

1 **Long-term alterations in brain and behavior after postnatal Zika virus infections in infant**
2 **macaques**

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1 **Abstract**

2 Considering the impact that Zika virus (ZIKV) infection has on the fetal nervous system and
3 given that the postnatal period is also a time of rapid brain growth, it is important to understand
4 the potential neurobehavioral consequences of ZIKV infection during infancy. Postnatal ZIKV
5 infection in a rhesus macaque (RM) model resulted in long-term behavioral, motor, and
6 cognitive changes, including increased emotional reactivity, decreased social contact, loss of
7 balance, and deficits in visual recognition memory at one year of age. Structural and functional
8 MRI showed that ZIKV-infected infant RMs had persistent enlargement of lateral ventricles,
9 smaller volumes and altered functional connectivity between brain areas important for
10 socioemotional behavior, cognitive, and motor function (e.g. amygdala, hippocampus,
11 cerebellum). Neuropathological changes corresponded with neuroimaging results and were
12 consistent with the behavioral and memory deficits. Overall, this study demonstrates that
13 postnatal ZIKV infection of infants in this model has long lasting neurodevelopmental
14 consequences.

1 Main

2 Zika virus (ZIKV) is a neurotropic flavivirus that was first isolated in 1947 from rhesus macaques
3 (RMs) in Uganda¹ and then from humans in 1952². As of May 2019, ZIKV had infected
4 individuals in 89 countries and territories, including the United States³. ZIKV is transmitted
5 primarily by the bite of an *Aedes* genus mosquito, but also through sexual contact, blood
6 transfusion, organ transplantation, and from mother to fetus during gestation. While the majority
7 of ZIKV infections in adults are either asymptomatic or result in mild symptoms, ZIKV is
8 associated with severe birth defects when women are infected during pregnancy⁴⁻⁶. The
9 passage of ZIKV into the brain and its ability to infect human cortical neural progenitor cells⁷,
10 induces neuropathological changes that have been reported since the late 1950s⁸⁻¹⁰. However,
11 it was not until the 2015-2016 outbreak of ZIKV in Brazil that the link to microcephaly was first
12 established⁵, leading to the declaration of a global health emergency¹¹. Congenital infection with
13 ZIKV occurs throughout gestation with resultant microcephaly and other brain malformations¹²⁻¹⁵
14 that are thought to be the consequence of ZIKV infection of neural progenitor cells as well as
15 activation of innate immune responses^{7,16}. Congenital ZIKV syndrome is a pattern of birth
16 defects that includes severe microcephaly, thinning of the cerebral cortex with subcortical
17 calcifications, macular scarring and retinal mottling, congenital contractures, and hypertonicity⁶.
18 Infants born with this syndrome can develop seizures, hearing and vision problems, feeding
19 difficulties, and gross motor abnormalities¹⁷. Several reports have documented the postnatal
20 onset of microcephaly, neurologic dysfunction, and neurodevelopmental abnormalities in infants
21 infected prenatally but without overt symptoms at birth¹⁸⁻²². A recent follow-up of 216 toddlers
22 with prenatal ZIKV exposure reported microcephaly in only 3.7% cases, yet despite no signs of
23 congenital ZIKV syndrome 40.4% had below average scores on the Bayley-III
24 neurodevelopmental evaluation²³. Together these studies highlight the potential of ZIKV to
25 cause ongoing damage in the postnatal period.

26 Infants and children can also acquire ZIKV infection postnatally through mosquito bites and
27 possibly breast milk²⁴. As ZIKV has adapted to persistent endemic transmission²⁵, continued
28 exposures of the young are likely, evidenced by 9% of children aged 1-4 years testing ZIKV
29 seropositive in Indonesia²⁶. There have been conflicting reports on the neurologic complications
30 and neurodevelopmental outcomes of children exposed to ZIKV in the peripartum and postnatal
31 periods²⁷⁻³¹. Studies with large numbers of cases have yet to be published, while research in
32 animal models suggest that ZIKV infection during the postnatal period may also have a
33 deleterious impact on development^{32,33}. We recently demonstrated in infant rhesus macaques
34 (RMs) that postnatal infection with ZIKV disseminates to the central and peripheral nervous
35 systems with histologic features in the post-mortem brain similar to prenatal ZIKV infection³².
36 Despite clearance of virus from blood after 7 days, brain structural and functional anomalies
37 were detected up to 6 months after postnatal infection, including ventriculomegaly, blunted
38 hippocampal growth, and weaker amygdala-hippocampus functional connectivity (FC)
39 compared to uninfected infants. Further, the altered FC between these brain limbic structures
40 was associated with atypical behavioral responses. Yet, little is known about the longer-term
41 consequences of postnatal ZIKV infection on brain and behavior. Given the quick clearance of
42 ZIKV in the periphery and the considerable protracted development of the primate brain, there is
43 considerable opportunity for plasticity and compensation of function. Therefore, the current
44 study examined the impact of early postnatal ZIKV infection on brain and behavior up to 12
45 months of age (equivalent to 4-5 years in human age) and permitted a longitudinal comparison
46 to results previously obtained at 3 and 6 months of age. We demonstrate that postnatal ZIKV

1 infection resulted in long-term behavioral differences, abnormal brain structure and function, and
2 corresponding brain pathological changes, therefore suggesting a lack of compensatory
3 plasticity or functional recovery.

4 **Persistent Changes in Socioemotional Behaviors:**

5 Two infant RMs (ZIKV-5 and ZIKV-6) were challenged subcutaneously with 10^5 plaque-forming
6 units (pfu) of ZIKV PRVABC59 at 5 weeks of age (equivalent to a 4 month old human infant³⁴)
7 and two infant RMs served as age-, sex-, and rearing-matched uninfected controls (Control-1
8 and Control-2). To assess the long-term impact of postnatal ZIKV infection on brain
9 development and socioemotional behavior, at 12 months of age, we placed the familiar pairs of
10 RM infants in a novel social play cage (390 cubic feet) on 6 separate testing days for 4.5 hours
11 of total observations of social interactions. Both groups habituated to the novel environment
12 over time, but ZIKV-infected RMs took longer to enter the novel play cage (Fig 1A), primarily
13 stayed on the perimeter of the cage (Fig 1B) and rarely explored the center of the cage (Fig 1C)
14 as compared to controls. The large play cage provided the RM infants room to run, jump, swing,
15 and play, as well as created opportunities for infants to exhibit uncoordinated movements
16 resulting in losses of balance. Both groups spent similar amounts of time playing (Supplemental
17 Table 1), but as they became more comfortable in this large novel environment both groups
18 exhibited an increased number of losses of balance or missteps when jumping and landing long
19 distances (5 - 8 feet) in the play cage. ZIKV-infected RM infants tended to exhibit more losses of
20 balance compared to controls (Fig 1D). On the first day, both pairs spent most of their time near
21 each other (Fig 1E), but by the second day ZIKV-infected infants began spending most of their
22 time away from their familiar partner (Fig 1F; average across all days $M = 34.8$ and $M = 10.98$,
23 for ZIKV and controls respectively). This decreased prosocial behavior was also evident by
24 ZIKV-infected infants exhibiting less affiliative behaviors as compared to controls (Fig 1G).
25 Interestingly, ZIKV-5 exhibited increased hostility across testing days (Fig 1H), which resulted in
26 increased submissive behaviors from ZIKV-6 by the last day of testing (Fig 1I). Controls and
27 ZIKV-infected RMs exhibited similar levels of anxiety, vocalizations, and stereotypies
28 (Supplemental Table 1). These results show that ZIKV infection at 5 weeks of age resulted in
29 atypical socioemotional behavior and impaired motor function at 12 months of age (equivalent to
30 4-5 years in humans). Taken together these findings suggest that ZIKV infection during infancy
31 resulted in quantifiable behavioral alterations at 12 months of age that may be consequences of
32 the brain growth and function effects reported below.

