LASTING MEMORY AND BEHAVIORAL DEFICITS IN MICE
SURVIVING AN ENTEROVIRUS INFECTION DURING THE NEONATAL PERIOD

A Thesis
Presented to the
Faculty of
San Diego State University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
with a Concentration in
Molecular Biology

by
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Spring 2012
SAN DIEGO STATE UNIVERSITY

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DEDICATION

This Master’s Thesis is dedicated to my Mother and Father, family, and friends. The research within the thesis is dedicated to science and people who are mentally ill.
Dare to be GREAT but be humble.

—Donn A. Van Deren, Jr.
ABSTRACT OF THE THESIS

Lasting Memory and Behavioral Deficits in Mice Surviving an Enterovirus Infection During the Neonatal Period
by
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San Diego State University, 2012

Enterovirus infections remain a serious health issue in developing countries and may result in severe impairment, especially in young children. Coxsackieviruses, members of the enterovirus genus, are significant human pathogens, and the neonatal central nervous system (CNS) is a major target for infection. Despite the extreme susceptibility of newborn infants to coxsackievirus infection, distinct tropism for the CNS, and a relatively high infection rate among infants, few studies have been aimed at determining the long-term consequences of infection on the developing CNS. We recently described a neonatal mouse model of coxsackievirus B3 (CVB3) infection, and discovered that proliferating stem cells in the CNS were preferentially targeted for infection. Nestin+ cells in neurogenic regions of the CNS underwent apoptosis following inoculation with a recombinant virus expressing eGFP (eGFP-CVB3), indicating that resident neural stem cells may be depleted following infection. Furthermore, virus persistence, chronic neuroinflammation, and reduced brain wet weights were observed in mice surviving neonatal infection. Therefore, mice surviving neonatal infection were tested for behavioral abnormalities and memory impairments utilizing the Morris Water Maze Task. We hypothesized that memory deficiencies might be detected in surviving mice given a relatively low viral inoculum with no obvious histological signs of CNS disease. Mock-infected mice were compared to two groups of mice inoculated with either high (10^7 pfu) or low (10^5 pfu) levels of eGFP-CVB3, and all animals were analyzed for memory impairments 90 days post-inoculation. Longer time achievements to the platform during swim trials were prominent in high-titer and low-titer infected mice, as compared to mock-infected control mice. The brains of infected mice showed signs of hippocampal pathology and astrogliosis/microgliosis. We suggest that developmental defects induced by a relatively common viral infection during the neonatal period may be long-lasting. With this in mind, long-term neurological sequelae might be expected following neonatal CVB3 infection.
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CHAPTER 1

INTRODUCTION

Childhood infections are a major health concern especially in developing countries. Key players in these infections include members of the enterovirus family. Enterovirus infections are widespread and common during early childhood, and newborn infants are extremely susceptible to infection (1, 2). Coxsackieviruses, members of the enterovirus family, are positive sense-stranded RNA viruses that cause human disease, including dilated cardiomyopathy (3, 4), pancreatitis (5, 6), meningo-encephalitis (7, 8) and aseptic meningitis (9). Despite the damaging effects of coxsackievirus infection in newborns (10-15), long-term studies of chronic infection remain largely unknown.

Recently, our laboratory has shown that infection of coxsackievirus B3 (CVB3) preferentially targets neural stem cells (NSC) of the central nervous system (CNS) in a neonatal mouse model of infection (13). More specifically, CVB3-infected mice displayed an increase in apoptosis of nestin+ cells and a reduction in Ki67+ cells within neurogenic sites. Moreover, infectious CVB3 was cleared by the host immune system, although CVB3 RNA persisted in the CNS. During persistent infection, CNS alterations were revealed by an observed increase in astrogliosis and reactive microgliosis. Astrogliosis may indicate persistent neuronal damage (16), suggesting possible CNS abnormalities in mice surviving CVB3 infection. As a result, neurodevelopmental disturbances may lead to behavioral impairments during adulthood, although the lasting consequences of persistent CVB3 infection are currently unknown.

The hippocampus, a major site for CVB3 infection, is also essential for learning and memory function (17, 18). There are two important regions of the hippocampus targeted by CVB3 infection (13, 19). The dentate gyrus (DG), a site for neurogenesis, is involved with processing geometric figures from the environment and recalls short-term memory in cooperation with the CA3 region (20). Similarly, the CA3 region is responsible for processing the geometry of the environment, encoding new spatial information, and retrieving short-term memory (21, 22). Given that neurodevelopment is impacted at
neurogenic sites (13, 15), particularly the DG in the juvenile brain, lasting neuropathology may render behavioral impairments in the adult brain.

Here we examined the long-term effects of CVB3 infection in the surviving host. We hypothesized that long-term neurological and behavioral alterations may occur following CVB3 infection during the neonatal period. We inspected adult neuropathology of CNS development by examining histopathology, viral persistence, neural stem cell proliferation, and levels of astrogliosis/reactive microgliosis in CVB3-infected mice. We then evaluated behavioral function using the Morris Watermaze Task in an attempt to link long-term behavioral function to neuropathology following CVB3 infection.
CHAPTER 2

MATERIALS AND METHODS

In this chapter, I discuss the materials and methods utilized to gather the data for histopathology and behavioral analysis.

