THE EFFECT OF A HIGH FAT DIET ON THE BRAIN FOLLOWING HSV-1 INFECTION

by

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Abstract

Children from low socioeconomic status have an increased risk of developing anxiety. Additionally, both obesity and herpes simplex virus 1 (HSV-1) infection have an increased prevalence among children from lower socioeconomic status, and may be environmental factors that contribute to the development of anxiety disorders. This study aimed to determine if a high fat diet following HSV-1 infection increased inflammation in the hippocampus and altered the phenotypes of monocytes in the blood. Female C57BL/6 mice were placed on a 10% LF diet for one week prior to intranasal HSV-1 or mock infection. Mice were randomized to either the 45% HF diet or remain on the LF diet 14 days post infection. Mice were sacrificed at either 4-5 months or 9 months, and hippocampus samples were obtained. RNA extraction, reverse transcription and qPCR were performed to examine expression of IL-6, IL-1β, TNFα, MHCII and CCL2. Flow cytometry was utilized to determine the percentage of CCR2+ and Cx3CR1+ monocytes in the blood. The infected LF and HF groups saw a significant decrease in IL-6 expression with age. Expression of the inflammatory monocyte CCR2+ was highest in the lymphocytes of young, LF uninfected mice, and significantly lower in the older, HF infected group. In conclusion, the level of inflammation seen in these female mice was lower than that seen in the larger study using male mice, suggesting that other factors, such as weight, development of metabolic syndrome, or estrogen interactions may affect neuroinflammation.
Chapter 1: Study Aims and Hypotheses

Children from low socioeconomic backgrounds have a greater risk of developing anxiety and learning problems\textsuperscript{19}, but the biological factors that contribute to this increased risk are not well understood. Early exposure to certain environmental factors that affect the brain may play a role in the development of these mental health disorders. Two examples of these factors are the high rates of herpes simplex virus (HSV)-1 and the prevalence of obesity in children from low socio-economic backgrounds\textsuperscript{20,21}. We have developed a mouse model that mimics this environment. My project is part of a larger study to test the hypothesis that obesity (induced by a diet high in saturated fatty acids) in mice latently infected with HSV-1 results in neuroinflammation and increased anxiety in these mice.

The main study focuses on the response in male mice, here we utilize female (C57BL/6) mice; this is unique in that females are more prone to anxiety and also constitute an understudied population (both in rodent and human studies). In order to study the effect of a high fat diet on perpetuating neuroinflammation induced by HSV-1 infection, mice were randomly assigned to HSV-1 infection or mock infection. The mice were allowed to recover from the infection (and the virus became latent). After 14 days, the mice were then randomized to a high-fat or a low-fat diet. After the mice were sacrificed, blood samples and brain tissue samples were analyzed to investigate differences in the immune response among the mice.

Neuroinflammation is associated with increases in anxiety and is a product of cytokines made by microglia in the brain. In addition, recent evidence suggests that inflammatory monocytes (CCR2+) can enter the brain and increase anxiety by exacerbating inflammation\textsuperscript{(36)}. Therefore, my study will examine these aims:
**Specific Aim 1:** To determine if HF diet following HSV-1 infection increases inflammation in the hippocampus of the brain, an area that is key to the development of anxiety

- qRT-PCR: IL-6, IL-1β, TNFα, MHCII and CCL2 (a chemokine that attracts CCR2+ monocytes)

**Specific Aim 2:** To determine if HF diet following HSV-1 infection alters the monocyte phenotypes in the blood

1. Genotyping mice: CCR2 and Cx3Cr1 heterozygous
2. Flow cytometry: CCR2 and Cx3Cr1 expression monocytes to quantify “inflammatory” monocytes.

**Hypothesis:** After HSV-1 infection, mice on high-fat diet will have an increased inflammatory response in the brain and increased CCR2+ monocytes in the blood, which are associated with increased anxiety behaviors.

