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Microglia ablation in the hippocampus affects mouse behavior

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Joan Ellen Danver

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We, the thesis committee for the above candidate for the

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Abstract of the Thesis

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Microglia are the resident immune cells of the central nervous system (CNS). They are involved in the maintenance of brain homeostasis not only as activated rapid responders to pathological changes in the CNS, but also when non-activated through continually extending and retracting of processes to survey the brain parenchyma and make contacts with neuronal synapses. Recent work has shown that microglia regulate the number of presynaptic terminals and glutamate receptor expression. Elimination of microglia in hippocampal brain slices results in an increase in the frequency of excitatory postsynaptic current (EPSC), and replenishment of microglia restores the EPSC frequency to normal levels. To asses if these changes in synaptic physiology with and without microglia have an effect on behavior we inject clodronate or PBS into the CA1 hippocampus of mice. Clodronate is internalized by macrophages and causes their selective apoptosis. Behavioral studies revealed that, compared to vehicle-injected mice, clodronate-injected mice exhibited a decrease in artificially induced grooming behavior and rate of spatial learning. Clodronate-injected mice did not exhibit any difference in activity level or rearings. Together with the previous data, our findings demonstrate that the non-activated microglia in the hippocampus modulate synaptic activity by regulating number of functional synapses and affect animal behavior.

Dedication Page

To my fiancé, Nathan, my ever present cheerleader, whose love and support made this possible. To my parents who constantly pushed and supported me throughout my life and who helped inspire me to continue my education and still inspire me to do more everyday.

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List of Abbreviations

AMPAR – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptor

BBB – Blood-Brain Barrier

CA – Cornu Ammonis

CA1 – Cornu Ammonis 1

CA2 – Cornu Ammonis 2

CA3 – Cornu Ammonis 3

CNS – Central Nervous System

DG – Dentate Gyrus

EGFP – Enhanced Green Fluorescent Protein

EPSC – Excitatory Postsynaptic Currents

i.p. - intraperitoneally

mEPSC – Miniature Excitatory Postsynaptic Currents

PBS – Phosphate Buffered Saline

PNS – Peripheral Nervous System

sEPSC – Spontaneous Excitatory Postsynaptic Currents

WT – Wild-Type Mice (C57BL6)

YS - Yolk Sac

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I want to thank my parents for their love and support and inspiring me to pursue further education. Lastly I would like to thank Nathan Cook for his unwavering support and love. For whose presence in my life made me want to push myself harder and do more than I could have alone.

I. Introduction

1.1 The Central Nervous System and Microglia

1.1.1 Central Nervous System

The central nervous system (CNS) consists of the brain and spinal cord. Together with the peripheral nervous system (PNS), it plays a fundamental role in the control of behavior. The CNS is composed of two main types of cells: nerve cells (or neurons) and glia. There are two types of glia: macroglia and microglia. Macroglia are composed of astrocytes, oligodendrocytes, ependymal cells and radial glia. The number of glial cells in the central nervous system far exceeds that of neurons and they are crucial for proper neural function. Together glial cells maintain homeostasis, form myelin and provide support and protection for neurons.

Another unique feature of the CNS is that it is separated from the circulating blood in the body by a structure known as the blood-brain barrier (BBB). The BBB is formed by tight junctions between endothelial cells of capillaries, pericytes and astrocytes and regulates the movement of ions, molecules and cells between the blood and CNS including, but not limited to, toxins, pathogens and immune cells (Daneman, 2012). This fact would suggest that since immune cells are not allowed into the CNS it would be an environment with no cells to participate in an immune response. This however is not the case because microglia function as the immune-competent cells of the CNS.

1.1.2 Microglia

"Microglia are phagocytes that are mobilized after injury, infection, or disease. They arise from macrophages and are physiologically and embryologically unrelated to the other cell types of the nervous system. We shall therefore not consider the microglia further" (Kandel, 1991).

Pío del Río-Hortega originally described the "microglia cell" in 1919, yet three sentences were all the authors of a textbook published in 1991 felt were sufficient to cover microglia (Rio-Hortega, 1939). Twenty two years later it has become quite apparent that microglia should be

considered much further. In the simple terms of the quote above, microglia are the macrophages in the CNS, but in reality this is an oversimplification of the multitude of functions they perform.

In order to understand the role of microglia in the CNS it is important to understand where they originate. Some studies suggest that microglia were derived from macrophage progenitors in the blood island of the embryonic yolk sac (YS), which seed the brain rudiment (Takahashi et al., 1989). The YS is attached to the embryo and provides early nourishment. It has an outside layer of extra-embryonic mesenchyme membrane derived from the mesoderm which surrounds an endoderm membrane. Another more recent study showed that bone-marrow derived cells can contribute to the post-natal microglial population(Beers et al., 2006). The initial study involved the use of a knockout mouse where no endogenous embryonic microglia were present and was challenged by a further study which confirmed that in a healthy adult the majority of microglia cells originate from YS progenitors and not definitive hematopoiesis (Ginhoux et al., 2010). Once embryonic microglia are present in the brain they remain and maintain themselves by continuing to proliferate throughout adulthood (Ginhoux et al., 2013).

Microglia have long been recognized as rapid responders to pathological changes, injury, disease and neurodegeneration in the CNS and perform a broad range of functions such as phagocytosis of cellular material and invading organisms; as well as release of inflammatory signals that facilitate the return to tissue homeostasis (Kreutzberg, 1996). Concomitant with the release of these inflammatory signals microglia rapidly change their phenotype to states that are commonly referred to as “activated”, which are accompanied by changes in their morphology and expression of cell surface antigens. Figure 1.1A shows a microglial cell in the normal baseline, branched, resting state, and Figure 1.1B shows a cell in an activated state. These states have different effector functions and can either release pro-inflammatory mediators, which are destructive to the cell, or anti-inflammatory, which possess neuroprotective properties (Kreutzberg, 1996). Disregulation of these activation states along with microglia-mediated inflammation can directly contribute to neuron function and progression of different CNS diseases and pathologies (Kettenmann et al., 2011; Perry et al., 2010).

