Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter now, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Muskan Ali March 23, 2024

The Impact of Zika Virus Infection During Infancy on Attachment in a Rhesus Macaque Model

by

Muskan Ali

Jessica Raper
Adviser

Neuroscience and Behavioral Biology

Jessica Raper

Adviser

Ann Chahroudi

Committee Member

Andrew Kazama

Committee Member

2024

The Impact of Zika Virus Infection During Infancy on Attachment in a Rhesus Macaque Model

by

Muskan Ali

Jessica Raper

Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Science with Honors

Neuroscience and Behavioral Biology

2024

Abstract

The Impact of Zika Virus Infection During Infancy on Attachment

in a Rhesus Macaque Model

by Muskan Ali

ZIKV infection in rhesus macaques (RMs) during infancy has been linked with increased ventricle size and decreased amygdala and hippocampal volumes, areas important for social and emotional development, compared to controls (Mavinger, et al, 2018; Raper, et al, 2020). Previous studies have found that attachment to a primary caregiver can impact social and emotional development in later life (Harlow & Zimmermann, 1959; Hofer, 1994; Sullivan, Perry, Sloan, Kleinhaus, & Burtchen, 2011). RMs with neonatal lesions to the amygdala and hippocampus have previously been shown to have subtle alterations in bond strength to a primary caregiver. This study assesses the impact of ZIKV infection during early infancy on bonding with a primary human caregiver (PHC) in RMs at 7-8 months of age. ZIKV infection did not impact the development of a preference for the PHC over a familiar human (FH) stimulus compared to uninfected and poly-IC controls (PIC). There was a decreased frequency of reactive vocalizations in the ZIKV group compared with the PIC group and an increased frequency of affiliative behaviors in females from the ZIKV group compared to females from the PIC group. These data suggest that brain damage caused by ZIKV infection during early infancy is less severe than a neonatal amygdala lesion.

The Impact of Zika Virus Infection During Infancy on Attachment in a Rhesus Macaque Model

By

Muskan Ali

Jessica Raper

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Science with Honors

Neuroscience and Behavioral Biology

2024

Acknowledgements

I would like to thank Dr. Jessica Raper for her support and guidance throughout this project and my entire time with her lab. I would like to thank all members of the Raper lab for their support throughout this project and my entire time with the Raper lab.

Table of Contents

Hypothesis:

The present study hypothesized that Zika virus (ZIKV) infection in rhesus macaques (RMs) during infancy would lead to a weaker bond with the primary human caregiver at 7-8 months of age when compared with age-, sex-, and rearing-matched poly-IC and uninfected controls. Specifically, there would be decreased affiliative behaviors in ZIKV infected infants when compared with control infants although the index of preference score is expected to remain consistent between all groups.

Purpose and Rationale:

This project was part of a larger study to understand the potential impact of ZIKV neurotropism on early brain development in a RM model. The current project aimed to investigate whether social attachment bonds would be altered by early postnatal ZIKV infection. Prior studies have found that RMs with ZIKV infection during infancy exhibited altered brain development, including increased ventricle size and decreased volume of the hippocampus and amygdala (brain areas important for social and emotional behavior) as compared with uninfected controls (Mavinger, et al, 2018; Raper, et al, 2020). Additionally, early postnatal lesion studies in RMs have found that amygdala damage is associated with decreased attachment bond strength with the primary caregiver when compared to normally developing controls (Bauman et al, 2004, Goursand & Bachevalier 2007; Goursand et al, 2014). Alterations in the attachment of an infant to a caregiver can impact long-term neurodevelopment, including emotionality, cognition and mental health later in life (Harlow & Zimmermann, 1959; Hofer, 1994; Sullivan, Perry, Sloan, Kleinhaus, & Burtchen, 2011). To assess whether altered brain

development after postnatal ZIKV infection leads to deficits in attachment bond strength, similar to neonatal lesions, attachment to a primary human caregiver was assessed for infant RMs infected with ZIKV at one month of age as compared to age-, sex-, and rearing-matched viral mimic (poly-IC) and uninfected controls.

Background and History of Zika Virus:

Zika virus (ZIKV) is a Flavivirus related to Japanese encephalitis, West Nile, dengue, and yellow fever viruses, which are all primarily transmitted by blood-sucking mosquitoes. ZIKV was first isolated in 1947 from a febrile sentinel RM in Uganda in Zika forest (Gubler et al, 2017). In 1948, ZIKV was isolated from a pool of *Aedes africanus* mosquitoes near the same site (Gubler et al, 2017). The first confirmed human ZIKV infection is suggested to also be in Uganda in 1962-1963 (Wikan & Smith 2007). Since its isolation, ZIKV spread from equatorial Africa and Asia to the Pacific Islands, then further to South and Central America and the Caribbean (Yun & Lee 2017). Currently, ZIKV is circulating in Latin America, the Pacific Islands, and Southeast Asia (Yun & Lee 2017). Over the past decade, ZIKV has gained more attention due to outbreaks.

The first major outbreak of ZIKV outside of Africa and Asia occurred in April 2007 (Song et al, 2017). After this outbreak, most cases of ZIKV infection occurred in small clusters until the second major outbreak in October 2013 in French Polynesia (Cao-Lormeau et al, 2016). In early 2015, ZIKV was detected in Brazil after which it spread across South and Central America and the Caribbean Islands (Song et al, 2017). While ZIKV infection generally causes only mild symptoms in most infected individuals, it is associated with a range of neuroimmunological disorders, including Guillain-Barré

syndrome, meningoencephalitis, and myelitis (Song et al, 2017). Despite not causing major complications in most infected people, during the 2015 outbreak in South America, it was discovered that ZIKV infection during pregnancy results in congenital Zika syndrome (CZS) which has a range of symptoms including microcephaly and loss of brain tissue, ventriculomegaly and increased extra-axial fluid, brain tissue calcifications, cortical thinning and decreased myelination, seizures, eye defects, hearing impairments, movement and feeding difficulties, and impaired growth (Moore, et al., 2017).

