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Diet-induced obesity prolongs neuroinflammation and recruits CCR2⁺ monocytes to the brain following herpes simplex virus (HSV)-1 latency in mice

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Abstract

Herpes simplex virus (HSV)-1 is a ubiquitous human infection, with increased prevalence in obese populations. Obesity has been linked to increased inflammation, susceptibility to infection, and higher rates of anxiety disorder and cognitive impairment. To determine how obesity alters neuroinflammation and behavior following infection, we infected weanling C57BL/6 or CCR2^{RFP/+}/CX3CR1^{GFP/+} mice with a very low dose of HSV-1. Following viral latency (14 days post infection (d p.i.)), mice were randomly assigned to remain on the low fat (LF) diet or switched to a 45% high fat (HF) diet. Eight weeks post diet shift, latently infected mice on the HF diet (HSV-HF) had greater microglial activation and infiltration of inflammatory CCR2⁺ monocytes in the hypothalamus and dentate gyrus, in comparison to both HSV-LF mice and uninfected mice on LF and HF diets. VCAM staining was present in hypothalamus and hippocampus of the HSV-HF mice in the areas of monocyte infiltration. Infiltrating monocytes also produced proinflammatory cytokines demonstrating that, along with activated microglia, monocytes contribute to sustained neuroinflammation in latently infected obese mice. Utilizing a light-dark preference test, we found that HSV-HF mice had increased anxiety-like behavior. In the marble-burying test, HF diet and HSV infection resulted in increased numbers of buried marbles. Together, these mice provide a useful, testable model to study the biobehavioral effects of obesity and latent HSV-1 infection in regards to anxiety and may provide a tool for studying diet intervention programs in the future.

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Conflict of Interest

The authors have declared that no conflict of interest exists.

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Keywords

HSV-1; obesity; neuroinflammation; anxiety; microglia and macrophage

1. Introduction

Herpes simplex virus (HSV)-1 is a ubiquitous human virus that predominately causes oral lesions, though there is an increase in the prevalence of genital HSV-1 infections (Gupta et al., 2007). During HSV-1 infection, microglia, the resident immune cells of the CNS, are activated and produce cytokines and chemokines in order to inhibit viral replication and to recruit other immune cells to the brain. The controlled and limited production of cytokines by microglia in response to insult or injury is necessary for clearing infections in the CNS and can be neuroprotective (Streit, 2002). However, excessive production of proinflammatory cytokines can result in neurotoxicity (Cunningham et al., 2009; Teeling and Perry). In response to microglial-derived chemokine, T Ly6C^{hi} monocytes infiltrate the brain and differentiate into macrophages in conjunction with microglia, recruit T cells to the brain to resolve the infection (Marques et al., 2008; Terry et al., 2012). After a primary infection with HSV-1, the virus becomes latent and persists in neurons of the sensory and autonomic ganglia, including the trigeminal (TG). National Health and Nutrition Examination Survey (NHANES) data from 2005–2010 suggests that 30.1% of adolescents age 14–19 in the US are seropositive for HSV-1 (Bradley et al., 2014), while the prevalence of HSV-1 in children ages 6–16 is higher in children of lower socioeconomic status regardless of race (Dowd et al., 2009). The prevalence of HSV-1 infection continues to increase throughout life and by 49 years old (y.o.), 65% of all US residents are seropositive (Kasubi et al., 2006). In a small study of postmortem TG samples, HSV-1 DNA was present in 60–85% samples of people 0–20 y.o. (Hill et al., 2008). Together, these data indicate that most primary infections with HSV-1 occur early in life and that the presence of latent HSV-1 in the CNS is a lifelong condition for most humans.

Like HSV-1 infection, obesity is also a significant public health concern for both adults and children (Johnson et al., 2014). Infiltration of inflammatory macrophage into adipose tissue is a hallmark of obesity and is believed to play a role in the development of a number of comorbidities, including Type 2 diabetes. These infiltrating macrophage have an M1 or proinflammatory phenotype and as individuals become increasingly obese, macrophage phenotypes polarize to a proinflammatory/M1 state. This polarization occurs as the cells are activated by IFN γ , TNF α and IL-6, induced by saturated fatty acid stimulation via TLR 2 and 4 receptors (Lumeng et al., 2007a; Lumeng et al., 2007b; Nguyen et al., 2005; Suganami et al., 2007). While the infiltration of macrophage into adipose has been widely studied, the infiltration of these cells into the brain of obese mice from the periphery was recently discovered (Buckman et al., 2013). The consequence of the macrophage infiltration has not been elucidated. High saturated fat diets also results in activated microglia in the hypothalamus and hippocampus of rodents (Huang et al., 2013; Hwang et al., 2009; Milanski et al., 2009) and in obese mice, these microglia are more proinflammatory (Aguzzi et al., 2013). Overall, high fat diets and obesity result in neuroinflammation.

