

CCL11 Inhibits Neurogenesis after Sepsis

Abstract

The mechanisms of brain injury in sepsis are not well characterized despite the significant brain dysfunction that sepsis-survivors experience. CCL11 levels are dysregulated in a number of neurodegenerative disorders and aging¹. Our research aims to better understand whether CCL11 plays a role in regulating brain homeostasis in sepsis, given the current literature does not have much information on CCL11 in this context. We find that our CCL11 levels are elevated in the brain after sepsis in a murine model. We show that both Ki-67 and Mcm2 expression, markers of cell proliferation, are decreased in the neurogenic subventricular zone and dentate gyrus after sepsis, and rescued by neutralization of CCL11. By quantifying Iba1 expression, we find that CCL11 also increases microglial activation and neuroinflammation. Together this shows that CCL11 not only has a role in inhibiting neurogenesis but also for activating inflammatory immune cells in the neurogenic niche.

Introduction

Sepsis is characterized by a set of systemic inflammatory reactions in response to intensive infection that cannot be locally contained by the host². Over 1.3 million patients survive sepsis every year in the United States alone, but survivorship comes with the burdens of long-term brain dysfunction, mood disorders, and chronic illness^{3,4}. The mechanisms of brain injury in sepsis are not well characterized despite the significant brain dysfunction that those that have sepsis may experience. Given the significant disability that sepsis survivors experience, studying the pathologic processes that continue after illness is necessary to reduce the effects of sepsis.

Patients who die of sepsis have multiple forms of brain pathology, including inflammation and breakdown of the blood brain barrier (BBB)⁵. However, it is not possible to study these processes in sepsis survivors because they require examining brain tissue. Animal models have demonstrated long-lasting inflammation in the brains of animals that survive sepsis⁶⁻⁸. Our laboratory currently uses a model of abdominal sepsis, cecal ligation and puncture (CLP) to study the long-term effects of sepsis in mice⁶. Given the public health impact of sepsis survival, comparatively little research has been done to understand the mechanisms that sustain brain dysfunction after sepsis, and there have been no trials of interventions to decrease the long-term effects of sepsis after it has been resolved⁹.

Our aims strive to improve upon the current research of sepsis that exists. In our current model of murine sepsis, we induce sepsis in mice through CLP and study the brains of animals that recover from this illness⁶. This allows us to measure gene and protein expression throughout the whole brain and view the infiltration of inflammatory cells. CLP has shown to stimulate the innate immune response, which includes significant activation of microglia and the expression of cytokines¹⁰. Monocytes and astrocytes are also important sources of cytokines and may possibly play a role in ongoing neuroinflammation¹¹. By studying these different cells through the stimulation of sepsis through a murine model, the goal is to understand the mechanisms of brain injury in patients who survive sepsis or critical illness.

In our laboratory's study of brain inflammation after sepsis, we have found that levels of the cytokine CCL11, also known as eotaxin, are elevated in the brains of sepsis survivor mice for at

least two weeks after sepsis. In both mice and humans, CCL11 levels also increase in plasma and cerebrospinal fluid with aging. Recent studies have shown that chemokines such as CCL11 may directly contribute to degenerative processes in the central nervous system (CNS) ^{1,12–16}. Not only has it been suggested that CCL11 is dysregulated during neurodegenerative disorders, but also that increasing the systemic level of CCL11 causes inhibition of adult neurogenesis and impairment of learning and memory^{1,12}. When compared to healthy controls, levels of CCL11 are elevated throughout the plasma in Alzheimer's disease, Huntington's disease, progressive multiple sclerosis, and schizophrenia^{12,16}. By identifying if CCL11 signaling is active after sepsis, steps can be made to identify the pathways for sepsis-induced brain dysfunction treatment, especially because inhibitors of CCL11 signaling have been tested in other diseases and are safe for use in humans¹⁷. The goal of the study was to determine if CCL11 during active sepsis contributes to the suppression of neurogenesis. Given CCL11's potential role in suppressing neurogenesis, further research must be done to identify the mechanism in which this chemokine impacts sepsis survivors.

Background

Sepsis and the Innate Immune System

Sepsis is a clinical syndrome that results from a complex interaction between the host and infectious agents and is characterized by the activation of multiple inflammatory pathways creating a systemic inflammatory response to infection¹⁸. During sepsis, the innate immune system is dysregulated and cellular and metabolic changes occur¹⁸. Cells and tissues such as the vascular endothelium, the heart, and the brain that are not routinely exposed to pathogens produce a significant inflammatory burst upon initial exposure and are subsequently followed by monocytes and macrophages infiltration into tissue¹⁹. Monocytes and macrophages produce cytokines and chemokines through the induction of early response genes such as TNF- α and IL-6. These cytokines, including CCL11, amplify the early inflammatory response by recruiting neutrophils to the infected site, which express pathogen killing oxygen-free radicals¹⁹. It is a disruption in the normal immune response that causes excessive pro-inflammatory cytokine response and eventually sepsis. To induce sepsis in rodents, our laboratory uses a reproducible, titratable, naturalistic CLP model that imitates the clinically relevant human condition^{2,6}. Our previous research shows that sepsis survivor mice continue to experience significant neuroinflammation for months after CLP and that brain dysfunction persists at 50 days post-CLP⁶.

Regions of Neurogenesis in Rodents

Studies have shown that neurogenesis is primarily present in young rodents and drastically decreases with aging ^{22–24}. Mammalian adult neurogenesis occurs primarily in two regions of the brain: the subventricular zone (SVZ) along the lateral ventricle and the subgranular zone of the dentate gyrus (DG) of the hippocampus^{20,25}. The neuronal precursor cells in these areas migrate and differentiate into new neurons of the olfactory bulb in SVZ neurogenesis and the hippocampus in the DG neurogenesis²⁵.

Neuronal Cell Cycle and Biochemical Markers

For a cell to develop into a neuron, there must be cell division and cell differentiation. Both of these processes are very carefully regulated and coordinated by molecules that direct the cell

cycle^{26,27}. Our research tested Ki-67, Mcm2, and 5'-bromo-2'-deoxyuridine (BrdU) as markers of dividing cells.