33

34 **Deficits in Memory Function:**

35 Next, we examined the potential impact on cognition using a visual recognition memory task.
36 RM infants were trained to sit in a primate chair and viewed color images for the visual paired
37 comparison task (VPC) for object and visuospatial memory, and their looking patterns were
38 recorded using a Tobii T60/T120 eye tracker (Tobii Technology). The VPC task (Fig 2A)
39 presents a sample image for an accumulative 15s of 'looking time', followed by a delay ranging
40 from 10s-120s. In the retention test, the 'familiar' sample and a novel image are presented side-
41 by-side for 5s, and then re-presented with the left/right positions of the images switched. The
42 eyetracker calculated total duration of fixation times to the familiar or novel stimulus, which was
43 used to calculate a novelty preference score [$\text{looking time}(\text{novel})/\text{total looking time}(\text{novel} +$
44 $\text{familiar}) \times 100$], as macaques, like humans, have a natural preference for novelty.

1 As shown in Figure 2B, the RM infants showed similar novelty preference across the 4 delays.
2 However, control infants' performance differed reliably from chance even at the 120s delay,
3 whereas the ZIKV-infected infants showed a drop in performance at the 120s delay, such that
4 their performance did not differ from chance at that delay, suggesting a problem with memory
5 retention in this object recognition task.

6 Although object recognition can be demonstrated early in life, spatial relational memory matures
7 between 12-18 months in RM³⁵. That is, the ability to learn not only the identities of a set of co-
8 presented items, but also to learn and remember their spatial arrangement. We assessed
9 spatial relational memory using the Object in Place vs. Object Control variants of the VPC task
10 (Fig 2A). For this task, an array of 5 objects is presented during the Sample phase as for
11 standard VPC. The array disappears for a brief 5s delay, and then reappears paired with the
12 same array with either 3 objects repositioned (Object in Place (OIP)), or 3 of the objects
13 replaced with novel ones (Object Control (OC)). In the present study, following completion of the
14 Object Recognition VPC task, the RM infants were presented with mixed trials of OIP and OC.
15 As is expected for this age, all 4 RM infants performed at chance on the OIP trials. Although the
16 two control infants had a higher novelty preference on OC trials, ZIKV-infected infants remained
17 at chance (Fig 2C). Given that ZIKV infants exhibited normal performance at short delays on
18 the standard VPC task, this latter finding suggests a specific deficit in visuospatial processing.
19 This result could reflect that ZIKV-infected infants are unable to perform with an increasing
20 memory load, or it is possible that ZIKV-infected infants may not have adequately sampled all
21 items resulting in lower performance. Overall, the impaired cognitive function detected on the
22 VPC task suggest that postnatal ZIKV infection may have negatively impacted the development
23 of brain areas important for visual learning and memory.

24

25 **Persistent structural changes in specific brain regions:**

26 Given the alterations detected in socioemotional behavior and cognitive functioning at 12
27 months of age, we then conducted magnetic resonance imaging (MRI) to determine potential
28 structural alterations caused by postnatal ZIKV infection in brain regions that control these
29 functions. MRI findings were considered descriptive in nature due to the small sample size;
30 however, a consistent effect was considered potentially clinically relevant. T1- and T2-weighted
31 structural MRI scans revealed no differences between ZIKV-infected RMs and controls in total
32 brain volume (TBV) or intracranial volume (ICV; Supplemental Table 2). Despite similar TBV,
33 the lateral ventricle volumes were larger in ZIKV-infected infant RMs than controls (Fig 3A-C;
34 Supplemental Fig 1). In contrast to MRI findings we reported in these RM infants at 3 and 6
35 months of age³², groups did not differ in GM or WM volumes at 12 months of age, which implies
36 potential neural recovery after postnatal ZIKV insult.

37 Temporal lobe structures, such as the amygdala and hippocampus, play an important role in
38 socioemotional behavior. They also exhibit a prolonged postnatal development which can make
39 them particularly vulnerable to early CNS insults, including ZIKV infection. Amygdala volume in
40 RMs increases rapidly from birth to 8 months of age, but continues growing into adulthood³⁶⁻⁴⁰.
41 Amygdala volumes were similar to controls at 3 and 6 months of age³²; however, ZIKV-infected
42 RM infants exhibited smaller amygdala volume than controls at 12 months of age (Fig 3D).
43 Postnatal development of the hippocampus is more protracted than the amygdala, with the
44 hippocampus doubling its volume between 1 week and 2 years of life in RMs⁴⁰. Here, ZIKV-

1 infected RM infants also exhibited smaller hippocampal volumes compared to controls at 12
2 months of life (Fig 3E). Decreased hippocampal volumes were previously detected at 3 and 6
3 months of age³², and the magnitude of the observed volumetric difference appears to lessen
4 with age, which again may suggest a potential for recovery or compensation during this period
5 of neural plasticity and protracted brain development.

6 The dorsal striatum (e.g., caudate and putamen) receives inputs from amygdala, hippocampus,
7 prefrontal cortex, ventral tegmental area and substantia nigra, making it an important interface
8 for the motor control, reward, and addiction circuitry⁴¹. The striatum has also been implicated in
9 learning and memory and inhibitory control of action⁴²⁻⁴⁵. Interestingly, ZIKV-infected RM infants
10 showed smaller putamen volumes compared to controls (Fig 3F), but no difference in the
11 caudate measurements (Supplemental Table 2). Coordination of motor function is also
12 controlled by the cerebellum, which has been shown to exhibit hypoplasia in cases of congenital
13 Zika syndrome⁴⁶. Our results are consistent with those reports, since at 12 months of age, ZIKV-
14 infected RM infants had decreased cerebellar GM volume than controls (Fig 3G), although WM
15 volume was unaffected (Supplemental Table 2).

16 While visual areas are well developed at birth in RMs, synaptic density in visual cortex
17 increases in the first few months of life, followed by a sharp pruning before the first year⁴⁷⁻⁴⁹.
18 Visual areas undergo experience-based refinements throughout the first few years of age,
19 indicating selective survival of certain synapses^{49,50}. ZIKV-infected infants had larger GM
20 volumes in the right occipital lobe (Supplemental Table 2), suggesting that they may be
21 experiencing less pruning/refinement in visual areas compared to uninfected normally
22 developing controls.

23 In summary, these structural MRI results indicate that ZIKV infection during infancy can lead to
24 alterations in brain development and growth that are detected as early as 3 months of age³² and
25 many of which persist through 12 months of age, with other regions showing evidence of
26 recovery.