Increasing epidemiological evidence has shown that early childhood events associated with poverty can affect health throughout life. Specifically, low socioeconomic status (SES) in childhood has been correlated with adult anxiety and major depressive disorders (McLaughlin et al., 2011). Two health factors often associated with lower SES are obesity and increased infectious burden, particularly with latent viruses, including HSV-1 (Beydoun et al., 2010; Frederick, 2014). Latent HSV-1 infection has been correlated with cognitive impairment during childhood, schizophrenia and dementia/Alzheimer's disease (AD) in adulthood (Deleidi and Isacson, 2012; Tarter et al., 2013). Until recently, the rates of childhood/adolescent obesity had been increasing at the same rate across all SES levels. However, obesity rates have begun to level off in all but lower SES groups, where they continue to increase at a significant rate (Frederick, 2014). Obesity has been associated with increased risk for dementia/AD, mood disorders and anxiety. A positive association between obesity and HSV-1 seropositivity has been made (Fernandez-Real et al., 2007; Karjala et al., 2011). Brain regions associated with anxiety, such as the hypothalamus, hippocampus, medial amygdala and the bed nucleus of the stria terminalis are sites of HSV-1 antigen following infection (Armien et al., 2010; Mori et al., 2005; Mori et al., 2006). During HSV-1 infection, in conjunction with T cells, Ly6C^{hi} monocytes infiltrate the brain and differentiate into macrophages in order to resolve the infection (Marques et al., 2008; Terry et al., 2012). A growing body of literature demonstrates that early life infection results in increases in social avoidance and anxiety in an animal model (Bilbo, 2010; Bilbo and Schwarz, 2009; Fernandez-Real et al., 2007; Karjala et al., 2011). In addition, HF diets and obesity can increase anxiety in both rodents and humans (Farr et al., 2008; Fitzpatrick et al., 2009; Holden et al., 2009; Kaczmarczyk et al., 2013). Microglial activation and the infiltration of macrophages into regions associated with anxiety may play a role in the development of anxiety in certain stress paradigms (Wohleb et al., 2014; Wohleb et al., 2013). Therefore, we sought to determine if HF diet following HSV-1 infection and latency results in increased neuroinflammation via microglia activation and proinflammatory monocyte/macrophage infiltration in regions associated with anxiety, as well as increased anxiety in mice.

2. Materials and methods

2.1. Mice and Infection

Weanling male, C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were housed at the University of North Carolina Animal Facility, which is fully accredited by the American Association for Laboratory Animal Care. Mice were maintained under protocols approved by the Institutional Animal Use and Care Committee. All mice were placed on the LF diet when they arrived and were allowed to acclimate for 1 week prior to infection. Cages of mice were randomized to either mock (saline) or HSV-1 infection via the intranasal route; this model of infection in mice best mimics infection in humans (Shivkumar et al., 2013). For infection, mice were lightly anesthetized with a ketamine (0.6mg/kg)/xylazine (0.35mg/kg) solution and infected intranasally (i.n.) with 2.5×10^5 pfu HSV-1 in 10 μ l in PBS. This dose of HSV-1 does not result in encephalitis or significant symptoms of sickness, but does result in HSV-1 latency in the TG. Following latency at 14 days post infection (p.i.) the cages were randomized again to remain on the LF diet or switched to the HF diet. Mice were housed 4/cage with free access to food and water.

CCR2^{RFP/+}/CX3CR1^{GFP/+} mice were generated by crossing CCR2^{RFP/RFP} (B6.129(Cg)-Ccr2^{tm2.1Ifc/J}) mice with CX3CR1^{GFP/GFP} (B6.129P-Cx3cr1^{tm1Litt/J}, Jackson Labs, Bar Harbor, ME) (Mizutani et al., 2012; Saederup et al., 2010). CCR2^{RFP/+}/CX3CR1^{GFP/+} mice carry one functioning copy of CCR2 and CX3CR1 and one copy of CCR2-RFP and CX3CR1-GFP. CCR2^{RFP/+}/CX3CR1^{GFP/+} mice have been used to differentiate microglia from infiltrating mononuclear cells as microglia express CX3CR1 throughout development and this can be monitored via GFP (and lack RFP expression) (Mizutani et al., 2012). The infiltrating CCR2⁺ monocytes in turn express RFP and are easily distinguishable from the microglia (Mizutani et al., 2012; Saederup et al., 2010). CCR2⁺ monocytes are proinflammatory, M1 precursors and the infiltration of these cells is associated with neuroinflammation (Shi and Pamer, 2011). After weaning at 23 d the procedures described above were followed.

2.2. HSV-1

HSV-1 McIntyre (ATCC, Manassas, VA) stocks were propagated in Vero cells (ATCC, Manassas, VA), collected, centrifuged and stored at -80°C .

2.3. Diets

We used D12451 (Research Diets, New Brunswick, NJ) a 45% fat by kcal diet with soybean oil and lard as the fat source. The LF control diet D12450B is a 10% fat diet with the same source of fat. These diets were chosen because the HF diet contains fat sources that yield a diet that is 16% saturated fat, 20% monounsaturated fat and 8% polyunsaturated fat. An analysis of the US diet conducted with NHANES data from 2005–6 demonstrates that the average American diet is approximately 33% fat, composed of 11% saturated fat, 12% monounsaturated fat and 7% polyunsaturated fat (U.S. Department of Agriculture, 2008).

2.4. Assessment of pathology and body weight measurement

During the course of infection, mice were observed 3 times daily for symptoms of HSV-induced illness and encephalitis as previously described (Sheridan and Beck, 2008). At 3 months p.i., mice were weighed on the day of sacrifice.

2.5. Brain mononuclear cell isolation

Microglia were isolated following a previously published method (Henry et al., 2009). Briefly, brains were removed and homogenized in HBSS. Single cells suspensions were filtered through 70 μM nylon mesh and centrifuged at 500g for 5 min. The cells were resuspended in 70% Isotonic Percoll (Sigma-Aldrich, St. Louis, MO) and then layered with 50%, 35% and 0% isotonic Percoll. The Percoll gradients were centrifuged at 2000g for 20min at room temperature. The mononuclear layer located at the 70/50% interphase was carefully collected and washed 2 times with HBSS and finally resuspended in complete RPMI. Previous studies have demonstrated that this method results in the isolation of 6×10^5 viable cells that are 89–96% pure from uninfected mice. Importantly, this method does not cause the activation of microglia (Henry et al., 2009).

