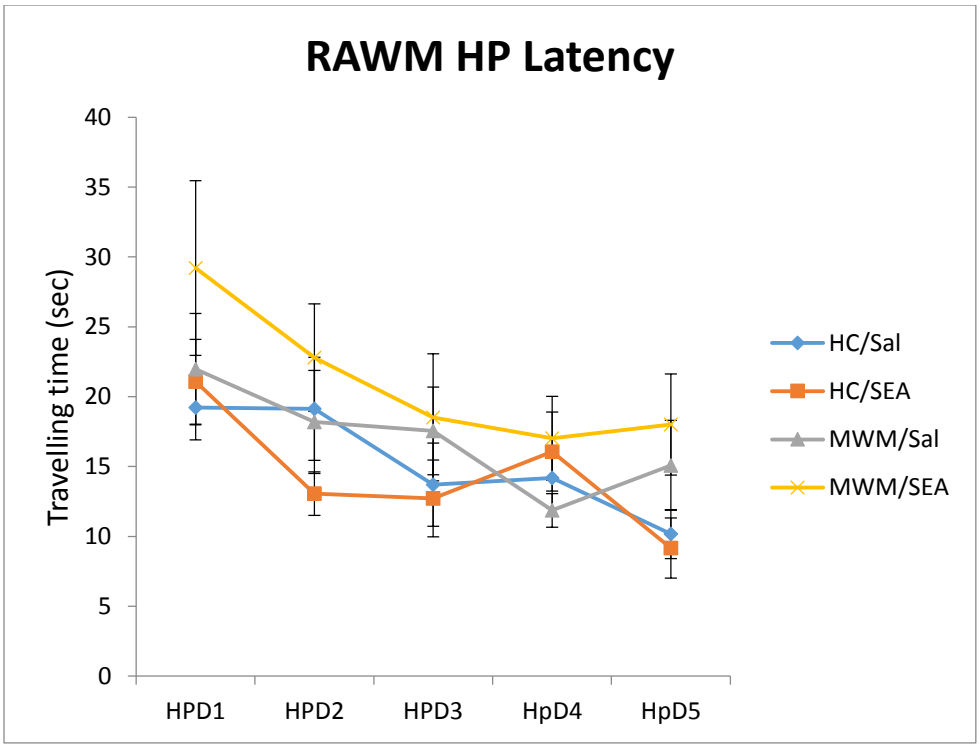

Figure 8. Experiment 2: Latency to reach escape platform in hidden platform training of wRAM. No difference was found between groups.

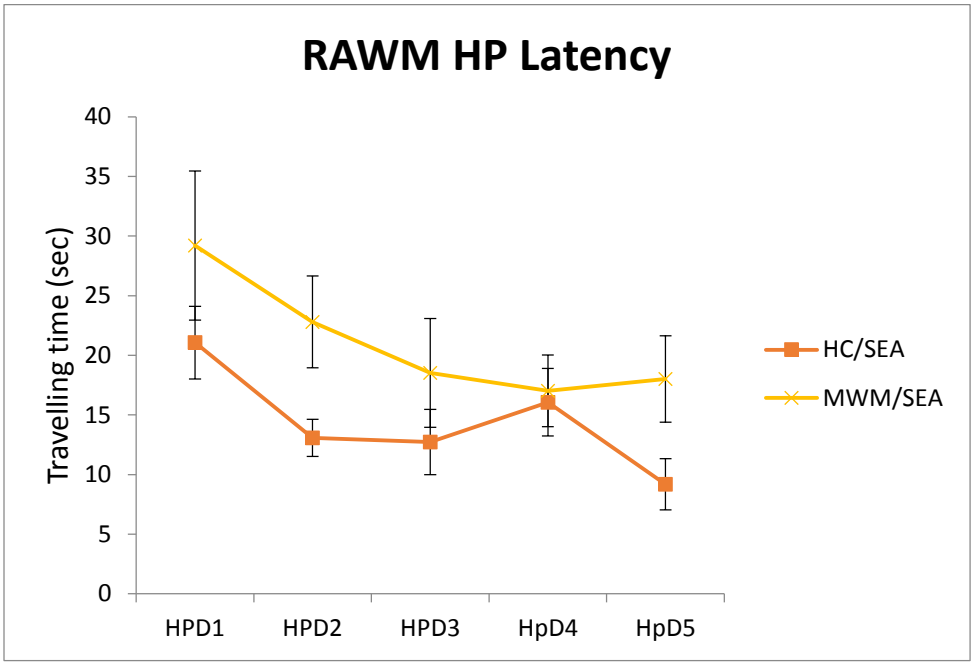

Figure 9. Experiment 1: Latency to reach platform in HP training of SEA groups. The two SEA treated groups are compared for their learning curve to locate the HP. No difference was found between groups.