Ki-67 has been used frequently as a marker of cell proliferation, as it is expressed in dividing cells for the majority of the mitotic process (G1, S, G2, and M phases)²⁸. Ki-67 is a large nuclear protein that undergoes phosphorylation and dephosphorylation during mitosis and shares structural similarities with other cell-cycle regulatory proteins²⁹. While the function of Ki-67 is not known, it has been used as a reliable marker of mitosis due to its consistent expression during mitosis, confinement to the nucleus, and short half-life. Most importantly, studies have shown that Ki-67 is expressed during mitosis in all mammalian species, including rodents and humans.

Mcm2 has been reported as a marker of cells actively undergoing proliferation³⁰. Mcm2 is a protein that is involved in the formation of the pre-replicative complex necessary for the initiation of DNA synthesis. Mcm2 has been seen to be expressed during the G1 and S phase³⁰. Studies have reported that there is a high correlation between Mcm2 and Ki-67 but immunohistochemistry for Mcm2 consistently identifies a higher proportion of dividing cells than Ki-67. Given the rate of neurogenesis in adult mammals is relatively low, Mcm2 might prove useful to identify a greater cell population.

BrdU is the third marker of cell proliferation that we used. Rodents injected with BrdU have the thymidine analog incorporated into their DNA at the time of DNA synthesis, allowing for birth dating of cells. By injecting mice with BrdU the days leading up to euthanasia and then staining brain tissue for the marker, observation of how and when proliferating cells are being generated from dividing their respective progenitor cells in the regions previously identified can be done²⁹. One drawback of using BrdU is the stressful injection procedure and uncertain uniformity in BrdU expression in targeted cells.

Impacts of Neuroinflammation on Neurogenesis

When the inflammatory response occurs in the CNS, it is known as neuroinflammation²⁰. This process is characterized by the activation of microglia and astrocytes, subsequent increase in cytokine and chemokines, and eventual disruption of the BBB that leads to the migration of circulating immune cells to the injury site. While neuroinflammation can be protective in the short-term, chronic inflammation due to lipopolysaccharide infusion can lead to neuronal damage and impairment of neurogenesis²⁰. It has been shown that activated microglia, which secrete TNF- α , IL-6, and IL-1 β , lead to decreased proliferation, survival and neuronal differentiation.

More recently CCL11, which historically has been studied in an allergic reaction framework, has been reported to be linked to adult neurogenesis and aging, leading to questions regarding the chemokine's presentation in sepsis survivors which have decreased neurogenesis¹. We hypothesized that CCL11 has a role in microglial activation and tested this by measuring Iba1 immunoreactivity.

Emerging Literature on the Importance of CCL11 in Neurogenesis

The potential importance of CCL11 came to our attention when Villeda's *The Ageing Systemic Milieu Negatively Regulates Neurogenesis and Cognitive Function* was published in *Nature*¹. By using heterochronic parabiosis, the study showed that blood-borne factors, including CCL11, present in the systemic milieu could inhibit neurogenesis in an age-dependent fashion in mice. The study further concluded that increasing levels of CCL11 was shown to be correlated with reduced neurogenesis in heterochronic parabionts and aged mice. They also showed that increasing peripheral CCL11 chemokine levels (*in vivo*) in young mice decreased adult neurogenesis and impaired learning and memory¹.

In recent years, there has been an emergence of literature related to CCL11^{12–16}. Smith's *The Systemic Environment: At the Interface of Aging and Adult Neurogenesis* synthesized and reviewed current research on how age-related changes in blood, blood-borne factors and peripheral immune cells contribute to the decline in adult neurogenesis and potential neurogenic niche-mediated mechanisms¹⁵. He refers to Villeda's study as a forefront driver of demonstrating how the immune-related molecular and cellular changes in the aging systemic environment negatively regulate adult neurogenesis. The review also highlights how age-related decline in neurogenesis can be ameliorated by exposure to the young systemic environment and may lead to potential treatments in the future. While the mechanisms driving the decline in neurogenesis with aging are not well known, Smith hypothesizes that circulating factors directly modulate neural stem/progenitor cell function or by indirectly altering signaling in the local neurogenic niche. Huber's research supports this mechanism by concluding that since CCL11 is capable of crossing the blood-brain barrier of normal mice then CCL11 produced in the periphery mostly likely exerts physiological and pathological actions in the CNS¹².

Other studies that have built off of Villeda's initial work include a group that found that young bone marrow transplantation preserves learning and memory in older mice¹⁴. Studies have reported that CCL11 levels were lower in young bone marrow recipients and led to the preservation of synaptic connections and reduced microglial activation in the hippocampus. On the other hand, when young mice were administered with CCL11, the opposite effect occurred and these mice had reduced synapses and increased microglial activation. Clinical studies of the effects of CCL11 have begun in a number of neurodegenerative disorders and psychiatric disorders including schizophrenia¹⁶. It was reported the CCL11 levels had a strong impact on memory and sustained attention including development in formal thought disorders and psychopathology and that further research should be conducted to see if CCL11 inhibitors could be a novel therapy.

All of these previous studies have solidified the importance of prolonged exposure of CCL11 in aging. Looking at sepsis as an acute disease state, we are interested in CCL11 exposure in a much shorter time frame – including questions of if the amount of CCL11 that is physiologically produced can have a significant effect in a short period of time and is CCL11 an important driver of changes of neurogenesis post-sepsis compared to the myriad of cytokines that are also produced. Despite the large burden of long-term brain dysfunction that sepsis survivors experience, there is a lack of literature characterizing the role of CCL11 in inhibiting neurogenesis after sepsis. Through our research, we hope to fill this research gap and eventually propose potential therapeutic methods in combating cognitive decline after a severe illness

Approach

Our aim is to determine how long after sepsis neurogenesis is suppressed and if CCL11 during acute sepsis contributes to suppression of neurogenesis. Trials were conducted with several cohorts of mice, examining markers of early neurogenesis such as Ki-67, Mcm2, and BrdU. Microglial activation and neuroinflammation were measured with Iba1 expression. Mice were also tested at both a more acute and longer time point. Naive or post-CLP mice were treated with isotype or anti-CCL11 antibody. Cohorts of animals were then examined at 96-hours then brains were stained for previously indicated markers. This may be repeated for a cohort of mice that are euthanized at 14 days and 6 weeks post-CLP in the future.