The techniques used for this study are RNA extraction, reverse transcription and qPCR to examine inflammation. DNA extraction and PCR were utilized to genotype the CCR2<sup>RFP</sup>+/Cx3CR1<sup>eGFP</sup>+/ mice and flow cytometry to determine the percentage of CCR2+ and Cx3CR1+ monocytes in the blood.
Chapter 2: Introduction

With a 12-month prevalence of 18.1%, anxiety and anxiety related disorders are the most common mental health problem in the United States\textsuperscript{25}. The burden and impairment caused by anxiety and anxiety disorders is significant and comparable to other chronic medical illnesses, such as diabetes\textsuperscript{26}. Additionally, anxiety disorders are highly comorbid with other mental illnesses\textsuperscript{26}. Genetic factors play a role in the development of anxiety disorders, and heritability is estimated to be around 30\%\textsuperscript{26}. However, environmental factors also have a significant role in the development of anxiety\textsuperscript{26}.

Previous studies have suggested a correlation between mental disorders and obesity\textsuperscript{27}. Additionally, recent literature has suggested that high fat diets and obesity can contribute to cognitive impairment in both rodents and humans\textsuperscript{10,12-24}. Recent evidence has shown that obesity results in a state of chronic inflammation. Specifically, it has been shown that diet-induced obesity causes a shift in the state of macrophages in adipocytes to a more inflammatory state\textsuperscript{6}. Furthermore, obese individuals have higher circulating levels of inflammatory cytokines such as TNF\textalpha, IL-1\beta and IL-6, which are thought to contribute to this chronic inflammation\textsuperscript{2,8}. A correlation between cytokines released by adipocytes and inflammatory markers indicates that these cytokines may cause higher secretion of inflammatory proteins in liver\textsuperscript{7}. Recent evidence has suggested a role for cytokines beyond basic signaling mechanisms and the mediation of inflammatory response\textsuperscript{12}. They may have a role in the development of certain mental illnesses including major depression, schizophrenia, and Alzheimer’s disease\textsuperscript{9,12}. How this inflammatory state relates to immune response in the brain upon acute infection deserves more attention considering that neuroinflammation may have a significant effect on cognition and mental health.
The relationship between diet and neuroinflammation also warrants more attention. It has been shown that in instances of diet-induced obesity, hypertriglyceridemia contributes to cognitive impairment\(^\text{10}\). Additionally, a diet high in saturated fat or hydrogenated fats has a positive correlation with risk of developing Alzheimer’s disease\(^\text{11}\).

A better understanding of the implications of diet-induced obesity on neural inflammation is relevant today as obesity becomes increasingly common in our society. Furthermore, both obesity and HSV-1 seropositivity are prevalent in low socioeconomic (SES) conditions. A recent study found that obese individuals were significantly more likely to be HSV-1 positive\(^\text{4}\). A separate study found a correlation between fat mass and HSV-1 seropositivity in middle aged men\(^\text{5}\). One study found an association between HSV-1 seropositivity and impaired cognitive function among children ages 6-16\(^\text{29}\).

This study will examine the relationship between diet-induced obesity, inflammation, and anxiety. Recently, childhood obesity has decreased in children among higher socioeconomic classes, but increased in children from low socioeconomic status\(^\text{20}\). Additionally, children ages 6-16 in lower socioeconomic classes have a higher prevalence of HSV-1 infection\(^\text{28}\). Both obesity and HSV-1 infection have been implicated in cognitive impairment. Therefore this mouse model mimics the daily environment of children living in poverty in order to analyze the effects of obesity and HSV-1 infection on the development of anxiety behavior. As part of a larger study, this study focused on examining inflammation in the hippocampus, an area of the brain important to the development of anxiety. The expression of proinflammatory cytokines IL-6, IL-1\(\beta\), TNF\(\alpha\), MHCII and CCL2 was analyzed to evaluate the effect of a high fat diet and HSV-1 infection on neuroinflammation. Additionally, the composition of monocytes in the blood was analyzed in order to determine if the phenotypes of monocytes in the blood is altered due to a
high-fat diet following HSV-1 infection. We hypothesized that after HSV-1 infection, mice on high-fat diet will have an increased inflammatory response in the brain and increased CCR2+ monocytes in the blood, which are associated with increases in anxiety.
Chapter 3: Methods

Animals

In all of these experiments, weanling female C57Bl/6 CCR2<sup>RFP/+</sup>/CX3CR1<sup>eGFP/+</sup> heterozygous mice were placed on a 10% LF diet and allowed to acclimate for one week prior to intranasal HSV-1 or mock infection. Fourteen days post infection (p.i.), mice were randomized to either the 45% HF diet or remain on the LF diet. The mice were sacrificed at either 4-5 months or 9 months. The mice sacrificed at 4 and 5 months were categorized as “young,” and the mice sacrificed at 9 months were categorized as the “old” group.