The activated state is not the baseline state of microglia in normal CNS function, yet the majority of the research has focused on the contribution of these activated cells to disease. Less

is known about the role of microglia in their resting/ramified state when the CNS environment is void of pathological changes, injury, disease or neurodegeneration. This is partially because any change in homeostasis caused to the CNS will result in activation of microglia making it challenging to study them physiologically when "resting". Recently, a careful surgical technique called the thin-skulled transcranial approach was used with two-photon microscopy on anesthetized mice to visualize microglia tagged by enhanced green fluorescent protein (EGFP). With this technique researchers were able to show that resting microglia were not "resting" at all (Davalos et al., 2005; Nimmerjahn et al., 2005). Instead they were dynamically and continuously surveying their environment, extending and retracting their processes at such a rate that microglia had the capacity to interact with the entire brain parenchyma within a few hours (Nimmerjahn et al., 2005). Studies have focused on possible explanations for this large output of energy and movement and have shown that microglia are involved in neuronal proliferation and differentiation (Graeber, 2010). During postnatal development microglia engulf presynaptic retinal inputs undergoing synaptic pruning in response to neuronal activity (Graeber, 2010; Schafer et al., 2012). In adults they are constantly surveying their environment and making contact with neuronal synapses, where they have been shown to modify and eliminate synaptic structures (Tremblay et al., 2010). Thus, with the combination of their active and resting states, microglia have been shown to be important for both maintenance and protection of the CNS.

1.2 Depletion of Microglia in the Brain and Change in Behavior

1.2.1 Depletion of Microglia in the Hippocampus

The hippocampus is the region of the brain associated with learning, memory (specifically storage) and spatial navigation. It is part of the limbic system, which modulates the activity of the autonomic nervous system, and is composed of two mirror imaged structures across the interhemispheric fissure. In humans it is located in the medial temporal lobe of the brain. The hippocampus has two main areas: the dentate gyrus (DG) and the cornu ammonis (CA). The DG contains neurons and granule cells and is a region known to perform neurogenesis, creation of new neurons, in the adult brain. The CA is subdivided into three sections typically known as CA1, CA2 and CA3. CA3 is located right next to the DG which

sends inputs to it from granule cells along the mossy fiber pathway. The pyramidal cells in the CA3 project their axons to CA2, a small strip of cells, and to the CA1 via the Schaffer collateral.

The CA1 connects to the subiculum and the deep entorhinal cortex. The combination of all these areas makes up the hippocampal circuit. Signals pass from the entorhinal cortex layer II and III into the DG, CA1 and CA3 in some combination (Fig 1.2). All signals then pass through the CA1 to the subiculum or the deep entrohinal cortex (Andersen et al., 2000; Steward, 1976). The critical role of the hippocampus in learning, memory and spatial awareness combined with the knowledge of the hippocampal circuit and cell types make the hippocampus an important area of the brain to study.

Along the pyramidal neuronal cell layer in the hippocampus, there is a significant microglial population. This makes the CA1 an ideal location to study the interaction of microglia with neuronal synapses. The CA1 also offers the advantage that its pyramidal cells have functionally distinct inputs that may be activated by large stimulation electrodes specifically (Arrigoni and Greene, 2004). Ji et al., in previous work done by our lab, used recordings from CA1 pyramidal neurons in organotypic hippocampal slices to measure synaptic properties of this region with and without microglia. Synaptic activity was measured through spontaneous and miniature excitatory postsynaptic currents (s- and m-EPSCs) mediated by AMPARs (**α**-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor). AMPARs are receptors for glutamate and are responsible for most of the fast synaptic transmissions in the CNS (Platt, 2007). There was an increase in the frequency of AMPAR-mediated mEPSCs in slices where microglia had been ablated. When microglia were added back to the hippocampal slices the frequency returned to that of the pre-ablated slice. This result was also shown to be true in co-cultures of neurons and microglial cells. GluA1, a glutamate receptor protein, was increased in slices lacking microglia along with levels of synaptic adhesion molecules. This result indicates that microglia are interacting with and modifying these synapses. This finding was further corroborated by evidence that these molecules were found in proximity with microglia and that microglia themselves were shown to be in proximity of neurons and engulfing neuronal material. These results combined suggest that resting microglia are directly affecting neurons as they actively regulate synaptic density of glutamatergic synapses, thus modifying their activity (Ji et al.,

2013). Suboptimal pruning may lead to changes in neuronal activity in adult animals which could in turn affect behavior.

1.2.2 Microglia and Change in Behavior

Since the hippocampus is so important for memory, learning and spatial recognition, it is the appropriate region to study in vivo effects of microglial ablation on cognitive behavior in mouse models. Previous results from Ji et al. showed that loss of microglia leads to a change in synaptic activity in the CA1. Based upon these results, we predicted that by ablating microglia in the CA1 of the hippocampus in mice we may observe a change in behavior. Using behavior tests that assess overall activity level, spatial learning and memory, and grooming, we report that ablation of microglia of the CA1 of the hippocampus does indeed lead to changes in behavior. Our results, combined with the results previously shown by Ji et al. in vitro (Ji et al., 2013), suggest that modifying or suppressing resting microglial function affects neuronal properties in the mature brain and directly leads to behavior changes. These data demonstrate that resting microglia are important for maintaining correct communication and function of the CNS and indicate another way that microglial dysfunction could lead to disease.

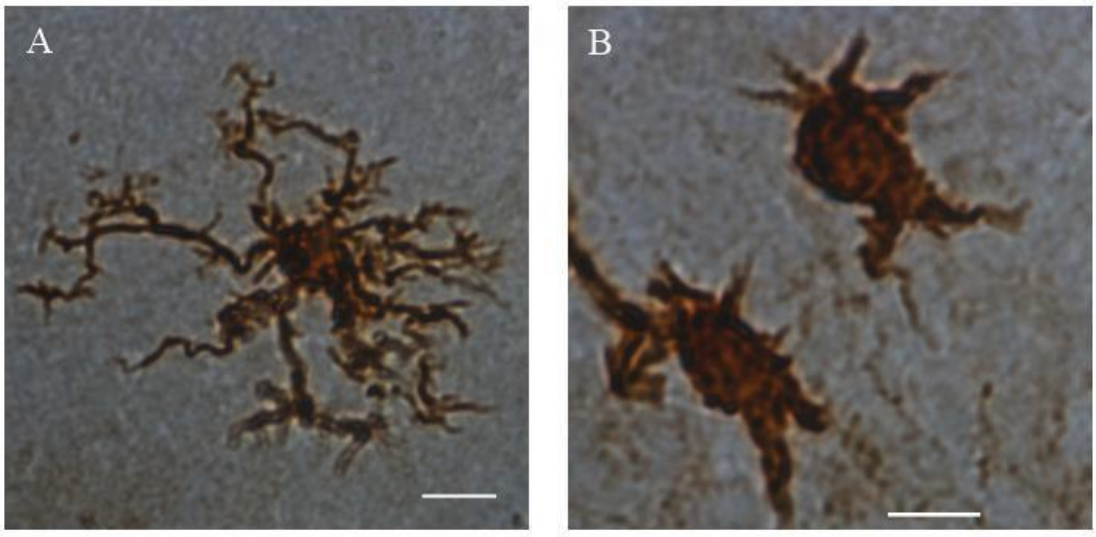

Figure 1.1: Active and Resting Microglia. Microglia shown by immunohistochemistry using anti-Iba-1 primary antibody, followed by biotinylated anti-rabbit IgG secondary antibody and visualized with ABC Kit and DAB staining under bright field microscope at 100x. Scale bars 10 μm **A.** Resting microglia with lots of processes. **B.** Microglia with drawn-in processes and large body representative of an activated state.

