THE NEURODEVELOPMENTAL IMPACT OF

PRENATAL ZIKA VIRUS INFECTION IN A RAT MODEL:

A LONGITUDINAL MRI STUDY

by

Rita Patel

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ABSTRACT

The widespread epidemic of Zika infection (ZIKV) first reported in the Americas, has uncovered the devastating impact of ZIKV infection especially in pregnant women. ZIKV has emerged as a major challenge for global health agencies due to its ability to cause congenital Zika syndrome which is characterized by brain abnormalities and microcephaly in neonates as well as cognitive developmental challenges in young children (Adachi and Nielsen-Saines, 2019; Trevathan, 2016). Although, the causal link between ZIKV prenatal infection and serious brain abnormalities seems unquestionable, present findings only offer a limited scope of ZIKV pathogenicity. Pediatricians are now reporting an increased risk of seizures, irritability, and cognitive developmental delays in ZIKV affected children, some of whom appeared asymptomatic at birth (Wheeler, 2018). Currently, our lab has developed a rat model of prenatal ZIKV infection which results in vertical transmission of the virus to the fetus, producing a significant increase in cell death in the cortex and hippocampus of surviving affected offspring (Sherer et al., 2019). Using this unique model in conjunction with longitudinal magnetic resonance imaging, we explore the long-term neurodevelopmental outcomes of prenatal ZIKV infection in rodent offspring. In this study, we demonstrate that prenatal ZIKV infection significantly affects long-term neurodevelopment in hippocampal regions therefore potentially inducing long-term neurological consequences.

Chapter 1

INTRODUCTION

1.1   Zika Virus

Zika virus (ZIKV) is an arthropod-borne Flavivirus, closely related to dengue, yellow fever, Japanese encephalitis, and West Nile viruses (Hayes, 2009). Similar to other flaviviruses, Zika virus is a positive-stranded RNA virus which allows the virus to directly serve as messenger RNA so that it can be translated into protein in the host cell (Sager et al., 2018). Zika virus is primarily transmitted through a sylvatic infection cycle by the *Aedes aegypti* mosquito which thrive in tropical and sub-tropical regions such as Asia, Africa, and the Americas (Braack et al., 2018). ZIKV has more recently been shown to be transmitted through other routes including sexual transmission, transfusion of blood products, breast milk feeding, as well as vertical transmission from the pregnant mother to the fetus (Mann et al., 2018). Typically, ZIKV infections are asymptomatic, as only 20% of ZIKV infection result in the presentation of symptoms (Pomar et al., 2019). Moreover, the symptoms are mild and resolve in less than two weeks (Rombi et al., 2020). Clinical symptoms of ZIKV infection in adults are similar to other flavivirus infections and include fever, headache, joint pain, muscle pain, and maculopapular rash. More severe clinical outcomes of ZIKV infection have been reported in adult humans. These include Guillain-Barré syndrome, acute myelitis, encephalomyelitis, encephalitis, meningoencephalitis, and sensory polyneuropathy, however these neurological consequences are comparatively very rare (Figueiredo et al., 2019; Rombi et al., 2020). Since its emergence in the Americas,

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there has been renewed interest in understanding the pathogenicity of ZIKV in order to develop vaccines and therapeutic strategies able to combat infection. Nevertheless, no effective therapies currently exist.

The namesake of Zika virus comes from the Zika Forest located in Uganda, where the virus was first isolated in 1947 through a study of yellow fever. For the next 70 years, Zika virus would be silently circulating in Africa and Asia with only 14 cases of ZIKV infection reported (Herrara.et al., 2017). That is, until April 2007, when the first outbreak outside of Africa and Asia on the island of Yap in the Federated States of Micronesia occurred (Herrara.et al., 2017). Thereafter the virus rapidly spread, resulting in an estimated 5,000 infections among the total population of 6,700 (Peterson et al., 2016). The outbreak of Zika fever in Micronesia marked a pivotal turning point in recognizing the transmission potential of the virus. Subsequent outbreaks would later occur in New Caledonia, French Polynesia and other Pacific islands (Peterson et al., 2016). However, it was not until March 2015, when an outbreak in Brazil would uncover the devastating impact of ZIKV infection in pregnant women.

ZIKV emerged as a major challenge for global health agencies due to its ability to cause Congenital Zika Syndrome (CZS) which is characterized by brain abnormalities and microcephaly in neonates as well as cognitive developmental challenges in young children. Zika soon became the first major infectious disease linked to human birth defects to be discovered in more than half a century which created such global alarm that the World Health Organization (WHO) would declare a Public Health Emergency of International Concern (Lowe et al., 2018; Peterson et al., 2016). In fact, there has been nearly a 20-fold increase in the incidence of

microcephaly and birth defects seen among women giving birth in Brazil between 2014-2015 (Campos et al., 2018; Fiorentino and Montero, 2016), leading to the Centers for Disease Control and Prevention (CDC) to officially declare a causal link between prenatal ZIKV infection and the serious brain abnormalities seen in affected infants. Although, the link between ZIKV prenatal infection and serious brain abnormalities seems unquestionable, present findings likely only offer a limited scope of ZIKV's full pathogenicity.

ZIKV has now been firmly established as a cause of microcephaly, and subsequent studies continue to show that the ZIKV is highly neurotropic; however, only an estimated 10% of ZIKV infected fetuses present some kind of malformation when born (Reynolds et al., 2017). In fact, a recent study has shown that a far greater number of children are likely to be affected by ZIKV neuropathologies other than microcephaly (Wheeler, 2018). Therefore, it has been proposed that microcephaly may just be the "tip of the iceberg" in terms of symptoms associated with ZIKV exposure. Many infants exposed to Zika in utero are born with a normal head size but then quickly display serious cognitive deficiencies and motor impairments as they age. Pediatricians are now reporting an increased risk of seizures, irritability, and cognitive developmental delays in ZIKV affected children, some of whom appeared asymptomatic at birth (Adachi and Nielsen-Saines, 2019; Trevathan, 2016). Moreover, the CDC is recommending postnatal testing, which is more likely to detect lesser viral injuries through cranial ultrasound imaging (and/or MRI, if feasible), eye examination, auditory, longitudinal developmental assessments, and close monitoring of growth. Clinics in Brazil have found one way to implement this postnatal testing by obtaining scores of Bayley Scales of Infant and Toddler Development measuring motor,

cognitive and language domain skills (Einspieler et al., 2019). Unfortunately, many infants with prenatal ZIKV exposure do not receive all of the recommended early assessments and are not followed longitudinally (Rice et al., 2018). Normal-appearing ZIKV-exposed children may unfortunately go unnoticed, yet may have lower neurodevelopmental scores. Understanding the greater spectrum of ZIKV-associated consequences is essential so that children do not go undiagnosed or untreated.

1.2   Evidence of Neurological Sequelae Associated With Zika Virus

Although ZIKV demonstrates a wide range of neuronal and non-neuronal tropism, infection rate is highest in intermediate progenitor cells and immature neurons in human fetal tissue, suggesting that ZIKV preferentially targets both immature neurons and neural progenitor cells (Ho et al., 2017) Previous *in vitro* and *in vivo* studies have demonstrated the ability of Zika virus to infect neural progenitor cells, inducing cell death in both central and peripheral neuronal cell populations (Chavali et al., 2017; Cugola et. al., 2016; Li et al., 2016; Tang et. al, 2016; Wu et al., 2016). Furthermore, research has also shown ZIKV infection attenuates the growth of human neural progenitor cells (hNPCs) (Xu et al., 2019). These hNPCs not only play critical roles during fetal brain development, but also persist in adult brain throughout life (Bond, Ming, and Song, 2015), suggesting that the ZIKV-mediated growth retardation potentially contributes to long-term neurodevelopmental defects through exposure to the virus. Moreover, these studies also suggest ZIKV targets different brain cells throughout the course of brain development (van del Pol et al., 2017), preferentially targeting neural progenitor cells that seed and pattern the developing cortex (Wu et. al, 2016). The neurotropic properties of ZIKV and its impact on developing neural cells raise concerns about the potential long-term neurological

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sequelae of ZIKV infection. There is a growing body of evidence which raises questions about the possibility of undetected neurological impairment among children with acquired ZIKV infection that may not be discovered unless clinicians and researchers take a closer look.