Like all Flaviviruses, ZIKV infiltrates host cells through clathrin-mediated endocytosis, a vesicular trafficking process that transports the virus from the cell surface into the cytoplasm (see review Agrelli, et al, 2019). Importantly, *in vivo* and *in vitro* studies have shown that multiple cell types are susceptible to ZIKV infection, including skin fibroblasts, uterine fibroblasts, primary placental trophoblasts and Hofbauer cells, endometrial stromal cells, male reproductive cells (e.g. spermatogonia, Sertoli and Leydig cells), as well as cerebral cortex, and neural progenitor cells (Agrelli, et al, 2019). Once the host is infected, ZIKV spreads almost exclusively cell-to-cell and may spread through exosomes and through tunneling nanotubes to neighboring cells (Ferraris et al, 2019). *In vivo* studies of newborn mice found that microinjection of ZIKV intracerebrally can infect the hippocampus, cerebellum and cerebral cortex (Wang, et al, 2017), whereas unilateral injection into one hemisphere of the somatosensory cortex resulted in a mirror infection in the other hemisphere indicating the virus can move by axonal transport to synaptically coupled brain loci (van den Pol et al, 2017). ZIKV also differentially infects human neural progenitor cells according to their state of

differentiation leading to dysregulated neurogenesis (Ferraris et al, 2019). Therefore, CZS may be caused by impaired brain development via affecting neural progenitor cells (Caires-Júnior et al, 2018). Taken together these data suggest that ZIKV targets the CNS and may target different cells at different stages of fetal brain development. However, the brain is not fully mature at birth and undergoes rapid postnatal growth, but little is known about the potential impact of ZIKV infection during early postnatal development.

Postnatal Development and Neonatal Lesions:

Brain development occurs across the lifespan. The brain starts forming a few weeks after conception but does not reach full maturity until early adulthood (Tierney & Nelson 2009). The basic structure of the brain is laid down during the prenatal and early childhood period, while the formation and refinement of neural networks occurs over time (Tierney & Nelson 2009). The brain undergoes rapid postnatal development over the first year of life, including increased myelination, synaptogenesis, and maturation of brain structures important for complex behaviors (van Dyck & Morrow 2017). Basic sensation and perception systems are fully developed around five years of age, while other systems such as those involved in memory, decision making, social and emotional regulation continue to develop into early adulthood (Tierney & Nelson 2009). Therefore, the principles of anatomical change are not only essential to the development of the brain but are also responsible for the development of behaviors. Synaptic pruning and myelination contribute to improved precision and speed of coordinated movement and the development of cognitive skills (Luna 2009). The foundations of neural systems critical to social behavior and emotional skill are formed in early childhood and strongly

influenced by experiences during this time. Brain areas important for social and emotional behavior exhibit a particularly protracted development, including the amygdala and hippocampus. In RMs the amygdala reaches a peak linear growth around 8 months of age, but continues to develop into adulthood (Payne et al, 2010; Schumann et al, 2019). Hippocampal development is more extended, reaching its peak linear growth after 2 years of age (Payne et al, 2010; Hunsaker et al, 2014. Refinement in social and emotional behavior appears to coincide with the development of these brain areas (Kalin et al, 1991; Mendelson et al, 1982; Raper et al, 2013). The amygdala contributes to the processing of socially and emotionally relevant information (Šimić 2021), and the hippocampus coordinates the activity of regions involved in social and affective processing (Immordino-Yang & Singh, 2013). Considering the critical nature of the early infancy period in the development of the brain which in turn impacts behavior and socioemotional regulation, assault to the brain during early life could have deleterious impacts on brain development and subsequently on behavior and socioemotional regulation.

Researchers have used selective lesion studies in animal models to understand the long-term impact of early postnatal brain insults. Previous studies of RMs with temporal lobe lesions during infancy found that early postnatal damage to the amygdala or hippocampus did not impact their preference for primary caregivers (either human caregiver or mother) (Goursaud & Bachevalier, 2007; Goursaud, et al, 2014), others found that early amygdala damage resulted lower social attachment to a primary caregiver (i.e. mother) compared to controls (Bauman, et al, 2004). Interestingly, despite lacking a strong preference for their primary caregiver (mother) infant RMs with

early amygdala lesions exhibited increased physical contact with their primary caregiver (mother) during home cage and social group observations (Bauman et al, 2004). These data suggest that infant monkeys with amygdala lesions lack the ability to discern the level of threat under different environmental conditions. Follow-up studies revealed the long-term effects of early amygdala lesions resulted in a lack of ability to modulate their emotional reactivity based on the level of threat presented during the human intruder paradigm (Raper et al, 2013), suggesting that early amygdala damage alters threat detection and behavioral modulation. In contrast, the long-term impact of hippocampal lesions resulted in persistent increased anxiety-related behaviors on multiple tasks (Raper et al, 2017; McKeon et al, 2022), suggesting that despite a normal social attachment hippocampal development plays an important role in emotional reactivity and anxiety overall. Taken together these data suggest that insults to the brain during this vulnerable period pose a risk for altered behavioral expression later in life. Thus, considering the neurotropic nature of ZIKV it is possible that infection during early postnatal development could have a deleterious impact on brain and behavior development.

Postnatal ZIKV Infection:

Infants and children can be infected with ZIKV through mosquito bites and breast milk; however, there is little data on ZIKV in children (see review Raper and Chahroudi 2021). A meta-analysis of pediatric ZIKV infections found that most children have mild symptoms, but severe neurologic complications and death have also been reported (Ramond et al, 2020). A study of 20–30-month-old Colombian children who were infected with ZIKV during early infancy (between 1-12 months old), 15.0% had adverse

outcomes on the neurologic, hearing, or eye examination (Pacheco et al, 2017). These findings suggest that the neurotropism of ZIKV can lead to adverse neurodevelopment for young brains and indicate an urgency to further investigate the consequences of ZIKV infection during infancy. Considering how little is known about the long-term impact of ZIKV infection during infancy, animal models are greatly needed to help close that knowledge gap.