27

28 **Changes in Resting-state Functional Connectivity:**

29 To assess the long-term impact of postnatal ZIKV infection on functional connectivity (FC)
30 between brain regions, we conducted resting state functional MRI (rs-fMRI) scans at 12 months
31 of age. Regions of interests (ROIs) were defined using published anatomical parcellations^{51,52}
32 mapped onto the University of North Carolina (UNC)–Emory RM infant atlases registered to the
33 F99 space⁵³. First, we examined whether the regions showing structural changes above exhibit
34 parallel alterations in FC, as measured by resting state blood oxygen level–dependent (BOLD)
35 signal correlated fluctuations (Supplemental Table 3). Although both groups showed positive FC
36 between the amygdala and hippocampus, ZIKV-infected RM infants had weaker FC compared
37 to uninfected controls in the left hemisphere (Fig 4A, B), which was first detected at 6 months of
38 age³². Regions of the prefrontal cortex (PFC), including the orbitofrontal cortex (OFC),
39 dorsolateral PFC (dlPFC) and medial PFC (mPFC) play important roles in attention, emotional
40 regulation, as well as learning and memory function^{54,55}. Controls exhibited stronger FC
41 between right hippocampus and ventromedial OFC area 14 than ZIKV-infected RMs (with one
42 of the infants exhibiting an anticorrelation – negative FC between these regions; Fig 4C). Other
43 regions where ZIKV animals showed weaker FC than controls include right hippocampus with
44 dlPFC area 46 (Fig 4D) and between inferior temporal cortex area TEO and putamen (Fig 4E).

1 In contrast, stronger FC was found in ZIKV-infected than control infants between the right
2 amygdala and dIPFC area 46 (Fig 4F) and between right amygdala and caudate (Fig 4G).
3 These rs-fMRI data suggest that postnatal ZIKV infection leads to long-term impact in FC
4 between cortico-limbic regions, including PFC, amygdala, hippocampus and striatal areas,
5 which may affect the functions they control (i.e. emotional regulation, social motivation, motor
6 control, and cognitive maturation).

7

8 **Neurohistopathological Alterations after Postnatal ZIKV Infection:**

9 Once behavioral and neuroimaging assessments were completed the four infant RMs were
10 euthanized to assess neurohistopathology approximately one year after early postnatal ZIKV
11 infection. We have previously reported that ZIKV RNA was cleared from plasma in the two
12 infected RM infants within 7 days post-infection and, while ZIKV RNA was found in the brain at
13 14-15 days after infection, virus was not detected in the CSF³². In other experiments, ZIKV has
14 been detected in CSF and brain tissue up to 72 days post-infection and virus- or immune-
15 mediated pathology is apparent post-mortem^{32,56,57}.

16 Pathologic evaluation was performed on several regions of brain and spinal cord, including
17 prefrontal cortex, frontal cortex, parietal cortex, temporal cortex, piriform cortex, occipital cortex,
18 basal ganglia, amygdala, substantia nigra, hippocampus, cerebellum, brainstem and cervical,
19 thoracic, lumbar, and sacral spinal cord and cauda equina. Spinal cord and cauda equina
20 revealed no significant lesions and immunohistochemical analysis of glial, neuronal, and
21 apoptotic markers (GFAP, NeuN, and caspase 3, respectively) in the brain regions above were
22 similar in ZIKV-infected and control RMs. However, ventricle expansion in ZIKV-infected RMs
23 was apparent on neuropathological examination (Fig 5A, B) confirming the enlarged lateral
24 ventricles detected with MRI. In addition, neuropil and perivascular calcification was detected in
25 the putamen of ZIKV-5 (Fig 5B, C), which may explain the structural and functional changes
26 detected with *in vivo* MRI scans. Histological examination of ZIKV-infected RMs revealed
27 underdeveloped dendritic branching of immature neurons in the paralaminar nucleus of the
28 amygdala (Fig 6), while no abnormalities were observed in the hippocampus. These results,
29 obtained remote from the time of infection, differ from the increased astrogliosis, perivascular
30 inflammation, and apoptosis found in ZIKV-infected RMs after only 2 weeks of infection³².

31 Overall, these findings demonstrate that despite ZIKV being cleared from the periphery quickly,
32 the infection can result in brain lesions that impact the long-term growth and functioning of the
33 brain in the absence of ongoing inflammatory changes.

34

35 **Discussion:**

36 The current study demonstrates that ZIKV infection during the early postnatal period can have
37 long lasting impact on brain structure, function, behavior, and cognition in primates. To date,
38 most research has focused on congenital ZIKV syndrome^{4-6,58}, leaving many questions
39 unanswered about the potential impact of postnatal infection on the developing brain. Given the
40 protracted development of the brain in humans and nonhuman primates, there is considerable
41 opportunity for plasticity and functional compensation after early brain damage. Yet, the current

1 study suggests persistent alterations in brain growth and function as a result of damage from
2 early postnatal ZIKV infection with limited recovery.

3 While ZIKV infection did not impact the RM infants' ability to produce species typical social and
4 emotional behaviors, the expression of those behaviors was altered as compared to controls.
5 We previously reported that ZIKV-infected infant RMs exhibited altered emotional reactivity on
6 an acute stress test as compared to controls³². The current findings show that this increased
7 emotional reactivity persisted through the juvenile period, such that ZIKV-infected RMs took
8 longer to habituate to a novel social environment and avoided spending time in the center of the
9 cage as compared to controls. Beyond emotional responses, we were also able to detect
10 alterations in social interactions. Young RMs spend most of their time with other conspecifics
11 engaged in play and affiliative behaviors⁵⁹. Yet, ZIKV-infected RMs preferred to spend more
12 time apart and engaged in fewer affiliative behaviors with a highly familiar partner as compared
13 to controls. Pharmacologically increasing amygdala activity results in decreased social
14 interaction in monkeys⁶⁰, which provides a potential mechanism to explain our results showing
15 increased functional connectivity between the amygdala and prefrontal area 46, as well as the
16 amygdala and caudate among ZIKV-infected infant RMs. In addition, previous studies of RMs
17 with selective lesions of the amygdala or hippocampus during infancy report similar alterations
18 in socioemotional behavior⁶¹⁻⁶⁵, further supporting the notion that behavioral changes in the
19 current study may be the result of structural and functional alterations found in these limbic
20 regions after postnatal ZIKV infection. Overall, these findings indicate that ZIKV infection during
21 this early and vulnerable period of rapid postnatal brain development can have a lasting impact
22 on socioemotional behavior.

23 Neuroimaging at 12 months of age revealed a persistent ventricular enlargement in ZIKV-
24 infected infant RMs as compared to age-, sex-, and rearing-matched controls. Increased lateral
25 ventricle size was first reported on structural MRIs taken at 3 and 6 months of age³² and is still
26 present at 12 months of age. Lateral ventricle size was not related to individual variability in
27 brain size, since there was no difference in total brain volume. Importantly, enlarged ventricles
28 for ZIKV-infected infant RMs were also confirmed with neuropathology. These results are similar
29 to *in utero* ZIKV infection which reports more extensive ventriculomegaly in live born infants and
30 autopsied fetuses^{4,5,66,67}.

31 Interestingly, no differences were detected in total WM or GM between ZIKV-infected RMs and
32 controls at 12 months of age, unlike our previous MRI investigations at 3 and 6 months of age³².
33 Previous studies in human and nonhuman primates have identified changes in WM tracts after
34 early life adversity^{68,69} and other viral infections^{70,71}. Similarly, postnatal ZIKV infection could
35 result in microstructural changes in WM tracts. However, to detect such changes a more
36 sensitive neuroimaging approach, such as diffusion tensor imaging (DTI), would be needed.