The extent to which Zika virus infection in humans causes long-term neurological damage or cognitive effects is currently unknown. The most compelling evidence to date of long-term neurological sequelae associated with ZIKV infection comes from a study of infant rhesus macaques (RMs) by Mavigner et al. (2018). The study consisted of six infants who were postnatally inoculated with ZIKV (Mavigner et al., 2018). In the infected infants, Mavigner et al. (2018) discovered the presence of ZIKV in peripheral and CNS tissues for 14 to 15 days after infections. In the same study, magnetic resonance imaging (MRI) and functional MRI conducted in two infected and two control RMs at ages three and six months showed enlarged lateral ventricles, attenuated hippocampal development, a reduction in white matter volume, and altered connectivity between the amygdala, hippocampus, and orbital frontal cortex in ZIKV infected RMs compared with control RMs (Mavigner et al., 2018). The authors also reported six month RMs showed altered emotional behavior in response to acute stress (Mavigner et al., 2018). In spite of these findings, more research is required due to the small number of animals studied, rendering comparisons between groups to be only descriptive in nature. Overall, Mavigner et al.'s (2018) study suggests the potential for long term functional and structural neurological sequelae secondary to acquired ZIKV infection.

It is becoming increasingly apparent that solely investigating the consequences of Zika in children who show overt symptoms or physical deformities provides only a

limited snapshot of the potential consequences of this virus. Moreover, it has only been a few years since the 2015 outbreak in Brazil, so our ability to study the long-term consequences of Zika exposure is limited, and therein lies the dilemma. It is therefore necessary to utilize animal models that best mirror the symptoms, the transmission, and outcomes associated with this virus in order to understand the long-term neurological consequences of prenatal ZIKV infection in the affected offspring. In the future, this may allow clinicians to identify early on which individuals are at later risk for developmental delays and neurocognitive deficits, so that they can be target these individuals for therapeutic interventions

1.3   Rat Model of Zika Virus

Our lab was the first to develop a rat model of prenatal ZIKV infection which results in vertical transmission of the virus to the fetus and a significant increase in cell death in the cortex and hippocampus of surviving affected offspring. In the model, pregnant rats are inoculated on, late gestation on embryonic day 18 (E18), using a subcutaneous injection with ZIKV to mimic a mosquito bite. This resulted in prenatal ZIKV infection that significantly impacts the developing fetus (Sherer et al., 2019). The study demonstrated that ZIKV infections in non-pregnant female rats presented a significant febrile response to ZIKV compared to diluent controls. This significant febrile response in response to ZIKV did not occur in the pregnant female rats (Sherer et al., 2019). The absence of a febrile response in the pregnant female rats suggests that the immune system of the pregnant dam does not fully respond to the viral infection, allowing for vertical transmission of ZIKV. In line with these findings, Sherer et al. demonstrate that prenatal ZIKV infection resulted in a significant increase in pup mortality, an increase in cell death, and a decrease in hippocampal and cortical

volume in the offspring two days after birth (PND 2). Furthermore, ZIKV presence in the PND 2 brain along with evidence of cerebrocortical dysplasia (Sherer et al., 2019).The ability for maternal ZIKV infection to produce these brain malformations and associated neural cell death in the affected pups suggests that the virus is not cleared by the maternal immune system and was able disrupt normal brain development in the fetus. For the current thesis, this model was used to further investigate the long-term neurodevelopmental impact of prenatal ZIKV infection on the offspring.

1.4   Aim of the Current Study

While studies mentioned above demonstrate abnormalities in certain brain structures associated with ZIKV infection, it is not fully understood 1) how structural changes in the brain correlate with active infection, 2) when these changes begin to emerge during development, and 3) how they may correlate with or predict the onset of deficits in learning and memory.

The present study represents a longitudinal analysis of brain structure during important periods of rodent brain development in order to explore the long-term neurodevelopmental outcomes of prenatal ZIKV infection in rodent offspring. Here, we utilize the rat model of prenatal ZIKV infection in conjunction with non-invasive magnetic resonance imaging to identify changes in volume among key brain regions, throughout development. We hypothesize that prenatal ZIKV infection results in persistent alterations in brain structure volumes, affecting regional brain morphometry by producing volumetric differences. Moreover, we predict to see reduced volume in specific brain regions such as the hippocampal and cortical regions due to previous studies that show ZIKV preferentially infects the cortex and hippocampus through

progenitor cells (Figueiredo et al., 2019). We also potentially expect to see alterations in the amygdala and cerebellum, based on the Mavigner et al. study (2018), which demonstrated dysfunctional connectivity in between the amygdala and hippocampus as well as ZIKV presence in neurons within the cerebellum, in nonhuman primates.

Chapter 2

MATERIALS AND METHODS

2.1   Animals and Breeding

All experiments used Sprague Dawley rats, adult males were ordered from
Envigo Laboratories (Indianapolis, IN) and adult females were ordered from Charles
River Laboratories (Wilmington, MA). Animals were housed in same sex pairs in
clear, polypropylene cages (45cm x 20.5cm x 24cm) with ad libitum access to food
and water. The colony room maintained controlled temperature and humidity, at 22°C
under a 12:12 hour light:dark cycle. Thirty nulliparous females aged 54–56 days in
age were paired individually with male rats for breeding to generate litters in various
cohorts throughout the course of the experiment. The presence of a vaginal plug was
checked daily in order to confirm pregnancy and the date of conception, embryonic
day 0 (E0). Day of birth (DOB, approximately E23) was assigned as Postnatal day 0
(P0). Male breeders were removed from cages and females were moved to a Biosafety
Level 2 (BSL 2) animal isolation facility and were individually housed in clean cages
prior to infection of the pregnant females on E18. All experiments were approved by
the University of Delaware Institutional Animal Care and Use Committee (Animal
Use Protocol #1306) under the *Guide for the Care and Use of Laboratory Animals* of
the National Institute of Health.

2.2   ZIKV growth conditions

Zika virus (strain PRVABC059, Puerto Rico, Human, December 2015) stocks
were propagated in Vero cells and serially-passaged to yield high titer virus stocks.
Stocks prepared for infection studies were prepared as T75 flasks of Vero cells
infected at an MOI of ~1 with cells being incubated for 72 hrs prior to harvest (based

on observed cytopathic effects). At 72 hrs post-infection, flasks were sealed with parafilm and snap frozen at -80˚C. Cells underwent three rounds of snap freezing-rapid thawing at 37˚C in a water bath. Cell supernatant medium and lysates were clarified by centrifugation at 1,500 rpm and the supernatant was filtered through 0.45 µM filter to remove cellular debris. This supernatant medium was the source of infectious virus and viral titer was determined by serial dilution on Vero cells (10^2 – 10^9), in triplicate wells of 12-well dishes plated with Vero cells. Viral titers was counted by fixing of cell monolayersat 96 hrs post-infection with 1% paraformaldehyde in 1X PBS for 1 hr, followed by three washes with 1X PBS and IFA using rat anti-Zika antibody (rat #15, diluted 1:2000).

2.3   ZIKV Inactivation

Ultraviolet (UV) radiation inactivates viruses by chemically modifying their genome. In order to inactivate ZIKV, a portion (5 mls) of the total infectious supernatant medium was removed and placed in a 60 mm dish for UV inactivation. UV-inactivation was performed using a Stratlinker crosslinker by placing the 60 mm dish (lid removed) inside the crosslinker set to 2,000 joules, constant power for 10 min at room temperature. Following inactivation, the supernatant medium was collected and divided into 1 ml aliquots, and a portion was titrated on Vero cells, as above at 10-1 to 10-4 dilutions, in triplicate. As with infectious virus, monolayers were fixed at 96 hrs post-infection and stained by Indirect Immunofluorescence Assay (IFA), as described above. Loss of infectivity was confirmed as only individual cells were IFA positive due to inoculum uptake in the absence of replication and spread to other cells.

2.4 Inoculations

Pregnant females were inoculated subcutaneously at embryonic day 18 (E18) with either a diluent control (0.1 ml of the same culture media used to grow ZIKV), ZIKV (dose of 1×10^7 PFU in 0.1 ml culture media), or inactivated ZIKV (iZIKV) (dose of 1×10^7 PFU in 0.1 ml culture media). The dose was selected based on previously published studies in mouse models as well as work that was previously published in our lab. iZIKV control was used to distinguish whether outcomes of ZIKV relied on active viral replication or from simply the presence of viral particles.

Infection on E18 of gestation was selected for the current study based on previous work in our lab indicating that during late gestation, the immune system of the pregnant female is significantly compromised and therefore unable to respond effectively to an immune challenge (Sherer, Posillico, and Schwarz, 2017). Furthermore, late gestation (E18-23) in rats is a time point during which significant brain growth occurs, similar to the second trimester equivalent in humans (Robinson and Klein, 2012; Sherer et al., 2017). Rats were inoculated on E18 based on the presence of a sperm plug on E1.