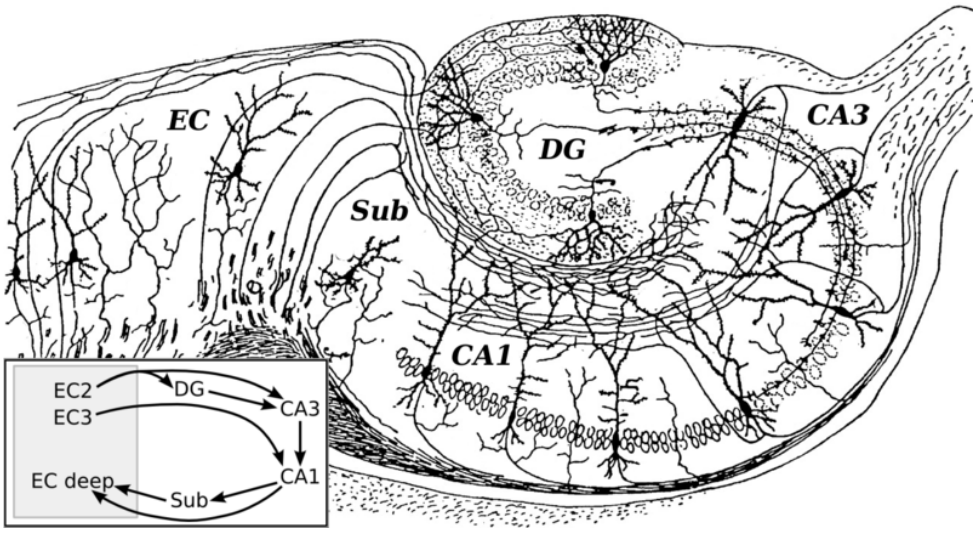

Figure 1.2: Neural circuitry of the rodent hippocampus. Modified version of the classical drawing of the hippocampal formation by Ramón y Cajal that shows the neural connections between the different neuronal types and the direction of the passage of signals throughout the hippocampus (Ramon y Cajal, 1911). Modification from Wikipedia.org/wiki/Hippocampus.

II. Materials and Methods

2.1 Animals

Male 6 week and older C57BL/6 (wild type, WT) and MacGreen mice, which express EGFP under the control of the microglial/macrophage-specific Csf1R promoter, in the C57BL/6 background were used in this study (Sasmono et al., 2007). Throughout the experiment, animals were housed individually in a light and temperature-controlled environment on a 12-hour light/12-hour dark cycle with free access to food and water. All animal procedures were approved by Stony Brook University Institutional Animal Care and Use Committee (IACUC).

2.2 Intrahippocampal Injections of clodronate

Injections were performed bilaterally in the hippocampus, using methods previously performed by the lab as described (Mirrione et al., 2010). Mice weighing 20-30g were deeply anesthetized with 1.25% Avertin (0.02 ml/g body weight delivered intraperitoneally, i.p.). A small burr hole was made at stereotaxic coordinates (Stoelting, Wood Dale, IL) -2.5 mm from bregma and -1.7 mm lateral. 2 μl phosphate buffered saline (PBS) or clodronate disodium salt (Calbiochem) in PBS at 10 mg/ml was delivered using a Hamilton syringe (0.485 mm I.D., Hamilton, Reno, NV) at a depth of 1.6 mm over 10 minutes with a motorized stereotaxic injector (Stoelting, Wood Dale, IL). To prevent reflux the needle remained in place for an extra 5 minutes. The same procedure was repeated at -2.5 mm from bregma and 1.7 mm lateral. Following surgery, animals were injected i.p. with the analgesic buprenorphine HCL (Bedford labs, Bedford, OH) (0.03 mg/kg). Five days after injection, and after behavior testing was completed, the mice were euthanized, the brains removed and fixed overnight in 4% paraformaldehyde.

2.3 Behavior Tests

2.3.1 Open Field

Following procedures previously established by the lab (Bukhari et al., 2011), vehicle and clodronate injected WT/MacGreen mice were placed in an empty rat cage (44 cm x 21 cm) situated inside an Opto-Varimex-Minor animal activity meter (Columbus Instruments). During a

5 minute observation period 15x15 infrared beams running in the x-y coordinates and 2 cm above the base of the machine recorded beam breaks associated with ambulatory (horizontal) activity (horizontal beam breaks) and total activity (total beam breaks). An observer, blind to genotype or treatments, manually recorded the animals' full rearings. Rearings were recorded when the animals used upper and lower limbs to reach up the side of a cage (supported) or stood up on its lower limbs without using any cage support (unsupported). These were combined together and recorded as total rearings. Activity measurements were recorded on day 5 after intrahippocampal injection.

2.3.2 Barnes Maze

The Barnes maze behavior test procedure and materials were followed from Harrison et al. with a few modifications (Harrison et al., 2006). The Barnes maze consisted of 8 equally spaced 5-cm diameter holes located 5 cm from the edge. All holes were open to the floor except for one, which had a black escape box placed below it. Trial data were recorded by an observer using a stop watch.

Each individual trial began with placement of an experimental mouse in the center of the maze facing the same wall and Barnes maze direction. The mouse was then allowed to explore the maze freely. The time was recorded when the mouse first came in contact with the open escape hole (primary latency) and when the mouse entered the open escape hole (latency to enter). The number of holes other than the escape hole the mouse visited before first contact with the escape hole (primary errors) and after contact were also recorded. Total errors were the sum of the primary errors and errors made thereafter. The mouse was left in the escape box for 50 seconds before it was returned to its cage. If after 300 seconds the mouse had not entered the escape hole on its own, it was picked up and placed over the target hole to enter where it was left for 50 seconds and then returned to its cage. The maze and escape box were cleaned after each test with 70% alcohol solution. 15 minutes after the initial trial the mouse was returned to the maze to repeat the experiment. These trials were performed for 5 days starting 24 hours post-surgery.

2.3.3 Grooming

Two methods were used to quantify grooming on day 5 after injection surgery: spontaneous grooming assay and induced grooming assay.

Spontaneous grooming assay. Test animals were placed in an empty rat cage (44 cm x 21 cm) and video recorded for 5 minutes. The video was observed and a stop watch was used to record time spent grooming. The duration of grooming events was also manually determined from the video (Xu et al., 2013).