2.6. DNA isolation, RNA Isolation and qRT-PCR

Bilateral hippocampi (with subregions remaining intact) were dissected following a published protocol and rapidly frozen in liquid nitrogen (Sultan, 2013). Total RNA was isolated using the TRIzol method (Invitrogen, Carlsbad, CA). Reverse transcription was carried out using Superscript II First Strand Synthesis kit (Invitrogen) with oligo (dT) primers. qRT-PCR was performed using TaqMan Universal Master Mix with the Taqman Assays on Demand Primer Probe pairs (Applied Biosystems, Foster City, CA). In order to determine HSV-1 latency, DNA and RNA were extracted from TG following the TRIzol dual isolation method. Reverse transcription was carried out as described above for mRNA and qPCR was performed using ICP0 Taqman Assays on Demand Primer Probe pair. The levels of mRNA for GAPDH were determined for all samples and used to normalize gene expression. In our experience, levels of GAPDH are constant in lung, brain and spleen during influenza, HSV-1 and coxsackie infections (Sheridan and Beck, 2008; Karlsson et al., 2009; Smith et al., 2007).

2.7. Flow cytometry

For flow cytometry, Percoll isolated cells were washed then resuspended in DMEM-5% FBS and incubated with anti-CD16/32 to block non-specific binding to Fc receptors. Cells were then surface stained with anti-CD45, anti-CD11b, anti-CD86 (eBiosciences, San Diego, CA) following previously published methods. After gating on live cells, macrophage were identified as CD11b⁺ and CD45^{hi}. After gating on the macrophage, CD86⁺ cells were identified (Wohleb et al., 2013). Appropriate isotype controls were used to determine positive staining. For flow cytometry on blood samples, whole blood was collected into EDTA-treated tubes. Cells were washed twice DMEM-5% HBS and then surface stained with anti-CD11b, Ly6c and Ly6G antibodies and examined by flow cytometry following previously published methods (Hettinger et al., 2013; Powell et al., 2013). Monocytes were identified as SSC^{lo}, CD11b⁺, Ly6G⁻, Ly6C^{hi} monocytes are proinflammatory while Ly6C^{lo} monocytes have a “patrolling/wound healing” phenotype (Shi and Pamer, 2011). Blood from CCR2^{RFP/+}/CX3CR1^{GFP/+} mice was surface stained with anti-CD11b. CD11b⁺ cells were gated and the % of RFP (CCR2) and GFP (Cx3CR1) positive cells were determined by flow cytometry. Appropriate isotype controls were used to determine positive staining. Data were collected on the BD Accuri Flow Cytometer (Franklin Lakes, NJ) and analyzed by FlowJo Software (Tree Star, Ashland, OR).

2.8. Perfusion and immunohistochemical staining

Mice were anesthetized deeply with avertin and transcardially perfused with 0.1M sodium phosphate buffer (pH 7.4) at 4°C followed by 4% paraformaldehyde. Brains were removed and immediately postfixed in 4% paraformaldehyde, then cryopreserved by overnight incubations in 15% then 30% sucrose. The brains were in OCT until frozen sectioning. Tissue sections were fluorescently stained using standard immunohistochemical technique. Briefly, tissues were incubated in blocking buffer solution for one hour (5% Goat Serum (Sigma G6767), 1% BSA (Fisher BP1600-100), 0.05% Sodium Azide-20% (RPI, S24080-500), 0.5% Triton X-100 (Fisher BP151-100), 1X PBS-10X, dH₂O). Tissues were incubated overnight with appropriate primary antibodies diluted in blocking buffer, washed

with PBS, and subsequently incubated for one hour with appropriate secondary antibodies diluted in blocking buffer. Specifically, for ionized calcium-binding adapter molecule (IBA)-1 immunolabeling with antibody diluted 1:250 (BioCare Medical, Concord, CA Cat #290), sections were stained as described by Williamson et al (2012). VCAM immunolabeling was performed with anti-goat primary antibody diluted 1:50 from Santa Cruz (Cat # SC-1504), followed by secondary staining with Alexa Fluor 350 Donkey Anti-Goat IgG (H+L) (Life Technologies, Carlsbad, CA, Cat#A-21081). Sections incubated with secondary antibody served as controls for non-specific staining, while brain sections from mice with HSV-encephalitis from a separate study were used as positive controls as they have significant inflammation and cellular infiltrate. Tissues were mounted with aqueous mounting media and cover-slipped. Slides were imaged on a Nikon Eclipse 90i microscopy system (Nikon Instruments, Melville, NY) using Nikon NIS-Elements Advanced Research software with fluorescent objectives magnified by the objective + 1X making the objective magnification (20X/40X) the actual magnification. Due to the low magnification, specific subregions of the hypothalamus/amygdala were not specifically imaged. Rather, these areas were imaged across their broad regions to obtain an average intensity for the region as a whole. To ensure images came from approximately the same regions across many tissues, tissue architecture was compared against the Allen Brain Atlas, as well as across tissue sections to ensure consistency. For GFAP, IBA-1, and VCAM, pixel density/intensity was determined via threshold analysis using Nikon NIS-Elements Advanced Research software.

2.9. CCL2 ELISA

Serum was collected from whole trunk blood and the CCL2 ELISA was performed following the manufacturer's protocol (eBioscience, San Diego, CA).

2.10. Behavior testing (light-dark preference and marble burying test)

Three months post infection mice (n=8/group) were tested for anxiety-like behaviors by the Mouse Behavioral Phenotyping Laboratory at UNC. In the light-dark preference test, mice were placed in the dark area of a two-chamber testing apparatus and activity was recorded for 10 min. Records were taken of the time spent in each side and entries into each side, measured by photobeam breaks by an automated system (Versamax, Accuscan Instruments, Columbus, OH). In the marble burying test, mice were placed in a Plexiglas cage located in a sound-attenuating chamber with ceiling light and fan. The cage contained 5 cm deep clean corncob bedding, with 20 black glass marbles (14 mm diameter) arranged in an equidistant 5 × 4 grid on top of the bedding. Animals were given access to the marbles for 30 min. Measures were taken of the number of buried marbles (2/3 of the marble covered by the bedding).