2.5 Pup Identification

Pup identification was critical to the experiment as our aim was to track developmental changes throughout each individual rat. Toe tattooing is a reliable and permanent identification method which is less invasive than alternatives. PND 2 pups were tattooed, along with the dam to prevent excessive maternal grooming of the tattoo. After being tattooed, the toes were gently wiped with dry gauze to remove any excess ink, and the animal was returned to its home cage.

2.6   Image Acquisition

During the scanning protocol, rats were anesthetized in order to prevent moving artifacts and allow for proper image acquisition. Before beginning any anesthesia procedures, all oxygen tanks and isoflurane levels were checked to ensure a sufficient amount for the day's experiments. Rats were placed in a chamber with isoflurane set to either 2.5L/min or 3.0L/min, based on age of the animal. Once anesthetized, rats were removed from the chamber and placed in the scanning bed, secured with nosecone and bite bar. The isoflurane was then adjusted down to 2.0L/min. Rats remained under anesthesia for no longer than 30 minutes for the completion of scanning.

Experiments were conducted using a 9.4 T Bruker Biospec 94/20 USR small animal MR system (Bruker BioSpin MRI, Ettlingen, Germany). Each animal was imaged on four different occasions: PND 2, PND 16, PND 24, and PND 60. Both mouse and rat brain 2 x 2 surface array coils were used, the mouse coil was used for the PND 2 and PND 16 time points, while the rat coil was used for the PND 24 and PND 60 time points. The correct positioning of each animal within the RF coil was confirmed through a series of scout images, localizing. T2-weighted imaging was chosen for the anatomical detail of the images. During, each imaging session a high-resolution anatomical data set was collected using the rapid acquisition with relaxation enhancement (RARE) pulse sequence 50 slice; 0.5 mm; field of vision [FOV] 3.0 cm; 256×256; repetition time (TR) 5 s; echo time (TE) 32 ms; 5 min acquisition time.

2.7   Brain Registration and Volumetric Analysis

We delineated 26 different ROIs by superimposing the T2 map images onto corresponding MRI Rat Brain Atlases, courtesy of Duke Center for In Vivo

Microscopy (http://www.civm.duhs.duke.edu/ratbraindevatlas/), and then extract the segmented boundaries for each individual subject. For the purposes of this experiment, we selected two significant ROIs comprising of: the hippocampus and neocortex.

Each voxel is tagged into the specific brain region based on the brain atlas registration, which is facilitated by a graphical user interface (Ekam Solutions LLC, Boston, MA). The Ekam Visualization and Analysis (EVA) tool allowing for the manipulation of images through three orthogonal views (axial, coronal and sagittal) interactively with real-time visual feedback and achieve accurate alignment with the help of internal and surface features.

Each subject voxel is mapped to a specific location within the atlas. The matrices that transformed the subject's anatomy to the atlas space were used to embed each slice within the atlas. Since atlas resolution (256×256×72) is higher than subject resolution (128×128×22), results in multiple atlas voxels may occupying a single subject voxel. To accurately classify these voxels with partial volume effects, ROIs with the greatest occupied volume within the subject is assigned to that voxel. Total volume of each region is calculated by multiplying unit volume of voxel in mm^3 by number of voxels. To account for different brain sizes all the ROI volumes were normalized by dividing each ROI volume by total brain volume for each rat.

2.8 Tissue Collection

All rats were administered an overdose of the barbiturate Euthasol (ANADA 200-071) via intraperitoneal injection. Sufficient anesthesia was assessed after the rat did not respond to a toe pinch using toothy forceps. Once anesthetized, rats were perfused with an ice cold 0.9% saline solution to remove blood from the brain tissue. After perfusion, the medial prefrontal cortex (mPFC), hippocampus (HC), half-of the

brain, spleen tissue, and serum samples were collected and were immediately flash frozen on dry ice. Tissue and serum samples were stored in a -80º freezer until further processing.

2.9   Real-Time PCR

RT-PCR was used to measure the presence of ZIKV in the UV-inactivated ZIKV (iZIKV). iZIKV PND 2 serum samples were extracted using the QIAamp Viral RNA Minikit. Viral RNA was quantified by qRT-PCR using the primers and probe previously published (Lanciotti et al., 2008): forward, 5′-CCGCTGCCCAACACAAG-3′; reverse, 5′-CCACTAACGTTCTTTTGCAGACAT-3′; probe, 5′-/56 FAM/AGCCTACCT/ZEN/TGACAAGCAATCAGACACTCAA/3IABkFQ/-3′ (Integrated DNA Technologies). The RT-PCR was performed using the iTaq Universal OneStep RT-qPCR kit on a CFX96Touch real time PCR machine. Viral presence was assessed by comparing samples to a standard curve generated using serial 10-fold dilutions of synthetic ZIKV RNA (ATCC).

2.10 Statistical Analysis

Whole brain volume (mm^3) data were analyzed using a two-way ANOVA, with inoculation as one factor (three levels: diluent, ZIKV and iZIKV) and sex as another factor (two levels: males and females). Similarly, a two-way ANOVA was used to assess volume differences in five ROIs, with inoculation and sex as factors. The ROIs included in the analysis are the hippocampus, amygdala, neocortex, cerebellum, and ventricles. Post-hoc pairwise comparisons were carried out, when appropriate, using Tukey's test with a Bonferroni correction. For the detailed ROI

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analyses, volumes were expressed as percent of whole brain and compared using two two-way ANOVAs as described above.

Chapter 3

EXAMINING ZIKV-INDUCED CHANGES IN REGIONAL BRAIN MORPHOLOGY

3.1 Experiment Overview

In this experiment, we longitudinally scanned a portion of both male and female pups from various litters representing three main treatment groups, diluent, ZIKV, and iZIKV (Figure 1). Treatment was prenatally administered on embryonic day 18 (E18) dams via subcutaneous injection. The diluent group represented a mock-infected control while the iZIKV group represented an inactivated viral presence control. On average, two males and two females were selected from each litter to become experimental subjects. Throughout the experiment, each individual rat was tracked via toe-tattooing and scanned across four time points, PND 2, PND 16, PND 24, and PND 60 (Figure 1). Rats were also weighed before each scan in order to track growth. Structural T2-weighted brain scans using a RARE pulse sequence were obtained over a period of 5 minutes using a, taking 50 slices of the whole brain from brainstem to olfactory bulb.

3.2 Experimental Design: Rationale

Longitudinal studies are particularly useful for evaluating development of disease, its relationship with various risk factors, and the outcomes of treatments over different lengths of time. Here, we are primarily interested in the role of prenatal ZIKV infection on neurodevelopment as well as long-term consequences of infection. Furthermore, it is particularly important to be able to discriminate between naturally-occurring, developmentally-dependent tissue changes and those alterations due to induced disease, within longitudinal studies. As individual organs, tissues and

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structures mature at different paces, choosing the age and observation period for the animal model of interest must be done carefully. Therefore, the time points within this experiment were chosen based on previous research that suggests their relevance in different developmental stages in rat and an estimation of corresponding human developmental stages as a comparison (Figure 2).

Additionally, it is becoming increasingly important to consider the sex as a biological variable, when applicable and relevant. Our primary motivation in using both males and females as experimental subjects was determine if there were any sex differences in neurodevelopment as a consequence of prenatal ZIKV infection. Interestingly, most studies have found males to be more susceptible to early life immune challenges than are females (Hanamsagar and Bilbo, 2016). Sex differences in microglial numbers and chemokine expression during critical developmental periods (Schwarz et al., 2012) may contribute to sexually dimorphic responses to infection early in life. Converging evidence also confirms that developmental outcomes of male fetuses exposed to prenatal and perinatal adversities are more highly impaired than those of female fetuses (Dipietro and Voegtline, 2017).