Materials and Methods

Mice

Wild-type mice used in the experiment were male C57BL/6 mice from Jackson Laboratory.

Cohort Set-Up and Injections

Using a murine model with three groups of mice, we determined the effects of CCL11 inhibition. Our groups include:

- Mouse (unoperated) + Control IgG
- Mouse + CLP + Control IgG
- Mouse + CLP + Neutralizing antibody against CCL11

Having three different cohorts of mice allows us to account for the effect of sepsis on brain inflammation, the specific effect of CCL11 neutralization, and the nonspecific effects of injecting mice with antibodies.

One of the cohorts was injected with an inhibitory antibody, specifically clone 42285, which targeted CCL11 and clear the cytokine from the circulation.³¹ As we speculate that CCL11 is coming from the circulation, inhibiting CCL11 itself rather than its receptor is likely to be effective even if the antibody does not pass the blood brain barrier. One of the other cohorts was injected with negative control mouse IgG which is used as a negative control for our inhibitory antibody. Negative control mouse IgG being used in place of the antibody allowed us to evaluate the nonspecific effects of injecting antibody on the immune system.³² All mice were injected with BrdU (100mg/kg) daily after CLP and before euthanization so that proliferating cells could later be stained. BrdU was given at this time point so that we could identify proliferating cells after inflammation was established but still early after sepsis, all in conjunction with the CCL11-antibody.

By modifying CCL11 levels by intraperitoneal injection of an anti-CCL11 antibody, which has previously been shown to decrease CCL11 levels and increase neurogenesis in aged animals in the setting of exogenous CCL11 administration, we were able to identify the continuous effect of the antibody during the early stages of sepsis.¹ We injected 50 mcg/kg of the antibody on the day of CLP and 48 hours after CLP (Figure 1).

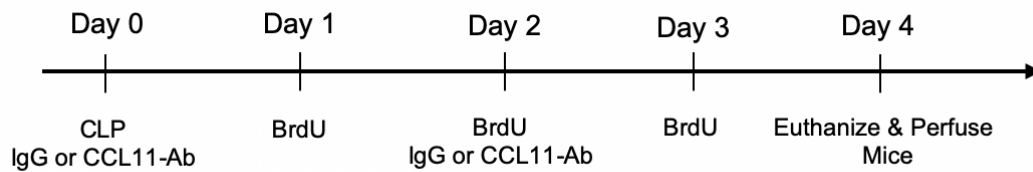


Figure 1. Timeline of Injections Given to Mice

Cecal Ligation and Puncture (CLP)

Cecal ligation puncture procedure (CLP) consists of the perforation of the cecum which allows the release of fecal material into the peritoneal cavity². This induces an immune response to a polymicrobial infection which simulates the multiple and overlapping mechanisms involved in sepsis. Most importantly, it is shown the mice share a similar cytokine profile seen in human sepsis when CLP is performed.² We used a 19 gauge needle in which we would then expect a 60% survival rate for the mice; this ensures that the survivor mice did indeed experience sepsis and minimizes survivor bias.

ELISA for CCL11

Mice were euthanized and transcardially perfused with PBS. Brain hemispheres were then frozen in liquid nitrogen. Protein containing homogenates were prepared by homogenizing frozen brain hemispheres with a rotor/status (Tissue Tearor) in RPMI medium. The raw homogenates were then cleared of particles by centrifugation at 20,000 x g for 30 minutes. CCL11 was quantified using anti-mouse CCL11 ELISA (R&D Systems).

Histology

Mice were euthanized and transcardially perfused with PBS and 4% paraformaldehyde at 96 hours post CLP. The brains were then removed, rinsed, and immersed in a buffered 30% sucrose solution until no longer buoyant. Cryoprotected tissues were then frozen on dry ice and stored at -80°C. Once the brains were needed for immunohistochemistry, we sectioned the brains into 40-micron sections using a cryostat. Tissue was stored at -20°C in cryoprotectant solution.

Immunofluorescence for CCL11

Sections were rinsed of cryoprotectant and then mounted on Superfrost Plus slides. Sections were then blocked and permeabilized (Tris-buffered saline with 10% serum and 0.4% Triton-X) and then incubated with anti-CCL11 antibody (capture antibody from R&D systems ELISA) overnight at 4°C. Sections were then washed and incubated with Alexa 488 conjugated secondary antibody for 2 hours, washed, and coverslipped in antifade medium.

Immunohistochemistry with DAB Peroxidase for Ki-67 and MCM2

Sections were first processed using a free-floating technique. Endogenous peroxidases were deactivated by treatment with 1% H₂O₂ and then washed with Tris buffer and TBS. Sections were then mounted to Superfrost Plus slides. The slides were then blocked and permeabilized by incubation with blocking buffer (Tris + 10% NGS/NHS + 0.4% Triton-X). Sections were incubated with anti-Ki-67 antibody (Cell Signaling D3b5, 1:400) or anti-MCM2 (Abcam, 1:200) overnight at room temperature. Sections were then washed and incubated with biotinylated horse anti-rabbit IgG secondary antibody (Vector, 1:200) for 90 minutes at room temperature. Sections

were washed again and then incubated with Vector Elite ABC solution for 1 hour. The tissue was reacted with DAB Peroxidase until fully developed. The sections were dried, dehydrated and cleared with washes in progressive (70%, 95%, 100%) ethanol solution and finally xylene. The tissue was coverslipped with Permount.

Immunohistochemistry with Tyramide Signal Application (TSA) for BrdU

Sections were processed using a free-floating technique. DNA was denatured with 2 hydrochloric acid for 30 min at 37°C and then neutralized with 0.1 M sodium borate for 10 min. Endogenous peroxidases were deactivated by treatment with 1% H₂O₂, followed by incubation with blocking buffer (10% NGS + 3% BSA + 1% glycine + 0.4% Triton-X in Tris-buffered saline). Sections were washed and mounted to Superfrost Plus slides. Sections were incubated with rat anti-BrdU antibody (AbD Serotec Clone: BU 1/75, 1:200) overnight at room temperature. Sections were then washed and incubated with biotinylated horse anti-rat and anti-rabbit IgG secondary antibody (Vector, 1:200) for 30 minutes at room temperature. To amplify the secondary antibody signal, we incubated each tissue section with 10 µl tyramide for 3 minutes. The sections were then washed and coverslipped in Antifade.