Artificially induced grooming assay. The same methods were followed as the spontaneous grooming assay protocol with the exception that test animals were sprayed 4 times with water mist near the head to induce grooming (Xu et al., 2013). Mice were then placed in an empty rate cage (44 cm x 21 cm), video recorded for 5 minutes and grooming stints and time spent observed and recorded.

2.4 Tissue Collection

5 days post injection and after completion of the last behavior test, mice were anesthetized with an i.p. injection of Avertin and then rapidly transcardially perfused using PBS followed by 25-50 ml of 4% PFA in PBS (pH 7.4). Brains were removed, post fixed overnight in 4% PFA at 4º C, and then placed in 30% sucrose at 4º C until fully cryoprotected (usually 24 hours). Tissue was removed from 30% sucrose, rinsed in PBS and brain stem removed with a straight cut. The tissue was then frozen embedded in O.C.T. Compound (Tissue-Tek) for a minimum of 2 hours then sectioned on a Leica (Nussloch, Germany) cryostat into 25 μM coronal sections (Mirrione et al., 2010). Sections were either stored in 24 well plates in PBS at 4º C or mounted onto slides (Fisher Superfrost Plus; Fisher Scientific), air dried overnight, and stored at -80º C.

2.5 Cresyl Violet Staining

Fresh-frozen sections were dipped in 100% ethanol for 2 minutes to displace water, for 2 minutes in xylenes to dissolve the fats, then a serial hydration from 100%, 70% to 20% ethanol

and distilled water. The sections were then dipped in cresyl violet dye (2.5 gm of cresyl violet, 30 ml of 1M sodium acetate, 170 ml of 1M acetic acid, and 300 ml of dH_2O) for 5 minutes. Sections were then dipped twice in distilled water, differential 1 (270 ml 70% ethanol, 30 ml 10% acetic acid) and differential 2 (270 ml 100% ethanol, 30 ml 10% acetic acid), dehydrated with 100% ethanol and then defatted in xylenes. Sections were coverslipped (VWR micro cover glass 24x55 mm; VWR) with a solution of 40% xylenes and 60% Permount (Fisher Scientific, Houston, TX). This combination was used to reduce the presence and likelihood of bubble formation between slide and coverslip. The slides were dried flat overnight.

The stained sections were photographed digitally using a digital camera (Nikon CoolPix 990; Nikon, Tokyo, Japan) on a Nikon Eclipse E600 microscope under bright-field optics at 2x magnification.

2.6 Immunohistochemistry

Immunostaining was conducted on the floating coronal sections modified from previously established methods (Siao et al., 2003). All steps were performed at room temperature. The sections were first washed with PBS then endogenous peroxidase activity was quenched with peroxide treatment (3% H_2O_2 in PBS, 5 minutes). Blocking in serum of the host of the secondary antibody [3% normal goat serum in PBS-T (0.2% Triton X-100 in PBS)] was performed for 30 minutes followed by a wash with PBS-T. The primary antibodies used at the following concentrations, Iba1 1:500 (Wako Pure Chemical Industries, Ltd., Osaka, Japan), glial fibrillary acidic protein (GFAP) 1:1000 (Abcam Inc., Cambridge, MA), were added into 1% serum in PBS-T for 2 hours with agitation. After 3 10 minute washes with PBS-T, the appropriate biotinylated secondary antibody, biotinylated anti-rabbit IgG (H+L) (Vector Laboratories, Burlingame, CA), was added in 1% serum and PBS-T and incubated for 1 hour with agitation. The ABC reagent was added to conjugate avidin-peroxidase to the immune complex (Vector Laboratories, Burlingame, CA) according to the kit directions for 1 hour. The sections were again washed 3 times for 10 minutes with PBS-T. The signal was visualized using 3,3'-Diaminobenzidine (DAB) (Sigma Chemical Co, St. Louis, MO) solution (DAB/0.1M PB/H_2O_2) until desired strength was reached then washed 3 more times with 0.1M PB (or distilled water). The slices were removed from the wells, painted on slides and left to dry

overnight. Sections were dehydrated in graded ethanol (50%, 70%, 90%, 100%, 100%), defatted in xylenes, and cover slipped (VWR micro cover glass 24x55 mm; VWR) with 40% xylenes and 60% Permount (Fisher Scientific, Houston, TX). Slides were left to dry flat overnight.

Slides were photographed and digitized as described above.

2.7 Statistics

All statistics were performed using Statview (v4.0) or Graphpad Prism 6 for Windows, GraphPad Software (**http://www.graphpad.com**). Data were graphically presented using Prism. For Barnes maze data, the average for the two trials for each of the recorded latencies (primary and to enter) was calculated and Mann-Whitney U test used for the statistical analysis. Graphical data represent mean (± SEM) of the two trials per day. Open field and grooming data are presented as mean ± SEM and were analyzed by Student's t-tests with Welch's correction for samples having possible unequal variances.

III. Results

3.1 Verification of Microglia Cell Death and Neuronal Health in Hippocampus

To validate the hypothesis that loss of microglia in the hippocampus led to behavioral changes in our mice we first ensured that clodronate ablated microglia, but had no effect on other cells. Clodronate is engulfed by macrophages/microglia and causes selective apoptosis (Dehghani et al., 2004). Microglia were ablated through injection of clodronate (10 mg/ml) in the CA1 of one hemisphere the hippocampus of WT or MacGreen mice. Staining with anti-GFAP antibody revealed no apparent difference in the density and structure of astrocytes (Fig 3.1B). Cresyl violet staining showed healthy neurons in the control and experimental hemispheres of the hippocampus of brain slices (Fig 3.1A). Performing immunohistochemistry using anti-Iba1 antibody showed that the microglia were ablated in the experimental but not control hemispheres of the hippocampus in brain slices (Fig 3.1A). The microglia outside of the hippocampus retained their resting morphology (Fig. 1.1A, 3.2B), though within the hippocampus and near where the injection syringe pierced the meninges, some activated microglia were found (Fig 1.1B, 3.2A).

When injected into the CA1 of both hemispheres of the hippocampus clodronate ablated microglia in the hippocampus and parts of the cortex above the hippocampus (Fig 3.2A). It was observed that there was no significant difference between the morphology and number of microglia in the amygdala (Fig 3.2B, 3.2C). The amygdala is known to be involved with memory and emotional reactions and this ensured that any behavior change seen in the mice was due solely to lack of microglia in the hippocampus.