As an important control, we inoculated a separate set of pregnant female rats with inactivated ZIKV (iZIKV). Our rational for including this control is based on epidemiological research indicating maternal immune activation or prenatal infections are considered a risk factor for a number of neurodevelopmental disorders in the offspring including autism, seizures and schizophrenia (Estes and McAllister, 2016). Infections with a high risk to the developing fetus include influenza, rubella and cytomegalovirus (Vermillion and Klein, 2018). Thus there are a number of laboratories studying the impact of maternal immune activation on fetal

neurodevelopment (Kentner et al., 2019; Patterson, 2009). By inoculating dams with iZIKV at the same dose, we can test whether the more subtle effects of ZIKV infection on the fetal brain throughout development can be replicated by simple maternal immune activation alone and not an active infection transmitted from the dam to the fetus. That said, we do not expect to see these reduced volumes in the diluent or iZIKV group, on the premise that ZIKV injury to brain regions is driven by active viral infection that is replicating rather than simply the presence of viral particles stimulating a maternal immune response.
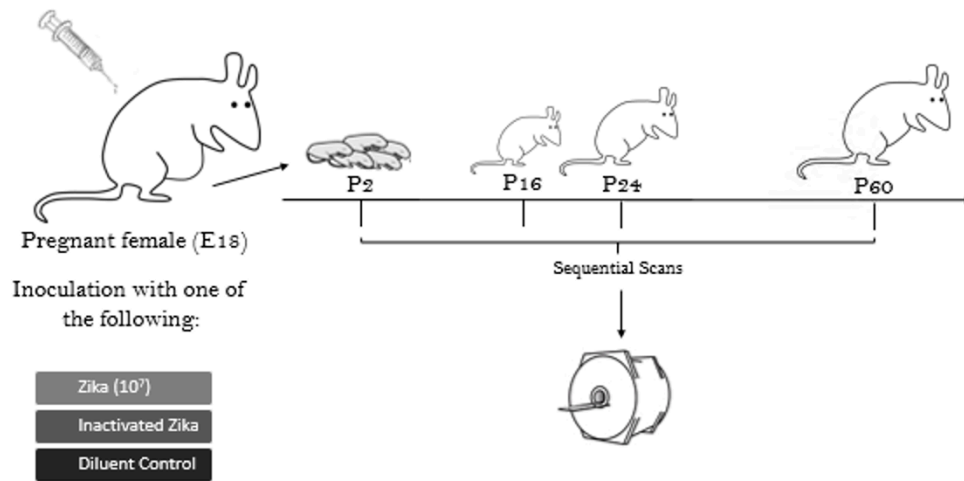
Figure 1. Experiment overview. E18 Pregnant females were inoculated with one of three possible treatments: diluent, ZIKV, or iZIKV. Once the females gave birth, litters were reduced to 8 pups, 4 per sex. Male and female offspring from each litter were then uniquely identified and tracked individually throughout the experiment. Structural MRI scans were collected on following time points: PND2, PND16, PND24, and PND60, for each rat.
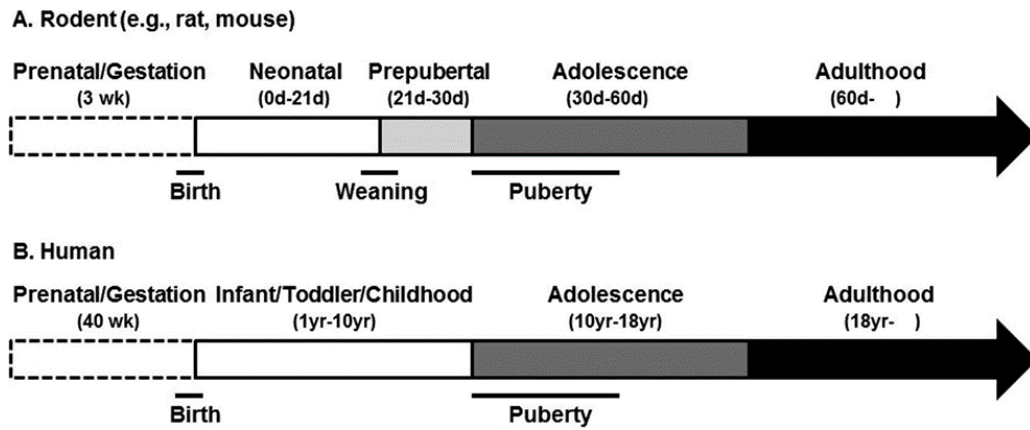
Figure 2. Timelines of development in rodents (e.g., rats and mice, A) and humans (B) along with approximate age ranges and commonly used terms for referring to those stages of development. Abbreviations: d, day; wk, week; yr, year. *Adapted from Eiland and Romeo, 2013*

3.3 Results

3.3.1 Whole Brain Volumes and ROI Analyses

Absolute volumes for segmented structures and the whole brain volume were calculated by multiplying number of the voxels belonging to the structure by voxel volume (Figures 3-6). Scaled (normalized) volumes were obtained by dividing structure volume by whole brain volume (Figures 7-8). Comparing whole brain volumes revealed no significant effect of treatment (Figure 6). Next, volumes of five ROIs were assessed in response to either diluent, ZIKV, or iZIKV treatment. The ROIs included in the analysis are the hippocampus, amygdala, neocortex, cerebellum, and ventricles. Only two ROIs, hippocampus and neocortex, yielded significant differences in volume in response to treatment. All ROI volumes were normalized by dividing each ROI volume by total brain volume for each rat. A main effect of ZIKV treatment on ROI volume was observed in the hippocampus and neocortex. There were no robust sex differences as a result of treatment.

3.3.1.1 Hippocampal Volumes

Here, we see reduced hippocampal volume at PND 16 ($F_{2,52} = 7.526$; p = 0.0013; Figure 7B), PND 24 ($F_{2,52} = 4.161$, p = 0.0211; Figure 7C), and PND 60 (: $F_{2,52} = 7.475$, p = 0.0014; Figure 7D). However, we did not see this reduced hippocampal volume at PND 2 ($F_{2,52} = 0.0781$, p = 0.9250; Figure 7A). Furthermore, we see these reductions in the hippocampus as a main effect of Treatment throughout the PND 16, PND 24, and PND 60 timepoints (Figure 7). However, it is important to note that there was no significant interaction of treatment and sex, PND 2 ($F_{2,52} = 2.828$, p = 0.0683; Figure 7A), PND 16 ($F_{2,52} = 2.034$, p = 0.1411; Figure 7B), PND 24 ($F_{2,52} = 0.2917$, p = 0.7482; Figure 7C), and PND 60 ($F_{2,52} = 0.6471$, p = 0.5277;

Figure 7D). Therefore, treatment had an effect that was not dependent upon sex. Overall, we find that the ZIKV-infected groups had smaller hippocampal volume than their diluent and inactivated control counterparts for the later timepoints (PND 16 - PND 60) (Figure 7). However, no significant hippocampal volume reduction was observed in the PND 2 group (Figure 7A).

3.3.1.2 Cortical Volumes

Cortical volumes were also analyzed alongside hippocampal volumes. Interestingly, we see significantly reduced cortical volume at PND 16 ($F_{2,52} = 7.881$, p = 0.0010; Figure 8B). We did not find a significant reduction in cortical volumes for the PND 2 (Treatment: $F_{2,52} = 2.812$, p = 0.0693; Figure 8A), PND 24 ($F_{2,52} = 2.417$, p = 0.0901; Figure 7C), and PND 60 ($F_{2,52} = 1.092$, p = 0.3423; Figure 8D). Although, we see a trending decrease in cortical volume for the PND 2 and PND 24 timepoints. Furthermore, we again emphasize that there was no significant interaction of treatment and sex for the cortex brain region, PND 2 ($F_{2,52} = 0.6769$, p = 0.5126; Figure 8A), PND 16 ($F_{2,52} = 0.5069$, p = 0.6053; Figure 8B), PND 24 ($F_{2,52} = 0.2374$, p = 0.7895; Figure 8C), and PND 60 ($F_{2,52} = 0.1966$, p = 0.8221; Figure 8D). Overall, we find that the ZIKV-infected groups had smaller cortical volumes than their diluent and inactivated control counterparts during the juvenile timepoint, PND 16 (Figure 8B). However, no significant hippocampal volume reduction was observed in the PND 2, PND 24, and PND 60 ZIKV-infected group (Figure 8).

