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Microglial Location, Morphology, and Cognitive Performance in Mold-Exposed Mice

Karen Marmon
CUNY Hunter College

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by

Karen Marmon

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Thesis Sponsor:

May 2, 2017	Cheryl Harding
Date	Signature
May 2, 2017	Carolyn Pytte
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Abstract

Moldy buildings are a public health threat. While there is evidence that exposure to mold causes severe health problems, including neurological and cognitive impairments, there is limited animal research exploring the mechanisms behind this process. We hypothesized that these effects are caused when mold activates the innate immune system, where responses include morphological changes to brain microglia, and that microglial activation would be associated with cognitive deficits. Additionally, we hypothesized that doxycycline, an antibiotic shown to inhibit microglial activation, would counteract the effects of the mold. We conducted a preliminary study in which we exposed a small number of mice to intact *Stachybotrys chartarum* spores or saline vehicle, and fed them either a mouse chow that included doxycycline or an otherwise identical control diet. We trained the mice on the Morris water maze to measure cognitive ability. Following perfusion, we quantified the microglia in each mouse's hippocampus according to morphology and location and performed correlations between the microglia data and cognitive performance. Due to small numbers, diet groups were combined to assess the effects of treatment, while treatment groups were combined to assess the effects of diet. Neither mold treatment nor doxycycline diet caused significant changes in microglial location or morphology. While most correlations between microglial characteristics and behavior were not significant, there were several interesting patterns in the data. When the platform was visible, greater numbers of microglia were associated with better performance for both vehicle-treated and mold-treated mice. However, once the platform was hidden and the task required spatial memory, greater numbers of microglia became associated with poorer performance for mold-treated mice. It was no surprise that increased numbers of ramified microglia, which maintain homeostasis and release positive growth factors, had strong associations with better performance in vehicle-treated mice for both learning and memory trials. In addition, improved performance in vehicle-treated mice was correlated with increased numbers of microglia in the molecular dentate gyrus, an area implicated in improved spatial learning. As expected, greater

numbers of primed and amoeboid microglia, which are implicated in brain inflammation, had strong associations with poorer memory performance in mold-treated mice. Impairments in memory performance in mold-treated mice were also correlated with greater numbers of microglia in the granular dentate gyrus. Following immune activation, increases in microglia in the granular dentate gyrus are linked to spatial learning impairments and decreased neurogenesis. Notably, increased numbers of microglia were mostly correlated with faster velocities for vehicle-treated mice and slower velocities for mold-treated mice in both visible and non-visible trials. While one of the strongest correlations with the speed of vehicle-treated mice was with the number of ramified microglia, the speed of mold-treated mice was strongly correlated with the number of amoeboid microglia. Amoeboid microglia are associated with malaise and lethargy, which could decrease locomotor ability or motivation. The doxycycline diet did not significantly reverse the effects of mold exposure. For mice on both the doxycycline and control diets, improved task performance was mostly associated with greater numbers of microglia for both visible and non-visible trials, though the reverse association with the number of microglia in the hilus was a notable exception. For both diets, greater numbers of microglia tended to be associated with faster velocities.

In summary, mice on each of the two diets showed similar relationships between their numbers of various types of microglia and MWM performance. Mold treatment did not significantly change the positive relationship between microglia and MWM performance during visible learning trials. However, while greater numbers of microglia in control-treated mice were associated with improved performance, greater numbers of microglia in mold-treated mice were linked to impairments in memory performance, suggesting that microglia can enhance learning under healthy conditions, but become detrimental following an immune challenge.

Introduction

It is well-established that humans exposed to the mold found in damp buildings experience many physical health problems, including upper respiratory tract symptoms, wheeze, cough, and exacerbation of asthma and other lung diseases (IOM, 2004; Mendell, Mirer, Cheung, Tong, & Douwes, 2011). Animal studies have also shown adverse health effects after mold exposure. *Stachybotrys chartarum* is a fairly common mold in water-damaged buildings. There is evidence that it causes acute inflammatory responses, macrophage cytotoxicity, and pulmonary hemorrhage (Lichtenstein et al., 2010). Satratoxin G, a common mycotoxin produced by some strains of *S. chartarum* (Islam, Shinozuka, Harkema, & Pestka, 2009), caused apoptosis of olfactory sensory neurons, rhinitis, and atrophy of the olfactory epithelium in both monkeys (Carey et al., 2012) and mice (Islam, Harkema, & Pestka, 2006). However, non-satratoxin-producing *S. chartarum* spores also caused inflammatory responses in murine lungs (Leino et al., 2003) suggesting that mold components other than satratoxins are harmful as well.

While studies concerning neurological or cognitive symptoms are less common, inhabitants of moldy environments reported symptoms such as memory loss, confusion, and difficulty with concentration (Shoemaker & House, 2006). Compared to control subjects, they had more visual abnormalities, increased reaction times, decreased balance, and decreased performance on cognitive and memory tests (Kilburn, 2009). Mold-exposed individuals also showed significant differences in brain structure with concurrent changes in inflammatory markers (Shoemaker, House, & Ryan, 2014).

Despite the evidence of mold-induced symptoms, the underlying mechanisms are not understood. We hypothesized that these effects are caused when mold activates the innate immune system and induces a dose-dependent response known as sickness behavior. When infectious bacteria or other pathogens invade the body, various immune receptors recognize and bind to specific pathogen-associated molecular patterns (PAMPs) on the infectious

microorganisms, which causes pro-inflammatory cytokines, including interleukin-1 beta (IL-1β, an endogenous pyrogen), to be produced locally (Dantzer, 2006). These peripheral cytokines relay this inflammatory signal to the central nervous system through a variety of pathways. Possible pathways include a neural route whereby the afferent vagus nerves transmit the signal from the periphery to the brain, and a humoral pathway which involves the synthesis of cytokines in the circumventricular organs and choroid plexus that transmit the signal either by activating neurons that project to the brain or by diffusing by volume transmission into the brain (Dantzer, 2001). Alternatively, cytokines may enter the brain by binding to receptors on vascular endothelial cells (Konsman, Parnet, & Dantzer, 2002), or be actively transported across the blood brain barrier by specialized transporters (Banks & Erickson, 2010). After reaching the brain, this signal activates microglia, the CNS-resident macrophages which are involved in immune surveillance. Activated microglia in the brain produce cytokines, which cause sickness behavior (Henry et al., 2008), a highly evolved strategy to fight infection and prioritize behaviors that promote recovery (Hart, 1988). Symptoms include malaise, lethargy, listlessness, loss of appetite, social withdrawal, and cognitive impairments, particularly in hippocampal-dependent tasks (Kelley et al., 2003).

The effects of mold on the immune system are similar to the immune activation caused by bacterial infection. Mold triggers innate immune responses leading to an influx of inflammatory cells in the lungs (Leino et al., 2003) and inflammation in the nose, lungs, and other peripheral tissues (Lichtenstein et al., 2006; Leino et al., 2003; Islam et al., 2006). The resulting physical symptoms and memory problems reported by mold-exposed patients mirror the effects of sickness behavior caused by bacterial infection. However, it has not yet been demonstrated that mold inhalation causes activation of microglia and the production of cytokines in the brain, or that exposure to identified mold can result in sickness behavior.

Since we hypothesized that mold exposure results in sickness behavior, we expected that exposed animals would have increased activation of microglia. Microglial activation

significantly changes a cell's morphology (Kettenmann, Hanisch, Noda, & Verkhratsky, 2011). Microglia have a ramified morphology in the healthy adult CNS, consisting of a small soma with thin cellular processes branching outward (Kettenmann et al., 2011). While ramified microglia were previously classified as "resting", it is now known that they constantly survey their local area for changes to the environment and maintain homeostasis (Kettenmann et al., 2011). In contrast to ramified microglia, activated microglia possess fewer cellular processes and a fat, rounded, amoeboid cell body (Walker et al., 2014). We predicted that we would see an increased number of amoeboid microglia in mold-exposed mice.

The hippocampus is critical to certain types of learning and memory (Squire, 1992). Animals with hippocampal lesions performed poorly on spatial memory and recognition tasks (Broadbent, Squire, & Clark, 2006; Zola et al., 2000). Hippocampal long-term potentiation (LTP), a strengthening of synapses that occurs with repeated stimulation and results in a long-lasting increase in synaptic responses, is required for spatial memory (Morris, Anderson, Lynch, & Baudry, 1986). LTP was inhibited by the cytokines IL-1β, Interleukin-6 (IL-6), and tumor necrosis factor (TNF) (Cunningham, Murray, O'Neill, Lynch, & O'Connor, 1996; Li, Katafuchi, Oda, Hori, & Oomura, 1997), which are commonly released during sickness behavior (Konsman, Parnet, & Dantzer, 2002). Cytokine-induced microglial activation was correlated with impaired adult hippocampal neurogenesis (Kohman, Bhattacharya, Kilby, Bucko, & Rhodes, 2013; Song & Wang, 2011). Many studies inducing sickness behavior in rodents using IL-1β or lipopolysaccharide (LPS), a component of bacterial cell walls, produced deficits in behavioral performance in the Morris water maze (Shaw, Commins, & O'Mara, 2000; Sparkman, Kohman, Scott, & Boehm, 2005; Gibertini, 1996), a hippocampal-dependent task commonly used to measure spatial learning in rodents (Brandeis, Brandys, & Yehuda, 1989). Therefore, we also expected mold-exposed animals to show learning impairments during the Morris water maze.

Doxycyline is a tetracycline-derived antibiotic that inhibits pro-inflammatory cytokines, microglia activation and increases neurogenesis (Sultan, Gebara, & Toni, 2013; Jantzie & Todd, 2010; DiCaprio, Lembo, Di Costanzo, Balato, & Monfrecola, 2015). It successfully improved LPS-induced depressive-like behaviors in mice (Mello et al., 2013). Minocycline, another tetracycline-derived antibiotic closely related to doxycyline, significantly improved water maze acquisition in aged mice (Kohman et al., 2013; Jiang et al., 2015). We expected that feeding a doxycycline diet to mold-exposed animals would counteract the effects of mold exposure. Compared to mold-exposed mice on a control diet, we predicted the mold-exposed mice on a doxycycline diet would have fewer amoeboid microglia and improved performance on the Morris water maze.

This paper will attempt to determine whether there is a relationship between learning deficits and changes in microglia caused by mold exposure. We will present an analysis of the Morris water maze performance compared to the population and distribution of microglia by phenotype among mice exposed to *Stachybotrys chartarum* as well as control mice exposed only to a saline vehicle. In addition, we will compare mice on a doxycycline diet to mice on a control diet. It is expected that the presence of microglia with an amoeboid morphology will be associated with cognitive deficiencies in learning the task.

Methods

Animals

Subjects were twelve adult male C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME). Mice were 13 weeks old on arrival. They were habituated for five weeks, including 11 days of human handling followed by three weeks of transport between the animal facility and testing rooms and habituation to various testing environments. Mice were housed singly in polycarbonate shoebox cages with paper bedding, a piece of PVC piping, and a cotton nestlet (Ancare, Bellmore, NY), with a 12-hour on/off light cycle. All animal methodology was approved

by the Institutional Animal Care & Use Committee of Hunter College and met all local and federal guidelines for animal research.

Treatment Conditions and Experimental Design

Mice were divided into four treatment groups using a stratified randomized block design controlling for body weight: saline-vehicle controls and control diet (VEH CD), saline-vehicle controls and doxycycline-augmented diet (VEH DD), intact *Stachybotrys* spores and control diet (IN CD), and intact *Stachybotrys* spores and doxycycline-augmented diet (IN DD). CD Mice were fed a standard chow diet throughout the experiment (Harlan-Teklad Research Diets #2016). DD mice were switched to a diet that was formulated to be identical except for the addition of 40 ppm doxycycline (Harlan-Teklad Research Diets #TD.120292) beginning during the seventh week post-arrival, two days before instillations began.