3.2 Effect of Ablation of Microglia on Activity

Open field was used to assess the baseline activity level of vehicle-injected and microglia ablated mice. The saline injected vehicle mice and clodronate injected mice had almost the exact same average number of horizontal (104 vehicle to 105.44 clodronate) and total beams breaks (171.33 vehicle and 171.55 clodronate) (Fig 3.3A, 3.3B). There was no significance difference in the number of rearings between the two conditions (24.88 vehicle and 30.33 clodronate) (Fig 3.3C). The combined open field tests showed that there was no significant difference between the

activity levels of the vehicle- or clodronate-injected mice. Loss of microglia did not cause the mice to be hyper- or hypoactive compared to the control animals. Therefore any difference in Barnes maze or grooming tests cannot be attributed to increased or decreased activity level.

3.3 Effect of Ablation of Microglia on Learning and Memory

To assess the effect of loss of microglia on spatial learning and memory the Barnes maze test was performed. The time in which vehicle- and clodronate-injected mice located the escape hole decreased each subsequent day of Barnes maze testing. After the first day, clodronate injected mice took consistently longer to find the escape hole compared to their control counterparts, and took significantly more time to find the escape hole on day 3 and 4 ($p=0.0499$ and $p=0.0388$) post injection. There was a trend towards significance on days 2 and 5 ($p=0.08$ and $p=0.07$) (Fig 3.4A). In contrast, there was no significant difference in the latency to enter the escape hole between the clodronate and vehicle mice (Fig 3.4B). There was the same trend of a decrease in amount of time each day of testing.

Vehicle mice committed fewer errors before initially finding the escape hole each day of testing. They also committed fewer errors between the time when they first found the escape hole and actually escaped. Though the clodronate mice did commit fewer primary errors and total errors on the last day compared to the first day, they did not follow the same consistent downwards trend as the saline injected mice. The difference between saline and clodronate injected mice, however, was not significant (Fig 3.4C, 3.4D).

3.4 Effect of Ablation of Microglia on Grooming

When mice were observed spontaneously grooming for 5 minutes in a new environment separate from their home cage, there was no significant difference between vehicle- and clodronate-injected mice in the amount of time spent grooming (unpaired t-test with Welch's correction, $p=0.3873$) (Fig 3.5A). The animals spent little time grooming during this period. In order to encourage additional bouts of grooming, mice were stimulated by water spray. The amount of time spent grooming in this induced condition was significantly reduced in the animals injected with clodronate compared to that of the animals injected with the vehicle (unpaired t-test with Welch's correction, $p=0.0292$) (Figure 3.5B).

All together these findings suggest that resting microglia are important for normal brain function. Their ablation in the hippocampus does not lead to change in activity levels, but the mice have spatial learning and memory deficits and spend less time grooming.

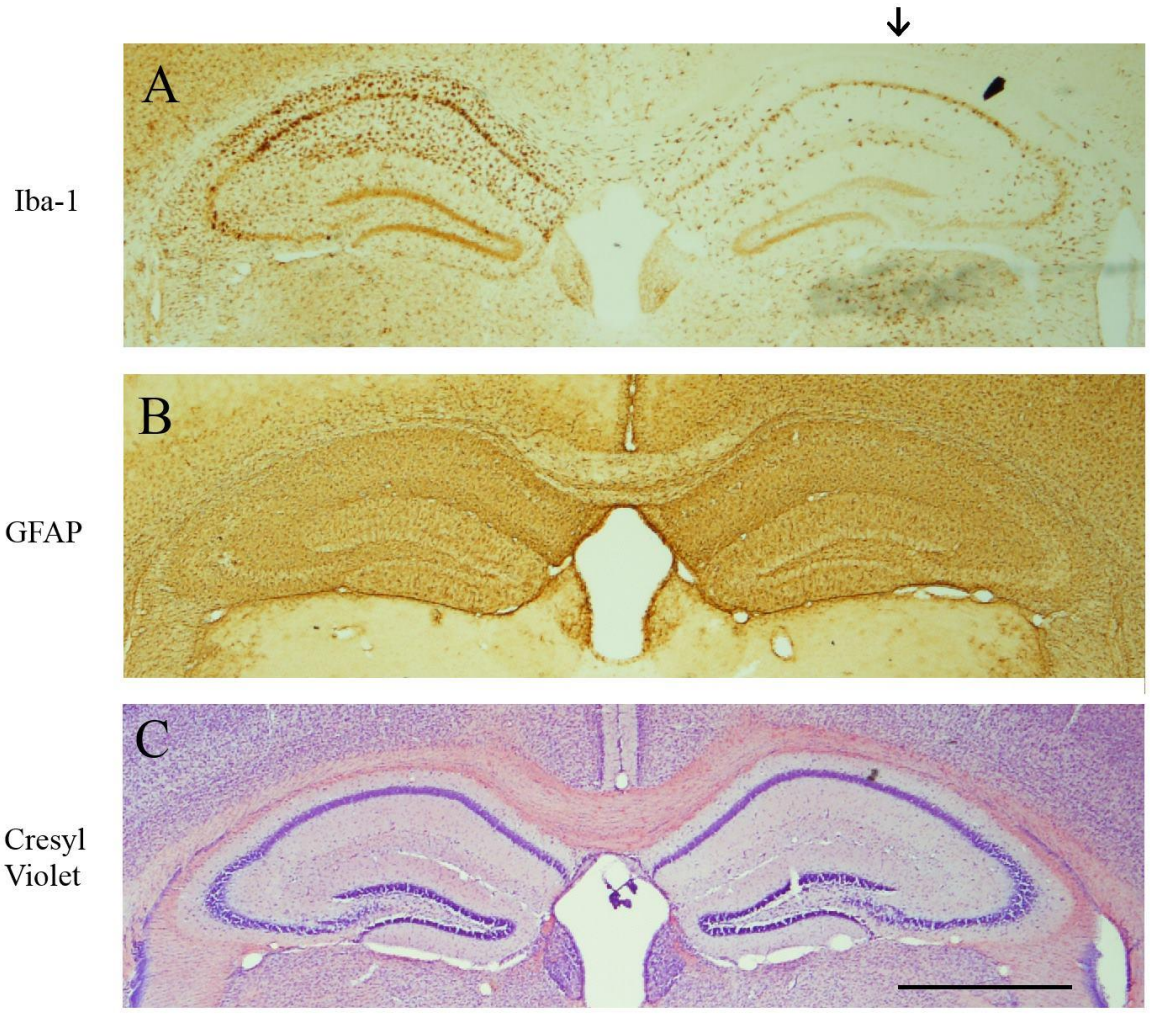

Figure 3.1: Clodronate ablates microglia but does not affect neurons or astrocytes in the hippocampus. A. Microglia were not present in the clodronate-injected hemisphere of the hippocampus as compared to the control hemisphere. There was no noticeable difference in the astrocytes and neurons between hemispheres. **B.** Astrocytes were stained with anti-GFAP and visualized with biotinylated anti-rabbit IgG with ABC Kit and DAB. **C.** Neurons were stained with cresyl violet. Bright field microscope at 2x magnification. Scale bar, 500 μm.