2.11. Statistics

Data were analyzed by 2-way ANOVA with the variables diet and infection using JMP statistical software (SAS, Cary, NC). A priori post hoc comparisons among groups were performed with Tukey-Kramer test and differences were considered significant if $P < 0.05$. Data are presented as mean $\pm$ SEM

3. Results

3.1. Intranasal infection results in HSV-1 latency in the trigeminal ganglion without symptoms of encephalitis

In order to determine if intranasal infection with HSV-1 would establish latency in the trigeminal ganglion, mice were either mock infected or infected with HSV-1. Mice were sacrificed at 14 d p.i. at this time point replicating virus was no longer detected in the TG of mice (no amplification of by 40 cycles of qRT-PCR), however, DNA for HSV-1 was present in the mice that had been infected (Table 1). These data confirm HSV-1 infection as well as latency by the time the mice are switched to the HF diet. No mice in these studies (C57Bl/6 or CCR2^{RFP/+}/CX3CR1^{GFP/+}) developed symptoms of encephalitis or any overt pathology.

3.2. 45% HF diet results in significant weight gain

At 3 m p.i. (2.5 m of diet) mice on the HF diet were significantly heavier than mice on the LF diet, regardless of infection (Fig. 1).

3.3. HF diet following HSV-1 infection results increased IBA-1 expression and proinflammatory cytokine expression

Priming of microglia may occur by various means, but ultimately primed microglia upregulate many cell surface markers including TLR2, TLR4, MHCII, CD11b, CD80 and CD86. The production of proinflammatory cytokines by the primed microglia and a change to an amoeboid morphology defines them as being in an “activated” state (Perry et al., 2007).

At 3 months p.i., we examined IBA-1 immunolabeling in the hypothalamus, amygdala, hippocampus (total region) and dentate gyrus (Fig. 2). Among the groups, there were no significant differences in the hypothalamus, nor hippocampus (data not shown), however, HF-diet alone resulted in increased IBA-1 in the amygdala. A significant increase in IBA-1 immunolabeling and microglia with larger cell bodies and shorter processes was observed in the dentate gyrus region of the HSV-HF mice, an indication of significant microglial activation.

In addition to its critical role in learning and memory, the hippocampus is also a primary relay center involved in anxiety-like behaviors (Burgess et al., 2002; Mineur et al., 2013). To further dissect the role of latent HSV infection and HF diet in activating microglia, we measured the upregulation of several cell surface markers and proinflammatory cytokines using qRT-PCR. Three months following the establishment of HSV latency, there were no differences in the expression of markers of glial priming and activation of HSV-LF mice compared to the LF uninfected controls (Table 2). While HF diet alone increased expression of MHCII and IL-10 (bolded black, $P < 0.05$ compared to LF and HSV-LF, but not HSV-HF) it is only in the latent HSV-HF fed group that significant changes were seen in the majority of markers of glial priming and activation (green, $P < 0.05$ compared to LF, HSV-LF and HF) (Table 2). It is important to note that we did not find mRNA for HSV-1 in the hippocampus nor TG in these mice, confirming that the virus had not reactivated and was not actively replicating.

3.4. HSV-1 infection results in a significant increase in monocytes/macrophage in the brain 3 m p.i

Buckman et al. demonstrated that monocytes/macrophage from the periphery enter the brain of obese mice (Buckman et al., 2013). In order to determine if there was increased monocyte/macrophage infiltration in the brains of the HSV-HF mice we isolated mononuclear cells from the whole brain and determined the percentage of CD45^{hi}CD11b⁺ macrophages by flow cytometry (Fig. 3). In these mice, HF diet alone did not significantly increase the percentage of macrophages in the brain, interestingly there was a significant increase in the percentage of CD45^{hi}CD11b⁺ cells in the brains of the HSV-HF mice (Fig. 3A). CD86 expression is an established marker of M1/classically activated macrophages (Kadl et al., 2010; Kigerl et al., 2009; Vogel et al., 2013). The percentage of CD45^{hi}CD11b⁺CD86⁺ cells was significantly increased in the HSV-HF mice compared to LF and HSV-LF mice (Fig. 3B). These data confirm that HF diet following HSV-1 infection results in M1-macrophage infiltration into the brain and that macrophages are maintained (or continuously recruited into the brain) of HSV-HF mice, however, macrophage that respond initially to the infection do not remain in HSV-LF mice. Together, these data indicate that the microenvironment created by both HSV and HF diet is required for the continued macrophage presence.

While we know that the HSV-HF mice have increased CD45^{hi}CD11b⁺ M1/classically activated macrophage, identifying their precise neuroanatomical location is difficult, as both macrophage and monocytes are CD45⁺ and CD11b⁺ and express overlapping sets of cell surface markers. Therefore, it is very difficult to differentiate these cells from one another by IHC. To identify the locations of infiltrating monocytes/macrophage, we utilized an innovating strategy in which the CCR2^{RFP/+}/CX3CR1^{GFP/+} mouse (Mizutani et al., 2012). The significant advantage of this system over the one described by Buckman et al is that it does not require bone marrow transplant using lethal irradiation that on its own can induce significant neuroinflammation (Buckman et al., 2014). In order to determine the location of the infiltrating cells, CCR2^{RFP/+}/CX3CR1^{GFP/+} mice were sacrifice at 3m p.i., (Fig. 4 and 5). While single RFP⁺ cells were found scattered throughout the brain, there was a significant and consistent infiltration of RFP⁺ cells in the hypothalamus and dentate gyrus of the HSV-HF mice (Fig. 4D and Fig. 5D). RFP⁺ cells were not found in the amygdala nor other regions of the hippocampus.