Immunofluorescence for Iba1

Sections were processed using a free-floating technique. Sections were washed with TBS to remove cryoprotectant and then mounted to Superfrost Plus slides. The slides were then blocked and permeabilized by incubation with blocking buffer (10% NHS + 3% BSA + 1% glycine + 0.4% Triton-X in Tris-buffered saline). Sections were incubated with anti-goat Iba1 antibody (Wako, 1:500) overnight at -4°C. Sections were then washed, blocked, and incubated with anti-goat Alexa 488 secondary antibody for 120 minutes at room temperature. The sections were then washed and coverslipped with Prolong Gold.

Counting of Ki67 and Mcm2 positive nuclei

The number of Ki67 or Mcm2 positive nuclei was counted in the SVZ and SGZ. Every 12th serial section was examined. For the SVZ, cells in sections from the anterior extent of the SVZ posteriorly to the fornix were counted. Cells were counted in the wall of the lateral ventricle and the rostral migratory stream. For the SGZ, cells were counted in all sections which demonstrated a distinct dentate hilus and granular cell layer. Cells were counted within a two-nucleus span from the border between the hilus and granular cell later. Cells were exhaustively counted through the entire thickness of each section, given the wide spacing of sections. Total counts per SVZ or SGZ were calculated by averaging counts from the left and right side of each section and then multiplying by 12.

Quantification of Iba1 staining

Iba1 stained sections at the level of the mid hippocampus were imaged using a Leica SP8 confocal microscope. 1-micron optical sections were taken through a 20-micron volume of each section and transformed into a maximum intensity projection. To determine the Iba1+ area of each section, images were thresholded at the level that identified the cell body of microglial cells. Single-pixel noise was eliminated by using the “erode” option in ImageJ, eliminating any pixel that is not adjacent to at least two other positive pixels.

Results

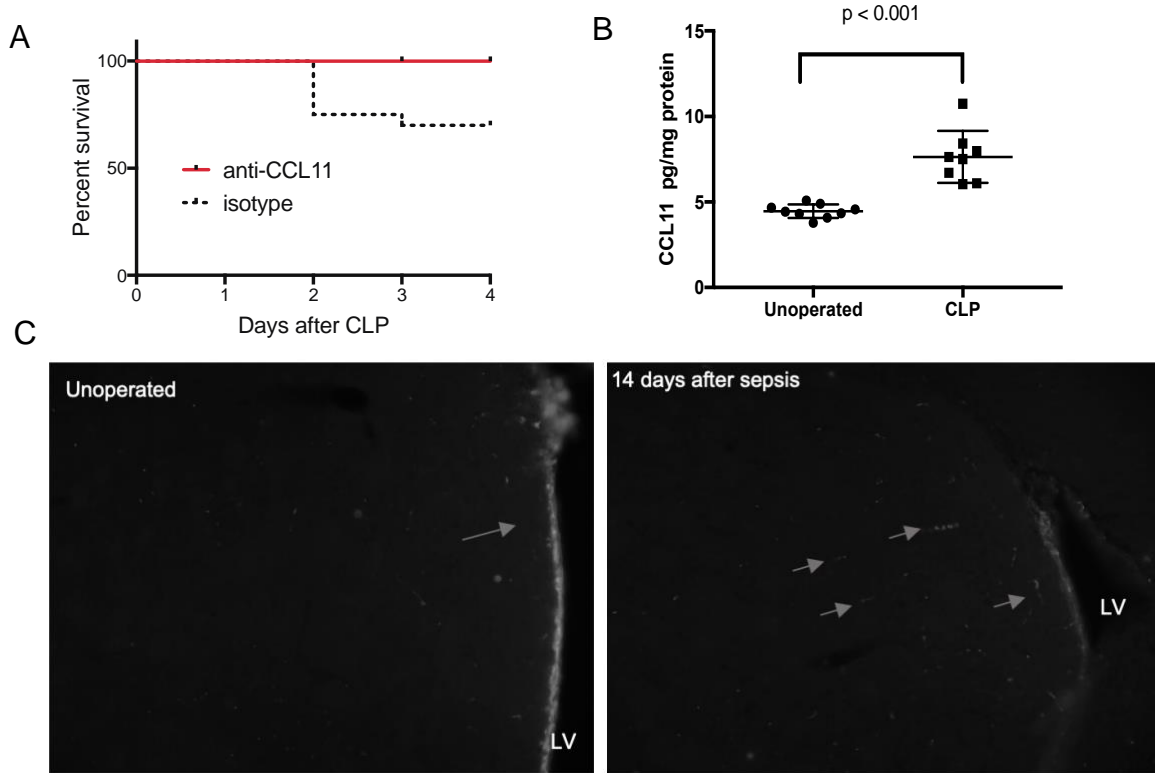


Figure 2. Sepsis survivor mice show an elevated expression of CCL11 due to recruitment and migration across the BBB.