Multiple studies focused the RM model for its immune system, brain development and complex behaviors which are similar to humans (Mavigner, et al, 2018; Raper et al, 2020). They found that despite a relatively mild infection (e.g. no fever or rash) with quick viral clearance from blood, ZIKV was present in brain tissues at 14 days post-infection resulting in astrogliosis and apoptosis in infant RM infected with ZIKV at 5 weeks of age (Mavigner et al 2018). Importantly, this neurotropism had a significant impact on long-term brain development resulting in structural and functional anomalies were detected up to 12 months after postnatal infection (Mavigner et al, 2018; Raper et al, 2020). ZIKV infection during infancy resulted in ventriculomegaly, blunted hippocampal growth, and weaker amygdala–hippocampus functional connectivity (FC) compared to uninfected age, sex, and rearing-matched control infant RMs (Raper et al, 2020) resulted in ventriculomegaly, blunted hippocampal growth, and weaker amygdala–hippocampus functional connectivity (FC) compared to uninfected age, sex, and rearing-matched control infant RMs (Raper et al, 2020).

Changes in brain development and function after postnatal ZIKV infection resulted in significant alterations in the behavior of the infant RMs. The most pronounced behavioral difference was increased emotional reactivity detected as early

as 6 months of age and persisted through the juvenile period at 12 months of age. Unlike controls, ZIKV-infected RMs lacked the species typical response on the human intruder task (Mavigner et al, 2018), took longer to habituate to a novel social play cage and avoided spending time in the center of the social play cage (Raper et al, 2020). Interestingly, ZIKV-infected RMs also preferred to spend more time apart and engaged in fewer affiliative behaviors with a highly familiar partner as compared to controls. Overall, these studies suggest that deficits in early neural development resulting from postnatal ZIKV infection leads to emotional dysregulation and decreased prosocial behaviors.

Considering that postnatal ZIKV infection impaired the development of the amygdala and hippocampus, it is possible that ZIKV during infancy may also lead to a weaker attachment bond with a PHC and result in increased emotional dysregulation.

Materials and Methods:

This study was conducted in strict accordance with U.S. Department of Agriculture regulations and the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, approved by the Emory University Institutional Animal Care and Use Committee, and conducted in an AAALAC accredited facility.

Animals:

Twenty-four Indian RMs (Macaca mulatta) were used in this study. Twelve were exposed to ZIKV at 1 month of age, 6 served as age-, sex-, and rearing-matched uninfected controls, and 6 served as age-, sex-, and rearing-matched innate immune

stimulated (poly-IC administration as a viral mimic) controls. The infants were delivered naturally by their dams while housed in indoor/outdoor social groups at the Emory National Primate Research Center (ENPRC) Field Station colony. Infants were then removed from their dams at 5–10 days of age and transported to the ENPRC Main Station nursery facility. Maternal factors (age, body weight, immune) have been shown to impact infant temperament, behavior, and immune responses, thus the dams in this study were similar between the groups (Bauman et al, 2014; Kong et al, 2018). Dams had a range in maternal age (4-16 years) and parity (1–13 infants), but this was spread equally between dams of control infants (maternal age: 4-14 years; parity: 1-9 infants), and dams of ZIKV-infected infants (maternal age: 4-16 years; parity: 2-13 infants). All dams had similar body weights during pregnancy (4.62-12.6 kg), were from the specific pathogen-free colony (negative for Herpes B, SIV, SRV, and STLV1), had not been previously used for infectious diseases or vaccine studies, and did not have any clinical signs of infection during pregnancy. All infants were pair-housed in warming incubators for the first 3 to 4 weeks with visual and auditory contact with additional conspecifics. Infants were hand-fed formula every 2–3 h for the first month, then via self-feeders for the next 3 months as per standard ENPRC protocol. Soft blankets, plush toys, and fleece surrogate were provided and changed daily. Soft chow and fruits were introduced starting at 1 month of age, and by 4 months, formula was discontinued, and all were fed a diet of Purina Primate Chow, Old World Monkey formulation, supplemented with daily fruits and vegetables. Water was provided ad libitum. At 4 weeks of age, infant pairs transitioned into age-appropriate caging with hanging fleece surrogate. At 7-8 weeks of age, they began socialization in larger groups of 4 to 6 age-matched peers. Infants were

pair housed according to their treatment group (ZIKV or control), as in other infectious diseases studies in infant RMs. Specifically, ZIKV-infected RM infants were paired together and likewise for the uninfected controls. ZIKV-infected infants were housed in an ABSL-2+ (Animal Biosafety Level 2) room until cleared of virus in blood and urine. Control infants were housed in the same room once the ZIKV-infection was cleared, which increased visual contact socialization and controlled for environmental factors. Precautions were taken to ensure that control and ZIKV-infected infants received similar housing, nutrition, and enrichment. In addition, control infants were subjected to the same sedation and collection procedures (blood and urine) as ZIKV-infected infants to minimize any differences in exposure to stressful experiences. All housing was indoors on a 12 h light–dark cycle.

Viral infection and clearance:

Twelve infant RMs were given a subcutaneous injection of 10^5 plaque-forming units (pfu) of the Puerto Rican strain of ZIKV (PRVABC59). Blood samples were collected at 2, 3, 5, 7, 10, and 14 days post-infection (dpi) to determine peak viral load and clearance via qPCR in the Suthar laboratory.

Behavioral Testing:

To examine social attachment and emotional behavior, subjects were presented with a two-choice discrimination task at approximately 8 months of age. The cage set up was a 10’ x 5’ rectangular social play cage similar to the mother preference cage previously published by Bauman et. al, 2004. The infant RM was taken from the home

cage and transported to the testing cage in a plastic animal transport box. The infant's primary human caregiver (PHC) sat on the outside at the end of the cage furthest away from the infant release door, and the familiar human (FH) was placed on the outside at the opposite end of the cage. At the onset of the trial, the infants are released into the testing cage from the transport box and allowed to freely move around the cage for 10 minutes. A 2.5' X 2.5' area inside the cage immediately in front of the PHC and FC were used as "proximity zones" for behavioral scoring.