37 Temporal lobe structures are particularly sensitive to early insults, which was also apparent with
38 early postnatal ZIKV infection. Although no differences were detected in amygdala volume at 3
39 and 6 months of age, by 12 months RM infants exposed to ZIKV had much smaller amygdalae
40 compared to controls. The rapid maturation of the amygdala seen in infant RMs may have
41 precluded the detection of group differences in amygdala volume until after the rate of growth
42 stabilized at around 8 months of age³⁶⁻⁴⁰. ZIKV-infected RMs also exhibited underdeveloped
43 dendritic branching of immature amygdala neurons, likely limiting their ability to be incorporated
44 into the neural network. These structural changes may explain the differences detected in
45 amygdala functional connectivity of ZIKV-infected infants, as well as their increased emotional

1 reactivity and decreased prosocial behavior. The hippocampus has a more protracted
2 development, doubling its volume over the first 2 years of life and continuing to grow into
3 adulthood in RMs^{36,40}. This prolonged neural plasticity in the hippocampus may explain the
4 relative lessening in hippocampal volume differences between RM infants infected with ZIKV
5 and controls at 12 months. ZIKV-infected infant RMs continued to have a smaller hippocampal
6 volume similar to our previous reports at 3 and 6 months of age³², but the magnitude of the
7 difference between the groups appears to be less pronounced by 12 months of age. While this
8 finding may suggest a potential for recovery or compensation during this period of neural
9 plasticity and rapid brain development, resting-state fMRI results demonstrate persistent altered
10 functional connectivity between the hippocampus and other areas important for socioemotional
11 and cognitive functioning, despite some structural normalization.

12 The current study also found structural and functional differences in the striatum (i.e. putamen
13 and caudate) on MRI, as well as calcification on neuropathology. Calcification in subcortical
14 areas have been reported in several studies of confirmed and suspected congenital Zika
15 syndrome cases in humans^{21,46,72}. These findings as well as the smaller cerebellum GM
16 volumes may explain the increased incidents of balance loss seen amongst the ZIKV-infected
17 infant RMs during their exploration of a novel social environment. Although we did not perform a
18 specific test of gross motor function, our current findings of uncoordinated movements resulting
19 in losses of balance suggest that postnatal ZIKV infection may have impacted motor
20 development and psychomotricity, which has also been demonstrated in a murine model of
21 postnatal ZIKV infection⁷³. Two factors could have contributed to the increased loss of balance
22 detected in the ZIKV-infected RMs over the six days of social observations. First, as the animals
23 habituated to the large novel play cage, they did more jumping and playing which provided more
24 opportunities for missteps and losses of balance when jumping and landing long distances (5 - 8
25 feet) in the play cage. Secondly, as shown in Fig 1H and 1I, ZIKV-5 began to exhibit increased
26 hostility while ZIKV-6 exhibited more submissive behaviors across the days of testing. The
27 hostile chasing and resultant fleeing we observed in the ZIKV-infected animals is a quick
28 behavioral sequence that may have also provided more opportunities for missteps and losses of
29 balance among the ZIKV-infected group.

30 Structural and functional changes in the hippocampus and striatum may also explain the
31 memory deficits detected on the VPC task at 12 months of age, which were not detected at 6
32 months of age³². In fact, infant RMs infected with ZIKV performed poorly only at 120s delays
33 indicating a delay-dependent deficit, which is similar to a late-developing delay-dependent
34 memory deficit detected in neonatal hippocampal lesioned RMs⁷⁴. Performance on spatial
35 relational VPC task (e.g. Object in Place) is late-developing and sensitive to neonatal and adult
36 hippocampal damage^{35,75,76}. The deficits in spatial relational memory observed in both control
37 and ZIKV-infected RMs were age-appropriate. However, the deficit on the Object Control task
38 for ZIKV-infected RMs could be due to the structural and functional changes detected in the
39 hippocampus and striatum, brain areas important for cognition. Despite the potential for neural
40 plasticity and recovery, postnatal ZIKV infection may have a long-term impact on cognitive
41 function that emerges with age. Considering the protracted development of the brain and
42 memory systems, we would further hypothesize that postnatal ZIKV infection may impact spatial
43 relational memory as well as other later developing memory systems, such as working memory.

44 There are limitations to the current study. First, the small number of animals studied made most
45 group comparisons descriptive in nature. As a result, this study should be viewed as an

1 exploratory, proof-of-concept experiment to inform future studies on the neurodevelopmental
2 outcomes of ZIKV infection after birth⁷⁷. The strength of this study does not reside in statistical
3 significance but rather in the integration of neuroscience experiments at multiple scales⁷⁸ and its
4 relevance to an understudied clinical problem. Second, our study focused on investigating a
5 single strain of ZIKV, PRVABC59, that has been widely used for RM studies and that is closely
6 related to isolates from Brazil⁷⁹, but which likely does not capture the entire spectrum of ZIKV
7 infections. Third, only female subjects were studied, and there may be sex differences in the
8 impact of ZIKV infection as has been recently reported in mice⁸⁰. Fourth, there are several
9 limitations to rs-fMRI which should be noted: (1) FC was obtained under anesthesia, but we
10 note that isoflurane doses were kept below those used in previous studies reporting patterns of
11 coherent BOLD fluctuations similar to the awake state, including in sensory and visual
12 systems⁸¹⁻⁸⁴; and (2) our design does not allow to establish causality between the alterations in
13 structural and functional brain measures and behavioral/cognitive alterations or to specific
14 physiological, cellular, or molecular brain maturational processes, which will need to be
15 addressed in future studies. Additional studies are needed to substantiate our findings regarding
16 postnatal ZIKV infection, which should include larger sample sizes of males and females and
17 multiple virus strains reflecting the diversity of this infection.

18 In summary, ZIKV infection during the early postnatal period is associated with long-term
19 alterations in socioemotional behavior and cognitive functioning, as well as changes in brain
20 growth and function. The impact of ZIKV on the brain was also apparent with
21 neurohistopathology conducted at approximately 12 months post-infection, further indicating the
22 long-lasting impact of the virus on neural development. The current study demonstrates that
23 behavioral and cognitive abnormalities become more apparent with age, however, future studies
24 are needed to understand the impact of early postnatal ZIKV infection during later stages of life
25 (e.g. adolescence and adulthood). This RM model sheds light on the potential outcomes of
26 human infants infected with ZIKV postnatally and provides a platform to test therapeutic
27 approaches to alleviate the neurologic consequences of ZIKV infection.

28

29 **Methods:**

30 **Study design**

31 The objective of this study was to evaluate the long-term neurodevelopmental outcome of
32 postnatal ZIKV infection of infant RMs. After infection, animals underwent virologic and
33 immunologic measurements, as well as neuroimaging, behavioral assessments, and
34 neurohistopathology. This was an exploratory pilot study of postnatal ZIKV infection in infant
35 RMs; therefore, a formal power analysis was not performed. Infants were randomized to either
36 the ZIKV or control groups based on the day of birth. Veterinary and animal resource staff were
37 not blinded to study group due to the necessary environmental precautions that were taken with
38 ZIKV. Laboratory staff were blinded to study group during the conduct of experiments and
39 analyses. Methods have been previously published³², but will be briefly described below.