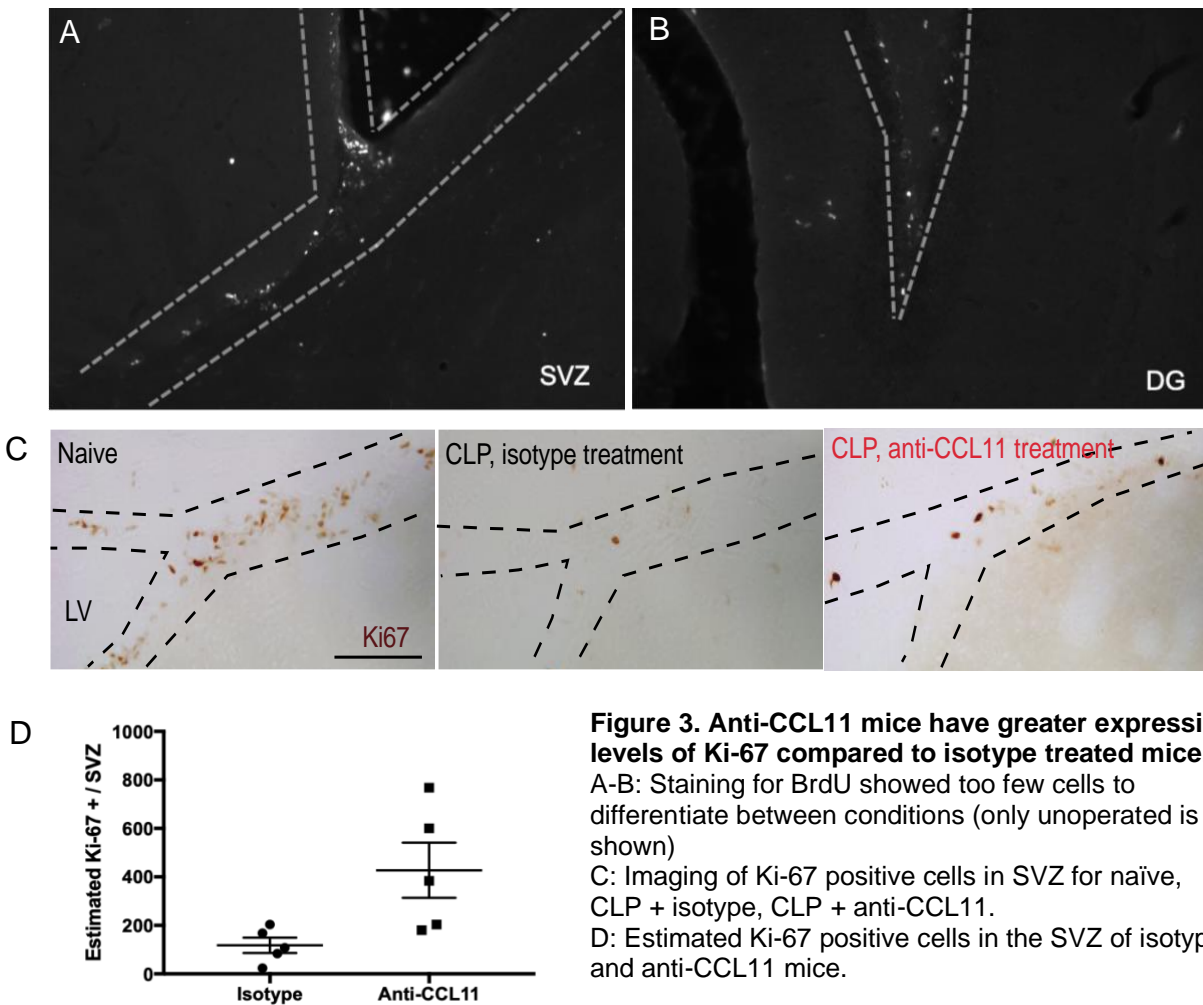
A: Survival data for post-CLP anti-CCL11 and isotype injected mice.

B: CCL11 expression levels in unoperated and CLP mice.

C: Immunofluorescent imaging shows that in post-CLP mice, there is greater diffuse perivascular CCL11 staining in the lateral ventricle.

CCL11 levels in the brain are elevated due to migration from the periphery rather than local production post-CLP.

We first sought to confirm that CCL11 levels are elevated in the brain post-CLP. After conducting CLP and injections, our data showed a 100% survival rate for anti-CCL11 injected mice and around a 60% survival rate for isotype injected mice (Figure 2A). Next by performing ELISAs, we found that CCL11 levels are elevated in mice that survive sepsis in comparison to unoperated healthy mice two weeks after sepsis (Figure 2B). In this same model, sepsis results in the infiltration of inflammatory cells such as neutrophils and monocytes that lasts for at least two weeks after sepsis. As CCL11 is capable of crossing the BBB in healthy mice, it is possible that CCL11 is produced in the periphery and has the ability to infiltrate and harm the CNS¹². Using immunohistochemistry, we wanted to determine if the CCL11 was being produced locally in the brain or was being recruited from the periphery. Current images show an increase of CCL11 found near blood vessels (Figure 2C). Increased diffuse perivascular staining, which is shown with the arrowheads, is prevalent in sepsis survivor mice compared to unoperated control mice. This data leads us to hypothesize that elevated levels of CCL11 is due to synthesis by vascular or perivascular cells and then migration across the BBB, rather than being produced by localized brain cells such as microglia.



Ki-67 expression is elevated in neurogenic SVZ in mice that were treated with anti-CCL11.

To analyze the impacts of CCL11 on neurogenesis, we conducted DAB/Ki-67 staining. Initially, we stained for BrdU with Tyramide but the samples showed too few cells to make a reliable statistical differentiation between conditions so we continued with other cell proliferation markers (Figure 3A-B). We counted serial sections in a blinded fashion that contained Ki-67 positive cells located in the SVZ (Figure 3C). We compared the cell counts by ANOVA. We found that there were statistically significant increases in Ki-67 expression in the neurogenic sub-ventricular zone in anti-CCL11 injected mice compared to isotype treated sepsis survivor mice (96 hours post-CLP), leading us to believe that the lack of neurogenesis in sepsis may be mediated by CCL11 (Figure 3D). However, while the cells in the SVZ demonstrated robust immunoreactivity for Ki67, only a few cells were seen in the SGZ, making this marker unsuitable for that region. We thus explored the utility of Mcm2 as a better marker of cell division in both neurogenic regions.