Behavioral Scoring and Preference Assessment:

Behaviors were scored using The Observer XT version 15 software (Noldus, Sterling, VA) and validated ethogram of infant rhesus macaque behaviors (Table 1). All attachment tests were scored by one trained observer (MA) with a high interrater reliability (Cohen's Kappa = 0.91) with another expert observer (JR). To assess whether the infant monkeys displayed a preference for their primary caregiver, we analyzed the following parameters: 1) index of preference, 2) total time in proximity to PHC and latency to approach PHC, and 3) frequency of affiliative behaviors. The index of preference is calculated by finding the difference between the time spent in each proximity zone and dividing by the total time spent in both proximity zones. This results in a preference score ranging from –1 to +1 which allows for assessing whether the infant has a preference for the PHC (+1) as opposed to the FH (-1) or no preference (0). Affiliative behaviors include cooing, grunting, and reaching and indicate the quality of the relationship with the PHC. Additionally, anxious and hostile behaviors including yawning, freezing, self-directed behaviors, and threat barks, were also scored.

Statistical Analysis:

Linear mixed models were used with Group (ZIKV-1mo, Uninfected Controls, Viral Mimic Control), and Sex (female, male) as fixed factors and individual subjects as a random variable. All analyses were conducted with SPSS 27 for Windows and significance was set at $p<0.05$.

Results:

Attachment bond strength was primarily measured using the Index of Preference, latency to enter the proximity zone of the PHC, and Affiliative behaviors. ZIKV infection did not impact the Index of Preference for the PHC as shown by the lack of a significant effect of Group ($F[2, 18]=2.13$, $p=0.15$; Figure 1A). There was also no effect of Sex ($F[1,18]=0.69$, $p=0.42$), or interaction between Group by Sex ($F[2,18]=0.60$, $p=0.56$) on Index of Preference. Latency to prox the PHC did not differ between ZIKV-infected and controls (Group: ($F[2,18]=1.17$, $p=0.33$; Figure 1B), as well as no difference by Sex ($F[1,18]=0.16$, $p=0.69$), or interaction between Group and Sex ($F[2,18]=0.62$, $p=0.55$). Interestingly, there was a significant Group by Sex interaction for affiliative behaviors ($F[2,18]=7.016$, $p=.006$; Figure 1C), such that ZIKV infected females exhibited more affiliative behaviors than poly-IC females ($p=0.035$), but not uninfected control females ($p=0.98$).

Potential changes in emotional regulation after postnatal ZIKV infection were examined using anxiety behaviors, duration of self-directed, and reactive vocalizations (i.e. screams and threat barks). Infant RMs infected with ZIKV did not differ from controls on their expression of anxiety behaviors (Group: $F[2,18]=1.19$, $p=0.33$; Figure

1D). Anxiety also did not differ by Sex ($F[1,18]=0.153$, $p=0.70$), and there was no significant Group by Sex interaction ($F[2,18]=2.62$, $p=0.10$). The amount of time that infant RMs spent engaged in self-directed behaviors did not differ between Group ($F[2,18]=1.06$, $p=0.37$; Figure 1E), Sex ($F[1,18]=0.012$, $p=0.92$), or interaction between Group and Sex ($F[2,18]=.01$, $p=0.99$). There was a significant main effect of Group ($F[2, 18]=4.11$, $p=0.034$; Figure 1F) for reactive vocalizations, such that ZIKV infected infants emitted fewer reactive vocalizations compared to poly-IC controls ($p=0.038$), but not uninfected controls ($p=0.31$). There was no significant Sex effect ($F[1,18]=0.040$, $p=0.84$) nor interaction between Group and Sex ($F[2,18]=0.64$, $p=0.54$) for reactive vocalizations emitted.

Discussion:

The present study revealed that postnatal ZIKV infection does not alter the social attachment bond in infant RMs at 7-8 months of age. ZIKV-infected, uninfected and viral mimic control infant RMs all demonstrated a strong preference for their primary caregiver, as shown by the index of preference and latency to enter the proximity zone. Although, the ZIKV infected females exhibited more affiliative behaviors than viral mimic females, there was no significant difference from the uninfected controls. ZIKV-infected infant RMs did not differ from controls in their anxiety expression in the current task. Lastly, ZIKV-infected infants emitted fewer reactive vocalizations than poly-IC infants. Taken together the present results suggest that postnatal ZIKV infection did not impact social attachment, but altered social and emotional behavior in subtle ways.

Attachment of an infant to a caregiver is critical during early development, as altered attachment can impact long-term neurodevelopment, including emotionality, cognition and mental health later in life (Harlow & Zimmermann, 1959; Hofer, 1994; Sullivan, Perry, Sloan, Kleinhaus, & Burtchen, 2011). The current study found that in infant RMs, ZIKV infection at 1 month of age did not impair their ability to form an attachment to a PHC, resulting in similar attachments compared to uninfected and viral mimic control infants reared in the same environment. ZIKV infection during infancy has previously been shown to result in ventriculomegaly, blunted amygdala and hippocampal growth, as well as weaker amygdala–hippocampus functional connectivity compared to uninfected age, sex, and rearing-matched control infant RMs (Raper et al, 2020). Unlike infant RMs with neonatal amygdala lesions that have weaker mother preference (Bauman et al, 2004), ZIKV-infected infant RMs exhibit a strong preference for their primary caregiver that is similar to that of uninfected and viral mimic controls. Given the inconsistency with the findings from Bauman and colleagues (2004), the current study suggests that ZIKV associated brain damage is less severe as compared to a direct lesion to the amygdala during infancy. Additionally, the lack of difference between uninfected and viral mimic controls suggests that early life immune activation alone is not sufficient to impact attachment formation in infant RMs.