40 **Animals**

41 Four female infant Indian RMs (*Macaca mulatta*) were used in this study, two were exposed to
42 ZIKV at 5 weeks of age, while the remaining two served as uninfected controls. The infants
43 were delivered naturally by their dams while housed in indoor/outdoor social groups. Infants

1 were then removed from their dams at 5 to 10 days of age and transported to the Yerkes
2 National Primate Research Center (YNPRC) nursery facility. All infants were singly housed in
3 warming incubators for the first 3 to 4 weeks, but had visual and auditory contact with
4 conspecifics. Infants were hand-fed formula every 2 to 3 hours for the first month, then via self-
5 feeders for the next 3 months as per standard YNPRC protocol. Soft blankets, plush toys, or
6 fleece were provided and changed daily. Soft chow and fruits were introduced starting at 1
7 month of age, and by 4 months, formula was discontinued and all were fed a diet of Purina
8 Primate Chow, Old World Monkey formulation, supplemented with daily fruits and vegetables.
9 Water was provided ad libitum. At 4 weeks of age, infants transitioned into age-appropriate
10 caging with visual and auditory contact with conspecifics. At 6 to 7 weeks of age, they began
11 socialization consisting of protected contact housing supplemented with 2 to 3 hours daily of full
12 contact with their age-matched peer. By 3 months of age, each pair was housed entirely
13 together. ZIKV-infected infants were housed in an ABSL-2+ (Animal Biosafety Level 2) room
14 until cleared of virus in blood and urine. Control infants were initially housed in a separate
15 ABSL-2 room, and then all infant pairs were housed together after ZIKV clearance with visual
16 contact to increase visual socialization and control for environmental factors. Precautions were
17 taken to ensure that control and ZIKV-infected infants received similar housing, nutrition, and
18 enrichment. In addition, control infants were subjected to the same sedation and collection
19 procedures (blood and urine) as ZIKV-infected infants to minimize any differences in exposure
20 to stressful experiences. All housing was indoors on a 12hour light-dark cycle. This study was
21 conducted in strict accordance with U.S. Department of Agriculture regulations and the
22 recommendations in the Guide for the Care and Use of Laboratory Animals of the National
23 Institutes of Health, approved by the Emory University Institutional Animal Care and Use
24 Committee, and conducted in an AAALAC accredited facility.

25 **Virus and infection**

26 The ZIKV of Puerto Rican origin (PRVABC59, GenBank accession number: KU501215.1) used
27 in this study had been passaged four times and titrated on Vero cells. Experimental infections
28 were performed via the subcutaneous route using 10^5 pfu of ZIKV PRVABC59.

29 **Behavioral Assessments**

30 Social Interactions: To examine social and emotional behavior, subjects were tested in highly
31 familiar dyads in a large novel social play cage (390 cubic feet). The social play cage was a
32 hexagonal nonhuman primate social cage (Flex-A-Gon; Britz and Company, Wheatland, WY).
33 There were 6 separate days of 45 minutes of testing for a total of 4.5 hours of behavioral
34 observations. Subjects entered the cage at the same time, but from separate doors that
35 alternated sides of the cage each day. Each test day was videotaped for behavioral coding later
36 using the Observer XT 14 software (Noldus Inc.) and a detailed ethogram (Supplemental Table
37 4). One experimenter coded all of the videotapes with a high degree of intra-rater reliability
38 Cohen's $\kappa = 0.98$; and an average inter-rater reliability of Cohen's $\kappa = 0.86$ with other trained
39 experimenters at YNPRC. Since the experimenter was not blind to the animals' treatment,
40 videos were coded without identifying information about the subject, which was revealed after
41 coding was complete.

42 Visual Paired Comparison (VPC) Task: A Tobii T60/T120 eye tracker (Tobii Technology) was
43 used for VPC testing. Subjects were acclimated to a size-appropriate nonhuman primate chair
44 and to facing the eye tracker monitor on which visual images were displayed. Visual stimuli

1 were displayed on the eye tracker monitor using Tobii Studio™ software. The tester running the
2 session was separated visually from the monkey by a curtain. The tester could monitor whether
3 the tracker was detecting the eyes, and a gaze-trail was displayed over a view of the presented
4 stimuli on the tester's laptop. Infants were calibrated to the eye tracker at the beginning of each
5 session.

6 Existing sets of visual stimuli for infant RMs⁸⁵ were used comprised of photo/clip-art images of
7 everyday junk objects (Nova Art Explosion 800,000 ClipArt). Selected images for each set were
8 chosen to be as different as possible. As shown in Fig. 2A, each testing trial consisted of a
9 Familiarization phase, in which a Sample image was presented in the center of the screen. The
10 image remained on screen until the monkey had cumulatively fixated within the boundaries of
11 the image for a total of 15s (as timed by the tester using a stopwatch). At this point, the image
12 disappeared and the screen remained black for the Delay time (10, 30, 60 or 120s). The Delay
13 period was followed by the first of two Retention tests, during which the Sample image and a
14 Novel image were placed on either side of the screen for 5s and the RM was free to look at
15 either. Both images then disappeared for another 5s, followed by the second Retention test in
16 which the left-right positions of the Sample and Novel images were switched to prevent against
17 side bias. Initial placement of the objects was determined pseudo randomly such that the
18 Sample object appeared equally often on either side of the screen for the first Retention test.
19 Each trial was separated by a 30s inter-trial interval. Subjects completed a total of 40 trials, 10
20 per delay.

21 Total duration of the fixation times to each predetermined region of interest (ROI) was
22 calculated using Tobii Studio™ software. Each ROI encompassed the image and the white
23 background and was categorized as Sample, for familiarization stimuli, and Novel or Familiar for
24 Retention stimuli. The total fixation duration time within each ROI was compared, yielding a total
25 time looking at Novel and/or Familiar stimuli. In this task, RMs (like humans) have an innate
26 tendency to look at the novel stimulus, and so Preference for Novelty is a standard measure of
27 memory. Preference for Novelty score was calculated as $[\text{Looking Time}(\text{Novel})/\text{Total Looking}$
28 $\text{Time}(\text{Novel} + \text{Familiar}) \times 100]$. Trials were included in the analysis if fixations were observed
29 during both Retentions and if no evidence of side bias was observed in that trial. Trials were
30 averaged per subject to yield a single score for each delay. On average, each subject
31 contributed 5 usable (range of 2-7) trials at each delay.

32

33 **Neuroimaging studies**

34 Neuroimaging data were collected 12 months of age using a Siemens 3T Tim Trio system
35 (Siemens Medical Solutions) with a Tx/Rx 8-channel volume coil. Data were acquired in a single
36 session, which included T1- and T2-weighted structural MRI scans, and two 15-min EPI rs-fMRI
37 (T2*-weighted) scans to measure temporal changes in regional BOLD signal. Animals were
38 scanned in the supine position in the same orientation, achieved by placement and
39 immobilization of the head in a custom-made head holder via ear bars and a mouthpiece. After
40 initial telazol induction and intubation, scans were collected under isoflurane anesthesia (0.8 to
41 1%, inhalation, to effect), kept at the lowest dose possible to minimize effects of anesthesia on
42 BOLD signal⁸¹⁻⁸⁴. End-tidal CO₂, inhaled CO₂, O₂ saturation, heart rate, respiratory rate, blood
43 pressure, and body temperature were monitored continuously and maintained during each MRI
44 session.