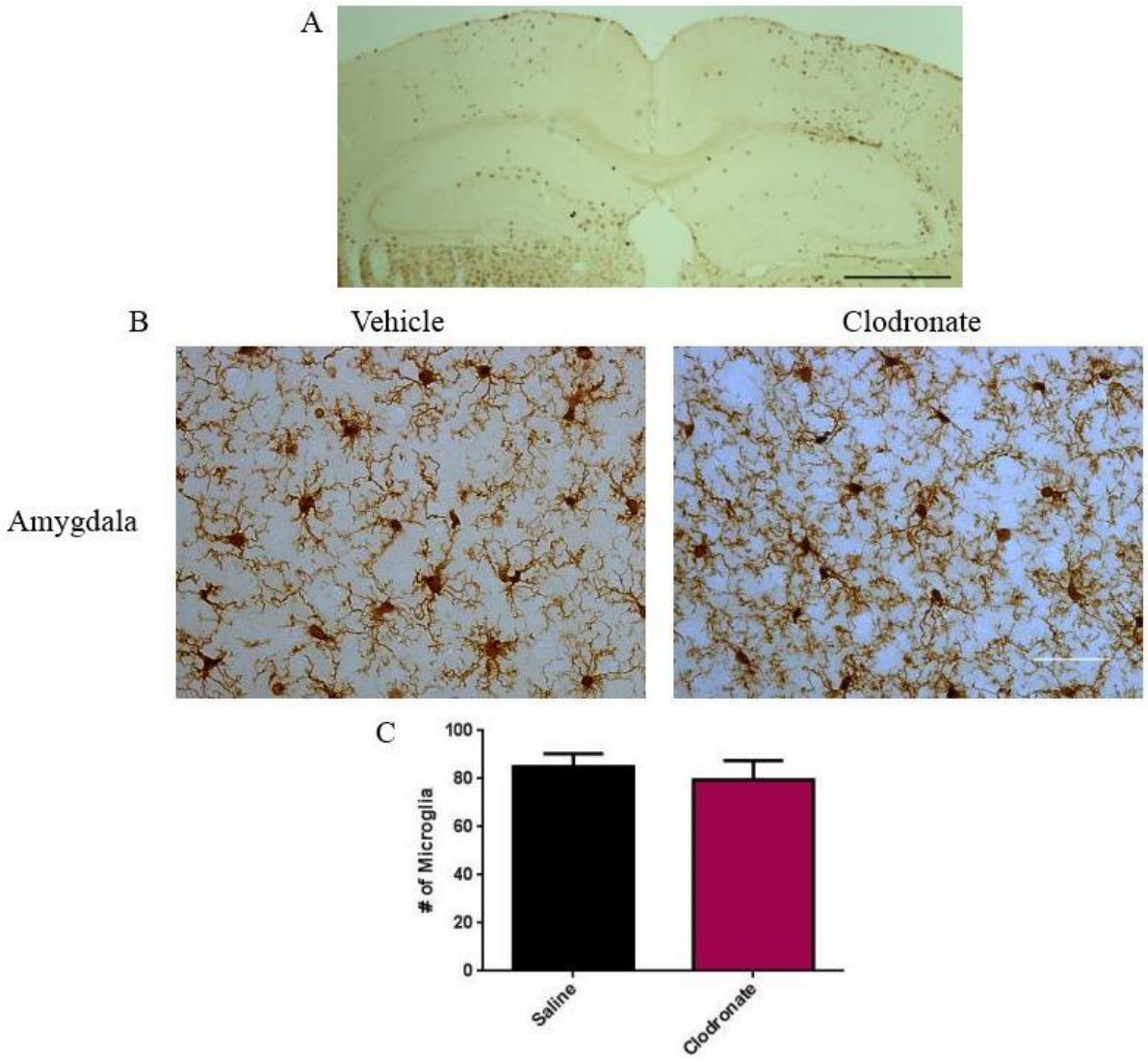

Figure 3.2: Injection of clodronate in CA1 of hippocampus does not result in change of morphology or number of microglia in amygdala. Stained with anti-Iba1 and visualized with biotinylated anti-rabbit IgG plus ABC kit and DAB. **A.** Injection of clodronate in the CA1 of both hemispheres results in almost full ablation of microglia in the CA region of the hippocampus. Bright field microscopy at 2x magnification. Scale bar, 500 μm. Clodronate injection does not affect the morphology (**B**) or number (**C**) of microglia in the amygdala. Bright field microscopy at 40x magnification. Scale bar, 50 μm.