3.3.2 Validating iZIKV Control

Here, we used qRT-PCR to measure iZIKV in the brain and serum of PND 2 offspring that were prenatally exposed to iZIKV. The PCR assay was able to detect the

inactivated ZIKV in the positive control samples, but it was not detected in any of the PND 2 brain tissue or serum samples. These results indicate that the virus did not transmit from mother to baby, in line with our predictions.
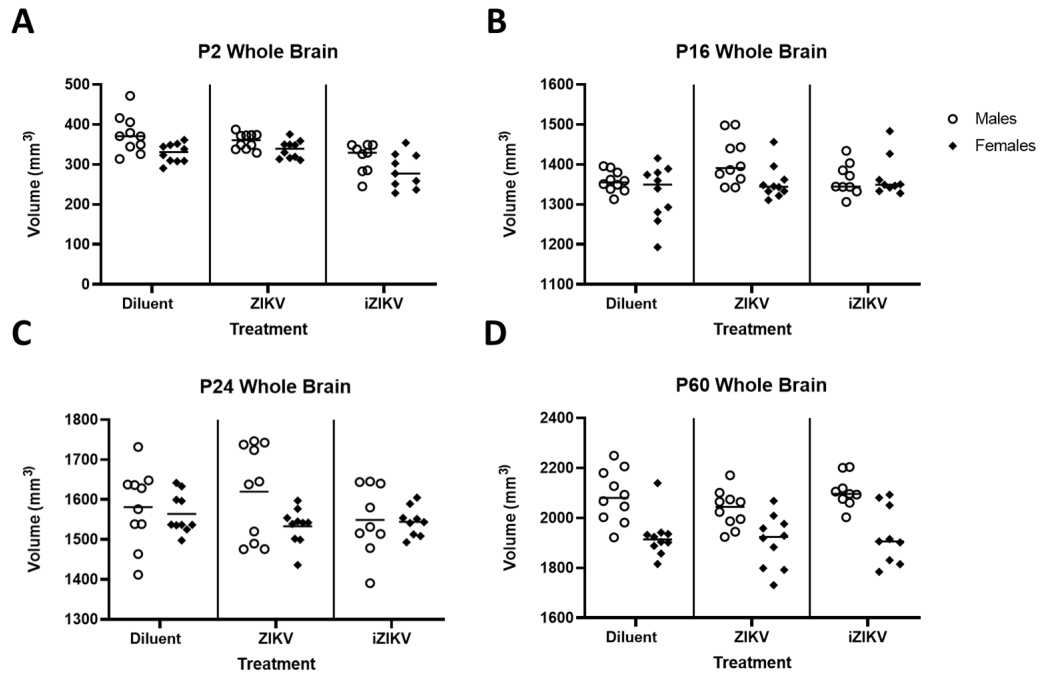
Figure 3. Absolute Whole Brain Volumes. Whole brain volume (mm³) for each individual rat throughout all experimental time points (PND 2-PND 60). Lines represent means, while each dot represents an individual value. (A) Postnatal day 2, (B) Postnatal day 16, (C) Postnatal day 24, (D) Postnatal day 60.
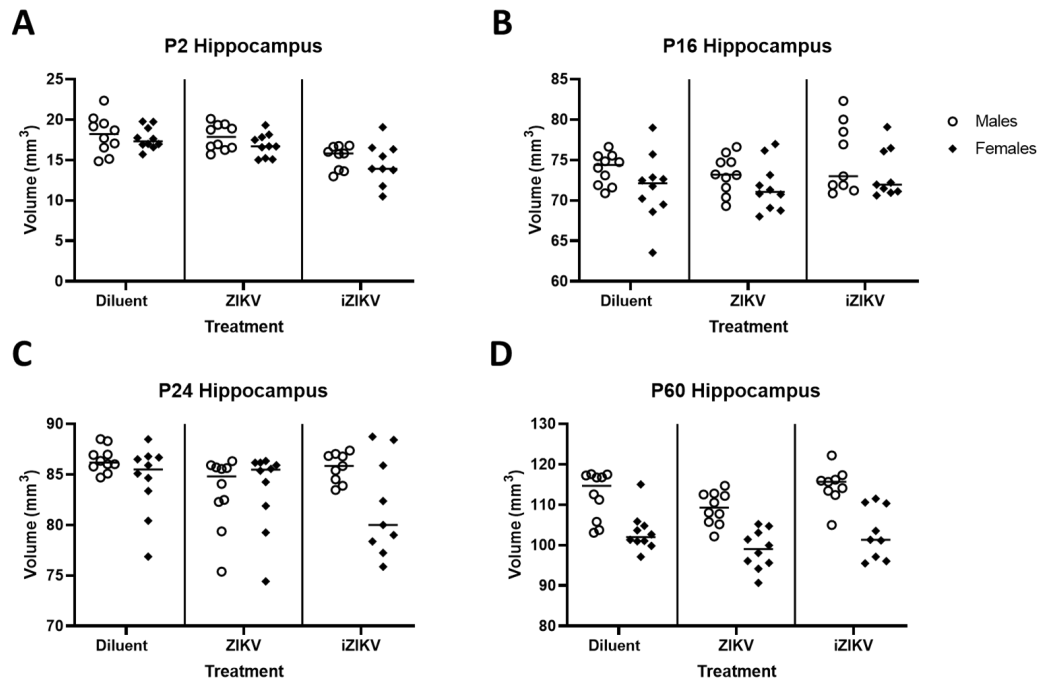
Figure 4. Absolute Hippocampal Volumes. Hippocampus volume (mm³) for each individual rat throughout all experimental time points (PND 2-PND 60). Lines represent means, while each dot represents an individual value. (A) Postnatal day 2, (B) Postnatal day 16, (C) Postnatal day 24, (D) Postnatal day 60.
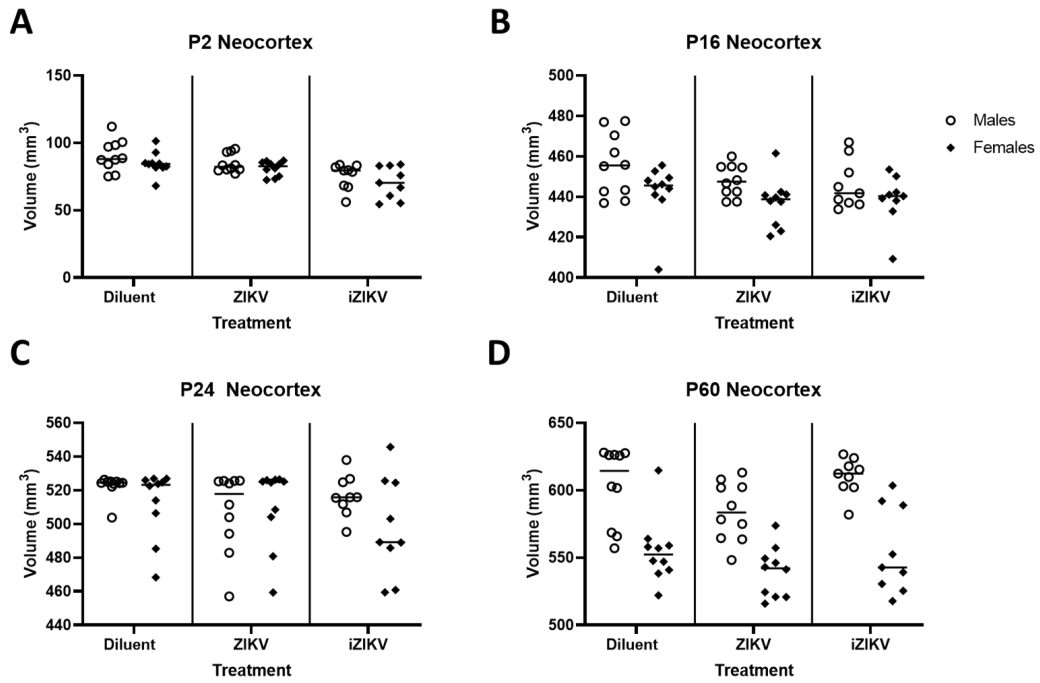
Figure 5. Absolute Cortical Volumes. Cortex volume (mm^3) for each individual rat throughout all experimental time points (PND 2-PND 60). Lines represent means, while each dot represents an individual value. (A) Postnatal day 2, (B) Postnatal day 16, (C) Postnatal day 24, (D) Postnatal day 60
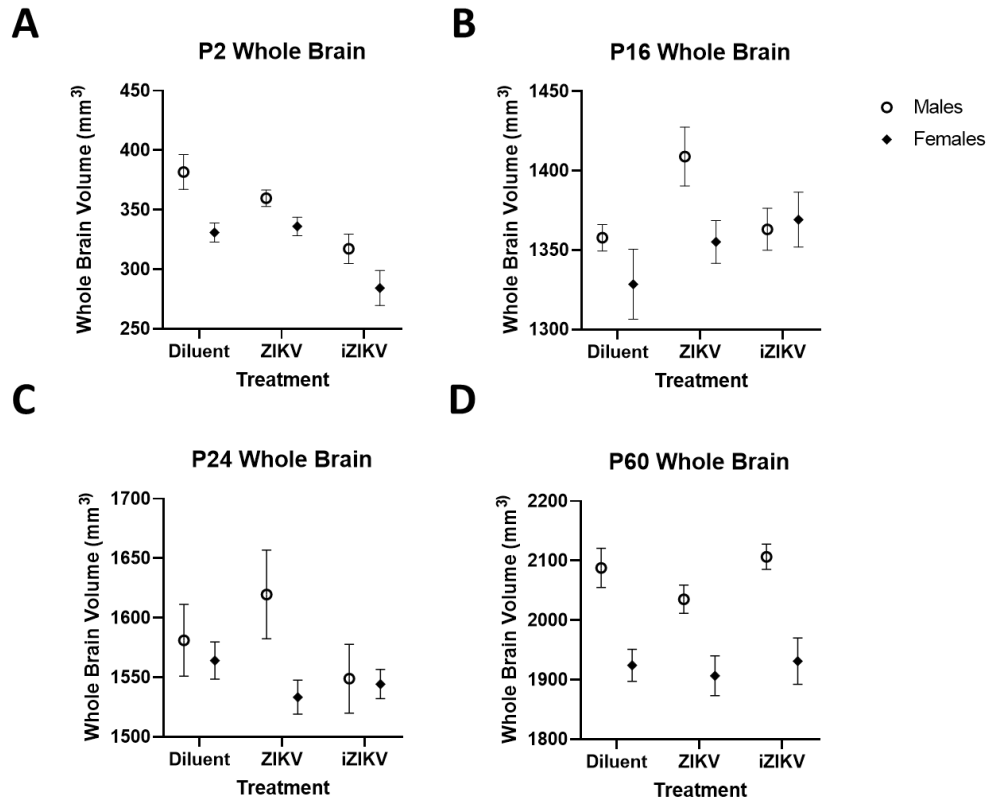
Figure 6. Absolute Whole Brain Volumes. Averaged brain volume (mm^3) data are shown for all experimental time points (PND 2-PND 60). Whole brain volumes were calculated by multiplying number of the voxels belonging to the brain structure by voxel volume (A) Postnatal day 2, Treatment: $F_{2,52} = 14.14$; $p < 0.0001$, Sex: $F_{1,52} = 15.71$; $p = 0.0002$, Treatment x Sex: $F_{2,52} = 0.8052$; $p = 0.4525$ (B) Postnatal day 16, Treatment: $F_{2,52} = 3.037$; $p = 0.0565$, Sex: $F_{1,52} = 3.787$; $p = 0.0571$, Treatment x Sex: $F_{2,52} = 1.694$; $p = 0.1937$ (C) Postnatal day 24, Treatment: $F_{2,52} = 0.8040$; $p = 0.4530$, Sex: $F_{1,52} = 3.063$; $p = 0.0860$, Treatment x Sex: $F_{2,52} = 1.542$; $p = 0.2236$ (D) Postnatal day 60, Treatment: $F_{2,52} = 1.357$; $p = 0.2663$, Sex: $F_{1,52} = 40.00$; $p < 0.0001$, Treatment x Sex: $F_{2,52} = 0.3297$; $p = 0.7206$. Statistical analysis revealed a significant no main effect of treatment in either males or females. Data in the figure represents means ± SEM, *p < 0.05 indicates the main effect of ZIKV infection on regional brain volume.