ZIKV-infected RM females exhibited more affiliative behaviors, demonstrated by reaching for the PHC, cooing, and grunting, compared to viral mimic (poly-IC) females. Behavioral sex differences after early brain damage have been previously detected, such that amygdala lesioned RM females exhibited an early emergence of independence from their mother during social group observations (Raper, et al, 2014).

Yet, the same amygdala lesioned RM infants reached for their mothers less than control animals during the attachment task (Goursand et al, 2014). The increased affiliative behavior seen in the current study is similar to hypersociability seen in adult monkeys with neonatal hippocampal lesions (Bliss-Moreau et al, 2013). However, behavioral changes after ZIKV may be more subtle than those of selective brain lesions. ZIKV neurotropism merely impairs growth and functional connectivity, which is less severe than a direct lesion. Importantly, the lack of significant difference between viral mimic and uninfected control females suggests that early immune activation alone is not sufficient to impact affiliative behaviors in infant RMs.

Previous studies have found that RMs with neonatal amygdala lesions produced fewer distress vocalizations compared to controls (Bauman et al, 2007; Goursand et al, 2014). A similar decrease in reactive vocalizations during the attachment task was found in postnatal ZIKV-infected RMs. Decreased aggressive signals have been found in RMs with neonatal amygdala lesions (Bliss-Moreau et al, 2013). The current findings in combination with previous lesions studies, suggest that postnatal ZIKV neurotropism does not lead to sufficient CNS damage to alter social attachment, but it does impact emotional vocal expressions. A longitudinal study in humans found a positive association between consistently high behavioral inhibition in childhood and adolescent anxiety symptoms among those with insecure attachment in infancy, suggesting a correlation between early attachment and emotional dysregulation later in life (Lewis-Morrarty et al, 2015). Our previous data found that ZIKV-infected infant RMs exhibit increased emotional reactivity and decreased social behavior as juveniles (Mavigner et al, 2018; Raper, et al, 2020). However, the current data suggests that the previous

findings of emotional dysregulation after postnatal ZIKV infection are not due to abnormal attachment among the ZIKV infected infant RMs. Instead, it appears that CNS damage from postnatal ZIKV infection leads to specific changes in emotional regulation outside of caregiver attachment.

Conclusions:

The current study found that postnatal ZIKV infection in infant RMs does not alter preference for a primary human caregiver when compared to uninfected or viral mimic controls, suggesting that damage caused by ZIKV infection during infancy is not sufficient to alter social attachment. ZIKV animals demonstrated decreased reactive vocalizations, as exhibited by fewer screams and threat barks, a finding that is consistent with previous neonatal amygdala lesion studies (Bauman et al, 2004; Goursand & Bachevalier 2007). Overall, these results suggest that there are subtle behavioral impacts of brain deficits caused by ZIKV infection during early infancy. Since ZIKV is endemic in mosquito populations and recent studies have indicated that ZIKV infection during early infancy may pose a threat for the rapidly developing postnatal brain, it is necessary to protect infants from ZIKV infection (Caires-Júnior et al, 2018; Raper et al, 2020; Ramond et al 2020; Pacheco et al 2021).

Table 1. Behavioral Ethogram

Behavior	Measurement	Definition
Proximity	Duration (seconds)	Coded when RMs approach a predetermined 2.5' X 2.5' area inside the cage immediately in front of the PHC or FC
Latency	Duration (seconds)	Amount of time before RMs approach the PHC after entering the testing cage
Affiliative Behaviors	Cumulative Frequency	Total frequency of grunts, coos, and reaches
Grunt	Frequency	Soft, guttural sound produced in affiliative encounters
Coo	Frequency	High-pitched, soft vocalization
Reach	Frequency	Subject stretches hand(s), arm(s) or leg(s) towards PHC or FH
Reactive Vocalizations	Cumulative Frequency	Total frequency of threat barks and screams
Threat Bark	Frequency	Low-pitched, guttural, rasping, low frequency sound often accompanied by some sort of threat posture
Scream	Frequency	High-pitched, high-intensity vocalization indicating fear or distress
Self-directed	Cumulative Duration	Self-directed behaviors such as eye poke or sucking thumb, plus self-groom and self-clasp
Self-Groom	Duration (seconds)	Use of hands to pick through or lick a fur or non-fur body part.