3.5. HSV-HF mice have significantly increased Ly6C⁺ and CCR2⁺ circulating monocytes

At 3m p.i. HSV-HF mice had significantly more circulating monocytes and the percentage of Ly6c^{hi} proinflammatory monocytes were significantly enriched with a corresponding decrease in Ly6C^{lo} monocytes (Fig. 6A and B). Ly6C^{hi} monocytes are CCR2⁺Cx3CR1⁻ while Ly6C^{lo} are CCR2⁺Cx3CR1⁻ (Shi and Pamer, 2011). We also examined the expression of RFP (CCR2) and GFP (Cx3CR1) on CD11b⁺ cells from the blood of CCR2^{RFP/+}/CX3CR1^{GFP/+} mice. As with Ly6C, there was a significant increase in the percentage of monocytes that were CCR2⁺Cx3CR1⁻ proinflammatory monocytes in the HSV-HF mice (Fig. 6C).

3.6. HF diet following HSV infection increased VCAM expression in areas with RFP⁺ cell infiltration

HF diet following HSV infection results in a unique microenvironment that promotes monocyte infiltration into the brain. During HSV-1 infection upregulation of the endothelial cell adhesion molecule, VCAM occurs allowing macrophages and T cells to enter the brain parenchyma (Terry et al., 2012). VCAM is upregulated by the production of IL-1 β and TNF α , because HSV-HF mice had increased mRNA expression of these cytokines in the hippocampus we hypothesized that they would have increased VCAM expression (Skelly et al., 2013). Unlike HSV-LF mice, HSV-HF brains have sustained macrophage infiltration/retention and this would suggest that chemokines and adhesion molecules (VCAM) continue to be upregulated in these mice. To determine if VCAM was upregulated, we immunolabeled sections with DAPI (a nuclear marker) and goat anti-mouse VCAM antibody. Consistent with previous findings, VCAM was not present in the hippocampus or hypothalamus of the LF mice, nor was it present in the HSV-LF or HF mice (Sawicki et al., 2015). However, VCAM was present in the hippocampus and hypothalamus of the HSV-HF mice (Fig. 7A–D; Additional images of VCAM immunolabeling Supp Fig. 1 A–F). Importantly, RFP⁺ cells co-localized or were in close proximity to VCAM-positive brain endothelia and suggest that regional VCAM expression allowed for extravasation of CCR2⁺ cells into the parenchyma (popouts Fig. 7 D and H).

3.7. HSV-HF mice have increased CCL2 in the hippocampus and serum

Several models of neuroinflammation suggest that CCL2 (MCP-1) produced by microglia attract the M1-type, LY6C^{hi} monocytes into the brain parenchyma. CCL2 is increased during inflammatory events including infection and obesity (Shi and Pamer, 2011; Takahashi et al., 2003). Relevant to our model, saturated fatty acids induce CCL2 expression in microglia of the hypothalamus (Valdearcos et al., 2014) and CCL2 is the main signal that recruits CCR2⁺/LY6C^{hi} monocytes from the bone marrow. We found that compared to LF controls, HSV-HF mice had significantly increased CCL2 expression in the hippocampus (Fig. 8A) and circulating serum CCL2 (Fig. 8B) at 3m p.i. Together, these data indicate that there is a unique microenvironment where increased circulating CCL2 recruits CCR2⁺/LY6C^{hi} monocytes from the bone marrow into circulation. The increase of CCL2 in the hippocampus, in conjunction with VCAM upregulation, allows for these cells to infiltrate the brain parenchyma.

3.8. Obesity and latent HSV-1 increase anxiety-like and repetitive behavior

Collectively, these data show that latent HSV and a HF diet increased microglial activation, CCR2⁺ macrophage infiltration and proinflammatory cytokine production in regions of the brain associated with anxiety-like behaviors. Therefore, we determined whether HSV-HF mice exhibited increased anxiety-like behaviors in a light-dark preference test and a marble bury assay. Other researchers have reported decreased exploration and increased marble-burying in mouse models of immune system activation and obesity (Andre et al., 2014; Bachis et al., 2016; Malkova et al., 2012; Paris et al., 2014; Pascual et al., 2015). At 3 m p.i., mice were tested by two robust behavior tests for anxiety: light-dark preference and marble burying. In the light-dark preference test anxious mice will spend less time in the lighted

side and make fewer crosses between the dark to light side (Bailey and Crawley, 2009). HSV-HF mice spent significantly less time in the light-side. There was a main effect of diet ($F(1, 31)=5.33$, $p=0.03$), but not infection ($F(1, 31)=0.0$, $p=0.99$) and there was no interaction ($F(1, 31)=0.67$, $p=.41$). Post hoc analysis revealed that HSV-HF mice spent less total time on the lighted-side than HSV-LF mice (Fig. 9A). There were differences in the number of transitions made between the dark and light sides. There was a main effect of diet ($F(1, 31)=4.03$, $p=0.05$), but not infection ($F(1, 31)=3.31$, $p=0.07$) and there was no interaction ($F(1, 31)=0.0$, $p=1.0$). Post hoc analysis revealed that only HSV-HF mice had significantly fewer transitions than LF mice (Fig. 9B). Marble burying is a test of both anxiety-like and repetitive behaviors (Thomas et al., 2009). The LF mice buried on average 12 marbles in 30 min, the expected number for mice of the C57Bl/6 strain (Thomas et al., 2009). There was a main effect of infection, but not diet (infection: $F(1, 31)=0.69$, $p=0.014$; diet: $F(1, 31)=2.9$, $p=0.09$ and no interaction: $F(1, 31)=2.48$, $p=0.13$). HF diet alone resulted in a trend towards increased marble burying, while, compared to LF mice, HSV-LF and HSV-HF mice buried more marbles, however, there was not a significant difference between the infection groups (Fig. 9C).