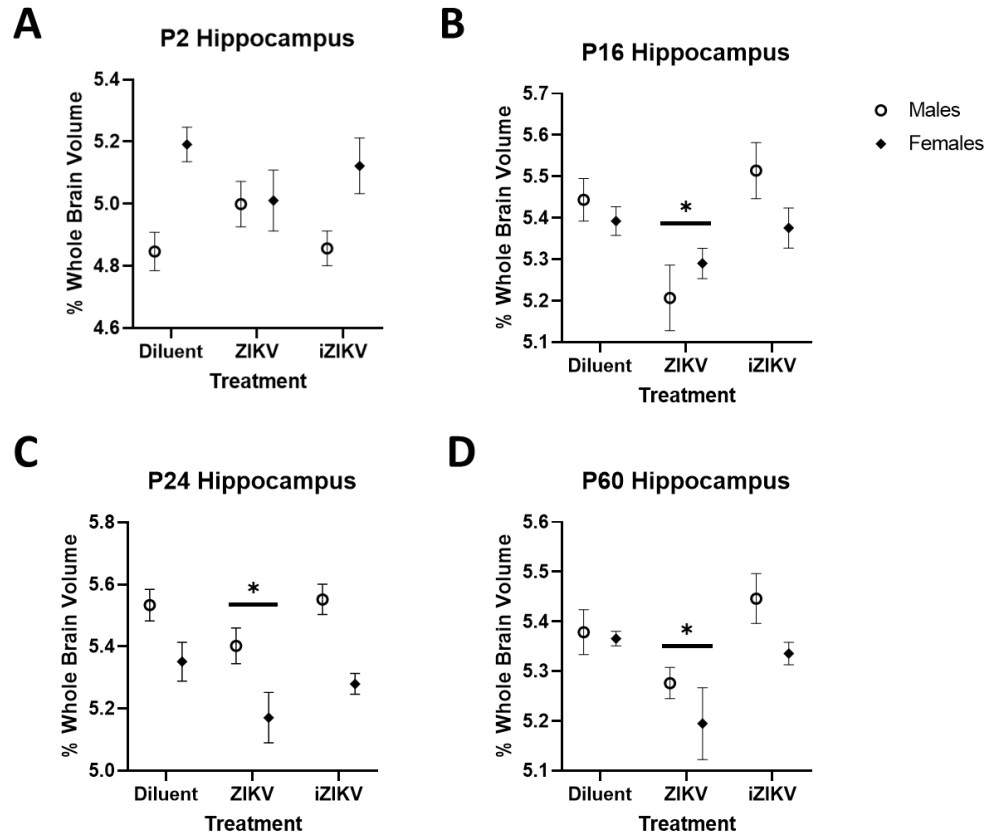
Figure 7. Relative Hippocampal Volumes. Hippocampal volume (mm^3) for each individual rat was divided by whole brain volume (mm^3) to normalize data, accounting for various brain sizes. Average relative hippocampal volumes expressed as percent whole brain volume, are shown for all experimental time points (PND 2-PND 60), for males and females in each of the three treatment groups. (A) Postnatal day 2, Treatment: $F_{2,52} = 0.0781$; $p = 0.9250$, Sex: $F_{1,52} = 11.68$; $p = 0.0012$, Treatment x Sex: $F_{2,52} = 2.828$; $p = 0.0683$ (B) Postnatal day 16, Treatment: $F_{2,52} = 7.526$; $p = 0.0013$, Sex: $F_{1,52} = 0.6177$; $p = 0.4355$, Treatment x Sex: $F_{2,52} = 2.034$; $p = 0.1411$ (C) Postnatal day 24, Treatment: $F_{2,52} = 4.161$; $p = 0.0211$, Sex: $F_{1,52} = 22.74$; $p < 0.0001$, Treatment x Sex: $F_{2,52} = 0.2917$; $p = 0.7482$ (D) Postnatal day 60, Treatment: $F_{2,52} = 7.475$; $p = 0.0014$, Sex: $F_{1,52} = 3.580$; $p = 0.0641$, Treatment x Sex: $F_{2,52} = 0.6471$; $p = 0.5277$. Statistical analysis revealed a significant decrease in cortex volume at PND 16, PND 24, and PND 60. Data in the figure represents means ± SEM, *$p < 0.05$ indicates the main effect of ZIKV infection on regional brain volume.
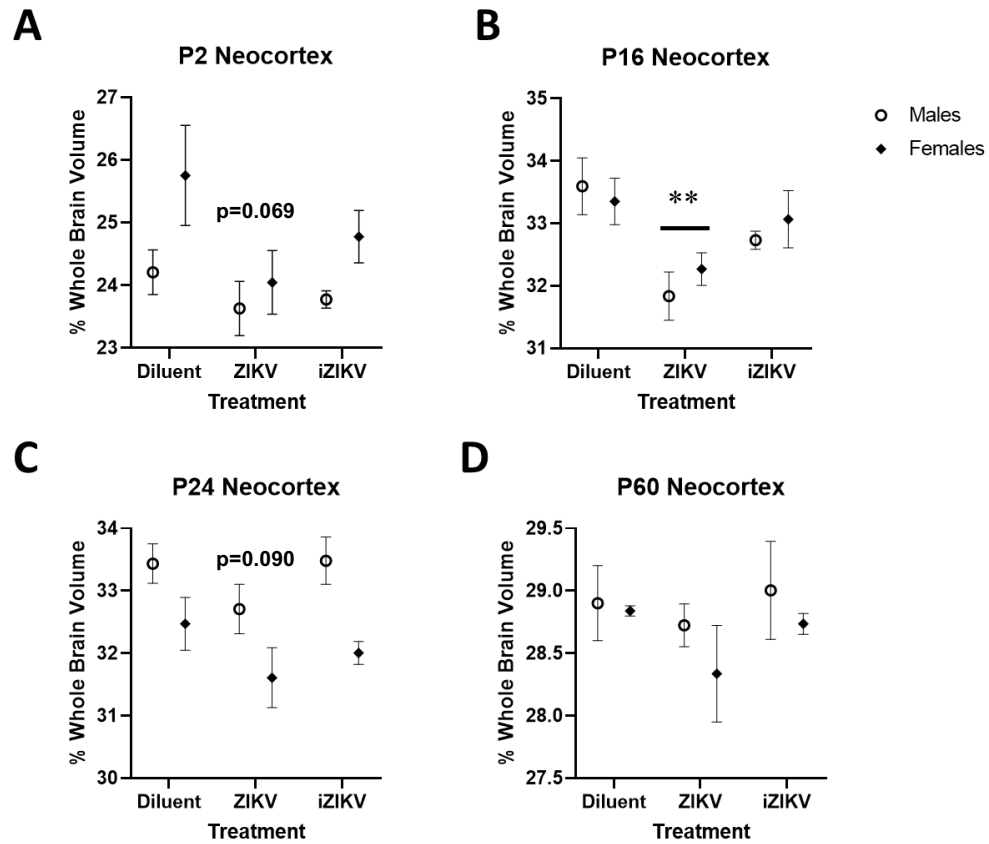
Figure 8. Relative Cortical Volumes. Cortex volume (mm^3) for each individual rat was divided by whole brain volume (mm^3) to normalize data, accounting for various brain sizes. Average relative cortical volumes expressed as percent whole brain volume are shown for all experimental time points (PND 2-PND 60), for males and females in each of the three treatment group.(A) Postnatal day 2, Treatment: $F_{2,52}$ = 2.812; p = 0.0693, Sex: $F_{1,52}$ = 5.980; p = 0.0179, Treatment x Sex: $F_{2,52}$ = 0.6769; p = 0.5126 (B) Postnatal day 16, Treatment: $F_{2,52}$ = 7.881; p = 0.0010, Sex: $F_{1,52}$ = 0.3424; p = 0.5610, Treatment x Sex: $F_{2,52}$ = 0.5069; p = 0.6053 (C) Postnatal day 24, Treatment: $F_{2,52}$ = 2.417; p = 0.0901, Sex: $F_{1,52}$ = 14.41; p = 0.0004, Treatment x Sex: $F_{2,52}$ = 0.2374; p = 0.7895 (D) Postnatal day 60, Treatment: $F_{2,52}$ = 1.092; p = 0.3423, Sex: $F_{1,52}$ = 1.201; p = 0.2782, Treatment x Sex: $F_{2,52}$ = 0.1966; p = 0.8221. Statistical analysis revealed a significant decrease in cortex volume at PND 16. Data in the figure represents means ± SEM, **: p<0.001 indicates the main effect of ZIKV infection on regional brain volume.