MICE AND VIRAL INOCULATIONS

C57BL/6 mice were obtained from Harlan Sprague Dawley (Harlan Laboratories, San Diego CA). Breeding mice ranged between 6 to 8 weeks of age, and breeding pairs were monitored daily to ensure that pups were identified within 24 hours (h) of birth. One or three days after the recorded date of birth, pups were inoculated intracranially (IC) with 1X DMEM, 10^5 pfu, or 10^7 pfu of eGFP-CVB3. The procedure for IC inoculation of pups has been described previously (13). Pups were euthanized by placing them in a bell jar with isoflurane followed by immediate decapitation. Alternatively, adult mice were euthanized by isoflurane followed by cervical dislocation. Brains were harvested, and brain wet weight values were recorded immediately. One half of each brain was fixed by immersion in 10% formaldehyde followed by paraffin embedding; the other half of the brain was placed into a tube and placed on dry ice. These samples were eventually used to determine viral RNA copies by real time RT-PCR.

ISOLATION AND PRODUCTION OF A RECOMBINANT COXSACKIEVIRUS

The generation of a recombinant coxsackievirus expressing eGFP has been described previously (23). Briefly, the CVB3 infectious clone (pH3) (obtained from Dr. Kirk Knowlton at the University of California at San Diego) was engineered to contain a unique SfiI site which facilitates the insertion of any foreign sequence into the CVB3 genome. All virus stocks were grown on HeLa RW cells maintained in Dulbecco’s modified Eagle’s medium (DMEM; Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum. Virus titrations were carried out as described previously (13).
BRAIN WET WEIGHT VALUES

Wet weight measurements were taken at time of harvest by placing the whole brain on a scale and recording the weight value. Wet weight values were analyzed using Graphpad Prism 3.0 software, and the data was displayed graphically.

VIRAL RNA EXTRACTION AND QUANTITATIVE REAL-TIME PCR

Viral RNA was isolated from brain tissue using TRIzol reagent (Gibco-BRL, Rockville, MD), as described by the manufacturer. Quantitative real-time RT-PCR procedure has been described previously (19). Briefly, reverse transcription reaction mixtures included 1µg of total RNA and were carried out using SuperScript III reverse transcriptase (Invitrogen Inc., Carlsbad, CA) following the procedure described by the manufacturer. There were separate RT reaction mixtures which contained the reverse primer or forward primer to quantify the number of positive and negative-sense viral genomes. The mixture contained 1µl of RNaseOUT (Invitrogen Inc.) and 1µl of RNase H (Invitrogen Inc.) to remove any RNA complementary to the cDNA. PCR amplification was done using Platinum quantitative PCR SuperMix-UDG ready-to-use cocktail (Invitrogen Inc.) which contained all the components needed, except the primers and probe, as described by the manufacturer. Quantitative analysis of viral RNA was carried out using an iQTM5 Real Time PCR Detection System in 96-well optical reaction plates heated to 50°C for 2 min to digest dUTP-containing contaminants, followed by 95°C for 2 min to deactivate uracil N-glycosylase and activate Platinum Taq DNA polymerase. Forty cycles of denaturation at 95°C for 15s and annealing and extension at 60°C for 30s were carried out. All samples were evaluated in triplicate amplification reactions. The procedure to normalize the amount of RNA in each sample has been described previously (15). CVB3 RNA generated via our infectious viral clone (pH3 plasmid) was used to generate the standard curve. The standard curve was based on threshold cycle (Cₜ) values and Cₜ values from unknown samples were compared to the standard curve to determine viral RNA copy numbers.
HISTOCHEMICAL AND IMMUNOFLUORESCENCE STAINING

Paraffin-embedded brain sections (4 μm thick) were stained by hematoxylin and eosin (H&E). For immunofluorescence microscopy, paraffin-embedded sections were deparaffinized with three washes of xylene, three ethanol washes (100%, 95%, and 70%), and followed by washes in PBS and water. The detection of GFAP, Iba1, Sox2, and BrDU required high-temperature antigen unmasking in low pH citrate buffer (Vector Labs, Burlingame, CA). Immunofluorescence staining procedures have been described previously (15). Briefly, sections were blocked with 10% normal goat serum (NGS) for 30 min and incubated overnight with the primary antibody at 4°C. All antibody dilutions were made in 2% NGS. The secondary antibody utilized was a biotinylated goat anti-rabbit IgG {heavy and light chains (H+L)} (Vector Laboratories, Burlingame, CA) diluted at 1:100 in 2% NGS and incubated on sections for 30 min. All sections were then washed twice with PBS and incubated for 30 min with a streptavidin-green complex (Molecular Probes/Invitrogen, Carlsbad, CA) or a streptavidin-red complex (Molecular Probes/Invitrogen, Carlsbad, CA) diluted at 1:500 in 2% NGS. Detection of nuclei was done using Vectashield mounting medium containing 4’, 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Sections were observed by fluorescence microscopy (Zeiss Axio Observer D1 with an attached Zeiss MRc camera; Zeiss, Oberkochen, Germany) for the indicated cellular marker (green or red), and DAPI (blue). Green, red and blue channel images were merged using AxioVision software (Zeiss). A black and white image of the channel used was converted to a binary format. Particles were quantified by ImageJ software (National Institute of Health) to determine protein expression levels. Bar graphs of protein expression levels were created by GraphPad Prism 3.0. P-values were determined by the unpaired student T test. A P-value < 0.05 was considered statistically significant.