4. Discussion

Our study demonstrates that obesity following HSV-1 latency lead to the potentiation of neuroinflammation including sustained microglial activation and the recruitment of inflammatory CCR2⁺ monocytes to the hypothalamus and hippocampus. The activated microglia and macrophages are likely the two sources of proinflammatory cytokines that are upregulated in the hippocampus of the HSV-HF mice. The CCR2⁺ monocytes/macrophages that were present in brain parenchyma of HSV-HF mice are of the M1-phenotype and come from CCR2⁺/Ly6C^{hi} monocytes in the periphery. HF diet following HSV latency clearing resulted in a unique microenvironment in the brain; VCAM was upregulated and inflammatory monocytes colocalized with the adhesion molecule and CCL2 mRNA is upregulated in the hippocampus. These changes correlated with increased anxiety-like behavior as measured by the Light-Dark test in HSV-HF mice, however HSV-HF, HSV-LF and HF mice exhibited increased anxiety-like/repetitive behaviors as measured by marble burying. For the HSV-LF and HF mice, increased repetitive behavior was seen in the absence of neuroinflammation or cellular infiltration in the hippocampus or hypothalamus (though, there was increase IBA-1 staining in the amygdala of HF mice).

Microglial activation and cytokine production are associated with anxiety behaviors in mice (Block et al., 2007). In this study we found that HSV latency was not sufficient to maintain microglial activation, and while obesity resulted in increased IBA-1 in the amygdala and MHCII and IL-10 expression in the hippocampus, only the combinatorial effects of HSV and HF diet lead to sustained microglial activation and proinflammatory cytokine and cell surface marker expression. Previous studies using the 45% fat lard-based diet have demonstrated that saturated fatty acids can stimulate TLR4 signaling in the hypothalamus and that this upregulation is predominantly found on activated microglia (Milanski et al., 2009). However, in these studies, unpurified chow diet was used as the low fat control diet. The utilization of chow diets as “control” diets are problematic as these diets vary in the amount and type of fat, as well as other nutrients, compared to purified defined diets.

In addition to microglial activation, the infiltration of macrophages in to regions associated with anxiety may play a role in the development of anxiety. This has been shown to occur in some experimental stress paradigms (Wohleb et al., 2014; Wohleb et al., 2013). During HSV-1 infection, Ly6C^{hi} monocytes infiltrate the brain and differentiate into macrophages in order to resolve the infection (Marques et al., 2008; Terry et al., 2012). These cells were not present in the HSV-LF mice, indicating that HF diet results in the retention of the cells or their continued recruitment in HSV-HF mice. While we can conclude that inflammatory bone marrow derived mononuclear cells infiltrate regions associated with anxiety behavior in HSV-HF mice, our current study does not allow us to determine if the HF diet results in the persistence of the macrophages that entered the brain during infection to stay or if it leads to the continual recruitment of CCR2⁺ cells. The increased circulating monocytes, as well as increased serum and brain CCL2 would suggest continual recruitment of CCR2⁺ cells to the brain. Additionally, other studies have suggested that macrophages recruited to the brain reduced CD45 expression and become more microglia-like (Ponomarev et al., 2011; Ritzel et al., 2015), again, an advantage of the CCR2^{RFP/+}/CX3CR1^{GFP/+} mice as CCR2/RFP expression is maintained. In addition, the flow cytometry data suggests that the HSV-HF brain contains a large proportion of CD11b⁺ cells are CD45^{hi}, suggesting newly recruited cells.

In the absence of latent infection, Buckman et al., demonstrated that HF diet is sufficient to allow macrophages to enter the brain (Buckman et al., 2014). In our study there were a few, sporadic CCR2⁺ cells in the HF mouse brain, but significantly fewer than in HSV-HF mice. The discrepancy between these studies is likely a result of the need for irradiation of the brain prior to the bone marrow transplant. By utilizing the CCR2^{RFP/+}/CX3CR1^{GFP/+} mouse model, we were able to avoid manipulation that could potentially alter macrophage infiltration or the inflammatory state of the brain beyond that induced by infection or the HF diet. Several studies have demonstrated that CCR2⁺ cell infiltration is responsible for damage following ischemia (Hsieh et al., 2014) and experimental autoimmune encephalomyelitis (Moreno et al., 2014) and of most consequence to our study, lack of CCR2 results in a failure of monocytes/macrophages to traffic to the brain (Wohleb et al., 2013). The inhibition of monocyte/macrophage trafficking to the CNS reverses stress-induced anxiety behaviors (Wohleb et al., 2013). Interestingly, obese CCR2 knockout mice have fewer adipose infiltrating macrophages and are resistant to the metabolic consequences of obesity (Weisberg et al., 2006).