Chapter 4

DISCUSSION

4.1 Brief Summary of Our Findings

This study presents a longitudinal analysis of brain structure after prenatal ZIKV infection. The overarching aim of this investigation was to determine how structural changes in the brain correlate with active ZIKV infection during important periods of brain development and when these changes begin to emerge throughout development. Using structural MRI scans, we segmented 26 different ROIs for each individual time point, further analyzing the hippocampus, amygdala, neocortex, cerebellum, ventricles, and whole brain volumes. Here, we report a persistent reduction in hippocampal volume in the ZIKV infected group, compared to age-matched controls for all time points except PND 2 (Figure 7). We also find the neocortex to be significantly reduced in the ZIKV infected group during the PND 16 time point (Figure 8). It is interesting to note that there were overall reductions in neocortex volume within the ZIKV infected group during the three other time points, PND2, PND 24 and PND 60; however, these reductions were not statistically significant. Moreover, these findings could be in line with increasing evidence from clinical and experimental studies suggest that ZIKV infection can lead to long-term neurological complications as a consequence of exposure. Our results also resonate with previous studies that demonstrate ZIKV preferentially infects progenitor cells in both the cortical and hippocampal subventricular zones (Cugola et al., 2016; Li et al., 2016; Tang et al., 2016; Wu et al., 2016).

4.2   Neurodevelopmental Impact of Prenatal Zika Virus Infection

Here, we analyzed 26 segmented brain regions based on the postnatal neurodevelopmental atlas from Duke Center for In Vivo Microscopy. We extensively analyzed structures that were previously implicated in ZIKV research as targets for viral injury. Comparing regional brain morphology, a reduction in the brain volume was seen in the hippocampal and cortical (neocortex) regions in prenatally infected ZIKV rats. We did not find significant volume reductions in the whole brain, amygdala, cerebellum, or ventricles. These finding suggest that the hippocampus and cortex are especially vulnerable to ZIKV infection during certain developmental periods.

Relative total cortex volume in the ZIKV treatment group was significantly smaller than in diluent and iZIKV controls for the PND 16 time point. This is particularly interesting, as we did not note significant volume reduction in the latter time points PND 24 and PND 60. Longitudinal MRI studies in rat brain have shown cortical thickness reaches final value at 1 month, while volume increases of cortex, striatum and whole brain end after two months (Mengler et al., 2014). Therefore the cortex is actively developing until PND 60, yet we did not observe any significant differences. Taken together with previous research demonstrating that ZIKV infects radial glial cells of dorsal ventricular zone (DVZ), which are the primary neural progenitors responsible for cortex development (Tang et al., 2016; Wu et al., 2016), we expected to see significant long term alterations in the cortex carried throughout PND 24 and PND 60. Could the effects of prenatal ZIKV infection be somehow partially rescued or alleviated by catch-up growth in the cortex through an unknown mechanism in later life? A more plausible explanation might point to limitations in analyzing the cortex as a whole rather than further segmenting the cortex into well-

defined regions. To that end, it would be interesting to apply cortex segmentation in order to determine if there is a reduction in almost all of cortical structures volume or few specific key regions, as a result from ZIKV exposure. Certain sub regions of the cortex may be more vulnerable to prenatal ZIKV infection than others.

Apart from the neocortex, reduced hippocampal volume was observed in the ZIKV treatment group when compared to both the diluent and iZIKV groups for all time points besides PND 2. This is contrary to previous results from our lab which demonstrate that prenatal ZIKV exposure leads to an increase in apoptosis in the hippocampus and cortex, as well as cerebrocortical dysplasia, in the PND 2 brain (Sherer et al., 2019). One possible explanation to account for these differences may be the unique challenge in employing *in vivo* MRI imaging at such a young time point. Our method of brain registration and volumetric analysis may not have sensitive enough to account for differences in such small regional brain structures as well as brain volumes. There is also exceptional variability in litter sizes and pup development at PND 0. While we controlled for differences in various brain sizes by normalizing brain structures to individual brain volumes, we also attempted to control variability in developmental rates by culling each litter to an *n* of 8 at PND 1. Emerging evidence indicates that culling, especially of large litters, can drastically change the feeding status of a pup, which can result in compensatory growth as well as rapid equalization of body weights (Agnish and Keller, 1997). However, this compensatory growth cannot happen within one day, as pups were scanned the very next day at PND 2. In similar fashion to the neocortex, the hippocampus was not further delineated into subregions such as the dentate gyrus, CA4, CA3, CA2, CA1, and the subiculum, denoting another possible limitation. Here, we hope to again apply further

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segmentation into well-defined regions in order to determine if certain sub regions are more susceptible to prenatal ZIKV infection.

4.2.1 Prenatal ZIKV Injury Requires Active Replication

Hippocampal and cortical reduction was not seen in the diluent or iZIKV control groups. It is particularly interesting to note there were no significant differences between the diluent and iZIKV group. PCR results confirmed our expectation that the inactivated virus would not transmit from mother to offspring. This finding suggests that ZIKV injury to these brain regions results from active infection that is driven by viral replication rather than maternal immune response to the presence of viral particles. In line with our predication, subtle effects of ZIKV infection on the fetal brain cannot be replicated by simple maternal immune activation alone.