GRANULAR CELL VALUES

The number of granular cells of the hippocampus was quantified using ImageJ software as stated above. A scatter plot was created by GraphPad Prism 3.0 and P-values were determined by the unpaired student T test. A P-value < 0.05 was considered statistically significant.
**PATHOLOGY SCORING**

Hippocampal pathology was assigned a score from 0 to 5 for representative mice (n = 15). A score of 0 exhibited no signs of histopathology and a 5 exhibited a severe degree of histopathology. Scoring was determined by the size of the hippocampus, inflammation, and lesions. A scatter bar graph was created using Graphpad Prism 3.0. P-values were determined by the unpaired student T test. A P-value < 0.05 was considered statistically significant.

**MORRIS WATERMAZE TASK**

A 2’ x 4’ tank was filled with opaque water at a recorded temperature of 22°C. A transparent platform, 5” in diameter, was submerged 1 cm below the water line. White curtains around the water tank prevented the mice from capturing distal objects from the room. Seven random objects were distributed evenly around the edge of the tank to serve as spatial cues for the mice during block trials. A total of 6 days were used for the Watermaze experiments. Days 1 and 2 were designated as pre-training runs in which mice were taught how to swim twice a day. At each block, mice swam for 8 trails and were allowed 60 seconds to reach the platform. The platform location was placed opposite of mouse placement. No spatial cues were present. Learning retention was carried out on days 3, 4, and 5 where each mouse swam twice a day (6 blocks total). At each block, mice swam for 3 – 4 trails and were allowed a maximum of 90 seconds to reach the platform. The platform location remained the same for each mouse but mice were placed in a new quadrant for each successive trail. Whether or not the mice found the platform, they were allotted 30 seconds to stay on the platform and immediately taken out to dry off and rest until the next trail. Day 6 was the probe test for memory retention. Each mouse swam once for 60 seconds. Spatial objects were present but the platform was removed. All blocks and the probe test were recorded by WinTV 2000 software. Recorded videos were analyzed by TopScan 2.0 behavioral software to determine the behavioral performances (duration, distance, and swim velocity) of each mouse. Trace images were exported from TopScan 2.0.

**CORRELATION GRAPHS**

Pathology scores of the hippocampus were compared to behavioral performance (duration, distance, and swim velocity) for representative mice (n = 15) using GraphPad
Prism 3.0. Trend lines were added and P-values were determined by using the unpaired student T test. A P-value < 0.05 was considered statistically significant.
CHAPTER 3

RESULTS

In this chapter, I discuss the histopathology and behavioral results of CVB3-infected mice during adulthood.

HIPPOCAMPAL LESIONS WERE OBSERVED IN THE CA3 REGION OF HIGH-INOCULUM INFECTED CVB3 MICE

Since the hippocampus is the primary site for learning and memory function (17, 24), we inspected the histopathology of infected mice at day 90 post-infection (p.i.). Mice challenged with a high-inoculum of eGFP-CVB3 exhibited a reduction in hippocampus size, enlargement of the lateral ventricle, and damage to the surrounding tissue (Figure 1C) when compared to mock control mice (Figure 1A). In contrast, mice challenged with a low-inoculum of eGFP-CVB3 exhibited little signs of histopathology when compared to mock control mice (Figure 1B). Furthermore, closer examination of the DG and CA3 region also presented no observable signs of histopathology when compared to mock controls. Higher magnification of the hippocampus of high-inoculum infected mice revealed observable alterations in DG formation (Figure 1F), a disruption in granular cell formation in the CA3 region, tissue loss (Figure 1C, black arrow), and lymphocyte recruitment to the sites of damage (Figure 1C, yellow arrows with black outline) compared to low-inoculum or mock control mice (Figure 1E and Figure 1H, Figure 1D and Figure 1G; respectively).

Previously we observed a reduction in brain wet weight values in 3 day-old BALB/c mice inoculated with high levels of eGFP-CVB3 (10^7 pfu IC) (12). Therefore, we expected to observe a similar reduction in brain wet weight values in C57BL/6 mice, although these mice are less susceptible to coxsackievirus-induced pathology. Low-inoculum and high-inoculum groups showed a reduction in brain wet weight values relative to the mock control mice (Figure 1J), although the reduction was not statistically significant. Even so, a statistically significant loss of granular cells in the hippocampi was observed in low-inoculum (P = 0.05) and high-inoculum mice (P = 0.0094) when compared to mock controls (Figure 1K). Taken together, these data
Figure 1. Hippocampal lesions are observed in the CA3 region. H & E staining of the hippocampi of mock-infected mice (A) and low-infected mice (B) show no signs of histopathology. (A – C: 5X magnification). In contrast, the hippocampi of high-infected mice (C) display lesions, inflammation (yellow arrows with black outline), and a disruption in granular cell morphology (black arrow) in the CA3 region. Dentate gyrus and CA3 region: 20X magnification. No significant reduction in brain wet weights was observed (D). A significant reduction in the number of granular cells is revealed in low (N = 3) and high-infected (N = 3) hippocampi (E). Group means ± SEM are shown. A P value < 0.05 was significant.