The spores were originally obtained from Dr. Dorr Dearborn (Case Western University) and were originally isolated from drywall from a home in Cleveland, OH (Yike, Rand, & Dearborn, 2005). Beginning seven weeks post-arrival, mice were anesthetized using isoflurane and instilled nasally three times a week for five weeks with either 15,000 spores/0.25 µl saline/gram of body weight (IN mice) or saline only (VEH mice), with half of the dose instilled in each nostril of each mouse. After instillation, each mouse was held vertically, nose up for two minutes to ensure maximum inhalation. All spore handling and nasal instillations were done in a Class B Biosafety Cabinet with all required safety precautions.

Behavioral Testing Apparatus and Procedures

The water maze was a circular pool 110 cm in diameter and 20 cm deep, with water 14 cm deep and water temperature between 24-26°C. Water was made opaque with nontoxic tempura paint. Each trial was recorded on video to be analyzed with a computerized behavioral tracking system (CleverSys Top Scan, CleverSys, Reston, VA). Sheets and posters were hung outside the maze as external cues and were not moved throughout the experiment. There were three rounds of testing with two consecutive days in each round. There were three types of

trials: 1) visible trials, where the platform was above the water surface and had a green cover, 2) non-visible trials, where the platform was one centimeter below the surface and had a white cover, and 3) probe trials, where the platform was removed. In each of the visible and non-visible platform trials, mice were released and allowed to swim to the platform. If they failed to find the platform in three minutes they were gently guided to the platform, where they were removed after 10 seconds. During probe trials, mice were allowed to swim freely for three minutes and then removed from the maze. For all trials, mice were placed in the maze and removed at specific positions according to an imaginary clock dial on the maze, with north at position 12 and south at position 6. Mice began each trial facing the wall of the maze, with the end of their noses approximately one inch from the wall. After removal, mice were returned to their cages and warmed under a heat lamp. The intertrial interval on each test day for each mouse was 30 minutes +/- 3 minutes.

The first round of water maze testing consisted of two days occurring five weeks post-arrival, before treatment with mold or doxycycline. For day 1, there were four visible trials with the platform in the north quadrant. For the four trials, mice were placed in the maze at positions 5, 3, 9, and 7, and removed from the maze at positions 3, 12, 6, and 9. During day 2, the platform remained in the north quadrant in the same position as day 1, but was below the surface for three non-visible trials, and then removed for one probe trial. During the non-visible trials, mice entered the maze at positions 9, 5, and 3, and exited at position 12, 9, and 6. During the probe trial, mice entered at position 7 and were removed at position 3. The second round of water maze testing occurred three weeks later, over two days during the second week of instillations. The first day began with another probe trial, followed by four visible trials with the platform in the south quadrant. Mice entered the maze at position 5 and exited at position 9 for the probe trial. For the four visible trials, mice entered at positions 9, 1, 11, and 3, and exited at 12, 3, 1, and 9. The second day was three non-visible trials with the platform in the south quadrant and then a probe trial. The three non-visible trials had mice entering at positions 1, 3,

and 9, and exiting at 12, 3, and 9. During the probe trial, mice entered at position 11 and exited at position 6. The third round of testing was during the fourth week of instillations, with a probe trial and four visible trials with the platform back in the north quadrant on the first day, and three non-visible trials with the platform in the north quadrant, ending with an additional probe trial on the second day. On day 1, mice entered at position 1 and exited at position 6 during the probe trial, and entered at positions 9, 3, 7, and 5 and exited at positions 12, 9, 1, and 3 during the visible trials. On day 2, mice entered at positions 7, 3, and 5 and exited at positions 3, 9, and 6 for the three non-visible trials, and entered at position 9 and exited at position 12 from the probe trial. Using the video software, the path length swum, latency to reach the platform, and velocity were determined for each trial and averaged across treatments and diets. Data presented in this paper were from the third round of testing. Of particular interest were the last visible trial on day 1 (D1V4), the first non-visible trial on day 2 (D2NV1), and the difference between the two trials (D1V4-D2NV1) for each variable. The measurements for D1V4 provided a baseline for each mouse's performance after learning the task, while the measurements for D2NV1 were a test of long-term memory after a 24-hour delay (Gulinello et al., 2009). A value close to zero for D1V4-D2NV1 suggests little impairment, while large negative values indicate a failure of long-term memory.

BrdU Treatment

Each mouse was injected intraperitoneally with BrdU (Sigma Aldrich B5002) in saline. Injections consisted of two doses per day for four days of 50 µg/g given five weeks prior to perfusion (to quantify mature neurons), and one dose of 200 µg/g given two hours prior to perfusion (to quantify neuron proliferation).

Perfusion and Tissue Preparation

Following five weeks of instillations, mice were sacrificed in the afternoon, approximately 24 hours following the last installation. Mice were first deeply anesthetized using 200 µg ketamine and 10 µg xylazine per gram of body weight, injected intraperitoneally. Each mouse

was perfused intracardially with 30 ml of 0.1M phosphate buffered saline (PBS) and then 30 ml of 4% paraformaldehyde in PBS. The brains were removed and post-fixed in 4% paraformaldehyde in PBS for an hour. They were placed in PBS overnight, then dehydrated with increasingly concentrated ethanol and embedded in polyethylene glycol (Polysciences). The brains were sliced into 6 µm sagittal sections and mounted on slides, allowed to dry overnight, and stored at -20^{0}C. Every fifth section throughout the dorsomedial hippocampus was labeled for visualizing Iba-1 expression, a specific marker for microglia (Imai, Ibata, Ito, Oshawa, & Kohsaka, 1996)

Immunohistochemistry

To label the sections for quantification of microglia, they were brought to room temperature and washed with PBS three times for 10 minutes per wash, after which the endogenous peroxidase was reacted with 2% hydrogen peroxide and 1% methanol in PBS for 30 minutes, followed by another three 10-minute PBS washes. The sections were blocked with 5% normal goat serum (Jackson ImmunoResearch) and 0.3% Triton X-100 in PBS at room temperature for one hour. Next, sections were exposed to rabbit Iba-1 antibody (1:500, 019-1941, Wako) at 4^{0}C for 72 hours, then washed three more times in PBS for 10 minutes each, then incubated in biotinylated goat anti-rabbit secondary antibody (1:500, 305-065-003, Jackson ImmunoResearch) in 2% normal goat serum (Jackson ImmunoResearch) in PBS for two hours. Tissue was subsequently labeled with avidin and biotin (ABC Elite kit, Vector Laboratories) for two hours followed by three 10-minute PBS washes. To visualize the secondary antibody, 3'3'-diaminobenzidine-tetrahydrochoride chromagen (IMMPact DAB, SK-4105, Vector Laboratories) was used, the tissue was rinsed in distilled water, dehydrated in increasingly concentrated ethanols, and coverslipped with Krystalon (EMD Chemicals).

Cell Quantification

A computer-yoked microscope (Olympus BX51; Lucivid microprojection, Neurolucida, Micro-BrightField, Inc.) was used to measure brain areas and count cells. Then dentate gyrus, hilus, and the hippocampus as a whole were outlined using anatomical markers in accordance with the mouse brain atlas (Franklin & Panxinos, 1997). The granular and subgranular zones were defined by their packing density and cell types present, whereas the molecular layer was identified by the comparative lack of cells (Amaral, Scharfman, & Lavenex, 2007). Neurolucida was used to mark all the Iba-ir cells. Cells were classified according to morphological characteristics as either ramified, amoeboid, primed, rod-like, motile, or monocyte-like. Microglia with a small soma and thin cellular processes branching outward were classified as ramified. Microglia possessing an enlarged, rounded soma and fewer cellular processes were classified as amoeboid. Primed microglia had a cell body size intermediate between ramified and amoeboid (Torres-Platas et al., 2014) and much thicker processes than ramified microglia. Microglia having a narrow cell soma, few planar processes, and highly polarized polar processes were classified as rod-like (Taylor, Morganti-Kossman, Lifshitz, & Ziebell, 2014). Microglia that appeared stretched and often had no processes were classified as motile. Microglia that were darkly shaded, with a round or oval shape and no processes were classified as monocyte-like. The number of all Iba-1 expressing cells, as well as the number of each cell type per mm^2 sampled was calculated for each area.

Statistics

Data were analyzed in GraphPad Prism (Versions 6 and 7 for PC). The D'Agostino & Pearson omnibus normality test determined that not all data were normally distributed. Not all normality issues could be fixed through transformation, so nonparametric Mann-Whitney U tests and Spearman's rank correlations were used. Correlations were performed between MWM data and microglia data for each treatment and diet. All tests were two-tailed. The false discovery rate was controlled using the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995).

Correlation coefficients were compared using Fisher's procedure (Fisher, 1921; Diedenhofen & Musch, 2015).

Results

Behavior

The latencies to reach the platform, path lengths, and velocities of vehicle-treated mice and mold-treated mice during the Morris water maze were compared (Fig. 1). Data was combined across diets to compare treatment effects. There were no significant differences between latencies, velocities, or path lengths during D1V4, when the platform was visible. There were significant differences between groups for D2NV1 and the difference scores between the two trials, but only for path lengths. Mold-treated mice had significantly shorter path lengths than vehicle-treated mice during D2NV1 (Fig. 1E). They also had significantly smaller differences in their path lengths from D1V4 to D2NV4 compared to vehicle-treated mice (Fig. 1F).

Behavioral measures of control diet and doxycycline diet mice during the Morris water maze were compared (Fig 2). Data was combined across treatments to assess the effects of diet. There were significant differences between mice on the control diet and mice on the doxycycline diet during D1V4, the last learning trial where the platform was visible. Doxycycline diet mice had significantly shorter latencies (Fig. 2A) and significantly shorter path lengths (Fig. 2D) during D1V4. There were no significant differences between latencies or path lengths during D2NV1, for the differences between latencies or path lengths, or for any velocity measure.

Effects of Treatments on Microglia

Numbers of each type of microglia from vehicle-treated and mold-treated mice were compared (Fig. 3). Data was lumped across diets to look at treatment effects. The only significant difference between treatment groups was that there were significantly more

monocyte-like microglia in mold-treated mice than in vehicle-treated mice. When numbers of microglia present in mice on the doxycycline diet and mice on the control diet were compared (Fig. 4), data was once again combined across treatments look at diet effects. There were no significant differences between diet groups for any type of microglia.

Behavior-Microglia Correlations across Treatments and Diets

When Spearman's rank correlations were calculated between behavioral data and numbers of microglia, initially many correlations appeared significant. Since there was an expected false discovery rate of 1/20, the Benjamini-Hochberg procedure was applied to correct for multiple comparisons, and most correlations were no longer significant.

Spearman's rank correlations were calculated for each mouse between the numbers of each type of microglia present and their latencies to reach the platform for both mold-treated and vehicle-treated mice (Fig. 5). During D1V4, the last training trial, correlations were mostly negative for both vehicle-treated and mold-treated mice (Fig. 5A). Greater numbers of microglia correlated with shorter latencies. During D2NV1, the first memory trial, correlations were mostly negative for vehicle-treated mice, but mostly positive for mold-treated mice (Fig. 5B). For mold-treated mice, greater numbers of microglia were correlated with longer latencies. There was a significant difference in correlation coefficients between the two groups for the correlations between their latencies during D2NV1 and their numbers of microglia in the molecular dentate gyrus. For the difference score of D1V4-D2NV1, correlations were mostly positive for vehicle-treated mice and mostly negative for mold-treated mice (Fig. 5C), the opposite of the correlations for D2NV1, since the values for latency were quite small for D1V4 and relatively large for D2NV1.