A large body of literature exists that has examined the importance of the CCR2⁺Ly6C^{hi} monocytes infiltration into the brain during inflammatory events. In general, the infiltration of these cells leads to increased and prolonged inflammation. During neuroinflammatory events, the chemokine CCL2 recruits CCR2⁺ monocytes that will become proinflammatory macrophages in the brain. In addition to being a significant source of proinflammatory cytokines, M1-like macrophages (from CCR2/Ly6Chi monocytes) produce the enzyme indoleamine-2,3-dioxygenase (IDO) (Murray et al., 2014). IDO diverts tryptophan from serotonin production into a pathway that produces kynurenine and quinolinic acid, both potentially neurotoxic agents (Schwarcz et al., 2012). IDO is expressed by several cells of the immune system, including macrophage and microglia, and neurons. IFN- γ , IL-1, IL-6 and TNF- α induce IDO production. Importantly, macrophages that have been activated to an

M1-like/proinflammatory state are significant producers of IDO (Murray et al., 2014). High levels of IDO and the subsequent decrease in tryptophan, serotonin and/or increase in kynurenine have been linked to depression, anxiety and memory impairment in mouse models (Haroon et al., 2012; O'Connor et al., 2009; Salazar et al., 2012). In a model of HF diet with systemic immune activation by LPS, Andre et al. showed that the ratio of kynurenine to tryptophan in the brain was increased with an increase in IDO mRNA on the hippocampus and hypothalamus of obese mice exposed to LPS. There was also a concomitant increase in IFN- γ and TNF α . Finally, these mice exhibited anxiety-like behaviors. As proinflammatory cytokines and IFN- γ are increased in the obese state and during HSV-1 infection, and induce IDO expression, this is a potential target as a mechanism that links HSV-1, obesity and anxiety in our model. Further studies will elucidate the role of IDO in our model.

Independent of infection, HF diets and obesity have been shown to increase anxiety (Andre et al., 2014; Krishna et al., 2015). While HSV-HF diet resulted in increased anxiety-like behaviors in light-dark testing, marble burying was increased by latent infection and HF diet independently. Our data are consistent with other studies showing that high fat diets have increase marble burying in C57BL/6 mice (Krishna et al., 2015). It is possible that there is a ceiling effect in the marble bury test as C57BL/6 mice routinely bury approximately 75% of the marbles (Deacon and Rawlins, 2005; Thomas et al., 2009). This may mean that it is not sensitive enough to determine if the addition of obesity to HSV latency increases the burying behavior. In addition, Thomas et al suggest that marble burying is a better reflection of repetitive behaviors as it does not correlate with the number of transitions between the sides in the light-dark test (Thomas et al., 2009). The neural circuit responsible for repetitive behaviors does not include the hippocampus or hypothalamus, therefore, it is possible that there are changes associated with infection and obesity in areas of the that we did not measure, however, CCR2⁺ cell infiltration was not present in other areas of the brain (Langen et al., 2011). Future studies may determine the mechanism resulting in increased marble burying.

While there are many strengths of the current study, there are several limitations. While we have mRNA data from the hippocampus, we do not have comparable data from the hypothalamus and amygdala despite showing increased microglial activation in these regions. Additionally, we have not measured IDO nor tryptophan metabolism in this study, however, future studies will evaluate both. Finally, while there has been no direct link between HSV-1 and anxiety in humans, several studies have highlighted the role of HSV-1 in cognitive function, one in the elderly, and one in children. (Katan et al., 2013; Tarter et al., 2013). This epidemiological evidence suggests that HSV-1 infection has a significant impact on brain function throughout the lifespan. We anticipate that as the HSV-HF mice age we will see increased age-related cognitive impairment and an exaggeration of anxiety-like behaviors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Neuroinflammation induced by obesity following HSV-1 infection is investigated

Obesity promotes microglial inflammation following HSV-1 latency

Obesity following HSV-1 latency results in recruitment of CCR2+ monocytes to the brain

Obesity and latent HSV result in increased anxiety-like behavior

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Fig. 1. 45% diet results in significant weight gain in both uninfected and latently infected mice (Diet, $P < 0.001$; Infection, $P = 0.11$; Diet x Infection, $P = 0.50$). Statistical analysis: two-way ANOVA. Letters above columns indicate statistical significance between groups by Tukey-Kramer post hoc test. Columns not sharing a letter differ significantly at $P < 0.05$, $n = 12$ /group. Data are presented as mean $\pm$ SEM.

Fig. 2. IBA-1 staining and quantification. (A) Representative sections from hypothalamus, amygdala and dentate gyrus at 20x magnification. Quantification of pixel density of IBA-1 in (B) hypothalamus (Diet, $P=NS$; Infection, $P=NS$; Diet x Infection, $P=NS$) (C) amygdala (Diet, $P=0.044$; Infection, $P=0.041$; Diet x Infection, $P=0.11$) and (D) dentate gyrus (Diet, $P=0.001$; Infection, $P=0.040$; Diet x Infection, $P=0.04$). Statistical analysis: two-way ANOVA. Letters above columns indicate statistical significance between groups by Tukey-Kramer post hoc test. Columns not sharing a letter differ significantly at $P<0.05$, $n=4$ /group. Data are presented as mean $\pm$ SEM.

Fig. 3. Increased CD45^{hi}CD11b⁺ M1-like macrophage infiltrate in the brain of HSV-HF mice. (A) % of CD45^{hi}CD11b⁺ macrophage (Diet, $P=0.017$; Infection, $P=0.08$; Diet x Infection, $P=0.14$) (B) % of CD45^{hi}CD11b⁺ that are CD86⁺ (Diet, $P=0.016$; Infection, $P=0.06$; Diet x Infection, $P=0.37$). Statistical analysis: two-way ANOVA. Letters above columns indicate statistical significance between groups by Tukey-Kramer post hoc test. Columns not sharing a letter differ significantly at $P<0.05$, $n=6/\text{group}$. Data are presented as mean $\pm$ SEM.

Fig. 4. CCR2⁺ (Red) cells infiltrate the hypothalamus of HSV-HF mice. Representative sections from the hypothalamus of CCR2^{RFP/+}/CX3CR1^{GFP/+} (20X) n=4/group. (A) LF (B) HF (C) HSV-LF and (D) HSV-HF.