suggest that high-inoculum infected mice displayed rigorous signs of histopathology and exhibited a significant loss of granular cells in the hippocampus. In addition, mice challenged with a low amount of CVB3 revealed a significant reduction of granular cells in the hippocampus although no apparent signs of histopathology were detected.

**CHRONIC ASTROGLIOSIS AT NEUROGENIC SITES OF HIGH-INOCULUM INFECTED MICE**

A global representation of damaged neurons following CVB3 infection at day 90 p.i. was examined using an antibody against glial fibrillary acidic protein (GFAP; astrocyte marker) to identify regions of astrogliosis in the brain. Higher levels of astrogliosis were observed in high-inoculum infected mice (Figure 2B) when compared to mock-infected control mice (Figure 2A).
Figure 2. Chronic astrogliosis occurs at neurogenic sites. Composite images of sagittal sections of a representative mock-infected brain (A) and high-infected brain (B) at day 90 p.i. labeled with GFAP (5X magnification). High magnification images of the OB, RMS, SVZ, and DG reveal an increase in GFAP signal in high-infected mice (B) when compared to mock mice (A). (20X magnification). Quantification of GFAP signal was significantly higher in the high-infected mice in the OB, RMS, and DG (C – F). Group means ± SEM are shown. (* P < 0.05, ** P < 0.005).

Also, high magnification images of the olfactory bulb (OB), rostral migratory stream (RMS), subventricular zone (SVZ), and dentate gyrus (DG) of high-inoculum infected mice revealed greater levels of astrogliosis when compared to mock controls. Expression levels of GFAP in the olfactory bulb (Figure 2C), rostral migratory stream (Figure 2D), and dentate gyrus (Figure 2F) were significantly higher than mock-infected control mice (P < 0.05). Also, GFAP expression was greater in the SVZ of high-inoculum infected mice, although these levels were not statistically significant (Figure 2E). Taken together, a prominent increase in astrogliosis occurred at numerous neurogenic sites of high-inoculum infected adult mice.
AN INCREASE IN ASTROGLIOSIS FOLLOWING CVB3 INFECTION IN THE HIPPOCAMPUS

Since the hippocampi of high-inoculum infected mice revealed granular cell loss and lesions in the DG and CA3 region, we hypothesized that inflammation might occur around damaged sites to form glial scars as described previously for BALB/c mice (19). We examined the degree of astrogliosis low and high-inoculum infected mice using an antibody against GFAP. As expected, mock-infected control mice displayed minimal signs of GFAP signal in the dentate gyrus (Figure 3A) and CA3 region (Figure 3B). In contrast, a greater level of GFAP signal was observed in the dentate gyrus and CA3 region of low-inoculum infected (P = 0.0028 and P = 0.0096; respectively) and high-inoculum infected mice (P = 0.0165 and P < 0.0001; respectively), as quantified in Figure 3C. A closer examination of the DG of a high-inoculum infected mouse showed an increased density of astrocytes. Overall, an increase of GFAP signal relative to the initial CVB3 inoculum suggested astrogliosis in the hippocampi of infected mice, perhaps in an attempt to protect tissue integrity following infection by the formation of glial scars.

REACTIVE MICROGLIOSIS FOLLOWING CVB3 INFECTION OCCURRED AT SITES OF DAMAGE

In parallel to the observed increase in astrogliosis in the hippocampi of CVB3-infected mice, microglia and macrophage activation was inspected by Iba1 staining. High-inoculum infected mice showed greater levels of Iba1 staining in the DG (Figure 3D) and CA3 regions (Figure 3E). A significant increase in Iba1 signal was observed in the dentate gyrus and CA3 region of low-inoculum infected (P = 0.0064 and P = 0.0047; respectively) and high-inoculum infected hippocampi (P = 0.0078 and P = 0.0003; respectively) (Figure 3F). Overall, these data suggest a significant involvement of the phagocytic cells to sites of damage in the hippocampi of infected mice.