1 Structural MRI acquisition: High-resolution T1-weighted MRI scans were acquired for
2 volumetrics and registration of the functional scans using a three-dimensional magnetization-
3 prepared rapid gradient echo (3D-MPRAGE) parallel image sequence [repetition time/echo time
4 (TR/TE) = 2600/3.46 ms; field of view (FOV), 116 mm × 116 mm; voxel size, 0.5 mm³ isotropic;
5 eight averages] with Generalized Autocalibrating Partially Parallel Acquisitions (GRAPPA)
6 acceleration factor of R = 2. T2-weighted MRI scans were collected using a 3D fast spin-echo
7 sequence (TR/TE = 3200/373 ms; FOV, 128 mm × 128 mm; voxel size, 0.5 mm³ isotropic; three
8 averages) with GRAPPA (R = 2) to aid with registration and delineation of anatomical borders.
9 Structural MRI data processing and analysis data sets were processed using AutoSeg_3.3.2
10 segmentation package⁸⁶ to get the volumes of brain WM and GM, CSF, and cortical (temporal
11 visual area, temporal auditory area, and prefrontal, frontal, parietal, occipital lobes, and
12 cerebellum) and subcortical (hippocampus, amygdala, caudate, and putamen) brain areas.
13 Image processing steps included the following: (i) averaging T1 and T2 images to improve
14 signal-to-noise ratio, (ii) intensity inhomogeneity correction, (iii) rigid-body registration of the
15 subject MRI to the 12-month UNC-Emory infant RM atlases⁵³, (iv) tissue segmentation and
16 skull stripping, (v) registration of the atlas to the subject's brain to generate cortical parcellations
17 (affine followed by deformable ANTS registration), and (vi) manual editing of the amygdala and
18 hippocampus done on all scans using previously published neuroanatomical boundaries⁴⁰. For
19 more detailed description of the structural MRI analysis, see^{86,87}. Cortical and subcortical
20 volume measurements (in cubic millimeters) were corrected by TBV and then multiplied by 100
21 (for example, hippocampal volume/TBV × 100).

22 RS-fMRI acquisition: BOLD-weighted functional images were collected using a single-shot
23 echo-planar imaging (EPI) sequence (2X400 volumes; TR/TE = 2060/25 ms, 2 × 15 min; voxel
24 size, 1.5 mm³ isotropic) after the T1-MRI scan. An additional short, reverse-phase encoding
25 scan was also acquired for unwarping susceptibility-induced distortions in the EPI images using
26 previously validated methods⁸⁸.

27 Functional MRI data preprocessing: Imaging data were preprocessed with an in-house pipeline
28 in Nipype⁸⁹ with modifications⁹⁰⁻⁹³, including adaptations for the RM brain⁹⁴. After the EPI
29 functional time series were concatenated and rigid-body registered to the subject's T1-weighted
30 structural image, this was transformed to conform to age-specific T1-weighted RM infant atlases
31⁵³ using nonlinear registration methods in FSL (FNIRT). These atlases were registered to the
32 112RM-SL atlas in F99 space^{95,96}. We registered the scans to the age-specific appropriate
33 infant atlas (12 months). Frames with displacement values greater than 0.2 mm were
34 removed^{97,98}, and data were visually inspected upon preprocessing completion to exclude series
35 with unsatisfactory co-registration or significant BOLD signal dropout.

36 FC analysis: FC between ROIs including the amygdala, hippocampus, PFC (dorsal, medial, and
37 ventral areas), OFC, and temporal cortical area (TEO) were analyzed. ROIs were defined on the
38 basis of published anatomical parcellations^{51,52} mapped onto the UNC-Emory RM infant atlases
39 registered to the F99 space⁵³. The left and right amygdala automatic label maps were manually
40 adjusted by experts using cytoarchitectonic maps in the existing UNC-Wisconsin adolescent
41 atlas⁹⁹, and other ROIs were manually edited in the infant atlases following established
42 anatomical landmarks^{100,101}. The time course of the BOLD signal was averaged across all voxels
43 within each ROI using a single ROI as the seed and then correlated with the time course of the
44 other ROIs. FC values are calculated as correlation coefficients (r values) between ROI BOLD
45 time courses, which were extracted from correlation matrices using MATLAB (MathWorks Inc.).

1 **Histopathology**

2 At the study end point, the animals were euthanized and a complete necropsy was performed.
3 For histopathologic examination, brain and spinal tissue samples were fixed in 10% neutral
4 buffered formalin, routinely processed, paraffin-embedded, sectioned at 4 μ m, and stained with
5 hematoxylin and eosin. To define specific cell types, we performed immunohistochemical (IHC)
6 analysis by using antibodies against Glial fibrillary acidic protein (Agilent Technologies, M0761),
7 NeuN (EMD Millipore, MAB377), caspase 3 (Cell Signaling, 9662), and doublecortin (Santa
8 Cruz, SC-8066). IHC staining on sections of brain from both ZIKV infected and control animals
9 was performed using a biotin-free polymer system (Biocare Medical). The paraffin-embedded
10 sections were subjected to deparaffinization in xylene, rehydration in graded series of ethanol,
11 and rinsed with double distilled water. The brain sections were incubated with rabbit anti-human
12 Glial fibrillary acidic protein and caspase 3 and mouse anti-human NeuN antibodies for 1 h after
13 heat-induced epitope retrieval. Antibodies labelling was visualized by development of the
14 chromogen (Warp Red or DAB Chromogen Kits; Biocare Medical). Amygdala and hippocampal
15 sections were pretreated (3% hydrogen peroxidase and 20% methanol in PBS) for 1 hours and
16 incubated overnight in anti-goat doublecortin (1:400) at room temperature. Sections were then
17 incubated in horse anti-goat secondary antibody (1:200; Vector Labs) for 1 hour, followed by an
18 hour in ABC (Vector, PK6100) and visualized with DAB (Sigma). Digital images of brain were
19 captured at 20 \times and 400 \times magnification with an Olympus BX43 microscope equipped with a
20 digital camera (DP26, Olympus) and evaluated using Cellsens® digital imaging software 1.15
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8

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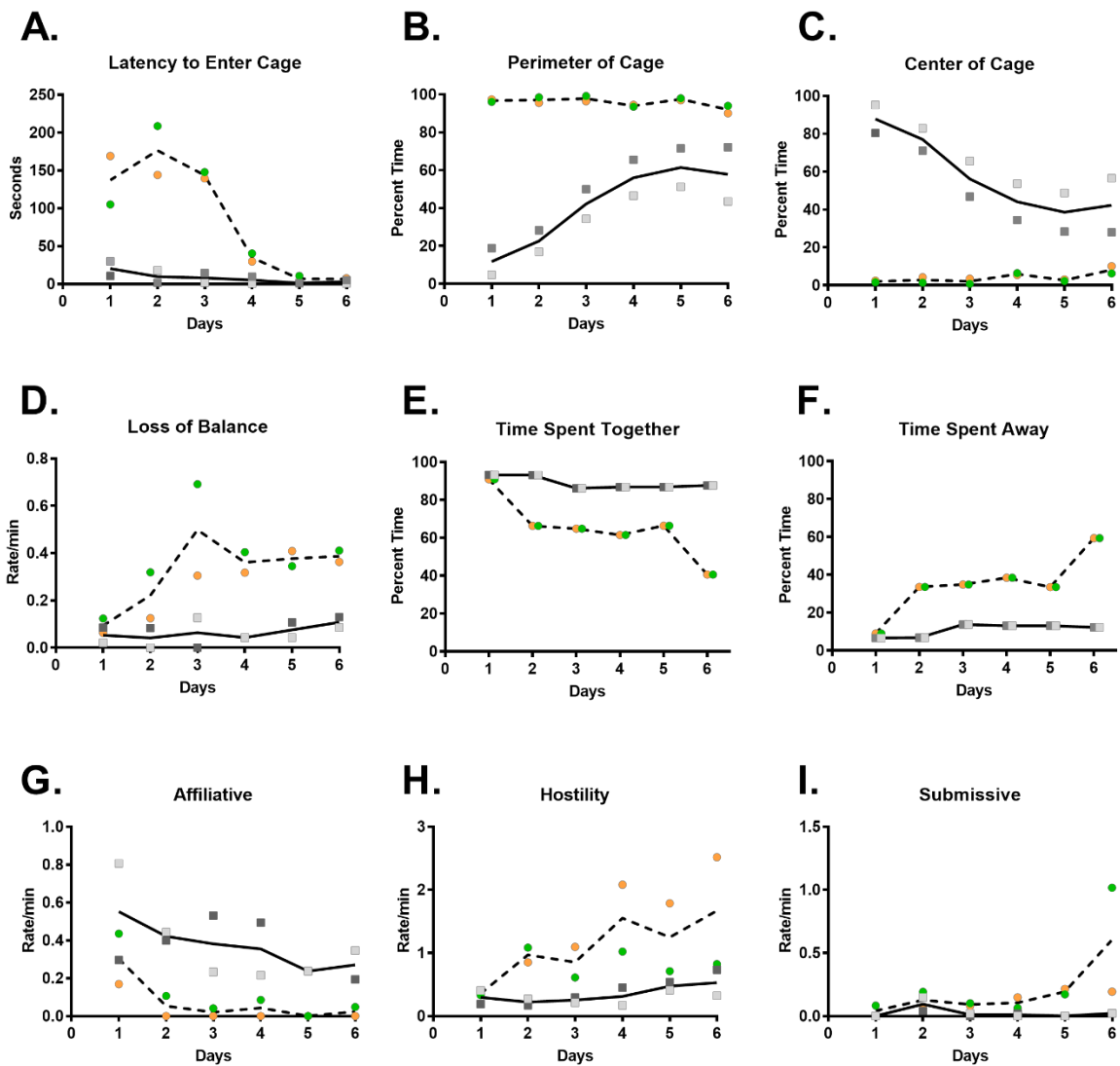