Spearman's rank correlations were calculated for the path lengths taken and the numbers of each type of microglia in vehicle-treated and mold-treated mice (Fig. 6). The pattern of correlations was the same as it was between microglia and latencies. The correlations were mostly negative for both vehicle-treated and mold-treated mice during D1V4, the last learning

trial (Fig. 6A), and still mostly negative for vehicle-treated mice but mostly positive for mold-treated mice during D2NV1, the first memory trial (Fig. 6B). For the differences between path lengths between the two trials (D1V4-D2NV1), correlations were mostly negative for vehicle-treated mice and mostly positive for mold-treated mice, again the reverse direction of the correlations for D2NV1 (Fig. 6C).

The correlations between measures of velocity and numbers of each type of microglia were calculated for both vehicle-treated and mold-treated mice (Fig. 7). Correlations were mostly positive for vehicle-treated mice and mostly negative for mold-treated mice during D1V4 (Fig. 7A). Correlations were mostly positive for vehicle-treated mice and mostly weak for mold-treated mice during D2NV1 (Fig. 7B). There was a significant correlation between the number of microglia in the granular dentate gyrus of vehicle-treated mice and their velocities during D2NV1, a correlation that remained significant after the Benjamini-Hochberg procedure. Correlations were mostly positive for vehicle-treated mice and mostly weak for mold-treated mice for D1V4-D2NV1 (Fig. 7C).

Spearman's rank correlations between latencies and numbers of microglia in each mouse were calculated for both diets (Fig. 8). Correlations between latencies during D1V4 and numbers of microglia were mostly negative for both control-diet and doxycycline-diet mice (Fig. 8A). Correlations were mostly negative between numbers of microglia in both groups and latency during D2NV1 (Fig. 8B). For the differences in latencies (D1V4-D2NV1), correlations with numbers of microglia were mostly positive for both groups (Fig. 8C).

The correlations between numbers of microglia in mice on each diet and their path lengths are shown in Fig. 9. During D1V4, correlations between path lengths and numbers of microglia were mostly negative for both control-diet and doxycycline-diet mice (Fig. 9A). Correlations were mostly negative between path lengths during D2NV1 and numbers of microglia in both diet groups (Fig. 9B). Correlations between the difference in path lengths and numbers of microglia were mostly positive for both groups (Fig. 9C).

The relationships between velocities and different types of microglia in the control-diet and doxycycline-diet mice are shown in Figure 10. Velocity during D1V4 was mostly positively correlated with numbers of microglia in both control-diet mice and doxycycline-diet mice (Fig. 10A). The correlation coefficients for control-diet and doxycycline-diet mice were significantly different for the correlations between their velocities during D1V4 and their numbers of microglia in the hilus. During D2NV1, correlations between velocities and numbers of microglia were mostly positive for both diet groups (Fig. 10B). For the difference in velocities between the two trials, correlations were mostly positive for both control-diet and doxycycline-diet mice (Fig. 10C). The correlation coefficient for the correlation between the number of microglia in the hilus of doxycycline-diet mice and their velocity difference scores was significantly different from the correlation coefficient for the correlation between the number of microglia in the hilus of control-diet mice and their velocity difference scores.

In order to examine the effects of doxycycline on mold-treated mice versus controls, we further separated both vehicle-treated and mold-treated mice into groups by diet. We calculated correlations between path lengths and numbers of microglia for vehicle-treated mice separated by diet (Fig. 11). Correlations between path lengths during D1V4 and numbers of microglia were mostly negative for vehicle-treated mice on both the control diet and the doxycycline diet (Fig. 11A). During D2NV1, correlations between path length and numbers of microglia were still mostly negative for vehicle-treated mice on both diets (Fig. 11B). For the difference scores between the path lengths of the two trials, correlations with numbers of microglia were mostly positive for vehicle-treated mice on both diets (Fig. 11C).

Correlations between path lengths and numbers of microglia were calculated for mold-treated mice separated by diet (Fig. 12). During D1V4, correlations between path lengths and numbers of microglia were mostly negative for both mold-treated mice on the control diet and mold-treated mice on the doxycycline diet (Fig. 12A). During D2NV1, correlations between path lengths and numbers of microglia were mostly positive for mold-treated mice on the control

diet and mixed for mold-treated mice on doxycycline diet (Fig. 12B). Correlations between the difference scores for path length and numbers of microglia for mold-treated mice on the control diet were mostly negative. Half of these correlations were very strong while those of mold-treated mice on the doxycycline diet were mostly mixed and weak (Fig. 12C).

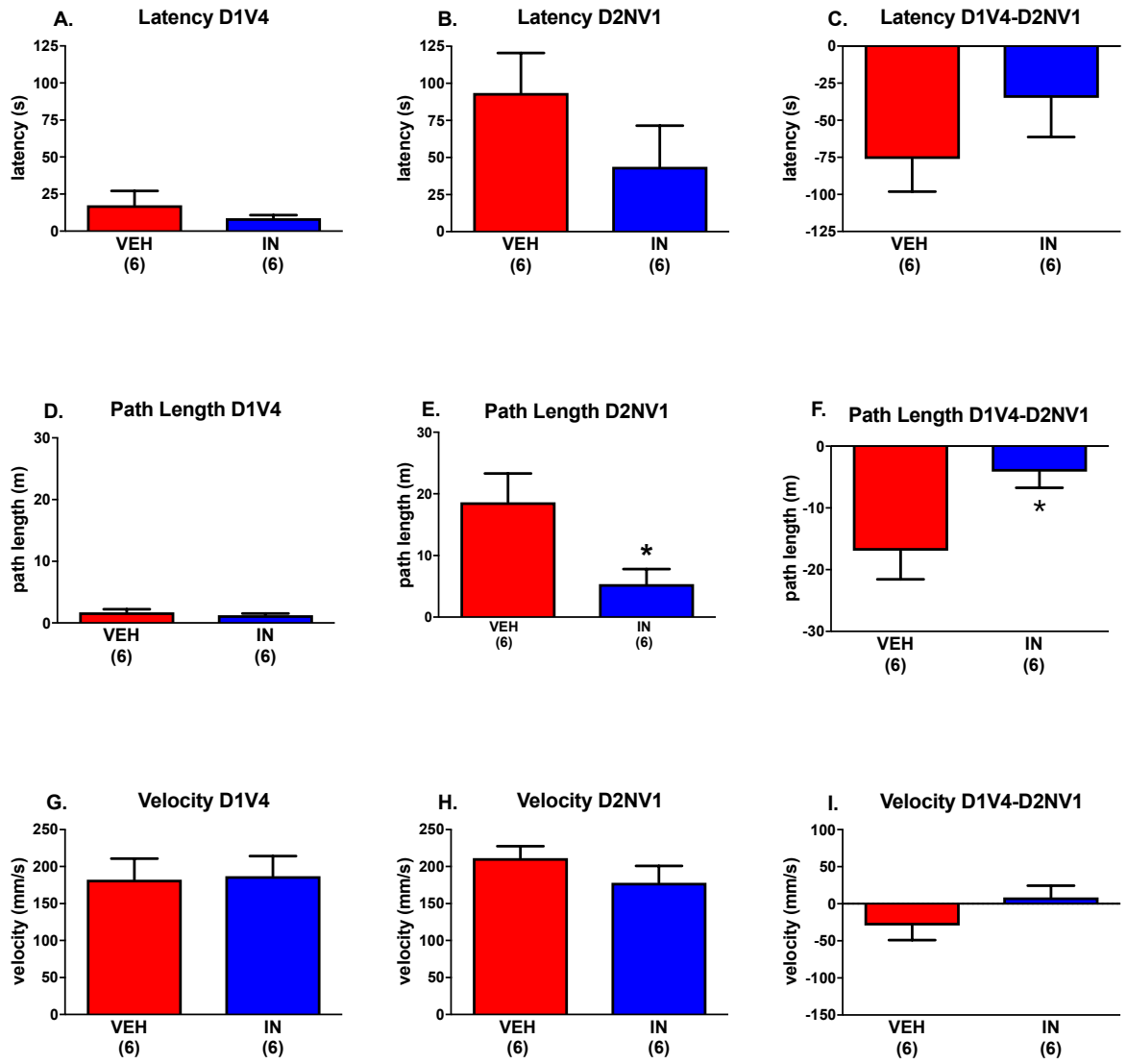


Figure 1 The differences in behavioral measures during the Morris water maze between mice on a control vehicle treatment (VEH) and mice treated with intact mold spores (IN) were compared. E. Mold-treated mice had significantly shorter path lengths during D2NV1 than vehicle-treated mice (Mann-Whitney $U = 5$, $p = 0.041$). F. Compared to vehicle-treated mice, mold-treated mice had significantly smaller differences in path length between D1V4 and D2NV1 (Mann-Whitney $U = 5$, $p = 0.041$).

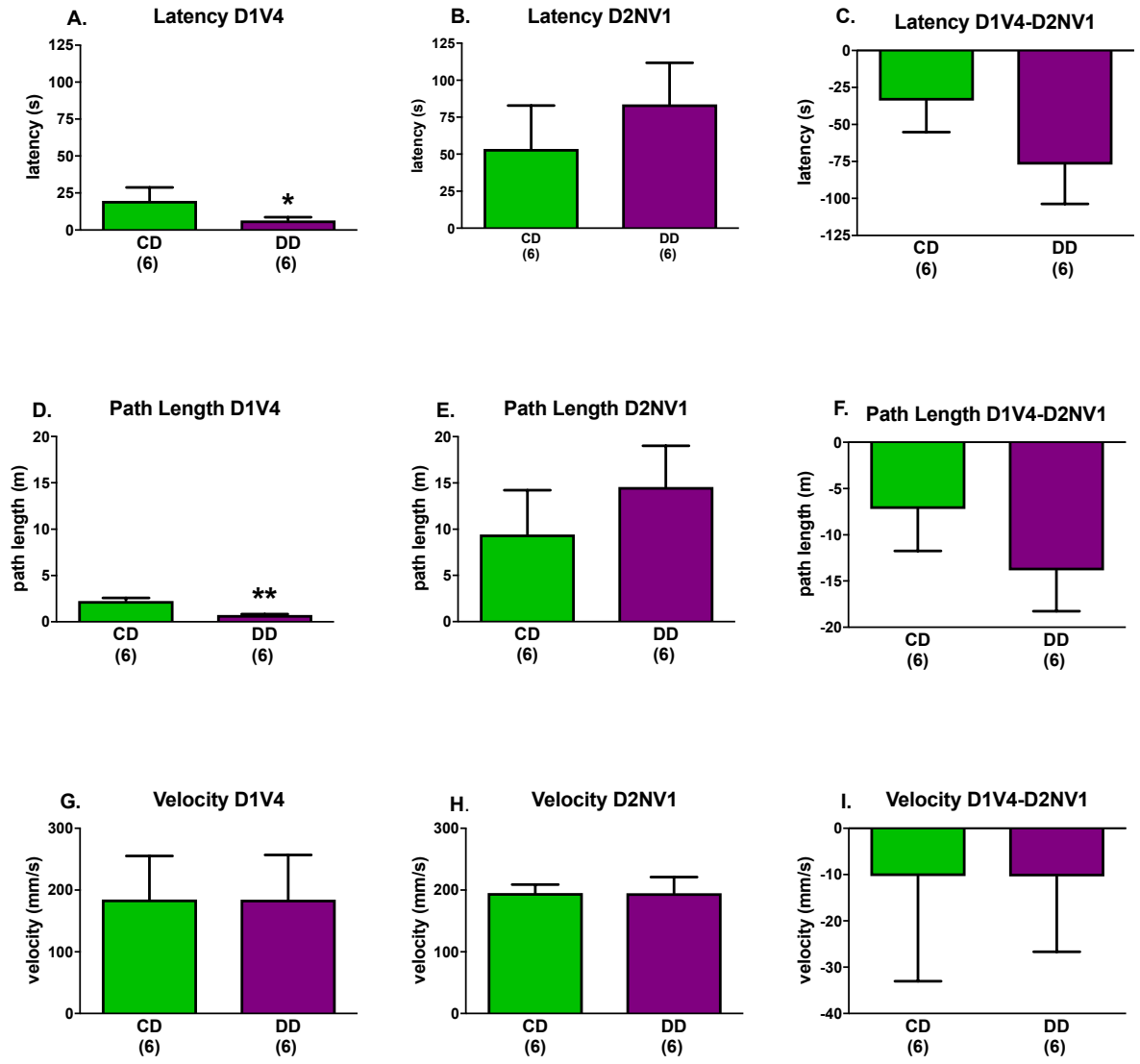


Figure 2 The differences in behavioral measures during the Morris water maze between mice on a control diet (CD) and mice on a doxycycline diet (DD) were compared. A. Latencies for doxycycline-diet mice were significantly shorter than for control-diet mice during D1V1 (Mann–Whitney $U = 5$, $p = 0.041$). D. The average path length for D1V4 was significantly shorter for mice on the doxycycline diet than for mice on the control diet (Mann–Whitney $U = 0$, $p = 0.0022$).