NEURAL STEM CELL RESERVOIRS WERE REDUCED FOLLOWING CVB3 INFECTION

During the neonatal period, a reduction of Ki67+ neural stem cells in the SVZ following CVB3 infection was previously reported by our laboratory (13). However, the
Figure 3. An increase in astroglosis and reactive microgliosis following CVB3 infection occurs in the hippocampus. The DG and CA3 region of low and high-infected mice (A – B: 20X magnification) exhibited a significant increase in GFAP signal (N ≥ 3). Group means ± SEM are shown. A P value < 0.05 was significant. (C) Quantification graph showing GFAP expression for mock, low, and high-infected mice at dentate gyrus and CA3. The DG and CA3 region of low and high-infected mice (D – E: 20X magnification) exhibited a significant increase in Iba1 signal (N ≥ 3). Group means ± SEM are shown. A P value < 0.05 was significant. (F) Quantification graph showing Iba1 expression for mock, low, and high-infected mice at dentate gyrus and CA3.

lasting effects of CVB3 infection on adult neurogenesis were not investigated. We examined the relative quantity of neural stem cells in the neonatal and adult CNS following infection (Figure 4A and Figure 4B; respectively) by inspecting levels of the transcription factor Sox2 in the DG and three additional neurogenic regions. As early as 24 hr p.i., a significant reduction in Sox2 signal was observed in the DG (P = 0.007), OB (P = 0.0062), SVZ (P =
Figure 4. Neural stem cell reservoirs are reduced during persistent CVB3 infection. A reduction in neural stem cell populations labeled with Sox2 are observed at the DG (A, B, C), OB (C), RMS (C), SVZ (C). (20X magnification). Day 1 high-infected neonatal mice showed a profound decrease in Sox2 signal (A, C). In addition, Day 90 mock and high-infected mice also exhibited a significant reduction in Sox2 signal. (B – C, 20X magnification). Group means ± SEM are shown. (* P < 0.05, ** P < 0.005, *** P < 0.0005). (N = 3 mice per group).

0.0376), and RMS of high-inoculum infected mice when compared to mock-infected control mice (Figure 4C). These data coincide with previous studies showing a reduction of Ki67+ neural stem cells following infection during the neonatal period (13). In adult mice surviving early CVB3 infection (day 90 p.i.), a reduction of Sox2 signal was observed in the DG and RMS, although these values were not statistically significant. Taken together, these data exhibit a significant reduction in neural stem cells at day 24 hr p.i. However, no statistically
significant effect on adult neurogenesis was seen in adult mice surviving early CVB3 infection.

**Cellular Proliferation in Neurogenic Regions was Significantly Reduced During Persistent CVB3 Infection**

Based on Sox2 staining data at day 1 p.i. described in Figure 4, we hypothesized that neural stem cell proliferation in adult mice surviving CVB3 infection might become affected. Mice were inoculated with BrDU nucleoside analog every two weeks following inoculation with eGFP-CVB3. The number of cells that had recently undergone proliferation was assessed at days 5, 30, and 90 p.i. by immunohistochemistry using an antibody against BrDU (Figure 5). Although a significant reduction in BrDU⁺ signal was observed for both mock and high-inoculum infected mice over time in the DG (P < 0.001), no reduction of BrDU⁺ signal was seen between mock and high-inoculum infected mice on day 30 and 90 p.i. (Figure 5A – D). In the SVZ of high-inoculum infected mice, no significant reduction of BrDU signal was observed at days 30 and 90 p.i. when compared to mock-infected control mice (Figure 5E – H). Overall, these data suggest that neural stem cell proliferation in the DG was drastically reduced by day 30 in both mock-infected and high-inoculum infected mice, although no greater reduction was specifically observed within infected animals.

**CVB3 Infection Leads to Long-Term Behavioral Abnormalities in Surviving Mice**

We evaluated the behavioral performances of CVB3-infected mice using the Morris Water Maze Task (Figure 6). A total of 6 training blocks over the course of 3 days (2 training blocks per day) were used for the Water Maze analyses. Representative trace images of infected mice displayed a less direct swimming route to find the submerged platform when compared to mock controls (Figure 6A).

Three functions of Water Maze analyses were measured for behavioral performance (Figure 6B): swim velocity, duration to reach submerged platform, and distance to reach submerged platform. For swim velocity, a 3 x 6 repeated measures analysis of variance (ANOVA) with group as a between group factor (mock, low-infected, high-infected) and block as a within group factor (1–6) was used to analyze groups differences in swim velocity as a function of block. The analysis revealed a statistically significant main effect of group
Figure 5. Neural stem cell replication is significantly reduced in the dentate gyrus. Stem cell replication was labeled with BrDU at days 5, 30, and 90 p.i. in the dentate gyrus and subventricular zone (A – H, 20X magnification). A significant reduction in Sox2 signal is seen in the dentate gyrus at days 30 and 90 p.i. (D). In the subventricular zone, no significant reduction was only seen with day 90 infected mice (H). Group means ± SEM are shown. (N = 3 mice per group).

(F(23) = 6.90; P < 0.01) and a significant group x block interaction (F{10, 155} = 2.38; P < 0.05). However, the main effect of block did not reach statistical significance (F{5, 155} = 0.90; P = 0.48).