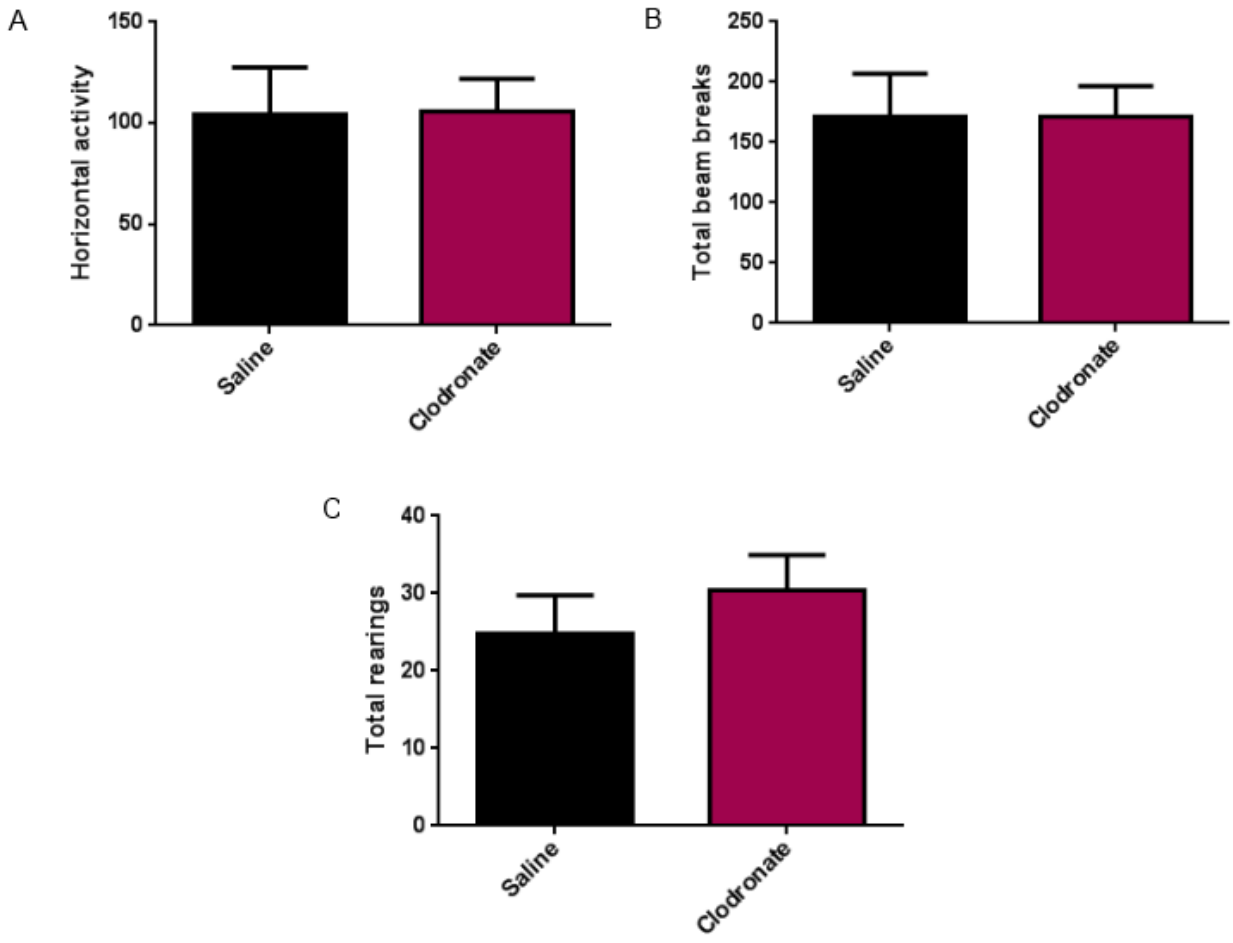

Figure 3.3: Vehicle and clodronate injected mice have the same activity level. In the open field test microglia ablated and vehicle mice display a similar amount of activity 5 days post-surgery. On average the mice had almost the same number of horizontal (104 vehicle to 105.44 clodronate) and total (171.33 vehicle and 171.55 clodronate) beam breaks (**A, B**) and similar number of rearings (**C**). Saline n = 9, Clodronate n = 9.

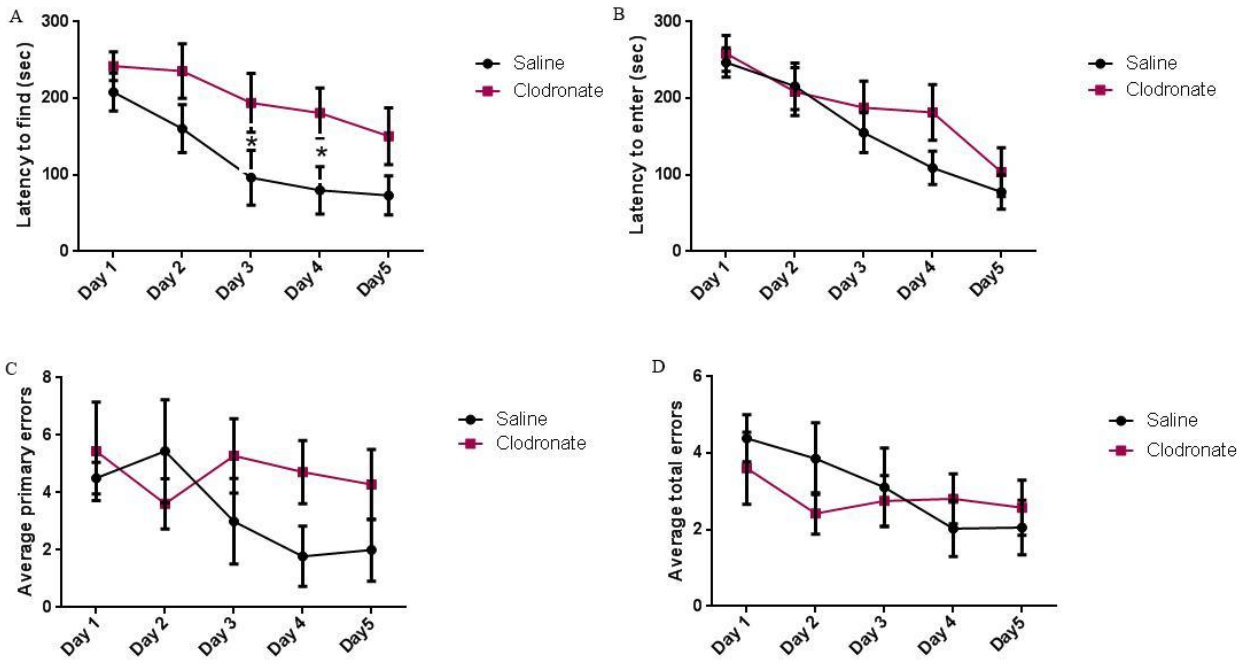

Figure 3.4: Clodronate injected mice take more time initially to find the escape hole than vehicle treated mice. A. The average of two Barnes maze trials (±SEM) over 5 days indicated that vehicle mice took significantly less time to find the escape hole on days 3 and 4 (p=0.0499 and p=0.0388) then clodronate injected mice. Days 2 and 5 were close to significant (p=0.08 and p=0.07). There was no significant difference in time spent between finding the escape hole and escaping (**B**) or the number of errors the mice made first locating (**C**) and between locating and entering the escape hole (**D**). Saline n = 9, Clodronate n = 9.