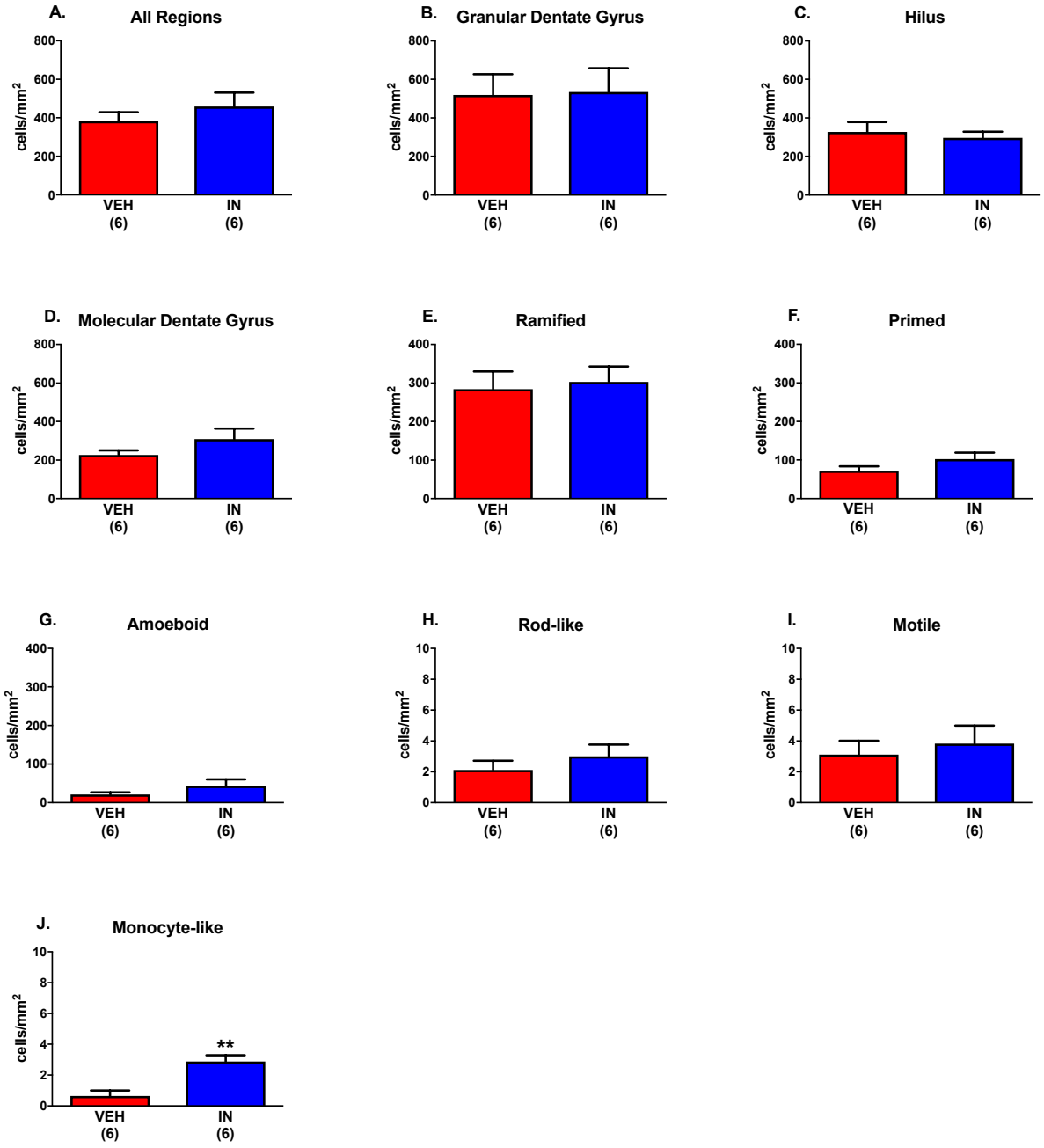


Figure 3 Microglia from mice who were treated with vehicle (VEH) or intact mold spores (IN) were classified according to brain area (A-D) or morphology (E-J) and the number of cells per square millimeter were compared. J. The mold-treated mice had significantly more monocyte-like microglia than vehicle-treated mice (Mann-Whitney $U = 2$, $p = 0.0087$). Maximal Y values are 800 for A-D, 400 for E-G, and 10 for H-J.

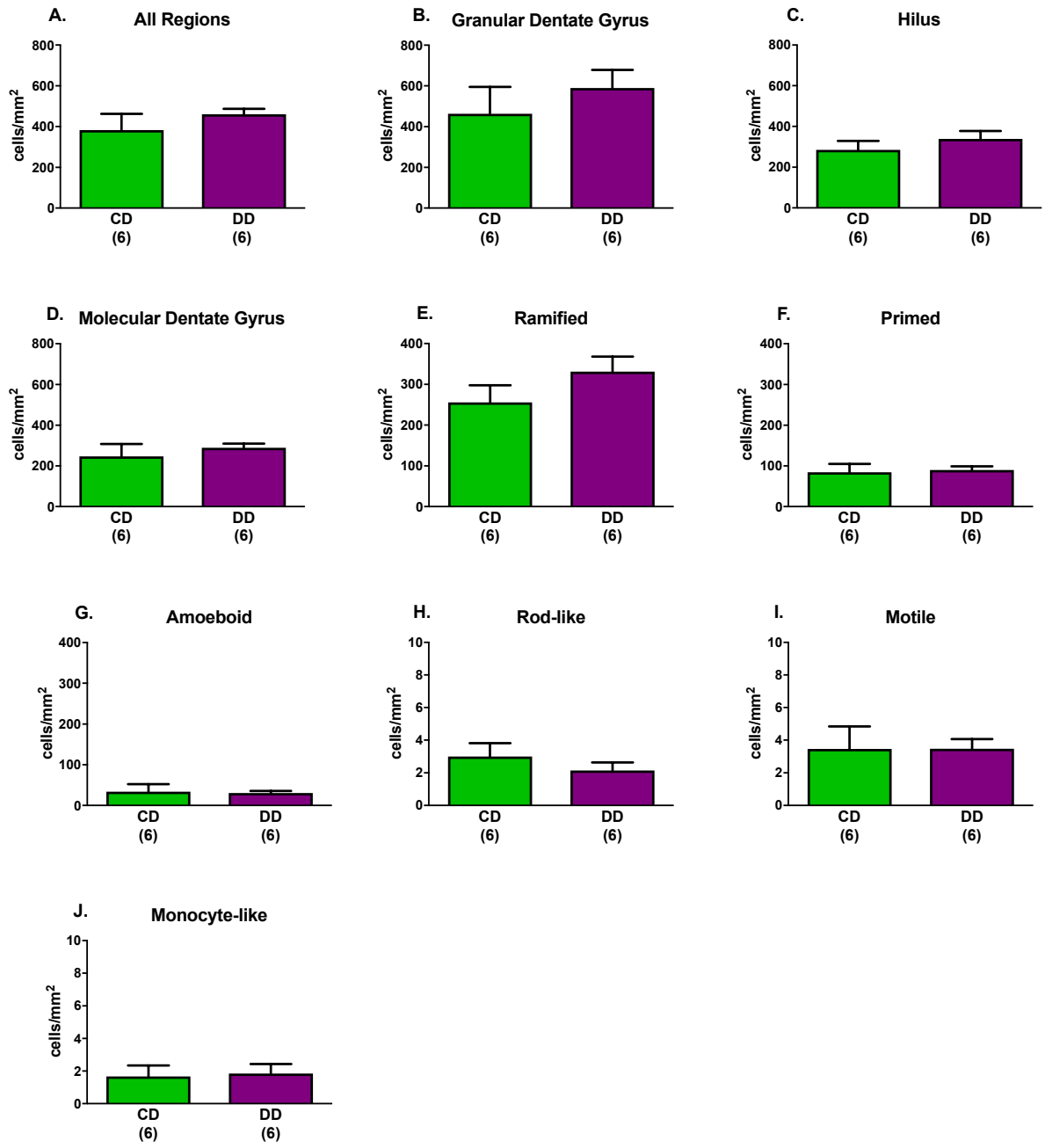


Figure 4 Microglia from mice who were fed a control diet (CD) or a doxycycline diet (DD) were classified according to brain area (A-D) or morphology (E-J) and the number of cells per square millimeter were compared. Maximal Y values are 800 for A-D, 400 for E-G, and 10 for H-J.

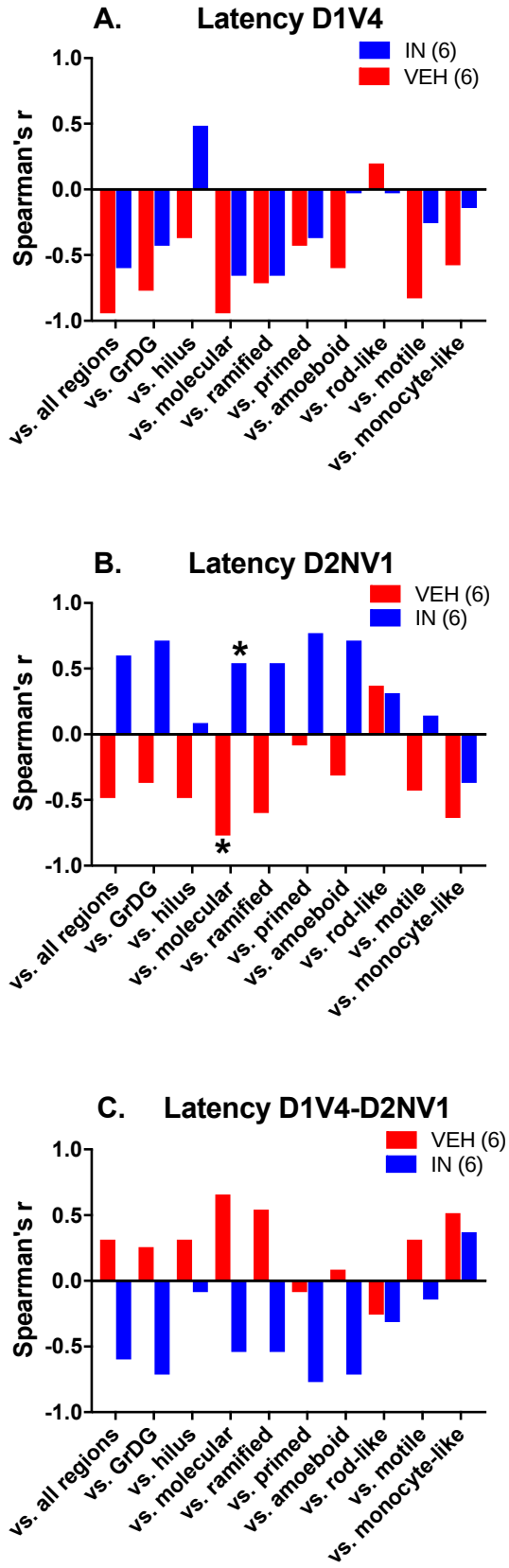


Figure 5 We correlated the latency measures (latency to reach the platform during D1V4, latency to reach the platform during D2NV1, and the difference score between the two trials) with various types of microglia for mice on a control vehicle treatment (VEH) or intact mold spores (IN). B. There was a significant difference between the correlation coefficents for mold-treated and vehicle-treated mice for latency during D2NV1 and microglia in the molecular dentate gyrus (Fisher's z = -2.00, p = 0.046).