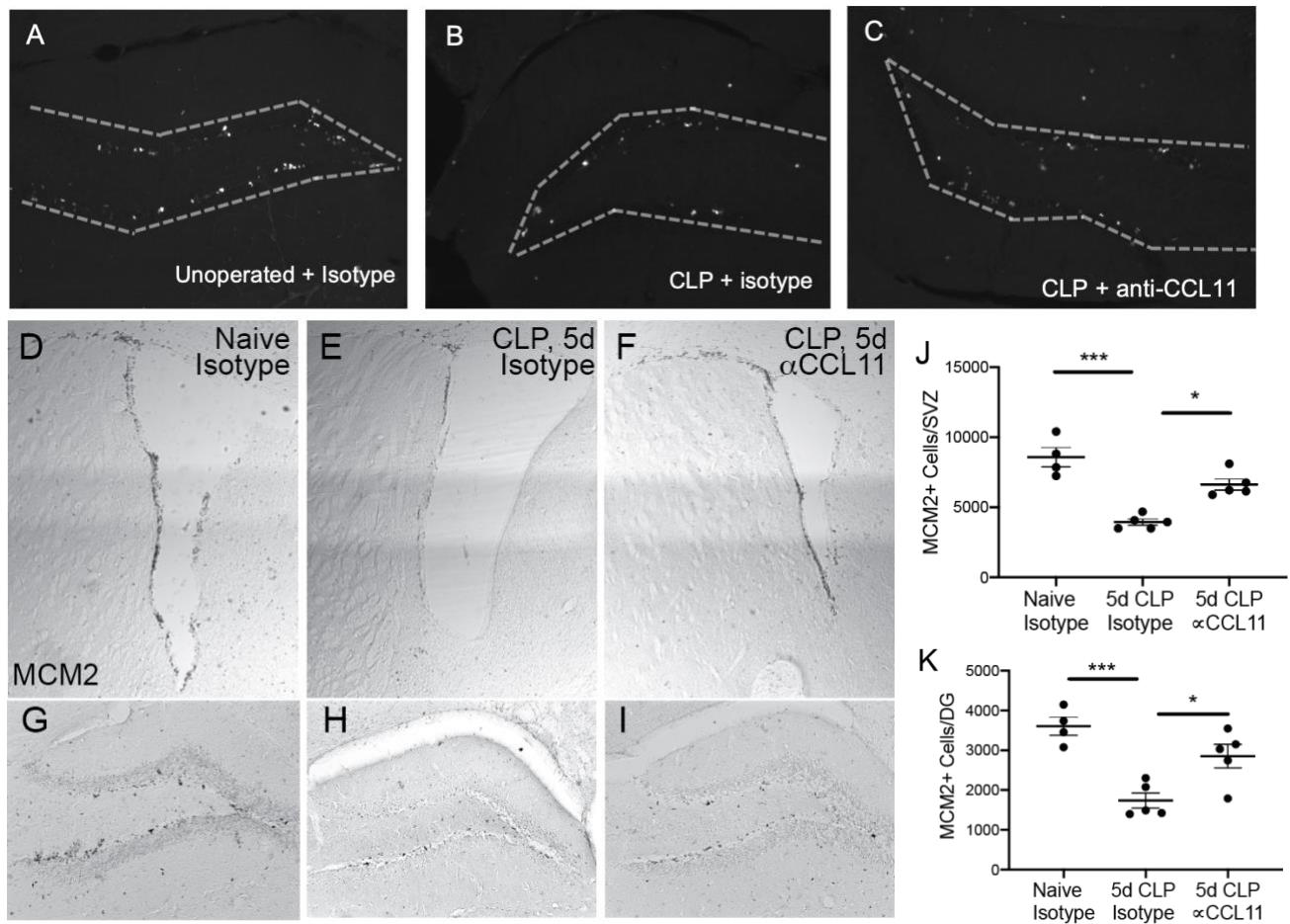


Figure 4. Anti-CCL11 treatment rescues neurogenesis in both the SVZ and DG.

A-C: Initial test of tyramide staining of Mcm2 positive cells in the SVZ in the three murine cohorts.

D-I: Imaging of Mcm2 positive cells in the SVZ and DG for all three murine cohorts. There is distinct rescue effect of Mcm2 positive cells in anti-CCL11 treated mice.

J-K: Mcm2 positive cell counts in the SVZ and DG for all three murine cohorts. There is statistically significant difference in cell counts between CLP + isotype and CLP + anti-CCL11 treatment, but not between naïve + isotype and CLP + anti-CCL11 treatment

Mcm2 expression is rescued in both neurogenic regions (SVZ, DG) for mice that are treated with anti-CCL11 antibody.

With our Tyramide and DAB immunohistochemistry protocol, we conducted Mcm2 staining and found that it showed the best staining results for both neurogenic regions out of all three of our potential cell proliferation markers (Figure A-I). We counted serial sections in a blinded fashion that contained Mcm2 positive cells located in the SVZ and DG using brightfield microscopy (Figure 4D-I). We then compared the cell counts by ANOVA. Imaging and cell counts show distinct differences between CLP + anti-CCL11 and CLP + isotype cohorts with Mcm2 positive cell expression being statistically higher and lower respectively. This leads us to conclude that blocking CCL11 in the brain leads to a rescuing effect for neurogenesis, as shown with elevated Ki-67 and Mcm2 expression levels in our anti-CCL11 treated mice.

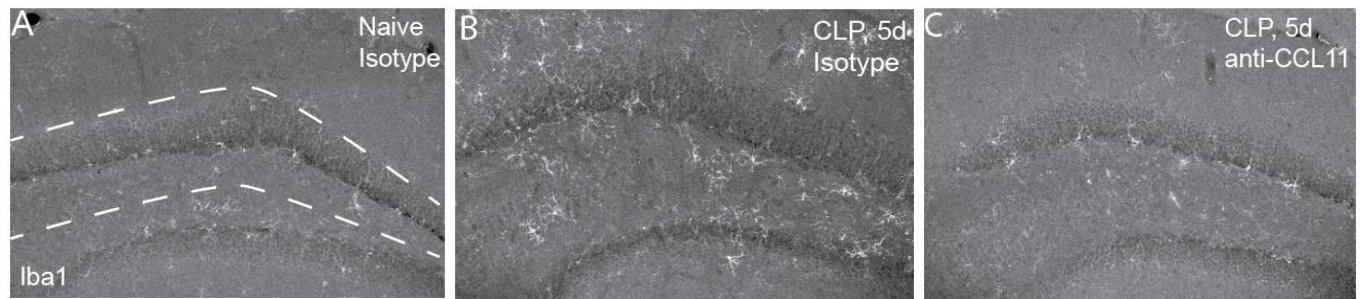


Figure 5. CCL11 plays a role in microglia activation, neuroinflammation and impairment of neurogenesis.

A-C: Imaging of Iba1 positive pixels in the DG for all three murine cohorts.