A Newman-Keuls posthoc comparison test of the main effect of group revealed that swim velocity was significantly higher (P < 0.05) in the mock-infected group compared to both the low-inoculum and high-inoculum infected groups. However, no significant differences in swim velocity were detected when comparing the low-inoculum and high-inoculum infected groups. A Newman-Keuls posthoc comparison test of the significant group x block interaction revealed no significant differences in swim velocity among the three groups on Block 1 of testing. However, swim velocities were significantly higher (P < 0.05) in the mock group compared to both the low-inoculum and high-inoculum infected groups on Blocks 2 – 6 (Figure 6B; blue asterisks). Overall, swim velocity impairment suggested that
Figure 6. CVB3 infection leads to long-term behavioral abnormalities. Representative trace images show the path routes of mock, low, and high-infected mice. Quadrants are designated I, II, III, and IV. Platform is colored with a white outline and black center (A). Swim velocity, duration, and distance to reach platform as a function of block (6 blocks = 3 days of training) display behavioral alterations and motor impairment in high-infected adult mice (B). (*, P < 0.05). No difference in memory retention is seen with infected mice during the probe trial (B). A significant GFAP signal is expressed in the cerebellums of high-infected mice when compared to mock mice (D – E). Group means ± SEM are shown. (N = 3 mice per group).

CVB3 infection may exhibit a possible effect on motor function in infected mice. We further evaluated swim velocity impairments by examining the levels of GFAP expression in the cerebellum of high-inoculum infected mice (Figure 6D). High-inoculum infected mice revealed a significantly higher level of GFAP expression (P = 0.0095) when compared to mock-infected control mice. Overall, astrogliosis appeared to be present in the cerebellum of high-inoculum infected mice, and such an effect may contribute to motor dysfunction.
For duration to reach submerged platform, a 3 x 6 repeated measures analysis of covariance (ANCOVA) with group as a between group factor (mock, low-inoculum infected, high-inoculum infected), block as a within group factor (1–6), and average swim velocity as a covariate was used to analyze group differences in duration to locate the submerged platform as a function of block. Since significant group differences in swim velocity were observed as described above, swim velocity was entered into the model as a covariate to statistically examine the effect of group differences in swim velocity on duration to locate the platform. The ANCOVA revealed a statistically significant group x block interaction, \( F_{10, 150} = 2.70; P < 0.01 \). However, the main effects of group (\( F(24) = 1.02; P = 0.37 \)), block (\( F(25) = 1.31; P = 0.26 \)), and velocity (\( F(22) = 0.31; P = 0.58 \)) did not reach significance. In addition, the block x velocity interaction failed to reach statistical significance \( (F(25) = 0.48; P = 0.79) \). As shown in Figure 6B (blue asterisk), a Newman-Keuls posthoc comparison test of the significant group x block interaction revealed that the duration of time required for the high-inoculum infected group to locate the submerged platform on Block 1 was significantly higher (\( P < 0.05 \)) compared to both the mock-infected control and low-inoculum infected groups. However, there were no statistically significant differences in duration to locate the platform between the mock and low-inoculum infected groups on Block 1. In addition, the analysis revealed that there were no significant group differences in duration to locate the platform on Blocks 2–6.

In a similar fashion for distance to reach submerged platform, a 3 x 6 repeated measures ANCOVA with group as a between group factor (mock-infected, low-inoculum infected, high-inoculum infected), block as a within group factor (1–6), and average swim velocity as a covariate was used to analyze group differences in distance traveled to locate the submerged platform as a function of block. Once again, swim velocity was entered into the model as a covariate to statistically examine the effect of group differences in swim velocity on distance traveled to locate the platform. The ANCOVA revealed statistically significant group x block \( (F_{10, 150} = 5.43; P < 0.001) \) and block x velocity \( (F(25) = 2.58; P < 0.05) \) interactions. The analysis also revealed a statistically significant main effect of velocity \( (F(22) = 41.84; P < 0.001) \). However, the main effects of group \( (F(24) = 2.05; P = 0.37) \) and block \( (F(25) = 1.18; P = 0.32) \) did not reach significance. A Newman-Keuls posthoc comparison test of the significant group x block interaction revealed that the distance
traveled by the high-inoculum infected group before locating the submerged platform on Block 1 was significantly ($P < 0.05$) longer compared to both the mock-infected and low-inoculum infected groups (Figure 6B). However, there were no statistically significant differences in distance traveled to locate the platform between the mock-infected and low-inoculum infected groups on Block 1. In addition, the analysis revealed that there were no significant group differences in distance traveled before locating the platform on Blocks 2–6. Taken together, mice infected with a high-inoculum of CVB3 showed a significant learning impairment in their distances to reach the platform in training block 1 when compared to mock-infected and low-inoculum infected mice.

A probe test was conducted to examine the average time spent during the probe trial in the quadrant that previously contained the platform to evaluate memory recall (Figure 6C). A one-way ANOVA with group as a between group factor (mock, low-inoculum infected, high-inoculum infected) revealed no significant group differences ($F_{2,33} = 1.01; P = 0.38$).

**Behavioral Deficits Correlate to Hippocampal Damage in CVB3-Infected Mice**

Pathology scores of the hippocampus were assigned to representative mice tested from the Morris Water Maze Task (Figure 7). The scores were based on three parameters for detecting histopathology: size of the hippocampus, number of lesions, and number of inflammatory cells. A score of 0 showed no signs of damage, and a score of 5 showed severe amounts of damage. Low-inoculum infected mice exhibited a relatively equivalent average histopathology score comparable with mock-infected control mice, while high-inoculum infected mice exhibited a significant average score of 2.4 ($P = 0.0001$) (Figure 7A and Figure 7B). Representative traces images of low and high-infected mice show that the learning task becomes more difficult as hippocampal damage increases.