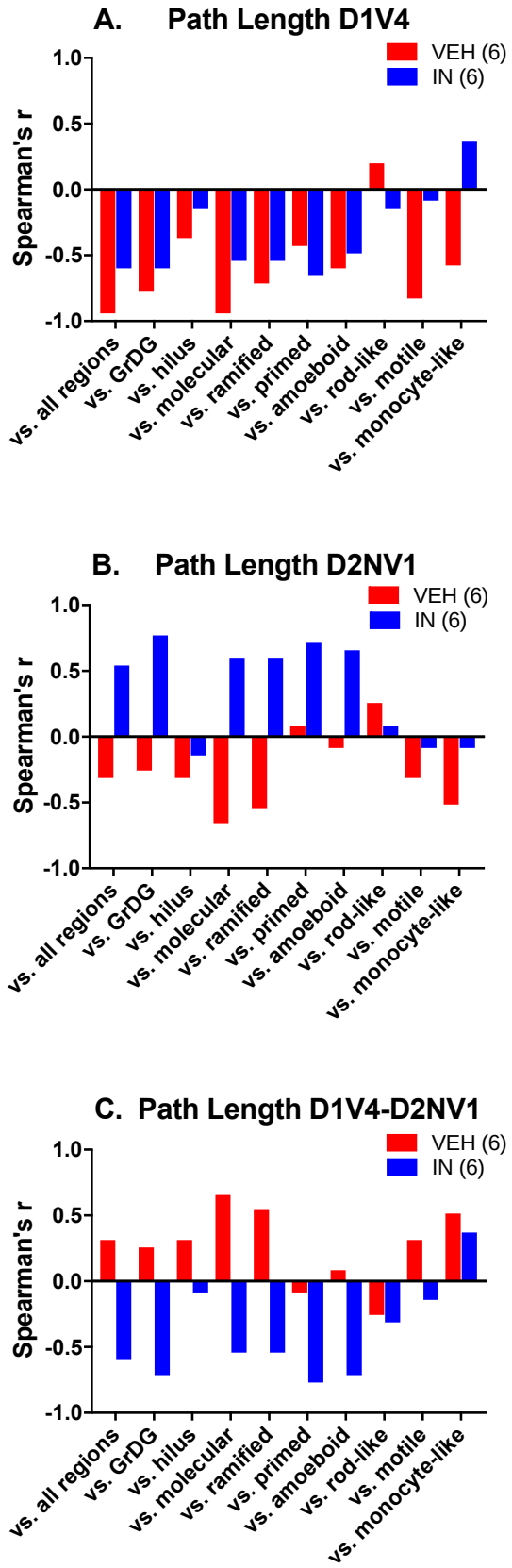


Figure 6 We correlated the path length measures (path length during D1V4, path length during D2NV1, and the difference score between the two trials) with various types of microglia for mice on a control vehicle treatment (VEH) or treated with intact mold spores (IN).

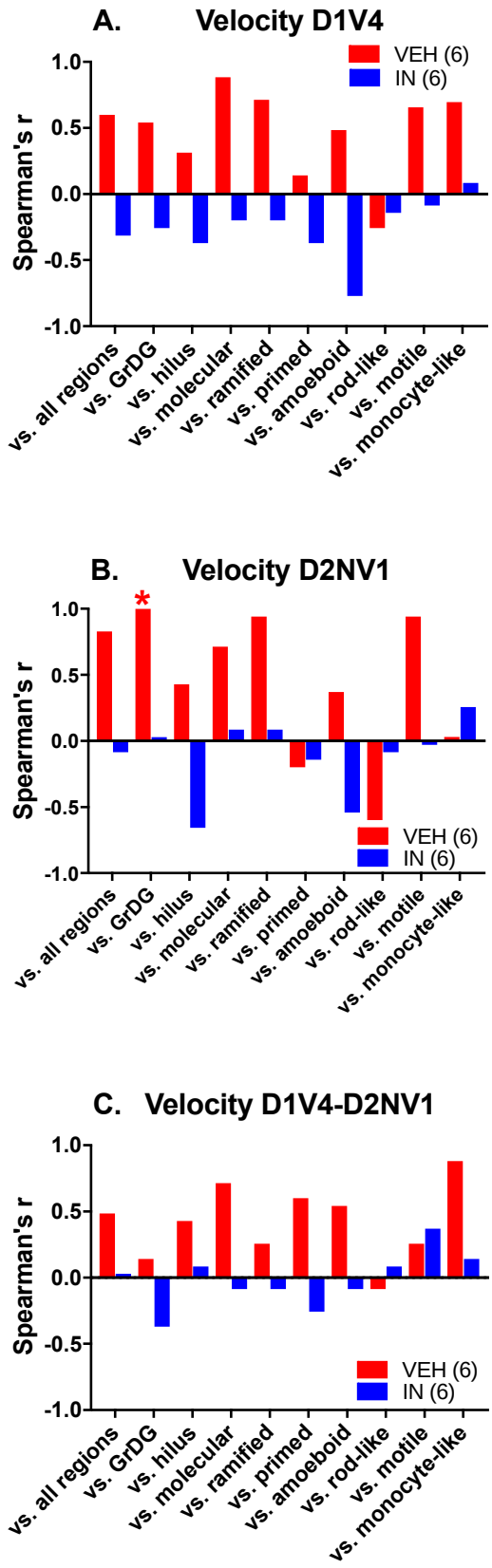
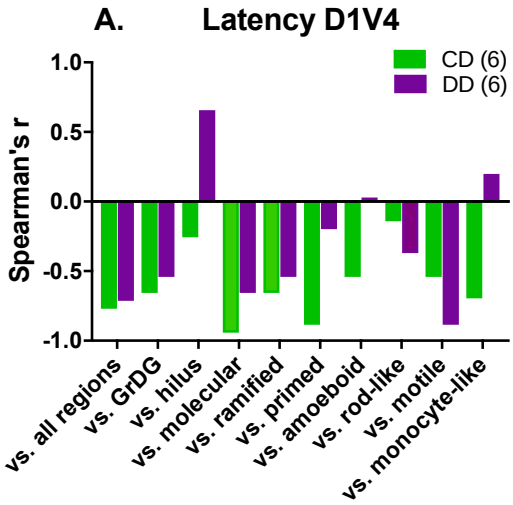
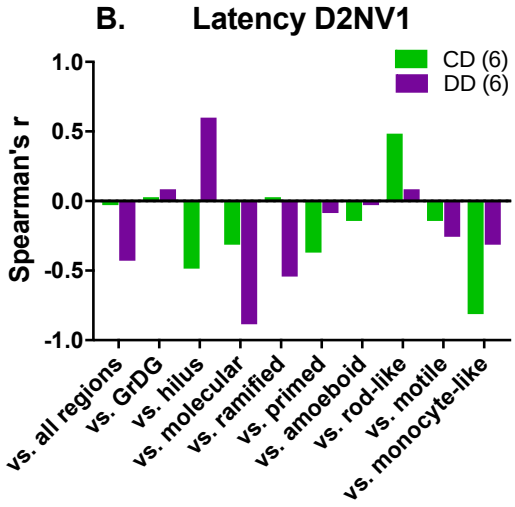


Figure 7 We correlated the velocity measures (velocity during D1V4, velocity during D2NV1, and the difference score between the two trials) with various types of microglia for mice on a control vehicle treatment (VEH) and mice treated with intact mold spores (IN). B. There was a significant correlation between the velocities of vehicle-treated mice and their numbers of microglia in the granular dentate gyrus (r_s = 1.0, p = 0.0028).





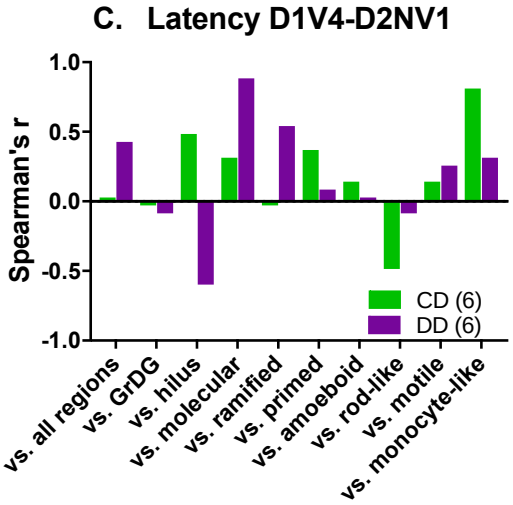


Figure 8 We correlated the latency measures (latency to reach the platform during D1V4, latency to reach the platform during D2NV1, and the difference score between the two trials) with various types of microglia for mice on the control diet (CD) and mice on the doxycycline diet (DD).

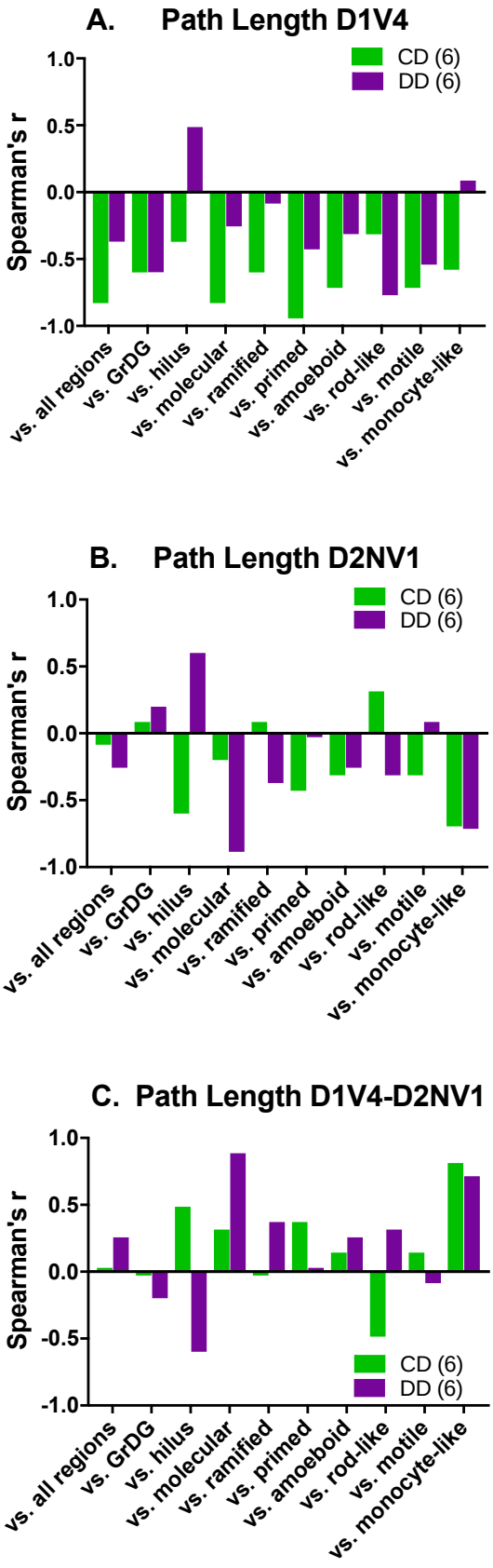


Figure 9 We correlated the path length measures (path length during D1V4, path length during D2NV1, and the difference score between the two trials) with various types of microglia for mice on the control diet (CD) and mice on the doxycycline diet (DD).