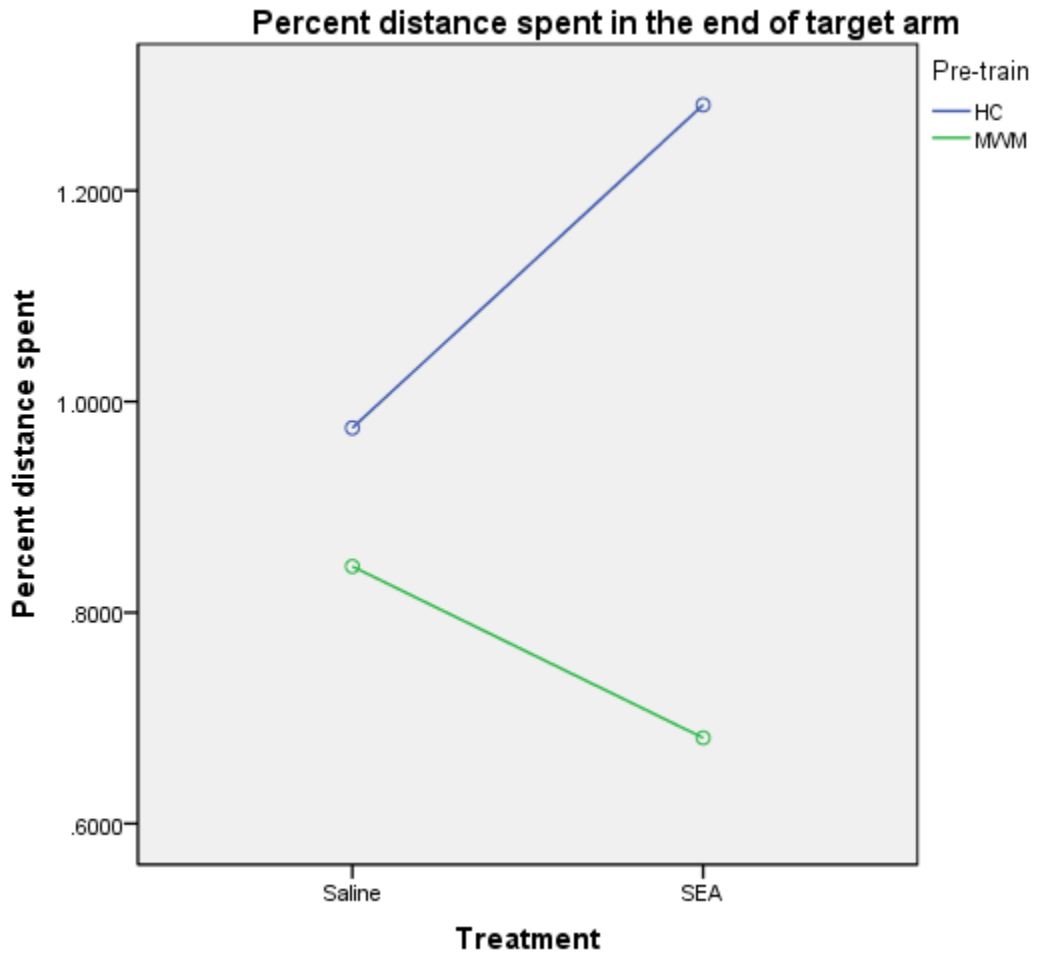

Figure 10. Experiment 2: The percentage of travelling distance in targeted area. The percentage of travelling distance in the end of the target arm over travelling distance of the 60 second probe test was tested. The Saline treated animals did not show a difference between the HC and MWM group, while the SEA treated animals showed worse memory with MWM pre exposure.