Genotyping

DNA was extracted from tail snips of mice bred to be heterozygous (CCR2<sup>RFP/+</sup>Cx3Cr1<sup>GFP/+</sup>) using the Qiagen DNeasy Quick-StartProtocol. The DNA extraction was performed according to manufacturer’s instructions for the DNeasy Blood and Tissue Kit, which included overnight lysing (in a 56°C water bath) of the tail snips in 180 µL Buffer ATL and 20 µL proteinase K. Next, 200 µL of Buffer AL and 200 µL of 96% ethanol was added. The 600 µL mixture was placed in a 2 mL collection tube, which was centrifuged at 8000 rpm for 1 minute. The flow-through and collection tube were discarded. The spin column was placed into a new 2 mL collection tube and 500 µL Buffer AW1 was added. The sample was centrifuged for 1 minute at 8000 rpm, and the flow-through and collection tube were discarded. The spin column was then placed into a new 2 mL collection tube, and 500 µL Buffer AW2 was added. The samples were centrifuged for 3 minutes at 14000 rpm and the flow-through and collection tubes were discarded. The spin columns were transferred to new 1.5 mL microcentrifuge tubes, and the DNA was eluted by adding 100 µL DEPC H₂O to the spin column. Samples were incubated for a
minute at room temperature, and then were centrifuged for 1 minute at 8000 rpm. This last step (DNA elution) was repeated using 100 µL DEPC H₂O to increase DNA yield.

PCR was used to amplify the DNA. The CCR2 primer set (wild-type, mutant, and common) was from Bioneer, as well as the Cx3Cr1 wild-type primer set (wild-type and common primers). The Cx3Cr1 mutant primer set (mutant and common primers) was from Invitrogen. One µL of DNA, 0.2 µL of each primer, 0.2 µL dNTP mix, 1 µL 10x Thermopol Buffer, and 0.5 Taq polymerase was used. The cycler program was set to denature the DNA at 94 °C, allow annealing at 50 °C, and then elongate the DNA at 72°C. Gel visualization (1% Ethidium Bromide gel) was performed using 1:8 dilutions of the amplified DNA.

RNA Isolation

Brain tissue samples were homogenized in 1mL of TRIzol, and then incubated for 5 minutes at room temperature. Next, 0.2 mL of chloroform was added and tubes were shaken vigorously by hand for 15 seconds. Samples were incubated for 2-3 minutes at room temperature, and then the samples were centrifuged at 4°C for 15 minutes at 12,000 x g. The aqueous phase was removed from the samples and placed in a new tube. The RNA was then isolated by adding 0.5mL of 100% isopropanol to the aqueous phase, and then incubating the samples at room temperature for 10 minutes. The samples were then centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was then removed from the tube, leaving an RNA pellet. The pellet was washed with 1 mL of 75% ethanol. The samples were briefly vortexed, and then centrifuged at 7500 x g for 5 minutes at 4°C. The wash was discarded, and the RNA pellet was allowed to air dry, and then was resuspended in 40 µL H₂O.

cDNA synthesis
The previously isolated RNA (1 µg in 8 µL water), 1 µL of 10x Reaction Buffer, and 1 µL Sigma DNase I, Amplification Grade (1 unit/ µL) were added to an RNase-free PCR tube. The solution was mixed gently, and then incubated for 15 minutes at room temperature. Next, 1 µL of Stop Solution was added, and the samples were heated at 70°C for 10 minutes to denature the DNase I and RNA. The solution was chilled on ice, and then 4 µL 5x iScript reaction mix, 1 µL iScript reverse transcriptase, and 4 µL nuclease-free H₂O was added to the solution. The reaction mix was incubated for 5 minutes at 25°C, 30 minutes at 42°C, and then for 5 minutes at 85°C.