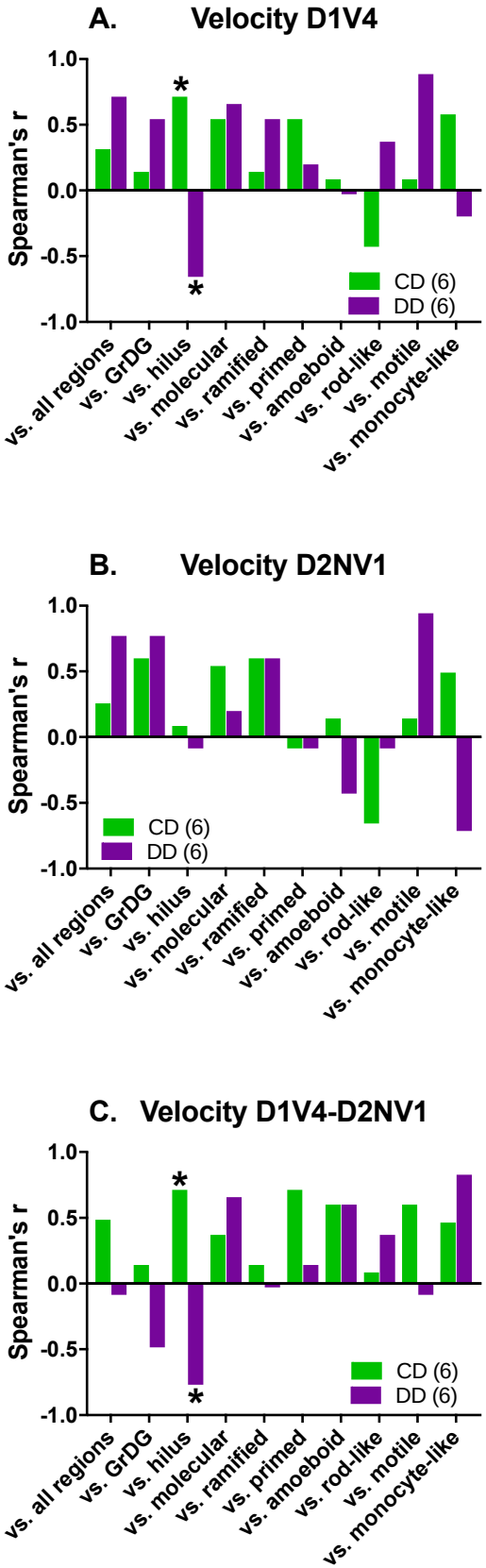


Figure 10 We correlated the velocity measures (velocity during D1V4, velocity during D2NV1, and the difference score between the two trials) with various types of microglia for mice on the control diet (CD) and mice on the doxycycline diet (DD). A. The coefficients for control-diet and doxycycline-diet mice were significantly different for correlations between the velocities during D1V4 and their numbers of microglia in the hilus (Fisher's z = 2.06, p = 0.039). C. There were significant differences between the coefficients of control-diet and doxycycline-diet mice for the correlations between their difference scores for velocity and their numbers of microglia in the hilus (Fisher's $z = 2.35$, $p = 0.019$).

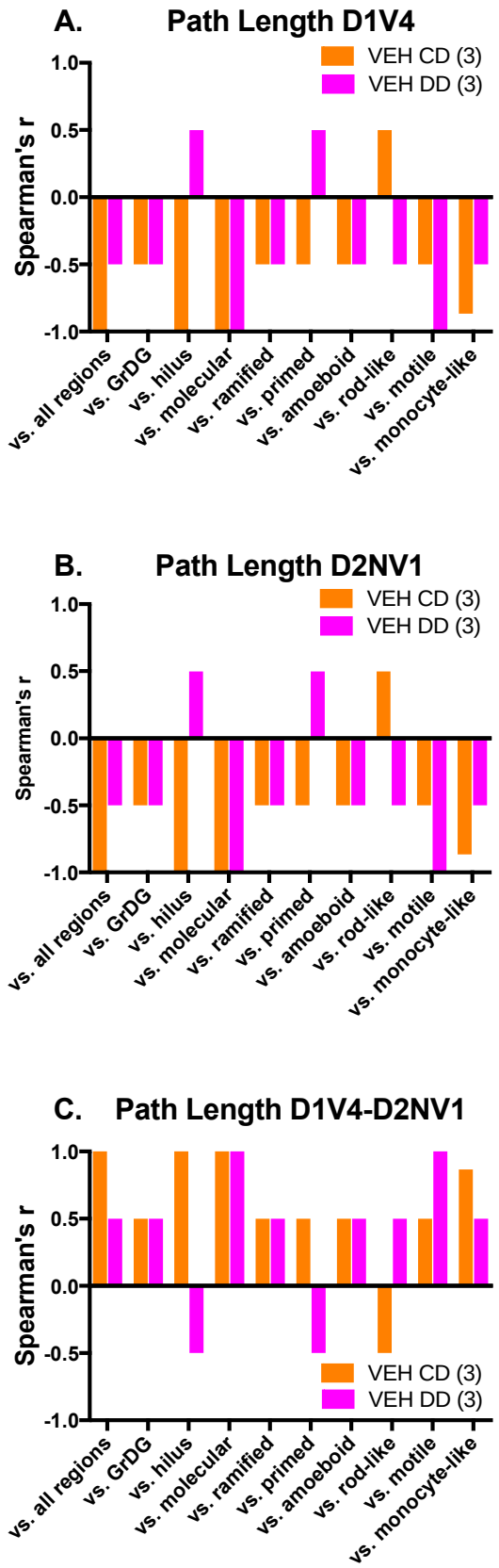


Figure 11 We correlated the path length measures (path length during D1V4, path length during D2NV1, and the difference score between the two trials) with various types of microglia for mice on vehicle treatments with a control diet (VEH CD) or vehicle treatments with a doxycycline diet (VEH DD).

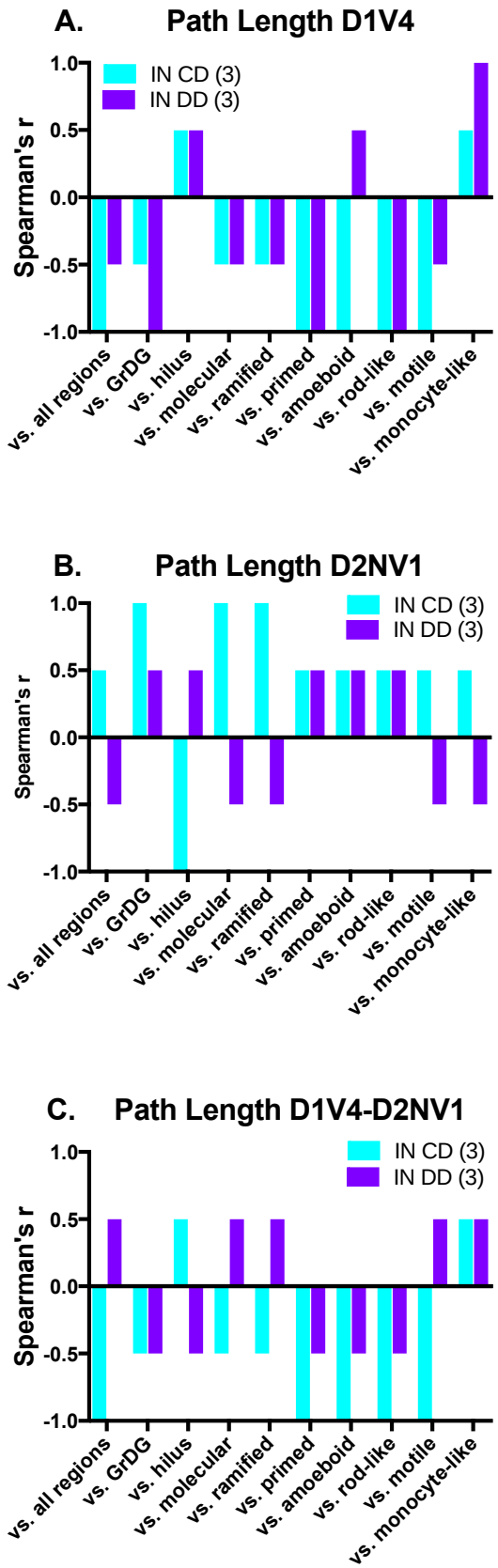


Figure 12 We correlated the path length measures (path length during D1V4, path length during D2NV1, and the difference score between the two trials) with various types of microglia for mice treated with intact mold spores and a control diet (IN CD) or intact mold spores with a doxycycline diet (IN DD).

Discussion

Throughout this line of research, our lab has hypothesized that mold exposure is initiating the immune response known as sickness behavior. As a result, we predicted that mold-treated mice would have increased activation of microglia compared to vehicle-treated mice. Also, we expected that mold-treated mice would have impaired learning and memory performance on the Morris water maze compared to controls, resulting in relatively longer latencies and path lengths. We predicted a correlation between the number of microglia and cognitive impairments. We anticipated that doxycycline would counteract the effects of mold, and that mold-exposed mice on the doxycycline diet would have fewer amoeboid microglia and improved performance compared to mold-exposed mice on the control diet. Neither mold treatment nor doxycycline diet caused significant changes in microglial location or morphology. Mold treatment did not adversely affect learning. The effect on path length to the hidden platform, a measure of performance, was in the opposite direction than expected, with vehicle-treated mice having significantly longer path lengths than mold-treated mice. Doxycyline-treated mice had significantly shorter latencies and path lengths than vehicle-treated mice during visible trials, but not when the platform was hidden. While there was no support for the initial hypotheses in terms of treatment, there were strong correlation patterns that suggest differences in microglial activation between the two groups that were consistent with the initial hypotheses. In terms of diets, in addition to the lack of support for the initial hypotheses, mice on both diets also showed similar correlations between performance and types of microglia.

Behavior

The Morris water maze is a task designed to assess spatial learning and memory in rodents (Morris, 1984). There is considerable evidence that the spatial aspects of Morris water maze learning require the involvement of the hippocampus. Rodents with hippocampal lesions were impaired in locating the hidden platform, but not a visible one (Morris, 1984). Nor were they impaired on non-spatial tasks such as operant conditioning (Gallagher & Holland, 1998) or

taste aversion discrimination (Skinner et al., 1994). Standard performance measures for the Morris water mazes usually include latency to reach the platform and/or path length. Many researchers have argued that path length is the most appropriate measure of cognitive function, since latency is affected by locomotor differences (Cunningham & Sanderson, 2008; Linder, Balch, & VanderMaelen, 1992). Measuring swimming velocity can assess locomotor function. The Morris water maze is a useful tool for investigating the behavioral and cognitive effects of inflammation and infection and is commonly used to assess the effects of LPS and IL-1β. While learning impairments can be compared to controls using path length, symptoms of sickness behavior could affect latency and swimming velocity. Potential symptoms of sickness behavior include decreased activity, general malaise, loss of energy, and feeling sick (Kelley et al., 2003) all of which could affect motivation and speed.

Rodents treated with LPS or IL-1β took more time than control animals to reach the platform in Morris water maze experiments (Gibertini, Newman, Friedman, & Klein, 1995; Arai, Matsuki, Ikegaya, & Nishiyama, 2001; Yirmiya, Winocur, & Goshen, 2002; Song & Horrobin, 2004). Increased latencies could result from cognitive impairments as well as other symptoms of sickness behavior. Since we expected mold exposure to cause immune activation and sickness behavior in our mice, we expected to see increased latencies to reach the platform compared to controls. However, we found no significant differences in latencies between mold-treated and vehicle-treated mice in our experiment. Though not significant, the mold-treated mice actually had decreased latencies compared to controls.