We compared the behavioral performances of low and high-inoculum infected mice at block 1 with their respective histopathology scores (Figure 7C). As expected, a significant correlation was observed for duration ($P = 0.0002$) and distance ($P = 0.0002$) performances when compared to hippocampal damage. In contrast, swim velocity ($P = 0.8602$) performances were not significant when compared to hippocampal damage. These data
Figure 7. Behavioral deficits correlate to hippocampal damage. Hippocampi of mock, low, and high-infected mice were assigned histopathology scores in the arbitrary range of 0 – 5. Hippocampi given a pathology score of 0 had no signs of histopathology and a pathology score of 5 had serious signs of pathology. Pathology scores were determined by size of hippocampus, number of lesions, and inflammation. Mock-infected controls and low-infected CVB3 mice had no significant signs of pathology. There were significant signs of pathology in the high-infected CVB3 mice (B). Representative hippocampi and trace images for mock, low, and high-infected mice are shown in conjunction with the average histopathology score of each group (A). Correlation graphs of block 1 between histopathology scores and behavioral performance indicate that a higher level of hippocampal damage results in a significant deficit in duration and distance performance (C). No significance is revealed with swim velocity and probe test performances (C). (D) CVB3 RNA levels were quantified by qRT-PCR. Group means ± SEM are shown. (N = 3 mice per group). (E) Correlation graphs of behavioral performance and hippocampal damage with CVB3 RNA displayed no significant correlation. (N = 3 mice per group).
suggest a strong link between behavioral impairments and hippocampal damage in CVB3-infected mice.

**HIPPOCAMPAL DAMAGE AND BEHAVIORAL PERFORMANCE ARE NOT INFLUENCED BY THE PRESENCE OF CVB3 RNA DURING PERSISTENT INFECTION**

To confirm behavioral impairments are strongly correlated with hippocampal damage, we compared hippocampal damage to CVB3 RNA levels at day 90 p.i. qRT-PCR analysis showed detectable levels of viral RNA in low and high-inoculum infected mouse brains (Figure 7D; P < 0.05). However, no significant correlations were observed for viral copies and either hippocampal damage or behavioral performance (Figure 7E; P < 0.05).

**CVB3 INFECTION IN THE ADULT MOUSE BRAIN**

Our adult mouse model (Figure 8) of chronic damage to the CNS induced by CVB3 infection proposes that lasting neurological deficits alter CNS development and affect behavior. Given that infectious particles of CVB3 are eradicated by day 10 p.i. (13), viral RNA may persist in the host stem cells or neurons thereby contributing to microgliosis and astrogliosis. As the infected mouse ages, alterations in neurodevelopment may highlight the cognitive impairments and motor dysfunction observed in adult mice infected with CVB3 during the neonatal period.
Figure 8. CVB3 infection in the adult mouse brain. Our adult model of CVB3 infection proposes that injury to the hippocampus alters normal development for learning and memory function. Although the virus is cleared by day 10 p.i., the immune and repair systems of the host activate microgliosis, macrophages, and astrogliosis to the sites of infection. In the chronic state, day 30 and beyond, activation of astrogliosis and microgliosis/macrophages continues and impairs behavioral function and motor abilities.
CHAPTER 4

DISCUSSION

Previous studies have found that early neurotrophic viral infections lead to disrupted memory and CNS alterations in murine models (13, 23, 25). However, the consequences of a human neurotrophic viral infection in a murine model have remained unknown. The Morris Water Maze task examines learning and memory function and has played a key role in behavioral abnormalities resulting from viral infections (23), cancer (26), radiation (27, 28), and brain trauma (29). We show for the first time that CVB3 infection during the neonatal period may cause a transient learning disability in adult mice. This learning disability was observed at block 1 but by block 6 no learning disability was noticed. Mice that spend more time in the training quadrant where the platform would be located have a higher percentage of memory recall. Intriguingly, memory recall from the probe test seemed to increase slightly in CVB3-infected mice.

Histopathology of high-inoculum infected mice displayed severe insult to the hippocampus. More specifically, lesions were observed in the CA3 and dentate gyrus where memory function and neurogenesis is governed. Lymphocyte recruitment was also noticed in these hippocampi where damage was present. Previously, we have shown a significant decrease in brain wet weights in CVB3-infected BALB/c mice (13). Although lower brain wet weights were also observed in C57BL/6 mice, these results were not statistically significant. A significant reduction in the number of granular cells and cellular RNA were noted in CVB3-infected C57BL/6 mice. Interestingly, high-inoculum infected mice displayed a low amount of persistent viral genome. Our qRT-PCR analysis of CVB3 detection coincides with previous reports that have indicated CVB3 establishes a persistent infection in the CNS during adulthood (13, 19). Low-inoculum infected mice displayed no detectable histopathology in the hippocampus. This is significant because we hypothesized that mice challenged with a low amount of CVB3 might harbor minimal histopathological signs, yet display behavioral impairments as the mice age. Contrary to our expectations, low-inoculum infected mice performed similarly to mock-infected mice. Notably, low-inoculum infected
mice swam significantly slower when compared to mock-infected mice which indicate that motor ability may be impaired following infection.