**qPCR**

For the purposes of this thesis, only qRT-PCR data from the hippocampus were analyzed and presented. Real-time qPCR was performed using the synthesized cDNA samples. The Real-Time PCR System used was Bio-Rad® CFX96™ and the thermal cycling protocol consisted of polymerase activation and DNA denaturation at 95°C for 30 seconds, amplification at 95°C for 5 seconds (for denaturation) and then 60°C for 30 seconds (for annealing and extension). The program cycled 39 more times for a total of 40 cycles. Each qPCR tube had a total volume of 20 µL, which included 10 µL TaqMan Universal Mix (2x), 1 µL of the desired primer (either GAPDH, IL-1, IL-6, MHCII, TNF-alpha), 6 µL of H₂O, and 3 µL of cDNA.

**Statistics**

Data was analyzed using a three-way ANOVA test and GraphPad Prism software.
Chapter 4: Results

Figure 1: Gel visualization showing that mice are heterozygous CCR2<sup>RFP/+</sup> Cx3Cr1<sup>GFP/+</sup>

Gel visualization using an ethidium bromide gel was used to confirm that the mice were heterozygous. Figure 1 shows a sample gel with all three primer sets. CCR2 heterozygotes display a band at 495 base pairs (bp), indicating that they possess the wild-type CCR2 gene. They also exhibit a band at 320 bp because they possess a knockout CCR2 gene (replaced with RFP). The Cx3Cr1 heterozygotes exhibit bands at 410 bp because they have one copy of the wild-type Cx3Cr1 gene, and bands at 500 bp because they have a Cx3Cr1 knockout (replaced with GFP). Successfully genotyping these mice was essential in order to further examine the percentages of CCR2+ and Cx3Cr1+ monocytes in the blood.

Figure 2 shows the levels expression for the various genes examined in this study. IL-1β is a proinflammatory cytokine produced by macrophages, and is normally found in brain tissues at low levels but expression increases in the event of disease, injury or inflammation<sup>17</sup>. In the hippocampus, we found that IL-1β levels increase among all groups with age, with the notable
exception of the HF infected mice, yet the increase was not significant (Fig. 2A). IL-6 is a proinflammatory cytokine secreted by adipocytes\(^ {18} \). Initially, the infected groups showed increased levels of IL-6, but levels of IL-6 decreased with age among all groups (Fig. 2B). The infected LF and HF groups saw a significant decrease in IL-6 expression (LF infected young mean=3.523E-05 ± 6.747E-06; LF infected old mean= 2.243E-05 ± 6.181E-06. HF infected young mean=3.215E-05 ± 7.064E-06; HF infected old mean= 1.517E-05 ± 3.976E-06). TNF\(\alpha\) is a proinflammatory cytokine produced in response to infection, as well as an adipokine that is
increased in the serum of obese subjects. TNFα levels increased with age across all groups, but the most notable increases were seen among the LF uninfected and infected groups (Fig. 2C). These differences were not statistically significant. MHCII is normally found on antigen presenting cells, including activated macrophages. The presence of MHCII in the brain is indicative of microglial activation, as it is not expressed on quiescent (non-activated) microglia. MHCII levels were similar among the young and old groups, with the exception of the HF uninfected young mice (no significant difference) (Fig. 2D). CCL2 is a chemokine that recruits inflammatory CCR2+ monocytes to the brain. Among the young mice, the expression of CCL2 was highest in the HF diet groups. But in the older mice, the LF infected group had the highest level of CCL2.