In contrast to latency, path length is a better measurement of learning, since it is independent of variations in swimming speed. Despite the advantages of using path length, latency is the more common measurement in the Morris water maze literature. However, Sparkman, Kohman, Scott, and Boehm (2005) measured path lengths and found that that LPS-treated mice had significantly longer path lengths than control mice. In our previous research, young adult mice treated with intact mold spores showed impaired performance compared to

controls in a contextual learning task, another hippocampal-dependent task, at 30 minutes and 24 hours after training (Harding et al, 2015). The same mice did not show any deficits in executing an auditory task, which requires the auditory cortex and not the hippocampus. In fact, the mold-treated mice showed a stronger memory for the association of tone and shock (as measured by an increase in freezing). The differences in performance between the two tasks suggested they were specifically deficient in memory retrieval during a hippocampal-dependent task. Based on these results, we expected to see memory impairments in mold-treated mice, which would cause longer path lengths to reach the platform. In our earlier Morris water maze experiment (Harding et al, 2015), mice treated with extracted mold spores had longer path lengths than vehicle-treated controls, but intact spore-treated mice did not show the same deficiency. In the current results, there were no significant differences between treatments in path lengths during D1V4, the visible platform trial. Mold treatment did not adversely affect learning. Vehicle-treated mice took significantly longer path lengths to reach the hidden platform than the mold-treated mice. The vehicle-treated mice either could not remember the location as well as the mold-treated mice, or they were less motivated to get there. However, we found that path lengths and latencies were highly correlated, so any motivational factors did not confound the latency measurements. In one result where pathogen exposure lead to shorter path lengths, Gibertini (1998) found that mice treated with high doses of IL-1β had consistently shorter path lengths than controls despite showing symptoms of sickness behavior. The sick mice may have been more highly motivated to escape the stress or discomfort of swimming in the pool, while the healthy mice were less likely to be equally motivated.

We expected that symptoms of sickness behavior would cause a decrease in swimming velocities of mold-treated mice compared to vehicle-treated mice. Several studies have shown an inhibition of behavioral or locomotor activity as a result of LPS or IL-1β administration (Hart 1988; Kent, Bluthe, Kelley, & Dantzer, 1992). There was a significant decrease in swim speed during Morris water maze in LPS-treated mice compared to controls (Sparkman, Martin, Calvert,

& Boehm, 2005). However, we found no significant differences in velocities between mold-treated and vehicle mice.

Some of the reasons that we didn't see clear differences in performance between the mold-treated mice and vehicle mice were possible differences in motivation between the two groups, inappropriate dosing to achieve results, possible strategies used by mold-treated mice to compensate for cognitive impairment. Also, combining diet groups when examining treatment effects and combining treatment groups when examining diet effects could have hidden or canceled out effects that would have been seen if the data was kept separate. Factors such as motivation and stress can confound the measurement of learning and memory. Mice that had been treated with IL-1β to provoke an immune response performed similarly to control mice when tested on the Morris water maze in cold water (18 degrees) (Gibertini, 1998). In warmer water (23 degrees), the IL-1β mice had significantly longer latencies than control mice to reach the platform, suggesting an impairment in motivation in warmer water and not an inability to learn. In studies that treated mice with IL-1β or LPS to induce inflammation and activate microglia, variations in dosage and timing were responsible for large variations in Morris water maze performance as measured by latencies and path lengths traveled (for a review, see Cunningham & Sanderson, 2008). Also, mice with hippocampal impairments could have used a strategy that was not reliant on spatial cues to find the hidden platform, such as thigmotaxis. In a separate experiment by Gilbertini (1998), IL-1β-treated mice had no significant differences in path lengths when finding the platform compared to controls. However, during probe trials, the control mice searched primarily in the target quadrant, while the IL-1β-treated mice did not, implying that only the control mice had truly learned the platform location.

We also expected to see improved performance in mold-treated mice that received doxycycline compared to mold-treated mice that were fed the control diet, resulting in shorter path lengths and decreased latencies to reach the platform in the doxycycline-treated group. We did not expect an effect of doxycycline on animals that were not treated with mold.

Treatment with minocycline, a doxycycline-related antibiotic, significantly ameliorated LPS-induced learning and memory deficits in rats in a passive avoidance task, but there was no difference when saline controls were treated with minocycline (Fan et al., 2005). Treatment with minocycline decreased both latencies and path lengths in LPS-treated mice during a Morris water maze experiment (Hou et al., 2016). The only significant differences we found were between the control-diet mice and the doxycycline-diet mice were during D1V4. Control-diet mice had significantly longer latencies and path lengths than doxycycline-diet mice. Since this difference was during the visible trial, this result suggested a difference in motivation to reach the platform and get out of the water rather than a difference in learning.

In prior experiments, doxycycline and related tetracyclines successfully improved many symptoms and impairments caused by LPS administration (Hou et al., 2016; Henry et al., 2008, Fan et al., 2005). Based on these data, we expected swimming velocities to be faster for the doxycycline-diet mice than the control-diet mice. We expected a decrease in speed in the mold-treated mice that would be reversed in the group that was then fed the doxycycline diet. We found that diet did not significantly affect the swimming velocities of the mice. Liu and colleagues (2007) found that minocycline also did not affect velocities of mice recovering from cerebral focal ischemia or control mice during Morris water maze testing.

Both mold and doxycycline have physiological effects that change the internal state of an animal. Similarly to how drugs can lead to state-dependent effects on learning (Overton, 1966; Zarrindast & Rezayof, 2004), an animal's internal state can be a contextual cue that affects memory retrieval (Shulz, Sosnik, Ego, Haidarliu, & Ahissar, 1999). While administration of cytokines (Kaster, Gadotti, Calixto, Santos, & Rodrigues, 2012) or LPS (Mello et al., 2013; Frenois et al., 2007) produced depressive-like behavior in mice, treatment with doxycycline (Mello et al., 2013) or minocycline (Frenois et al., 2007) prevented or reversed depressive-like symptoms. Mold treatments may have negatively affected the mouse's perception of the water maze experience, whereas the antidepressant effects of doxycycline may have improved

perception of the experience. While LPS caused increases in body temperature that could be inhibited by doxycycline or minocycline (Bastos, Merlo, Rocha, & Coelho, 2007), which could have affected perception of water temperature, it was likely that our water temperature (24-26°) was too warm to have an effect on motivation.

Effects of Treatments on Microglia

Neither the mold treatments nor the doxycycline diet significantly affected microglial morphology. There were no significant effects of mold or doxycycline on any population of microglia, except a significant increase in the number of monocyte-like microglia in mold-treated mice compared to vehicle-treated mice. Half of the vehicle-treated mice had no monocyte-like microglia at all, while the average number of monocyte-like microglia increased more than four-fold in the mold-treated mice. Following inflammation induced by LPS, mechanical or chemical lesions (Montero-Menei et al., 1996; Riva-Depaty, Fardeau, Mariani, Bouchaud, & Delhaye-Bouchaud, 1994), there was a strong recruitment of peripheral monocytes across the blood-brain barrier. Studies *in vitro* showed that adult monocytes from mice and humans can differentiate into microglia-like cells (Sievers, Parwaresch, & Wottge, 1994; Leone et al., 2006). While not significant, it is worth noting that the mold-treated mice had more primed and amoeboid microglia than the vehicle-treated mice, which agreed with previous findings that immune activation tended to increase numbers of primed and activated microglia (Kettenmann, et al., 2011).

Behavior-Microglia Correlations Across Treatments (Combined by Diet)

During D1V4, the latencies and path lengths of both vehicle-treated and mold-treated mice were mostly negatively correlated with numbers of microglia. In other words, greater numbers of microglia were associated with better performance—shorter latencies and path lengths. During D2NV1, the latencies and path lengths remained mostly negatively correlated with number of microglia for vehicle-treated mice, as they were in D1NV4. However, for mold-treated mice, the correlations were mostly positive. Unlike day one, on day two the platform

was not visible and the task required spatial memory and the involvement of the hippocampus. Instead of being associated with shorter latencies and path lengths, greater numbers of microglia were associated with longer latencies and path lengths for mold-treated mice on D2NV1. In addition, different groups of microglia correlated more strongly with performance for the two treatments. Vehicle-treated mice tended to have stronger correlations between behavioral measures and numbers of ramified microglia, whereas mold-treated mice tended to have stronger correlations between behavioral measures and the numbers of primed and amoeboid microglia, particularly during D2NV1. In the healthy central nervous system, ramified microglia produce growth factors such as insulin-like growth factor (IGF-1) and brain-derived neurotropic factor (BDNF) (Ziv and Schwartz, 2008). Treatment with BDNF-blocking antibodies impaired learning on hippocampal tasks such as Morris water maze (Mu, Li, Yao, & Zhou, 1999), contextual fear conditioning, and inhibitory avoidance (Bekinschtein et al., 2007). Systemic administration of IGF-1 improved cognitive impairment caused by IGF-1 deficiency (Lupien, Bluhm, & Ishii, 2003; Trejo et al., 2007). Primed and amoeboid microglia were the result of immune activation of ramified microglia. These physiological changes have been associated with increased synthesis of pro-inflammatory cytokines such as IL-1, tumor necrosis factor (TNF) and IL-6 (Perry & Holmes, 2014). Many researchers found that pro-inflammatory cytokines have detrimental effects on learning and memory (for a review, see Yirmiya & Goshen, 2011).

Total numbers of microglia in the molecular dentate gyrus were strongly correlated with behavioral measures in the vehicle mice. For every behavioral measure, these microglia were more strongly correlated with performance in the vehicle mice than in the mold-treated mice. Greater numbers of microglia in the molecular dentate gyrus were correlated with shorter latencies to the platform and shorter path lengths to the platform. The microglia population in the molecular dentate gyrus has been implicated in task performance. After training on spatial recognition tasks, rats demonstrated synapse remodeling in the molecular dentate gyrus

(Scully, Fedriani, Desouza, Murphy, & Regan, 2012). Task performance has also been linked to morphological features of microglia in the molecular dentate gyrus. In the molecular dentate gyrus of capuchin monkeys, two major morphological microglia phenotypes were identified (types I and II). Compared to type II, type I microglia were smaller and more ramified, with shorter and thinner branches, smaller surface areas, and a higher density of branches. Performance on visuospatial paired associated learning tasks was positively correlated with seven morphological features of type I microglia, including perimeter and area of the cell body (Santos-Filho et al., 2014). Better task performance was associated with having microglia with greater perimeter and area.

There were strong correlations between the number of microglia in the granular dentate gyrus of the mold-treated mice and behavioral measures during D2NV1. For mold-treated mice, more microglia in the granular dentate gyrus were associated with longer latencies and path lengths to the platform. The granular dentate gyrus has a very significant role in neurogenesis, since adult neurogenesis occurs only in the subventricular zone of the lateral ventricles and subgranular zone (SGZ) of the dentate gyrus in the hippocampus. In the SGZ, there is a progression from precursor cells to intermediate progenitor cells to neuroblasts, before becoming neurons (Ming & Song, 2011). Immature neurons migrate locally into the inner granule cell layer and differentiate into dentate granule cells. They develop dendrites that extend towards the molecular layer and project axons through the hilus towards CA3. Increased numbers of activated microglia in the granular dentate gyrus are associated with increased inflammation and decreased neurogenesis. Ekdahl, Claasen, Bonde, Kokaia, and Lindvall (2003) found that inducing inflammation with LPS in mice led to a significant increase in activated microglia in the granular dentate gyrus, and that the number of activated microglia was negatively correlated with the number of surviving neurons. Mice treated with intact mold spores had significantly fewer mature new neurons in the SGZ than controls (Harding et al.,

2015). Impairing neurogenesis in rodents can lead to interference with hippocampal-dependent tasks (Raber et al., 2004; Garthe & Kempermann, 2013; Burghardt, Park, Hen, & Fenton, 2012).