The neuropathology of CVB3-infected mice showed the recruitment of microgliosis and macrophages and the presence of astrogliosis. The presence of astrogliosis may serve as an indicator of inflammation (30, 31). We have found that a global increase of astrogliosis in the CNS is indirectly affected by CVB3 inoculum. Moreover, neurogenic sites, except the subventricular zone, showed a significant increase in astrogliosis when compared to mock-infected mice. A step-wise increase in astrogliosis was also noticed in the dentate gyrus and CA3 region of infected mice relative to viral inoculum. Similarly, microgliosis and macrophage recruitment also occurred in a step-wise fashion relative to viral inoculum. We have previously shown that reactive microgliosis occurred during adulthood following CVB3 infection (18).

Neural stem cell pools and proliferation were significantly reduced in CVB3-infected mice. During neonatal CNS development, active proliferation of neural stem cells occurs while neural stem cells pools are actively engaged in neurogenesis (32-35). Migration of de novo neurons from neurogenic sites occur via the rostral migratory stream to reach their destination (35). However, a viral infection may hinder this migration process and neural stem cell production (36). Our results have shown that neonatal CVB3-infected mice displayed a decrease in stem cell proliferation by day 5 p.i. in the dentate gyrus and subventricular zone, and these levels remained low during adulthood. Additionally, neural stem cell pools were significantly reduced, and our results indicate that neurogenesis may be hampered following CVB3 infection. Our data parallels previous reports that have indicated a reduction in NSC proliferation and pools following viral infection (13, 36).

CVB3-induced neuropathology appears to be linked to behavioral impairments in adult mice. We have shown positive correlations between hippocampal damage and behavioral performance in which a higher degree of hippocampal damage renders a poorer behavioral performance. The impaired learning ability of CVB3-infected mice may be due to the longer duration times and distances to find the platform. However, there seems to be retention of minimal memory over time because duration and distance performances improved in CVB3-infected mice. Interestingly, we did not expect to find swim velocity impairments in infected mice. Our data has shown that low and high-inoculum infected mice
consistently swam at a lower velocity over time when compared to mock-infected mice. Moreover, a high level of astrogliosis is present in the cerebellums of infected mice and may present a role in cerebellar diseases.

We have provided an overall examination of significant consequences of adult CNS development following CVB3 infection during the neonatal period. We have shown that (1) early CVB3 infection causes lasting histopathology in the adult CNS, (2) abnormal development of the CNS may be the result of the innate immune response and not CVB3 infection, (3) neural stem cell depletion is evident during the early and late stages of infection, and (4) impairments in behavioral performance is correlated to hippocampal damage following CVB3 infection.

Further investigation is needed to determine the type of cerebellar dysfunctions in infected mice and whether these dysfunctions are associated with CVB3 infection during the neonatal period. Additionally, drug treatment or exercise may alleviate the detrimental effects of CVB3-induced damage to the CNS. For instance, a common antiviral drug, Ribavirin, given to children with CVB3 infection could be used during adulthood in the hope of rectifying learning and memory function (37-41). Alternatively, rehabilitation exercise has been shown to restore neurogenesis through running in mice irradiated during childhood (42). It would be wise to attempt to restore neurogenesis in infected adult mice.
ACKNOWLEDGEMENTS

I would like to extend much gratitude to my committee members for their support, Drs. Ralph Feuer, Greg Harris, and Paul Gilbert. In particular, I thank Drs. Feuer and Gilbert for their knowledge, guidance, and care throughout my education as a Master’s student at San Diego State University. Dr. Feuer, a viral immunologist, has provided me essential techniques required for studies involving virus-host interactions and virus-mediated pathology. Dr. Gilbert, a behavioral psychologist, has broadened my perspective of behavioral neuroscience by utilizing the Morris Watermaze Task.

Let it be known that my family provided me their utmost support throughout undergraduate and graduate school. My Mother and Father spent countless hours listening about my current research. I honor their support and patience by sharing my Master’s Degree in Cell & Molecular Biology with them. Additionally, I truly thank Courtney Benson for being my best friend and #1 fan during my studies at San Diego State University.

This work was supported by National Institutes of Health (NIH) R01 Award NS054108 (to R.F.), an NIH Research Supplement to Promote Diversity in Health-Related Research Award 3R01NS054108-01A2S1 (to R.F. and S.M.R), and a National Institutes of Mental Health (NIMH) Minority Research Infrastructure Support Program (M-RISP) R24 Faculty Fellow Award MH065515 (to R.F.). Jenna M. Tabor-Godwin is a recipient of an Achievement Rewards for College Scientists Foundation Scholarship. Sonia Maciejewski is a recipient of the SDSU Minority Biomedical Research Support Program (NIGMS, NIH Grant 2R25GM058906-09A2). No conflicts of interest exist between the subject matter and the authors included in the manuscript.
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