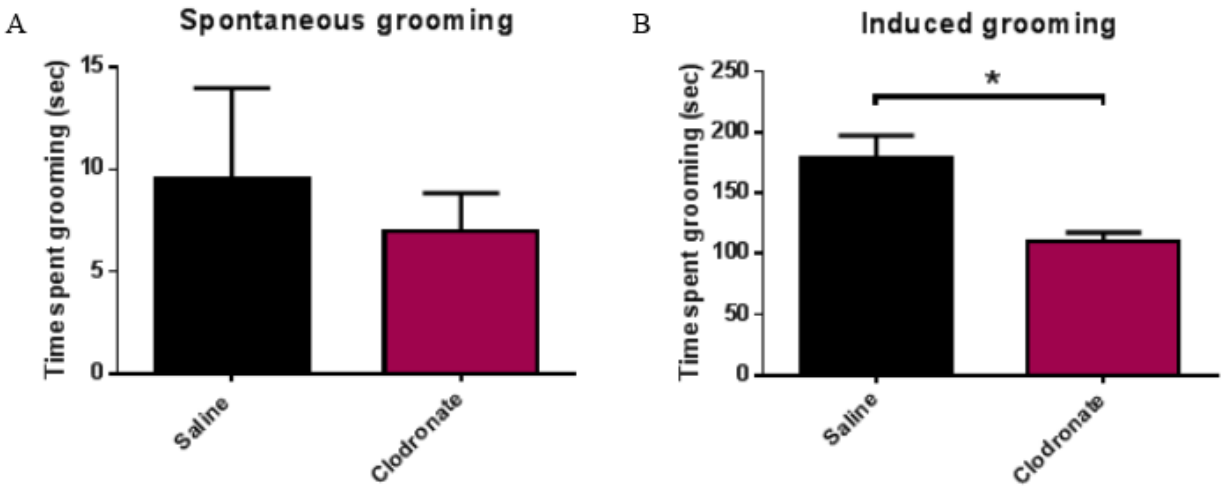

Figure 3.5: Microglia ablated mice spent less time grooming when artificially induced. Loss of microglia induces a decrease in grooming 5 days post-surgery. The difference in time spent spontaneously grooming between clodronate and vehicle mice was not significant (p=0.6118) (**A**) but when grooming was induced the clodronate injected mice groomed significantly less (p=0.0209) (**B**). Saline n = 5, Clodronate n = 5.

IV. Discussion

It was previously shown in Ji *et al.* that microglia affect synaptic activity by regulating synaptic numbers and glutamate receptor expression (Ji et al., 2013). A study by Etheron *et al.* knocked out the neurexin-1α gene and showed that a decrease in mEPSCs in acute slices from the CA1 region of the hippocampus correlated with an increase in grooming behavior and an improvement in motor learning, but no changes in social behaviors, locomotor activity, or spatial learning (Etherton et al., 2009). Though our models are not exactly the same (the Etheron *et al.* study was a knock out study that affected the whole brain not just the hippocampus), the Etheron et al. provides an example of another study making a correlation between a change in mEPSCs in the hippocampus and behavior. Through our study we provide evidence that loss of microglia in the hippocampus does not result in a change in locomotor activity levels, but accompanies a decrease in grooming behavior and the rate of spatial learning. These findings support the idea suggested in Ji *et al.* that the interaction of resting microglia with neurons in the mature brain leads to physical and functional consequences that can manifest themselves through changes in behavior (Ji et al., 2013).

The ablation of microglia in the mouse hippocampus does not lead to a change in their activity level. This finding suggests that all other behavior results were not affected by change in activity level between the two groups. The morphology and numbers of microglia in the amygdala were also comparable between vehicle- and clodronate-injected mice. This is important because the amygdalae are known to be involved with emotional reactions and memory. Had there been any difference between the two groups, it would have been difficult to determine if the change in behavior was directly due to the loss of microglia in the hippocampus, or if the behavior change was due to a change in the way the mice processed emotions, like fear.

With the knowledge that the amygdalae and energy levels are consistent between clodronate and control we can assess the Barnes maze results. Mice with ablation of microglia in the hippocampus took significantly more time to find the escape hole compared with the vehicle mice on days 3 and 4, with close to significance on days 2 and 5. On day 1 the microglia ablated mice were only slightly slower than the vehicle at finding the escape hole. On the first day, the mice do not have any previous knowledge of the environment. The fact that the speed by which

they find the escape hole the first day is not significant is good because it shows that both mice find the hole at similar speeds when they have not learned the environment. They exhibit similar base responses to their environment at similar activity levels as shown through the open field experiments. Each day thereafter, both groups decreased the amount of time it took to find the escape hole. This demonstrates that both the clodronate- and vehicle-injected mice were indeed learning, but the rate by which the clodronate-injected mice learned from day to day was different than the vehicle. Vehicle mice had a sharper decrease in time it took to locate the escape hole each day over the first few days, though their rate slowed in later days. Clodronate-injected mice on the other hand had a comparably decreased rate of learning each day consistently over the 5 days. Vehicle mice had a sharper decrease in the number primary errors, after the second day, compared to the clodronate-injected mice. Primary errors were the only other measurement of the Barnes maze that approached significance. Latency to enter and total error results were not significant.

There was a significant decrease in artificially induced grooming in clodronate-injected mice. This is an interesting result as grooming is sometimes considered to be a repetitive behavior and used to assess mouse models of human diseases of the brain like autism (Crawley, 2007). The behavioral implications of grooming however are much more complicated than just a repetitive behavior and I do not have the knowledge to discuss them here. The result however is significant and worth noting.

Our studies show that resting microglia, through their modification of synaptic numbers and glutamate receptor expression, modulate mEPSC in the hippocampus of the brain, which leads to quantifiable behavior changes. Mice lacking microglia in their hippocampus exhibit a significant decrease in time spent grooming when artificially induced and a decrease in rate of spatial learning. These results indicate that the function of resting microglia and their interactions with synapses can no longer be left out when discussing behavior paradigms. Our results also further confirm the difficulty in connecting synaptic activity and behavior. Increased synaptic activity does not necessarily lead to an increase in activity, grooming, or learning. It is much more complicated with many more factors involved and our study along with other studies, indicate that one cannot simply link synaptic activity directly with behavioral activity (Etherton et al., 2009). Are the increases in number of synapses caused by loss of microglia leading to an

increase in mEPSCs and synaptic activity because more synapses are firing but at lower efficiency? It is more complicated and while many different mouse mutants have characterized behavioral phenotypes there are not many that connect electrophysiology and behavior (Etherton et al., 2009).

Further experiments needs to be done to fully understand the behavioral phenotypes caused by lack of microglia in the hippocampus. First, in order to ensure that microglia are the direct cause of these behavior changes a recovery study needs to be performed. In Ji et al. this was done by adding microglia back into the slices (Ji et al., 2013). Microglia naturally proliferate in the brain and it is therefore not necessary to add microglia back through further intrahippocampal injections. Instead, as shown through previous data from the lab, by day 7 most of the microglia have returned to the hippocampus. We started recovery studies by performing the same behavior tests from day 15 to 20 post surgery. So far the sample size is too low to draw any conclusions. If the behavior is the same between clodronate and vehicle injected animals than we can assume that the loss of the microglia is the cause of the behavioral changes. Second, it might also be useful to do other behavior tests if we want to fully qualify the behavioral phenotypes associated with loss of microglia in the hippocampus. After the completion of these behavior tests we will have characterized the behaviors associated with loss of microglia in the hippocampus and associated them with an increase in synaptic activity through modification of synaptic numbers and glutamate receptor expression (Ji et al., 2013). We may not yet be able to connect this information to human disorders but we will be one step closer to understanding the connection between electrophysiology and behavior and the roles that resting microglia play in the CNS and how they can directly contribute to behavioral phenotypes.

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