D: Percent Iba1 positive pixels in the area for all three murine cohorts

CCL11 increases microglial activation

As stated earlier, neuroinflammation can be protective in an acute timeframe, but chronic inflammation can lead to impairment of neurogenesis. To complement the previous findings in CCL11's role in inhibiting neurogenesis, we wanted to see if CCL11 played a role in the regulation of microglial activation. We stained tissue from our three murine cohorts with Iba1, a known marker of microglia. Neutralizing CCL11 decreases microglial activation and neuroinflammation to a similar phenotype to unoperated mice. This confirms our hypothesis that CCL11 has a role in microglial activation and neuroinflammation post-sepsis.

Discussion

Long-term brain dysfunction, cognitive decline, and chronic disability are a few of the significant clinical problems that sepsis survivors experience. Despite the burden that sepsis survivors experience, the mechanisms of brain injury in sepsis are not well characterized. Our goal is to improve upon the current research of sepsis that exists, using a CLP model to study the acute inflammatory response in brains of mice that recover from this illness.

During our study of brain inflammation after sepsis, we found that levels of CCL11 were elevated in the brains of sepsis survivor mice. This intrigued us to see what literature exists regarding the potential importance of CCL11. As previously described, it has been suggested that increasing the levels of CCL11 leads to inhibition of adult neurogenesis¹¹⁻¹². Villeda's *The Ageing Systemic Milieu Negatively Regulates Neurogenesis and Cognitive Function* was a seminal work in showing that increasing peripheral CCL11 levels *in vivo* lead to decreased adult neurogenesis and impaired learning and memory¹¹. Building off of Villeda's work it has been hypothesized that circulating factors, such as cytokines, that are able to pass the BBB and directly regulate neurogenesis and stem cell function by altering the signaling that occurs within neurogenic regions. Further studies have focused on the administration of CCL11 in younger mice and saw increased neuroinflammation and decreased number of synapses¹⁴. Other clinical studies have begun to study the effects of CCL11 in schizophrenia and could be replicated in Alzheimer's,

Huntington's disease and progressive multiple sclerosis^{12,16}. Despite the newly emerging literature on CCL11, there is still a lack of literature characterizing the role of CCL11 in inhibiting neurogenesis after sepsis. Our goal was to fill this research gap and propose future directions which would eventually lead to potential therapeutic methods in combating cognitive decline after a severe illness.

Here, we showed that our CCL11 levels are elevated in the brain post-CLP due to migration from the periphery rather than local production. We also showed that both Ki-67 and Mcm2 expression is rescued in neurogenic SVZ and also for Mcm2 in neurogenic DG for mice that are treated with anti-CCL11. By quantifying Iba1 expression, we found that CCL11 also increases microglial activation. This shows that CCL11 not only has a role in inhibiting neurogenesis but also for activating inflammatory immune cells in the neurogenic niche.

There are a few limitations in our studies. While MCM2 and Ki67 are markers of the cell cycle, their expression is detectable for days and thus may not truly represent events that occur exactly at the time of induction of sepsis. Using BrdU to definitively label cells that divide at a specific time point after sepsis would be more precise. Although we administered BrdU to the mice examined in this study, we had difficulty visualizing sufficient cells to quantitatively compare the different treatment conditions. We are continuing to work on increasing the sensitivity of this staining protocol. A second limitation is that we have not determined whether the changes in rates of cell proliferation that we observed here will lead to changes in the number of newborn neurons several weeks after cell division. In order to do this, we would need to count the number of BrdU+ cells labeled during the period after sepsis that ultimately begin to express a neuron-specific marker, such as doublecortin, several weeks later. Finally, we have not determined how long suppression of neurogenesis persists after sepsis since we have only examined one early timepoint following CLP^{28-30,33,34}. We are currently continuing to conduct TSA staining for BrdU with a larger murine cohort euthanized at 14 days following CLP so that we can more accurately assay how long after sepsis neurogenesis is suppressed and if CCL11 during acute sepsis contributes to suppression of neurogenesis.

Someone researchers may question the magnitude and impact of neurogenesis in humans, given the role of new neurons in adult humans is yet to be determined^{25,35}. We propose that research regarding neurogenesis post-sepsis in rodent models of sepsis is important as neurogenesis may serve as a surrogate indicator of brain injury and homeostasis. Neurogenesis is an extremely sensitive process and is impacted before other downstream immune processes may occur. Furthermore, CCL11 signaling may directly or indirectly affect a broad variety of brain processes in addition to neurogenesis, as suggested by our finding that CCL11 neutralization modifies microglial reactivity.

While our research has shown that CCL11 inhibits neurogenesis after sepsis, there is still much to uncover, including (1) how long neurogenesis is suppressed after sepsis; (2) can the blockade of CCR3, the canonical receptor of CCL11, also rescue neurogenesis after sepsis; (3) are neurogenesis dependent behaviors altered due to sepsis? Once the mechanisms of CCL11 are better understood, therapeutic interventions such as a CCL11 or CCR3 inhibitor can be developed to treat the reversible components of long-term cognitive decline after sepsis.

Conclusion

We have found that CCL11 signaling in sepsis survivor mice contributes to decreased cell proliferation and neurogenic zones of the mouse brain and increases microglial reactivity. Further studies will determine how long this effect persists and whether acute decreases in cell proliferation after sepsis ultimately change the number of newborn neurons in the hippocampus.