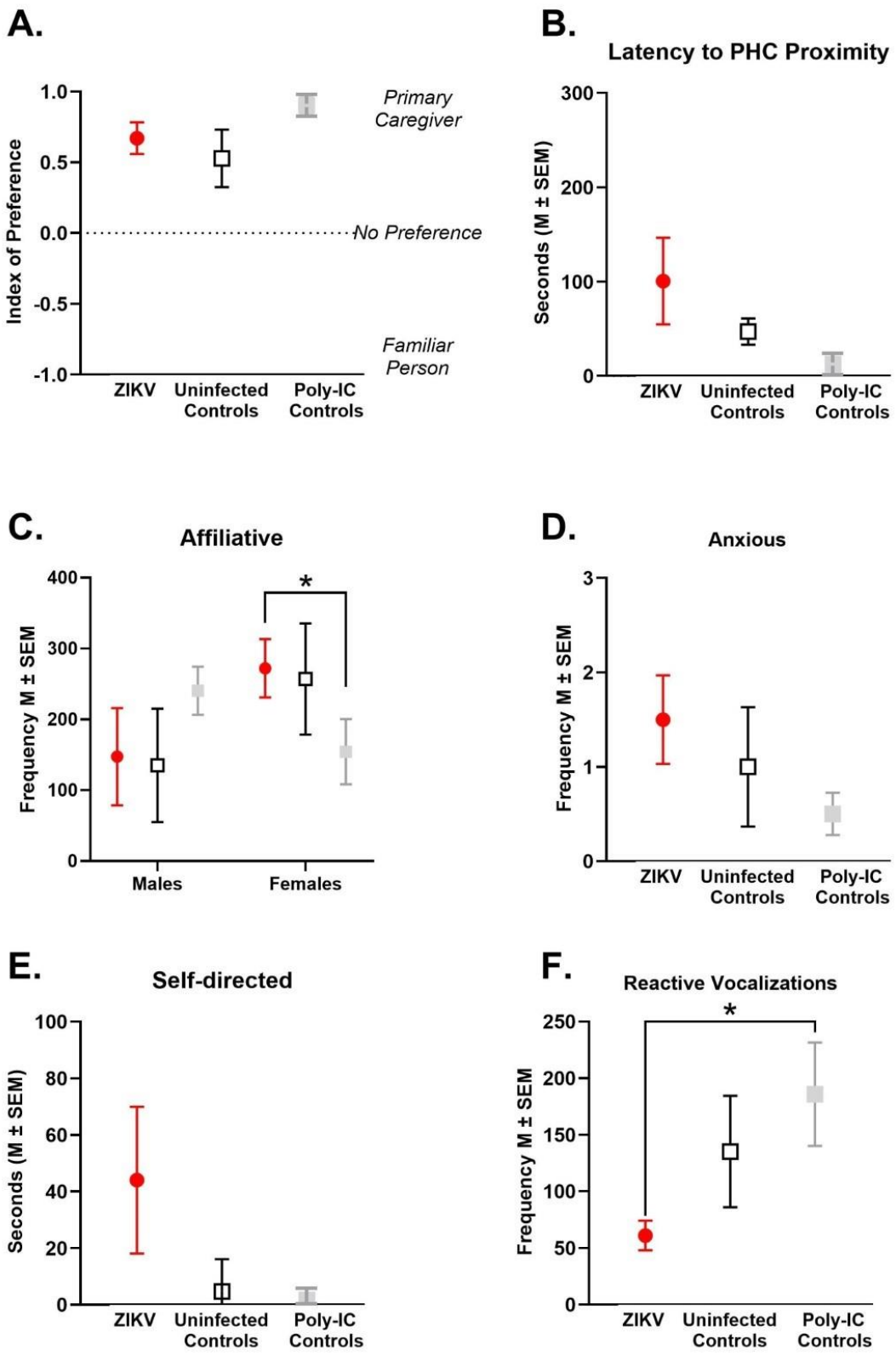
Self-Clasp	Duration (seconds)	Non-manipulatory enclosing or holding of a limb or body part with another body part
Anxiety	Cumulative Frequency	Total frequency of scratches and body shakes
Body Shake	Frequency	Rapid shaking of head and shoulders
Scratch	Frequency	Rapid hand movements, using fingers to scratch own body
Yawn	Frequency	Wide open mouth
Toothgrind[1]	Duration (seconds)	Repetitive audible rubbing of upper and lower teeth

[1] indicates behavior that was never detected during testing.

Figure Legends:

Figure 1. Index of Preference (A), latency to enter proximity zone of the primary human caregiver (PHC) (B), frequency of affiliative behaviors (C), frequency of anxious behaviors (D), duration of self-directed behaviors (E), frequency of reactive vocalizations (F). Rhesus macaques (RM) infected with Zika virus (ZIKV) are represented by shaded red circles, uninfected controls (UIC) are represented by unshaded black squares, and poly-IC (PIC) are represented by shaded gray squares. * indicates significant effect of group $p < 0.05$.

Figure 1.


A.
Index of Preference
1.0
0.5
0.0
-0.5
-1.0
Primary Caregiver
No Preference
Familiar Person
ZIKV
Uninfected Controls
Poly-IC Controls
B.
Latency to PHC Proximity
Seconds (M ± SEM)
300
200
100
0
ZIKV
Uninfected Controls
Poly-IC Controls
C.
Affiliative
Frequency M ± SEM
400
300
200
100
0
*
Males
Females
D.
Anxious
Frequency M ± SEM
3
2
1
0
ZIKV
Uninfected Controls
Poly-IC Controls
E.
Self-directed
Seconds (M ± SEM)
100
80
60
40
20
0
ZIKV
Uninfected Controls
Poly-IC Controls
F.
Reactive Vocalizations
Frequency M ± SEM
250
200
150
100
50
0
*
ZIKV
Uninfected Controls
Poly-IC Controls

References:

Bachevalier, J., Sanchez, M., Raper, J., Stephens, S. B. Z., & Wallen, K. (2016). The effects of neonatal amygdala lesions in rhesus monkeys living in a species-typical social environment. In D. G. Amaral & R. Adolphs (Eds.), *Living without an amygdala* (pp. 186–217). The Guilford Press.

Bauman, M. D., Lavenex, P., Mason, W. A., Capitanio, J. P., & Amaral, D. G. (2004). The development of mother-infant interactions after neonatal amygdala lesions in rhesus monkeys. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 24(3), 711–721. https://doi.org/10.1523/JNEUROSCI.3263-03.2004

Bauman, M. D., Iosif, A. M., Smith, S. E., Bregere, C., Amaral, D. G., & Patterson, P. H. (2014). Activation of the maternal immune system during pregnancy alters behavioral development of rhesus monkey offspring. *Biological psychiatry*, 75(4), 332–341. https://doi.org/10.1016/j.biopsych.2013.06.025

Bliss-Moreau, E., Moadab, G., Bauman, M. D., & Amaral, D. G. (2013). The impact of early amygdala damage on juvenile rhesus macaque social behavior. *Journal of cognitive neuroscience*, 25(12), 2124–2140. https://doi.org/10.1162/jocn_a_00483

Caires-Júnior, L. C., Goulart, E., Melo, U. S., Araujo, B. H. S., Alvizi, L., Soares-Schanoski, A., de Oliveira, D. F., Kobayashi, G. S., Griesi-Oliveira, K., Musso, C. M., Amaral, M. S., daSilva, L. F., Astray, R. M., Suárez-Patiño, S. F., Ventini, D. C., Gomes

da Silva, S., Yamamoto, G. L., Ezquina, S., Naslavsky, M. S., Telles-Silva, K. A., … Zatz, M. (2018). Discordant congenital Zika syndrome twins show differential in vitro viral susceptibility of neural progenitor cells. *Nature communications*, 9(1), 475. https://doi.org/10.1038/s41467-017-02790-9