Figure 1. Altered socioemotional behavior at 12 months of age in postnatal ZIKV infection of infant RMs. (A-I) Illustrates the change in behavioral responses of ZIKV-infected infant RMs (dashed line with circles; green = ZIKV-5 and orange = ZIKV-6) and controls (solid line with squares; dark grey = Control-1 and light grey = Control-2) across 6 days of testing in a large novel play cage. Latency to enter the cage (A) was measured in seconds, while specific behaviors were measured as rates per min (D, G-I) or as a percentage of total observation time (B-C, E-F).

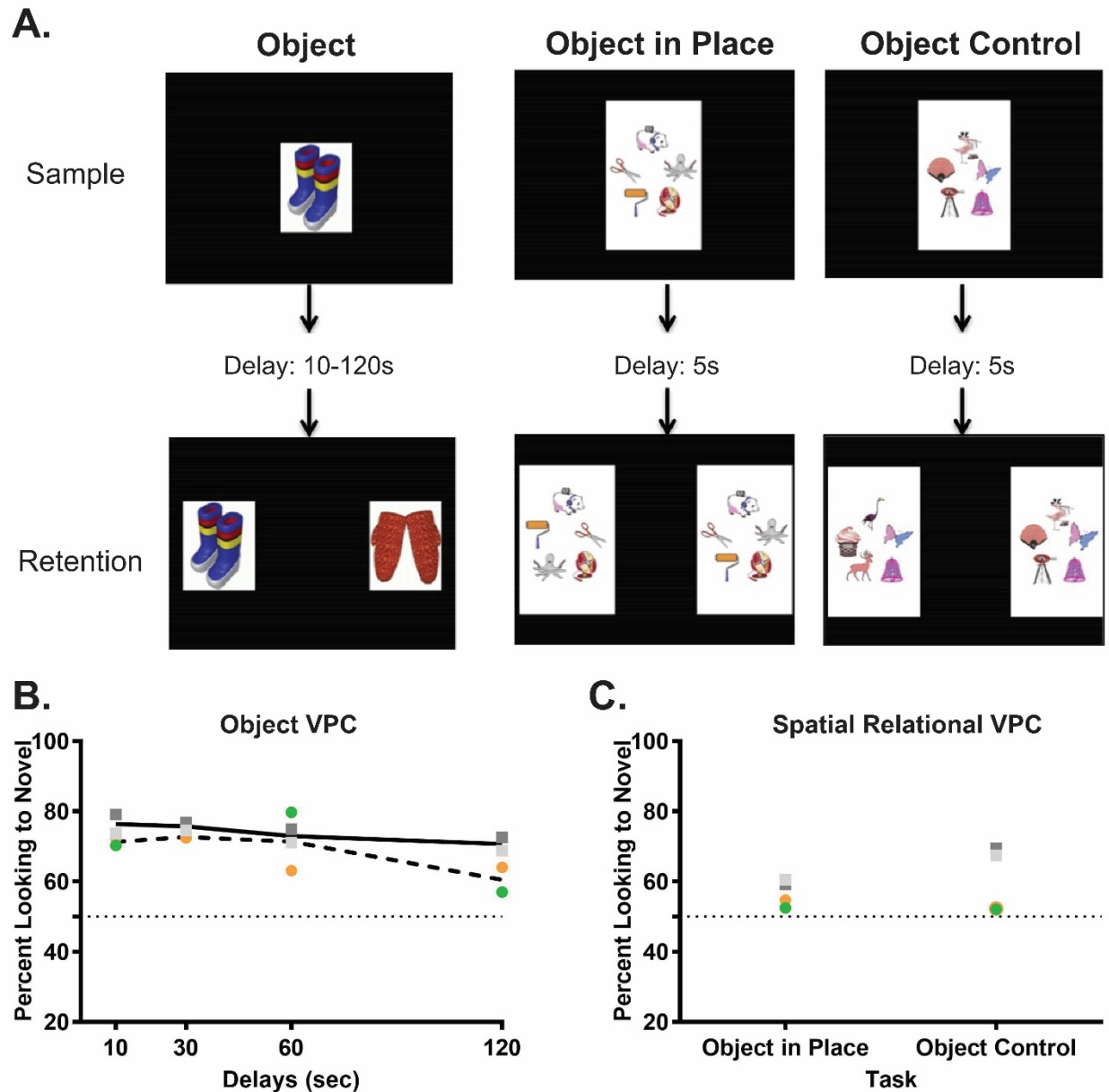


Figure 2. Impaired visual recognition memory at 12 months of age after postnatal ZIKV infection of infant RMs. (A) Illustrates an example trial for each of the three visual paired comparison (VPC) tasks used in this study. (B) Illustrates the percent looking toward the novel stimulus across delays for ZIKV-infected infant RMs and controls. (C) Compares the percent looking to novelty during object in place versus object control trials. Chance performance is depicted by a dotted line at 50 percent.