1. Villeda SA, Luo J, Mosher KI, et al. The ageing systemic milieu negatively regulates neurogenesis and cognitive function. *Naturefile:///Users/anniezhaio/Downloads/fnagi-09-00402.pdf*. 2011;477(7362):90-94. doi:10.1038/nature10357
2. Toscano MG, Ganea D, Gamero AM. Cecal ligation puncture procedure. *J Vis Exp*. 2011;(51):1-5. doi:10.3791/2860
3. Langa KM, Iwashyna T, T.J. I, E.W. E, D.M. S. Long-term cognitive impairment and functional disability among survivors of severe sepsis. *JAMA - J Am Med Assoc*. 2012;304(16):1787-1794. doi:10.1001/jama.2010.1553.Long-term
4. Elixhauser A, Friedman B, Stranges E. Septicemia in US hospitals, 2009. *Agency Healthc Res Qual*. 2011;348(62):1-13. <http://www.ncbi.nlm.nih.gov/books/NBK65391/>.
5. Sharshar T, Annane D, de la Grandmaison GL, Brouland JP, Hopkinson NS, Françoise G. The neuropathology of septic shock. *Brain Pathol*. 2004;14(1):21-33. doi:10.1111/j.1750-3639.2004.tb00494.x
6. Singer BH, Newstead MW, Zeng X, et al. Cecal Ligation and Puncture Results in Long-Term Central Nervous System Myeloid Inflammation. *PLoS One*. 2016;11(2):e0149136. doi:10.1371/journal.pone.0149136
7. Barichello T, Machado RA, Constantino L, et al. Antioxidant treatment prevented late memory impairment in an animal model of sepsis. *Crit Care Med*. 2007;35(9):2186-2190. doi:10.1097/01.CCM.0000281452.60683.96
8. Chavan SS, Huerta PT, Robbiati S, et al. HMGB1 mediates cognitive impairment in sepsis survivors. *Mol Med*. 2012;18(6):1. doi:10.2119/molmed.2012.00195
9. Sprung CL, Caralis P V, Marcial EH, et al. The effects of high-dose corticosteroids in patients with septic shock. A prospective, controlled study. *N Engl J Med*. 1984;311(18):1137-1143. doi:10.1056/NEJM198411013111801
10. Cuenca AG, Delano MJ, Kelly-Scumpia KM, Moldawer LL, Efron PA. Cecal ligation and puncture. *Curr Protoc Immunol*. 2010;(SUPPL.91). doi:10.1002/0471142735.im1913s91
11. Cazareth J, Guyon A, Heurteaux C, Chabry J, Petit-Paitel A. Molecular and cellular neuroinflammatory status of mouse brain after systemic lipopolysaccharide challenge: importance of CCR2/CCL2 signaling. *J Neuroinflammation*. 2014;11(1):132. doi:10.1186/1742-2094-11-132
12. Huber AK, Giles DA, Segal BM, Irani DN. An emerging role for eotaxins in neurodegenerative disease. *Clin Immunol*. 2016. doi:10.1016/j.clim.2016.09.010
13. Hoefler J, Luger M, Dal-pont C, Culig Z, Schennach H, Jochberger S. The “ Aging Factor ” Eotaxin-1 (CCL11) Is Detectable in Transfusion Blood Products and Increases with the Donor ’ s Age. 2017;9(December):1-8. doi:10.3389/fnagi.2017.00402
14. Das MM, Godoy M, Chen S, et al. Young bone marrow transplantation preserves learning and memory in old mice. *Commun Biol*. (2019):1-10. doi:10.1038/s42003-019-0298-5
15. Smith LK, Iii CWW, Villeda SA, Smith LK. The systemic environment : at the interface of aging and adult neurogenesis. 2018:105-113.
16. Sirivichayakul S, Kanchanatawan B, Thika S, Carvalho AF, Maes M. Eotaxin , an Endogenous Cognitive Deteriorating Chemokine (ECDC), Is a Major Contributor to Cognitive Decline in Normal People and to Executive , Memory , and Sustained Attention Deficits , Formal Thought Disorders , and Psychopathology in Schizophrenia Patients. 2019:122-138.
17. Neighbour H, Boulet LP, Lemiere C, et al. Safety and efficacy of an oral CCR3 antagonist

- in patients with asthma and eosinophilic bronchitis: A randomized, placebo-controlled clinical trial. *Clin Exp Allergy*. 2014;44(4):508-516. doi:10.1111/cea.12244
18. V P, Grigorean V, Popescu C. Molecular mechanisms in the pathogenesis of sepsis. 2014;7(2):38-41.
 19. Russell JA, Boyd J, Nakada T, Thair S, Walley KR. Molecular Mechanisms of Sepsis. 2011;17:48-85.
 20. Fuster-matanzo A, Llorens-martín M, Hernández F, Avila J. Role of Neuroinflammation in Adult Neurogenesis and Alzheimer Disease : Therapeutic Approaches. 2013;2013.
 21. Ito D. Microglia-specific localisation of a novel calcium binding protein , Iba1. 1998:1-9.
 22. Georg H, Gage FH. Neurogenesis in the Dentate Gyrus of the Adult Decrease of Neuronal Progenitor Proliferation Rat : Age-Related. 1996;76(6):2027-2033.
 23. Praag H Van, Kempermann G, Gage FH. Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. 1999;2(3).
 24. Jin K, Minami M, Lan JQ, et al. Neurogenesis in dentate subgranular zone and rostral subventricular zone after focal cerebral ischemia in the rat. 2001.
 25. Riksson PESE, Erfilieva EKP, Riksson THBJ, et al. Neurogenesis in the adult human hippocampus. 1998;4(11):1313-1317.
 26. Galderisi U, Jori FP, Giordano A. Cell cycle regulation and neural differentiation. 2003;5208-5219. doi:10.1038/sj.onc.1206558
 27. Rodier PM. Chronology of Neuron Development : Animal Studies and their Clinical Implications. 1980:525-545.
 28. Hospital JR, Brown DC, Gatter KC. Ki67 protein : the immaculate deception ? 2002:2-11.
 29. Wojtowicz JM, Kee N. BrdU assay for neurogenesis in rodents. 2006;1(3):1399-1405. doi:10.1038/nprot.2006.224
 30. Wharton SB, Chan KK, Anderson JR, Stoeber K, Williams GH. Replicative Mcm2 protein as a novel proliferation marker in oligodendrogliomas and its relationship to Ki67 labelling index , histological grade and prognosis. 2001.
 31. Parajuli B, Horiuchi H, Mizuno T, Takeuchi H, Suzumura A. CCL11 enhances excitotoxic neuronal death by producing reactive oxygen species in microglia. *Glia*. 2015;63(12):2274-2284. doi:10.1002/glia.22892
 32. Burry RW. Controls for immunocytochemistry: an update. *J Histochem Cytochem*. 2011;59(1):6-12. doi:10.1369/jhc.2010.956920
 33. Mu Y, Gage FH. Adult hippocampal neurogenesis and its role in Alzheimer ' s disease. 2011:1-9.
 34. Götz M, Huttner WB. THE CELL BIOLOGY OF NEUROGENESIS. 2005;6:777-789. doi:10.1038/nrm1739
 35. Praag H Van, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH. Functional neurogenesis in the adult hippocampus. 2002;415(February):1030-1034.