4.2.2 Sex Differences in Prenatal ZIKV Infection

We did not find any sex differences as a result of ZIKV infection impact on regional brain morphometry. Furthermore, sex differences in the neurobehavioral consequences of developmental ZIKV infection in humans have not yet been reported. However, there is still potential for conditions such as attention deficit hyperactivity disorder and impulsive behaviors, to emerge when the current population of children exposed to ZIKV in utero reaches 5–6 years of age. A recent study in mice suggests there are sex-dependent differences in long-term behavioral abnormalities, including hyperactivity, impulsiveness, and motor incoordination, stemming from ZIKV exposure (Snyder-Keller et al., 2019). Furthermore, study in mice, found ZIKV

infected females to be more affected in terms of social behavior, whereas males were more impaired during a motor task (Nem de Oliveira Souza et al., 2018).

4.3   Potential for Long-Term Neurocognitive Deficits

The hippocampus which is important for learning, memory, cognition, as well as emotion and stress response, begins to develop during mid- to late gestation and continues developing postnatally through childhood to young adulthood in humans (Boldrini et al., 2018; Kempermann et al., 2018; Sorrells et al., 2018; Spalding et al., 2013). In fact, adult hippocampal neurogenesis first observed in rodents (Altman, 1962), also is confirmed to exist in humans and non-human primates by several groups (Eriksson et al., 1998; Kempermann et al., 1997; Kuhn et al., 1996). Additionally, there is also emerging evidence indicating that ZIKV is capable of disrupting the function of neural progenitor cells or NPCs and causing neural stem cell death (Chavali et al., 2017; Cugola et. al., 2016; Li et al., 2016; Tang et. al, 2016). Furthermore, the areas of the brain that house these susceptible NPCs are the hippocampus and the anterior forebrain, responsible for learning, memory, and behavior (Li et al., 2016). Li et al. found that ZIKV infection reduced neuroprogenitor cells (NPCs) proliferation and induced apoptotic cell death in the subgranular zone of the hippocampus and the subventricular zone of the anterior forebrain of infected mice. While we did not investigate volumetric differences in the anterior forebrain, we observed blunted hippocampal growth in three of the four time points, (PND 16, PND 24, and PND P60). Furthermore, NPCs mature into brain cells through various stages of differentiation and maturation, much of this occurring during fetal brain development, but also continuing throughout childhood and well into adulthood (Stiles and Jernigan, 2010). The differentiation and maturation of NPCs carrying out

throughout adulthood taken to together with our observations, suggest the potential for long-term deficits in learning and memory.

As mounting evidence suggests, maternal-fetal ZIKV exposure lead to loss of neural stem and progenitor cells, causing neurogenic arrest (Adams Waldorf et al., 2018). It can also lead to the disruption of neural circuitry in the dentate gyrus (DG) a key hippocampal sub region (Adams Waldorf et al., 2018). In comparison, postnatal ZIKV exposure was associated with an overall alteration and arrest in hippocampal growth as well as dysfunctional connectivity with other brain regions such as the amygdala, leading to abnormal socioemotional behavior in older animals (Mavigner et al., 2018). Paradoxically, adult hippocampal neurogenesis is thought to only occur in the subgranular zone (SGZ) of the dentate gyrus of rodents and humans (Nicola, Fabel, and Kempermann, 2015). The dentate gyrus whose development is set apart from other hippocampal sub regions, has several unique features including, in some sense, 'never-ending' development (Nicola et al., 2015). Although similar structures exist in other species, the mammalian dentate gyrus is unique in its connectivity and involvement of adult neurogenesis (Kempermann et al., 2018). It is hypothesized that the dentate gyrus seen in modern mammals developed late phylogenetically and develops late ontogenetically (Kempermann et al., 2018). Taken altogether, prenatal ZIKV exposure leads to a disruption in neural circuitry in the dentate gyrus, of which the subgranular zone is responsible for life-long neurogenesis. We postulate this could be a possible mechanism explaining blunted hippocampal growth late into young adulthood, suggesting long term deficits in learning and memory.

Hippocampal injuries would not only lead to perturbations to later learning and memory development, but also be expected to correlate with the reported early onset

of seizures or epilepsy in human infants, and potentially even later-onset depression and premature age-related cognitive decline (Kempermann et al., 2018; Toda, Parylak, Linker, and Gage, 2018). In fact, early MRI studies measured hippocampal volume in various human subjects providing important information about several neuropsychiatric disorders (Geuze, Vermetten, and Bremner, 2005). Smaller hippocampal volumes have been reported in epilepsy, Alzheimer's disease, dementia, mild cognitive impairment, schizophrenia, major depression, borderline personality disorder, obsessive–compulsive disorder, antisocial personality disorder, among other disorders (Geuze et al., 2005). Unfortunately, specific types of hippocampal injuries are not easily detected in humans by standard prenatal MRI (Walker et al., 2019). Instead, requiring specialized and repeated postnatal MRI scans to monitor hippocampal developmental or perhaps postnatal clinical tests measuring learning and memory in ZIKV-exposed non-microcephalic infants. Here, our study does implicate long-term alterations in hippocampal development by demonstrating reduced hippocampal volumes over prolonged period of time.

While we observed long-term reduction in hippocampal volumes, we only reported significantly reduced cortical (neocortex) for the PND 16 time point. Despite this, we believe it is critical that nonmicrocephalic children exposed to Zika virus in utero or early life should also undergo long-term monitoring for higher order neurocognitive deficits. Previous research has discovered Zika virus infects radial glia cells (RGs) of dorsal ventricular zone of the fetuses, the primary neural progenitors responsible for cortex development (Tang et al., 2016). Research further indicated that ZIKV preferentially targets human neuronal precursor cells (NPCs) in both monolayer and cortical brain organoid culture systems and stunts their growth (Zhang et al.,

2019). Zhang et al. used a murine model to demonstration that prenatal ZIKV infection affects neural stem cells fate and leads to a thinner cortex and a smaller brain. More compelling evidence of potential cortical injury comes from the Mavigner et al. study (2018), where ZIKV presence was detected in frontal cortex, parietal cortex, and occipital cortex 14 days after postnatal ZIKV infection. Therefore, long-term neurocognitive consequences arising from ZIKV induced cortical injury should not be ruled out.

4.4   Conclusions

The findings in this thesis support the mounting body of evidence pointing to long-term neurological sequelae and reinforce the importance of long-term clinical surveillance in individuals exposed to ZIKV. Moreover, our work demonstrates persistent brain structural consequences arising from prenatal ZIKV infection. In line with previous findings, we show that prenatal ZIKV infection targeted hippocampal and cortical regions of the brain, demonstrated by significant volume reduction in these regions during certain periods of development. Of greater interest, is our finding showing distinct volume reductions in the hippocampal brain regions over a prolonged period of time, suggesting lasting hippocampal injury. This is particular concerning as hippocampal injury is associated with a myriad of adverse outcomes. Consequently, ZIKV-associated hippocampal injury may predispose individuals to learning disorders, developmental delay, and/or mental illnesses later childhood and adolescence. It is increasingly apparent that early detection and intervention in ZIKV exposure cases is crucial, alongside long-term clinical monitoring, allowing clinicians to provide the best neurodevelopmental outcome to those affected from ZIKV exposure. Overall, this research sheds further insight into potential long-term neurological consequences of

prenatal ZIKV infection. While we hope that this study will facilitate therapeutic approaches to alleviate the neurologic consequences of ZIKV infection, further longitudinal studies are needed to understand the far-reaching implications of ZIKV-exposure in affected individuals.

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Appendix

APPROVAL FOR THE USE OF ANIMAL SUBJECTS

UNIVERSITY OF DELAWARE. | Office of Laboratory Animal Medicine | Life Science Research Facility
79 E. Delaware Avenue
Newark, DE 19711
Phone: 302-831-2616
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To: Office of Graduate and Professional Education

From: Gwen Talham, DVM, Director, Animal Care Program

Subject: IACUC approval for Rita Patel

Date: 4/17/2020

Rita Patel was approved by the IACUC to work with animals on Jackie Schwarz's protocol #1306 "Effects of ZIKV Infection on the Fetal Brain". Please contact me at 831-2980 or gtalham@udel.edu with any additional questions.

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