The differences in latencies or path lengths between the two trials (D1V4-D2NV1) were mostly positively correlated with the numbers of microglia for vehicle mice and mostly negatively correlated for mold-treated mice. For both groups, the correlations were in the reverse direction from their respective correlations during D2NV1. The numbers of microglia in vehicle-treated mice were mostly negatively correlated for latencies and path lengths during D1V4, negatively correlated during D2NV4, and positively correlated for the difference score, D1V4-D2NV1. In contrast, the numbers of microglia in mold-treated mice were mostly negatively correlated with latencies and path lengths in D1V4, positively correlated during D2NV1, and negatively correlated for D1V4-D2NV1. For both treatment groups, the correlations for the difference scores of both latencies and path lengths were strongest for the same types of microglia that were most strongly correlated with latencies during D2NV1. This suggests that the results from D2NV1, when the platform was hidden and the time taken and distance traveled was longer, made a greater contribution to the correlations for D1V4-D2NV1 than the results from D1V4, when the platform was visible and the task was not hippocampal.

For the most part, swimming velocities during D1V4 and D2NV1 correlated positively with numbers of microglia for the vehicle-treated mice and negatively for the mold-treated mice. Greater numbers of microglia were correlated with faster speeds for vehicle-treated mice and slower speeds for mold-treated mice. Mold-treated mice with greater numbers of microglia were likely experiencing increased cytokine expression, which causes greater sickness behavior symptoms. Symptoms may include pain, decreased activity, decreased motivation, or increased stress, all of which could decrease swimming velocity. The r-values for correlations between velocities and numbers of microglia were much stronger for D2NV1 than D1V4 for vehicle-treated mice.

For the difference between the velocities between the two trials (D1V4-D2NV1), the correlations were mostly positive for the vehicle-treated mice, and weak for the mold-treated mice. For both vehicle-treated and mold-treated mice, correlations for D1V4-D2NV1 did not seem to be more strongly influenced by either D1V4 or D2NV1, suggesting similar contributions between the visible and non-visible trials, which was not surprising since the velocities were similar in both trial types.

Behavior-Microglia Correlations Across Diets (Combined by Treatments)

During both D1V4 and D2NV1, the correlations between latencies and path lengths of both control-diet mice and the doxycycline-diet mice and number of microglia were mostly negative. Each negative correlation means that greater numbers of microglia were associated with shorter latencies to the platform. The notable exception to the negative correlations was the positive correlation between latencies and the number of microglia in the hilus of doxycycline-diet mice.

For the differences in latency or path length between the two trials (D1V4-D2NV1), the correlations were mostly positive. For both diets and both latencies or path lengths, most of the strongest correlations for D1V4-D2NV1 were the same as the strongest correlations for D2NV1, with the opposite sign. This was the same pattern that was seen in the vehicle- and mold-treated mice, again suggesting that the results from the trial where the platform was hidden and the latencies and path lengths were greater in magnitude had more influence than the visible platform trial on the results for D1V4-D2NV1.

Swimming velocities during D1V4 were mostly positively correlated with the number of microglia of mice receiving both diets. For all positive correlations, possessing a greater number of microglia was associated with faster swimming speed. For both control-diet mice and doxycycline-diet mice, the correlations between velocities during D2NV1 and numbers of microglia were mostly positive.

For the differences in velocities between the two trials (D1V4-D2NV1), the correlations were mostly positive with the numbers of microglia for both diet groups. Neither the results from D1V4 nor D2NV1 seemed to be driving the results for D1V4-D2NV1, similarly to how neither appeared to be largely responsible for the results for difference in velocity between the visible and non-visible trials for the treatment groups.

There were many strong negative correlations between the numbers of microglia in the molecular dentate gyrus of doxycycline-diet mice and various behavioral measures, including latencies during D2NV1, the differences in latency between D1V4 and D2NV1 (D1V4-D2NV1), path lengths during D2NV1, and the differences in path length between D1V4 and D2NV1 (D1V4-D2NV1). Kohman and colleagues (2013) found a weak negative correlation between numbers of Iba-1 positive cells in the molecular layer and average path lengths in Morris water maze among adult mice treated with minocycline.

One reason why the correlations between the diet groups may look so similar is that both groups contain both vehicle-treated and mold-treated mice. While doxycycline and related compounds improved symptoms and impairments caused by inflammation, they did not often improve the performance of controls (Henry et al., 2008; Ekdahl et al., 2003).

For the doxycycline-diet mice, numbers of microglia in the hilus were often correlated in the opposite direction to the majority of the correlations. For trials D1NV4 and D2NV1, while path lengths and latencies were mostly negatively correlated with numbers of microglia of various types and in different areas, they were positively correlated with numbers of microglia in the hilus. For the differences in latencies between D1V4 and D2NV4 (D1V4-D2NV4), the differences in path length between D1V4 and D2NV4 (D1V4-D2NV1), and all measures of velocities, most other correlations with number of microglia were positive for the doxycycline-diet mice, while the correlations with the number of microglia in the hilus were negative. The more microglia were in the hilus, the longer the latencies were, the longer the path lengths traveled, and the slower the swim speeds were. Several studies found aberrant migration of

neurons into the hilus after seizure-like damage to the hippocampus, in association with activated microglia in the hilus (Yang et al., 2010; Parnet, Kelley, Bluthe, & Dantzer, 1997). If an increase in aberrant migration of neurons into the hilus was the cause of our result, it is not clear why it would be occurring in the doxycycline-diet mice and not the control-diet mice.

Behavior-Microglia Correlations Separated by Treatment and Diet

Since we hypothesized that the doxycycline diet would counteract the effects of mold treatment, we wanted to divide the data set further in order to compare the effects of each diet on each treatment. Despite our small sample size, we separated the data into groups corresponding to each combination of treatment and diet. The path length data for each group was correlated with their numbers of microglia. Historically, researchers have considered path length the best way to measure cognitive function in the Morris water maze. However, in our case path lengths and latencies were highly correlated, since there were no significant differences in velocities, so similar results would be expected if we used the latency data. The correlations between numbers of microglia and path lengths were mostly positive for D1V4 and D2NV1, and mostly negative for the difference score for both vehicle mice on the control diet and vehicle mice on the doxycycline diet. As with the combined vehicle group, greater numbers of microglia were mostly associated with shorter path lengths for the separated vehicle groups for both visible and non-visible trials. One of the exceptions was the correlation between path lengths and the numbers of microglia in the hilus of vehicle-treated mice on the doxycycline diet, which was the opposite of most other correlations, as was seen in the combined doxycycline diet group. The separated vehicle groups showed the same pattern of correlations for D2NV1 as for D1V4, and the pattern was the same with the opposite sign for the difference score. The correlations were rather similar for the two vehicle treatment groups. On each graph, seventy percent of the time, the correlation coefficient between path length and numbers of microglia had the same sign for vehicle mice on both diets, and forty percent of the time, the correlation coefficient was the same for vehicle mice on both diets. For D1V4, the correlations between

path lengths and numbers of microglia for the combined vehicle group were rather strong, and when the groups were divided, they were roughly the same strength, with the resulting correlations about equally likely to average stronger or weaker than the combined groups. For D2NV1 and the difference scores, the correlations between path length and numbers of microglia were not as strong for the combined vehicle group, and they were more likely than not to strengthen when the groups were divided. For all correlations with the path length measures of vehicle-treated mice, after dividing into groups by diet, the correlations with microglia in the molecular dentate gyrus were strong for both vehicle-treated mice on the control diet and vehicle-treated mice on the doxycycline diet. Additionally, there were strong correlations between path length measures and microglia in all regions, microglia in the hilus, and monocyte-like microglia for vehicle-treated mice on the control diet, and between path length measures and motile microglia for vehicle-treated mice on the doxycycline diet.

Numbers of microglia of mold-treated mice on the control diet were mostly negatively correlated with their path lengths during D1V4, mostly positively correlated with their path lengths during D2NV1, and mostly negatively correlated with their difference scores for path length. This was the same pattern we saw for the combined mold-treated group, where greater numbers of microglia were linked to shorter path lengths during visible trials and longer path lengths during non-visible trials. The correlations with the hilus were an exception. While correlations with microglia in the hilus previously appeared as an exception to the correlations around it, this effect was usually seen in mice on the doxycycline diet. Mold-treated mice had mostly negative correlations between their numbers of microglia and their path lengths during D1V4. They had a mixture of positive and negative correlations between their numbers of microglia and their path lengths during D2NV4, and between their numbers of microglia and their difference scores for path length. Greater numbers of microglia were linked to shorter path lengths during the visible trials, but there wasn't a clear pattern for the non-visible trials. However, it was striking that there were more negative correlations for mold-treated mice on the

doxycycline diet than for mold-treated mice on the control diet. Except for the hilus, the only negative correlations for the divided mold-treated groups were for mold-treated mice on the doxycycline diet. Whereas numbers of microglia were almost always associated with longer path lengths for mold-treated mice on the control diet, numbers of microglia were sometimes associated with shorter path lengths for mold-treated mice on the doxycycline diet. This supports our hypothesis that doxycycline would counteract the effects of mold exposure. The separated mold-treated groups behaved similarly during D1V4, where the correlation coefficients had the same sign nine times out of ten, but this was only true 40% of the time during D2NV1 and 50% of the time for the difference scores. For all three measures of path length, the correlations between the separated mold-treated groups were mostly much stronger than the combined correlations. For path lengths during D1V4, the average between the two correlations for the separate mold-treated groups was stronger than the correlation for the combined mold-treated group for eight out of ten correlations. In three cases, the stronger correlation of the divided groups was for mold-treated mice on the control diet, while in two cases the stronger correlation was for mold-treated mice on the doxycycline diet, and in three cases the correlations for both groups were equally strong. During D2NV4, the correlations between path lengths and microglia were stronger for the divided mold-treated groups than the combined group six out of ten times. In three instances, the stronger correlation was for mold-treated mice on the control diet and in the other three instances the correlation for both groups was equal in strength. For the difference scores, the correlations were stronger six out of ten times after splitting the mold-treated group by diet. Four times the stronger correlation was for mold-treated-mice on the control diet, and two times the correlations were equal for mold-treated mice on both diets.

Concluding Remarks

The present results suggest strong associations between specific populations of microglia and spatial learning performance following mold exposure or doxycycline

consumption. For vehicle-treated mice, the more microglia present in the hippocampus, the shorter the path lengths or latencies regardless of whether the platform was visible or not. However, for mold-treated mice, a greater number of microglia was associated with shorter path lengths or latencies when the platform was visible, but longer path lengths when the platform was non-visible and the task required hippocampus-dependent spatial memory recall and retrieval. The results suggest that microglial activation in response to mold exposure was related to differences in results between the vehicle-treated mice, where behavior was strongly correlated with the less-activated ramified microglia, and the mold-treated mice, where behavior was strongly correlated with the more-activated primed and amoeboid microglia. Further studies might examine cytokine levels to more accurately describe the relationship between the various states of microglia and the effects on learning and memory. Since the Morris water maze results can be affected by variations in motivation and stress caused by sickness behavior, it is essential to control for variables in performance in order to accurately measure differences in cognition, and to consider using other hippocampal tasks in addition and comparing results. A greater number of mice would be greatly helpful, to hopefully get significant results, and to allow a larger sample size. A larger sample size would be particularly useful when dividing into all four groups by treatment and diet.

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