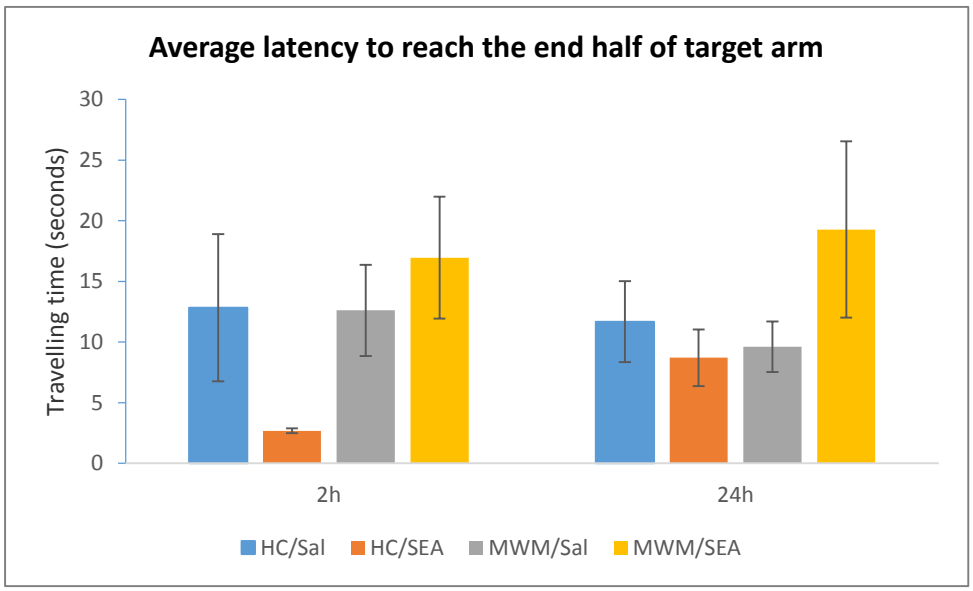

Figure 11. Experiment 2: Average latency to reach target zone. Average latency (±SE) to reach the end half of target arm in the probe test (N=8/group) was measured.

Reference

Bentley GA, Mariuzza RA. The structure of the T cell antigen receptor. Annu Rev Immunol. 1996; 14: 563–590.

Bluthé, R., Crestani, F., Kelley, K. W., & Dantzer, R. (1992). Mechanisms of the Behavioral Effects of Interleukin 1 Role of Prostaglandins and CRFa. Annals of the New York Academy of Sciences, 650(1), 268-275.

Bluthe, R. M., Pawlowski, M., Suarez, S., Parnet, P., Pittman, Q., Kelley, K. W., & Dantzer, R. (1994). Synergy between tumor necrosis factor α and interleukin-1 in the induction of sickness behavior in mice. Psychoneuroendocrinology, 19(2), 197-207.

Burghardt, N. S., Park, E. H., Hen, R., & Fenton, A. A. (2012). Adult ‐ born hippocampal neurons promote cognitive flexibility in mice. Hippocampus, 22(9), 1795-1808.

Butovsky, O., A. E. Talpalar, K. Ben-Yaakov, M. Schwartz. 2005. Activation of microglia by aggregated beta-amyloid or lipopolysaccharide impairs MHC-II expression and renders them cytotoxic whereas IFN-gamma and IL-4 render them protective. Mol. Cell. Neurosci. 29: 381–393.

Dantzer, R., O'Connor, J. C., Freund, G. G., Johnson, R. W., & Kelley, K. W. (2008). From inflammation to sickness and depression: when the immune system subjugates the brain. Nature reviews neuroscience, 9(1), 46-56.

Danzer, R. (2001). Cytokine-induced sickness behavior: Where do we stand. Brain Behav Immun, 15, 7-24.

Deng, W., Aimone, J. B., & Gage, F. H. (2010). New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory?. Nature Reviews Neuroscience, 11(5), 339-350.

Derecki, N. C., Cardani, A. N., Yang, C. H., Quinnies, K. M., Crihfield, A., Lynch, K. R., & Kipnis, J. (2010). Regulation of learning and memory by meningeal immunity: a key role for IL-4. The Journal of experimental medicine, 207(5), 1067-1080.

Fanselow, M. S., & Dong, H. W. (2010). Are the dorsal and ventral hippocampus functionally distinct structures?. Neuron, 65(1), 7-19.

Fraser, J. (1969). High affinity binding of staphylococcal enterotoxins A and B to HLA-DR. Nature 339, 221-223.

Germain, R. N. (2002). "T-cell development and the CD4-CD8 lineage decision." Nat Rev Immunol 2(5): 309-22.

Gould, E., Beylin, A., Tanapat, P., Reeves, A., & Shors, T. J. (1999). Learning enhances adult neurogenesis in the hippocampal region. Nature Neuroscience, 2, 260–265.

Kawabe, Y., and A. Ochi. 1990. Selective anergy of VB8+, CD4 + T cells in Staphylococcus enterotoxin B-primed mice. J. Exp. Med. 172:1065-1070. 25.

Jameson, S. C. and M. J. Bevan (1998). "T-cell selection." Curr Opin Immunol 10(2): 214-9.