As anxiety has been associated with the infiltration of bone-marrow derived monocytes from the blood to the brain, we examined the blood samples and determined the percentage of lymphocytes in the blood, as well as the percent of the lymphocytes that express CCR2+ or Cx3Cr1+ (Figure 3). CCR2+ monocytes are considered pro-inflammatory cytokines that enter the brain in response to inflammation\textsuperscript{16}. In contrast, Cx3Cr1+ is thought to be anti-inflammatory. In fact, deletion of Cx3Cr1 results in increased neuroinflammation and has been associated with decreased neurogenesis and cognitive impairment\textsuperscript{30}. In the young mice, the HF diet groups had the highest percent lymphocytes in the blood (Fig. 3A). However in the older mice, percentages were more similar. CCR2 was expressed at the highest level in the lymphocytes of young, LF uninfected mice (Fig. 3B) (LF uninfected young mean= 7.8 ± 1.7%). In the older mice, CCR2 expression was significantly lower in the HF infected group (HF infected old mean= 1.65 ± 0.4%). Among the young mice, the expression of Cx3Cr1 within lymphocytes was highest among the LF uninfected group (Fig. 3C) (LF uninfected young mean=4.6 ± 1.2%; LF
uninfected old mean = 1.03 ± 0.475%). The older mice showed similar expressions of Cx3Cr1 in their lymphocytes.
Chapter 5: Discussion

Previous studies have shown that elevated levels of the proinflammatory cytokines IL1-β, IL-6 and TNFα can result in increased anxiety among adult animals\textsuperscript{14}. The statistically significant decreases in IL-6 expression between the infected young mice and the infected old mice is contrary to what was expected. This may indicate that while infection provokes an increase in IL-6 expression initially, as the mice get older and infection progresses, the inflammatory response is less pronounced and IL-6 expression returns to normal levels. The finding of high IL-6 levels in infected young mice may have potential clinical implications due to evidence associating high levels of IL-6 with behavioral and cognitive changes\textsuperscript{13}.

More notable findings, although not statistically significant, include the differences in the young and old mice in CCL2 and TNF expression. It seems a HF diet may increase CCL2 expression, but with age, this agonistic effect no longer exists. CCL2 expression is most pronounced in older lean infected mice, indicating that its expression may increase after prolonged infection. However, the older mice on the HF diet did not have increased levels of CCL2, indicating that the inflammatory state of obesity may counteract the expression of CCL2 expression in these older mice. This may have serious implications on the inflammatory response in these mice because CCL2 is the chemokine that attracts the proinflammatory CCR2\textsuperscript{+} monocytes to the brain. Additionally, higher expression of TNFα in the older lean mice was contrary to what was expected, as the proinflammatory state of obesity is often accompanied by increased TNFα expression\textsuperscript{2}. This finding may indicate that another factor may affect the expression of TNFα in obese mice.

Cx3Cr1 is highly expressed in resident monocytes, whereas CCR2 is highly expressed in inflammatory monocytes\textsuperscript{16}. In previous studies, CCR2 has been implicated in having a role in
cognitive impairment\textsuperscript{15}. CCR2 is the receptor for CCL2\textsuperscript{15}, therefore the high expression of CCR2 in the lymphocytes of the younger lean mice, and low expression in the older HF mice may have affected the amount of IL-1\(\beta\), IL-6 and TNF\(\alpha\) found in the hippocampus.

An association between a high fat diet and increased neuroinflammation, which in turn contributes to behavioral changes, has previously been established in male mice in the larger study. However, in the female mice used in this part of the study, the inflammatory response was not as pronounced. As a result, if tested, these female mice may not exhibit the behavioral changes and increases in anxiety that were seen in male mice. The decreased inflammatory response may be due in part to the female C57BL/6 mice weighing less than the males, or the males developing metabolic syndrome earlier. Earlier onset of metabolic syndrome would contribute to an increased inflammatory state in these male mice. It is also possible that estrogen may have a role in brain inflammation. Women have an increased vulnerability to developing anxiety, and recent data suggests that female reproductive hormones may have a significant role\textsuperscript{31}. Additionally, there is evidence suggesting that estrogen plays a role in susceptibility to inflammation\textsuperscript{32}. One study even found increased expression of IL-1\(\beta\), IL-6 and TNF\(\alpha\) in male mice upon administration of estrogen\textsuperscript{32}. To adapt our model to examine the role of estrogen in neuroinflammation due to diet-induced obesity and HSV-1 infection, we could administer estrogen antagonists to female mice or treat male mice with estrogen. The discovery of a link between estrogen and inflammatory response may have vast implications for the field of psychiatry and the potential for this correlation warrants additional investigation.
References:


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