Cao-Lormeau, V. M., Blake, A., Mons, S., Lastère, S., Roche, C., Vanhomwegen, J., Dub, T., Baudouin, L., Teissier, A., Larre, P., Vial, A. L., Decam, C., Choumet, V., Halstead, S. K., Willison, H. J., Musset, L., Manuguerra, J. C., Despres, P., Fournier, E., Mallet, H. P., … Ghawché, F. (2016). Guillain-Barré Syndrome outbreak associated with Zika virus infection in French Polynesia: a case-control study. *Lancet* (London, England), 387(10027), 1531–1539. https://doi.org/10.1016/S0140-6736(16)00562-6

Duane J Gubler, Nikos Vasilakis, Didier Musso, History and Emergence of Zika Virus, *The Journal of Infectious Diseases*, Volume 216, Issue suppl_10, 15 December 2017, Pages S860–S867, https://doi.org/10.1093/infdis/jix451

Ferraris, P., Cochet, M., Hamel, R., Gladwyn-Ng, I., Alfano, C., Diop, F., Garcia, D., Talignani, L., Montero-Menei, C. N., Nougairède, A., Yssel, H., Nguyen, L., Coulpier, M., & Missé, D. (2019). Zika virus differentially infects human neural progenitor cells according to their state of differentiation and dysregulates neurogenesis through the Notch pathway. *Emerging microbes & infections*, 8(1), 1003–1016. https://doi.org/10.1080/22221751.2019.1637283

Goursaud, A. P., & Bachevalier, J. (2007). Social attachment in juvenile monkeys with neonatal lesion of the hippocampus, amygdala and orbital frontal cortex. *Behavioural brain research*, 176(1), 75–93. https://doi.org/10.1016/j.bbr.2006.09.020

Goursaud, A. P., Wallen, K., & Bachevalier, J. (2014). Mother recognition and preference after neonatal amygdala lesions in rhesus macaques (Macaca mulatta) raised in a semi-naturalistic environment. *Developmental psychobiology*, 56(8), 1723–1734. https://doi.org/10.1002/dev.21233

Harlow, H. F., & Zimmermann, R. R. (1959). Affectional responses in the infant monkey. *Science*, 130, 421–432. https://doi.org/10.1126/science.130.3373.421

Hofer, M. A. (1994). Hidden regulators in attachment, separation, and loss. *Monographs of the Society for Research in Child Development*, 59(2-3), 192–207, 250–283. https://doi.org/10.2307/1166146

Hunsaker, M. R., Scott, J. A., Bauman, M. D., Schumann, C. M., & Amaral, D. G. (2014). Postnatal development of the hippocampus in the Rhesus macaque (Macaca mulatta): a longitudinal magnetic resonance imaging study. *Hippocampus*, 24(7), 794–807. https://doi.org/10.1002/hipo.22271

Immordino-Yang, M. H., & Singh, V. (2013). Hippocampal contributions to the processing of social emotions. *Human brain mapping*, 34(4), 945–955. https://doi.org/10.1002/hbm.21485

Kalin, N. H., Shelton, S. E., & Takahashi, L. K. (1991). Defensive behaviors in infant rhesus monkeys: ontogeny and context-dependent selective expression. Child development, 62(5), 1175–1183.

Kong, L., Norstedt, G., Schalling, M., Gissler, M., & Lavebratt, C. (2018). The Risk of Offspring Psychiatric Disorders in the Setting of Maternal Obesity and Diabetes. *Pediatrics*, 142(3), e20180776. https://doi.org/10.1542/peds.2018-0776

Lewis-Morrarty, E., Degnan, K. A., Chronis-Tuscano, A., Pine, D. S., Henderson, H. A., & Fox, N. A. (2015). Infant attachment security and early childhood behavioral inhibition interact to predict adolescent social anxiety symptoms. *Child development*, 86(2), 598–613. https://doi.org/10.1111/cdev.12336

Luna B. (2009). Developmental changes in cognitive control through adolescence. *Advances in child development and behavior*, 37, 233–278. https://doi.org/10.1016/s0065-2407(09)03706-9

Mavigner, M., Habib, J., Deleage, C., Rosen, E., Mattingly, C., Bricker, K., Kashuba, A., Amblard, F., Schinazi, R. F., Lawson, B., Vanderford, T. H., Jean, S., Cohen, J.,

McGary, C., Paiardini, M., Wood, M. P., Sodora, D. L., Silvestri, G., Estes, J., & Chahroudi, A. (2018). Simian Immunodeficiency Virus Persistence in Cellular and Anatomic Reservoirs in Antiretroviral Therapy-Suppressed Infant Rhesus Macaques. *Journal of virology*, 92(18), e00562-18. https://doi.org/10.1128/JVI.00562-18

McKeon, J. W., Torres, J., Kazama, A. M., Bachevalier, J., & Raper, J. (2022). Differential responses toward conditioned and unconditioned stimuli, but decreased hypothalamic-pituitary-adrenal axis responsiveness in neonatal hippocampal lesioned monkeys. *Developmental cognitive neuroscience*, 58, 101165. https://doi.org/10.1016/j.dcn.2022.101165

Mendelson, M. J., Haith, M. M., & Goldman-Rakic, P. S. (1982). Face scanning and responsiveness to social cues in infant rhesus monkeys. *Developmental Psychology*, 18(2), 222–228. https://doi.org/10.1037/0012-1649.18.2.222

Moore, C. A., Staples, J. E., Dobyns, W. B., Pessoa, A., Ventura, C. V., Fonseca, E. B., Ribeiro, E. M., Ventura, L. O., Neto, N. N., Arena, J. F., & Rasmussen, S. A. (2017). Characterizing the Pattern of Anomalies in Congenital Zika Syndrome for Pediatric Clinicians. *JAMA pediatrics*, 171(3), 288–295. https://doi.org/10.1001/jamapediatrics.2016.3982