Kawashima, N. and A. W. Kusnecov (2002). "Effects of staphylococcal enterotoxin A on pituitary-adrenal activation and neophobic behavior in the C57BL/6 mouse." J Neuroimmunol 123(1-2): 41-9.

Kelley, K. W., Bluthé, R. M., Dantzer, R., Zhou, J. H., Shen, W. H., Johnson, R. W., & Broussard, S. R. (2003). Cytokine-induced sickness behavior. Brain, Behavior, and Immunity, 17(1), 112-118.

Kumar, S., & Jack, R. (2006). Invited review: origin of monocytes and their differentiation to macrophages and dendritic cells. Journal of endotoxin research, 12(5), 278-284.

Kusnecov, A. W., R. Liang, et al. (1999). "T-lymphocyte activation increases hypothalamic and amygdaloid expression of CRH mRNA and emotional reactivity to novelty." J Neurosci 19(11): 4533-43.

Leuner, B., & Gould, E. (2010). Structural plasticity and hippocampal function. Annual review of psychology, 61, 111.

McAfoose, J., & Baune, B. T. (2009). Evidence for a cytokine model of cognitive function. Neuroscience & Biobehavioral Reviews, 33(3), 355-366.

Mellman, I. and R. M. Steinman (2001). "Dendritic cells: specialized and regulated antigen processing machines." Cell 106(3): 255-8.

Murphy, K. M., & Reiner, S. L. (2002). The lineage decisions of helper T cells. Nature Reviews Immunology, 2(12), 933-944.

Mitsuyama, M., & Liu, D. (Ed.). (2008). Handbook of Listeria monocytogenes. CRC Press.

Nokia, M. S., Sisti, H. M., Choksi, M. R., & Shors, T. J. (2012). Learning to learn: theta oscillations predict new learning, which enhances related learning and neurogenesis. PloS one, 7(2), e31375.

Omoe K, Nunomura W, Kato H, Li ZJ, Igarashi O, Araake M, Sano K, Ono HK, Abe Y, Hu DL et al. (2010) High affinity of interaction between superantigen and T

cell receptor Vβ molecules induces a high level and prolonged expansion of superantigen-reactive CD4+ T cells. J Biol Chem 285, 30427–30435.

Proft, T. and J. D. Fraser (2003). "Bacterial superantigens." Clin Exp Immunol 133(3): 299-306.

Rossi-George, A., LeBlanc, F., Kaneta, T., Urbach, D., & Kusnecov, A. W. (2004). Effects of bacterial superantigens on behavior of mice in the elevated plus maze and light–dark box. Brain, behavior, and immunity, 18(1), 46-54.

Shors, T. J., Miesegaes, G., Beylin, A., Zhao, M., Rydel, T., & Gould, E. (2001). Neurogenesis in the adult is involved in the formation of trace memories. Nature, 410, 372–376.

Shors, T. J., Anderson, M. L., Curlik, D., & Nokia, M. S. (2012). Use it or lose it: how neurogenesis keeps the brain fit for learning. *Behavioural brain research*, *227*(2), 450-458.

Wolf, S. A., Steiner, B., Akpinarli, A., Kammertoens, T., Nassenstein, C., Braun, A., ... & Kempermann, G. (2009). CD4-positive T lymphocytes provide a neuroimmunological link in the control of adult hippocampal neurogenesis. The Journal of Immunology, 182(7), 3979-3984.

Wolf, S. A., Steiner, B., Wengner, A., Lipp, M., Kammertoens, T., & Kempermann, G. (2009). Adaptive peripheral immune response increases proliferation of neural precursor cells in the adult hippocampus. The FASEB Journal, 23(9), 3121-3128.

Woodruff, R. T. (2011). Effects of staphylococcal enterotoxin A on impulsivity and spatial cognition in C57BL/6 mice (Doctoral dissertation, Rutgers University-Graduate School-New Brunswick).

Vidlak, D., Mariani, M. M., Aldrich, A., Liu, S., & Kielian, T. (2011). Roles of Toll-like receptor 2 (TLR2) and superantigens on adaptive immune responses during CNS staphylococcal infection. Brain, behavior, and immunity, 25(5), 905-914.

Vitkovic, L., Konsman, J. P., Bockaert, J., Dantzer, R., Homburger, V., & Jacque, C. (2000). Cytokine signals propagate through the brain. Molecular psychiatry, 5(6), 604-615.

Ziv, Y., Ron, N., Butovsky, O., Landa, G., Sudai, E., Greenberg, N., ... & Schwartz, M. (2006). Immune cells contribute to the maintenance of neurogenesis and spatial learning abilities in adulthood. *Nature neuroscience*, *9*(2), 268-275.