Fig. 5. CCR2⁺ (red) cells infiltrate the hippocampus of HSV-HF mice. Representative sections from the hippocampus of CCR2^{RFP/+}/CX3CR1^{GFP/+} (20X) n=4/group. (A) LF (B) HF (C) HSV-LF and (D) HSV-HF. In D, white arrows indicate the location of CCR2⁺ cells.

Fig. 6.

Increased Ly6C^{hi} monocytes and CCR2⁺ monocytes in the blood of HSV-HF mice. (A) Percent circulating monocytes (SSC^{lo}CD11b⁺Ly6G⁻) (Diet, $P=0.048$; Infection, $P=0.070$; Diet x Infection, $P=0.07$) (B) Ly6C^{lo} monocytes (black bars) (Diet, $P=0.17$; Infection, $P=0.29$; Diet x Infection, $P=0.08$) and Ly6C^{hi} monocytes (white bars) (Diet, $P=0.27$; Infection, $P=0.12$; Diet x Infection, $P=0.20$) and (C) CD11b⁺CCR2⁺ monocytes (Diet, $P=0.10$; Infection, $P=0.26$; Diet x Infection, $P=0.04$). Statistical analysis: two-way ANOVA. *indicates a significant difference at $P<0.05$ from all other groups by Tukey-Kramer post hoc test, $n=6$ /group for A and B and $n=4$ /group for C. Data are presented as mean $\pm$ SEM.

Fig. 7.

VCAM is upregulated in HSV-HF mice and co-localizes with CCR2⁺ cells. (A–H) Representative sections from Hypothalamus and Hippocampus at 20x magnification. Insets are from D and H. Quantification of VCAM pixel density in (I) hippocampus (Diet, $P=0.13$; Infection, $P=0.05$; Diet x Infection, $P=0.07$) and (J) hypothalamus (Diet, $P=0.056$; Infection, $P=0.032$; Diet x Infection, $P=0.78$). Statistical analysis: two-way ANOVA. Letters above columns indicate statistical significance between groups by Tukey-Kramer post hoc test. Columns not sharing a letter differ significantly at $P<0.05$, $n=4$ /group. Data are presented as mean $\pm$ SEM.

Fig. 8.

Hippocampal CCL2 mRNA expression and serum CCL2 are increased in HSV-HF mice compared to LF controls. (A) Fold increase of CCL2 mRNA in the hippocampus (Diet, $P=0.02$; Infection, $P=0.12$; Diet x Infection, $P=0.70$) and (B) CCL2 protein in the serum of LF, HF, HSV-LF and HSV-HF mice (Diet, $P=0.12$; Infection, $P=0.049$; Diet x Infection, $P=0.11$). Statistical analysis: two-way ANOVA. Letters above columns indicate statistical significance between groups by Tukey-Kramer post hoc test. Columns not sharing a letter differ significantly at $P<0.05$, $n=6$ /group. Data are presented as mean $\pm$ SEM.

Fig. 9.

Alterations in Anxiety behavior in HSV-HF mice compared to LF controls. A–C: Light-Dark Test D: Open Field Test. (A) Total time spent on light side (Diet, $P=0.03$; Infection, $P=0.99$; Diet x Infection, $P=0.41$) (B) # of transitions between dark and light sides (Diet, $P=0.05$; Infection, $P=0.07$; Diet x Infection, $P=1.0$) (C) Number of marbles buried (Diet, $P=0.014$; Infection, $P=0.09$; Diet x Infection, $P=0.13$). Statistical analysis: two-way ANOVA. Letters above columns indicate statistical significance between groups. Columns not sharing a letter differ significantly at $P<0.05$, $n=8$ /group. Data are presented as mean $\pm$ SEM.

Table 1

Establishment of Latent HSV-1 in Mice via Intranasal Route

Infection	Diet	HSV mRNA TG D14 p.i.	HSV DNA TG D14p.i.
HSV	LH	0/6	6/6
HSV	HF	0/6	6/6
Mock	LF	0/6	0/6
Mock	HF	0/6	0/6

Table 2
Expression of Glial Markers in the Hippocampus Presented as Fold Increase from LF mice

(*post-hoc test*: **Bolded black**, $P < 0.05$ compared to LF and HSV-LF, but not HSV-HF; **Green** $P < 0.05$ compared to LF, HSV-LF, and HSV-HF; **Blue** $P < 0.05$ compared to LF, HSV-LF).

Gene	LF	HSV-LF	HF	HSV-HF	P_{diet}	$P_{infection}$	$P_{diet \times infection}$
<i>Markers of Primed Glia</i>							
TLR4	1.00±0.12	0.97±0.23	1.09±0.17	1.80±0.16	0.02	0.08	0.06
MHCII	1.00±0.08	1.12±0.14	1.74±0.30	1.80±0.20	0.00	0.58	0.95
CD80	1.00±0.16	0.89±0.20	1.29±0.21	1.96±0.28	0.01	0.24	0.11
CD86	1.00±0.18	0.85±0.25	1.26±0.21	2.08±0.34	0.02	0.24	0.10
CD11b	1.00±0.05	1.06±0.16	1.41±0.29	2.18±0.30	0.01	0.10	0.16
<i>Cytokines Released by Activated Glia</i>							
IL-1 β	1.00±0.15	1.18±0.18	1.40±0.24	2.50±0.46	0.02	0.06	0.17
IL-6	1.00±0.26	1.45±0.65	1.26±0.40	3.62±1.67	0.31	0.25	0.04
TNF α	1.00±0.08	0.87±0.13	1.42±0.25	1.52±0.18	0.01	0.93	0.53
IL-10	1.00±0.39	1.03±0.13	3.06±0.61	2.70±0.89	0.01	0.80	0.76