Pacheco, O., Newton, S. M., Daza, M., Cates, J. E., Reales, J. A. M., Burkel, V. K., Mercado, M., Godfred-Cato, S., Gonzalez, M., Anderson, K. N., Woodworth, K. R.,

Valencia, D., Tong, V. T., Gilboa, S. M., Osorio, M. B., Rodríguez, D. Y. S., Prieto-Alvarado, F. E., Moore, C. A., Honein, M. A., & Ospina Martínez, M. L. (2021). Neurodevelopmental findings in children 20-30 months of age with postnatal Zika infection at 1-12 months of age, Colombia, September-November 2017. *Paediatric and perinatal epidemiology*, 35(1), 92–97. https://doi.org/10.1111/ppe.12690

Payne, C., Machado, C. J., Bliwise, N. G., & Bachevalier, J. (2010). Maturation of the hippocampal formation and amygdala in Macaca mulatta: a volumetric magnetic resonance imaging study. *Hippocampus*, 20(8), 922–935. https://doi.org/10.1002/hipo.20688

Ramond, A., Lobkowicz, L., Clemente, N. S., Vaughan, A., Turchi, M. D., Wilder-Smith, A., & Brickley, E. B. (2020). Postnatal symptomatic Zika virus infections in children and adolescents: A systematic review. *PLoS neglected tropical diseases*, 14(10), e0008612. https://doi.org/10.1371/journal.pntd.0008612

Raper, J., & Chahroudi, A. (2021). Clinical and Preclinical Evidence for Adverse Neurodevelopment after Postnatal Zika Virus Infection. *Tropical medicine and infectious disease*, 6(1), 10. https://doi.org/10.3390/tropicalmed6010010

Raper, J., Kovacs-Balint, Z., Mavigner, M. et al. Long-term alterations in brain and behavior after postnatal Zika virus infection in infant macaques. *Nat Commun* 11, 2534 (2020). https://doi.org/10.1038/s41467-020-16320-7

Raper, J., Wilson, M., Sanchez, M., Payne, C., & Bachevalier, J. (2017). Increased anxiety-like behaviors, but blunted cortisol stress response after neonatal hippocampal lesions in monkeys. *Psychoneuroendocrinology*, 76, 57–66. https://doi.org/10.1016/j.psyneuen.2016.11.018

Raper, J., Stephens, S. B., Henry, A., Villarreal, T., Bachevalier, J., Wallen, K., & Sanchez, M. M. (2014). Neonatal amygdala lesions lead to increased activity of brain CRF systems and hypothalamic-pituitary-adrenal axis of juvenile rhesus monkeys. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *34*(34), 11452–11460. https://doi.org/10.1523/JNEUROSCI.0269-14.2014

Raper, J., Wilson, M., Sanchez, M., Machado, C. J., & Bachevalier, J. (2013). Pervasive alterations of emotional and neuroendocrine responses to an acute stressor after neonatal amygdala lesions in rhesus monkeys. *Psychoneuroendocrinology*, 38(7), 1021–1035. https://doi.org/10.1016/j.psyneuen.2012.10.008

Schumann, C. M., Scott, J. A., Lee, A., Bauman, M. D., & Amaral, D. G. (2019). Amygdala growth from youth to adulthood in the macaque monkey. *The Journal of comparative neurology*, 527(18), 3034–3045. https://doi.org/10.1002/cne.24728

Šimić, G., Tkalčić, M., Vukić, V., Mulc, D., Španić, E., Šagud, M., Olucha-Bordonau, F. E., Vukšić, M., & R Hof, P. (2021). Understanding Emotions: Origins and Roles of the Amygdala. *Biomolecules*, 11(6), 823. https://doi.org/10.3390/biom11060823

Song, B. H., Yun, S. I., Woolley, M., & Lee, Y. M. (2017). Zika virus: History, epidemiology, transmission, and clinical presentation. *Journal of neuroimmunology*, 308, 50–64. https://doi.org/10.1016/j.jneuroim.2017.03.001

Sullivan, R., Perry, R., Sloan, A., Kleinhaus, K., & Burtchen, N. (2011). Infant bonding and attachment to the caregiver: insights from basic and clinical science. *Clinics in perinatology*, 38(4), 643–655. https://doi.org/10.1016/j.clp.2011.08.011

Tierney, A. L., & Nelson, C. A., 3rd (2009). Brain Development and the Role of Experience in the Early Years. *Zero to three*, 30(2), 9–13.

van den Pol, Anthony N et al. “Zika Virus Targeting in the Developing Brain.” *The Journal of neuroscience : the official journal of the Society for Neuroscience* vol. 37,8 (2017): 2161-2175. doi:10.1523/JNEUROSCI.3124-16.2017

van Dyck, L. I., & Morrow, E. M. (2017). Genetic control of postnatal human brain growth. *Current opinion in neurology*, 30(1), 114–124. https://doi.org/10.1097/WCO.0000000000000405

Wang, Z. Y., Wang, Z., Zhen, Z. D., Feng, K. H., Guo, J., Gao, N., Fan, D. Y., Han, D. S., Wang, P. G., & An, J. (2017). Axl is not an indispensable factor for Zika virus

infection in mice. *The Journal of general virology*, 98(8), 2061–2068. https://doi.org/10.1099/jgv.0.000886

Wikan, N., & Smith, D. R. (2017). First published report of Zika virus infection in people: Simpson, not MacNamara. *The Lancet. Infectious diseases*, 17(1), 15–17. https://doi.org/10.1016/S1473-3099(16)30525-4

Yun, S. I., & Lee, Y. M. (2017). Zika virus: An emerging flavivirus. *Journal of microbiology* (Seoul, Korea), 55(3), 204–219. https://doi.org/10.1007/s12275-017-7063-6