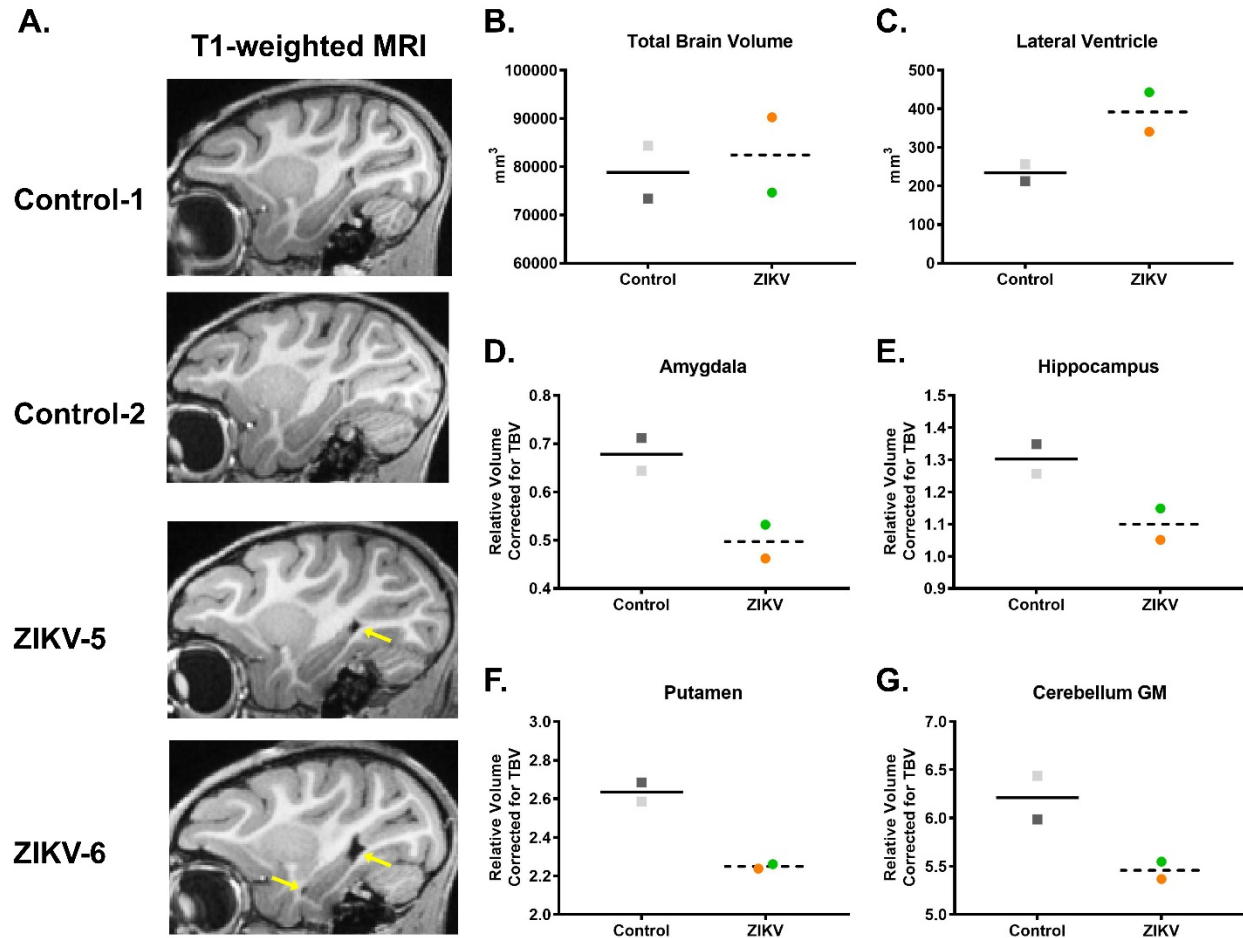


Figure 3. Postnatal ZIKV infection leads to lateral ventricle enlargement and reduced subcortical and cerebellar volumes at 12 months of age in infant RMs. (A) Sagittal T1-weighted structural MRI images through the hippocampus; yellow arrows illustrate the increased lateral ventricle volume in ZIKV-infected RMs. Volumetric measurements of (B) the total brain volume and (C) lateral ventricles in cubic millimeters, as well as (D) amygdala, (E) hippocampus, (F) putamen, and (G) cerebellum grey matter (GM) corrected by total brain volume. The correction used was specific brain region/TBV X 100.

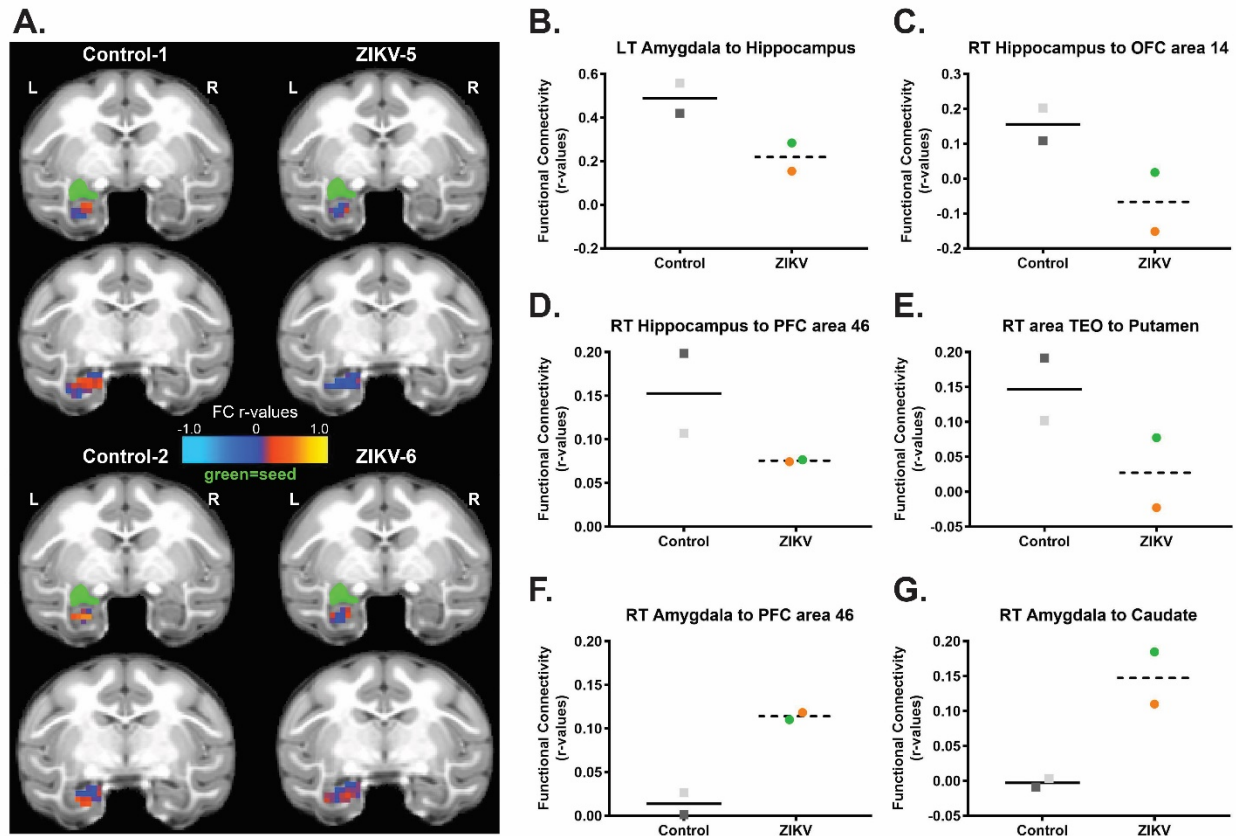


Figure 4. Altered functional brain connectivity at 12 months of age after postnatal ZIKV infection of infant RMs. (A) Depiction of amygdala-hippocampus resting-state FC measured by rs-fMRI displayed on the UNC-Emory RM infant atlas⁵³. Images show the same two coronal slice series in each subject, with the seed (green) placed in the left amygdala and the FC r values in the hippocampus colored according to the scale provided. L, left hemisphere; R, right hemisphere; r values, raw FC correlations. (B-G) Illustrate the rs-fMRI measurements of FC between specific brain regions in ZIKV-infected RM and controls.

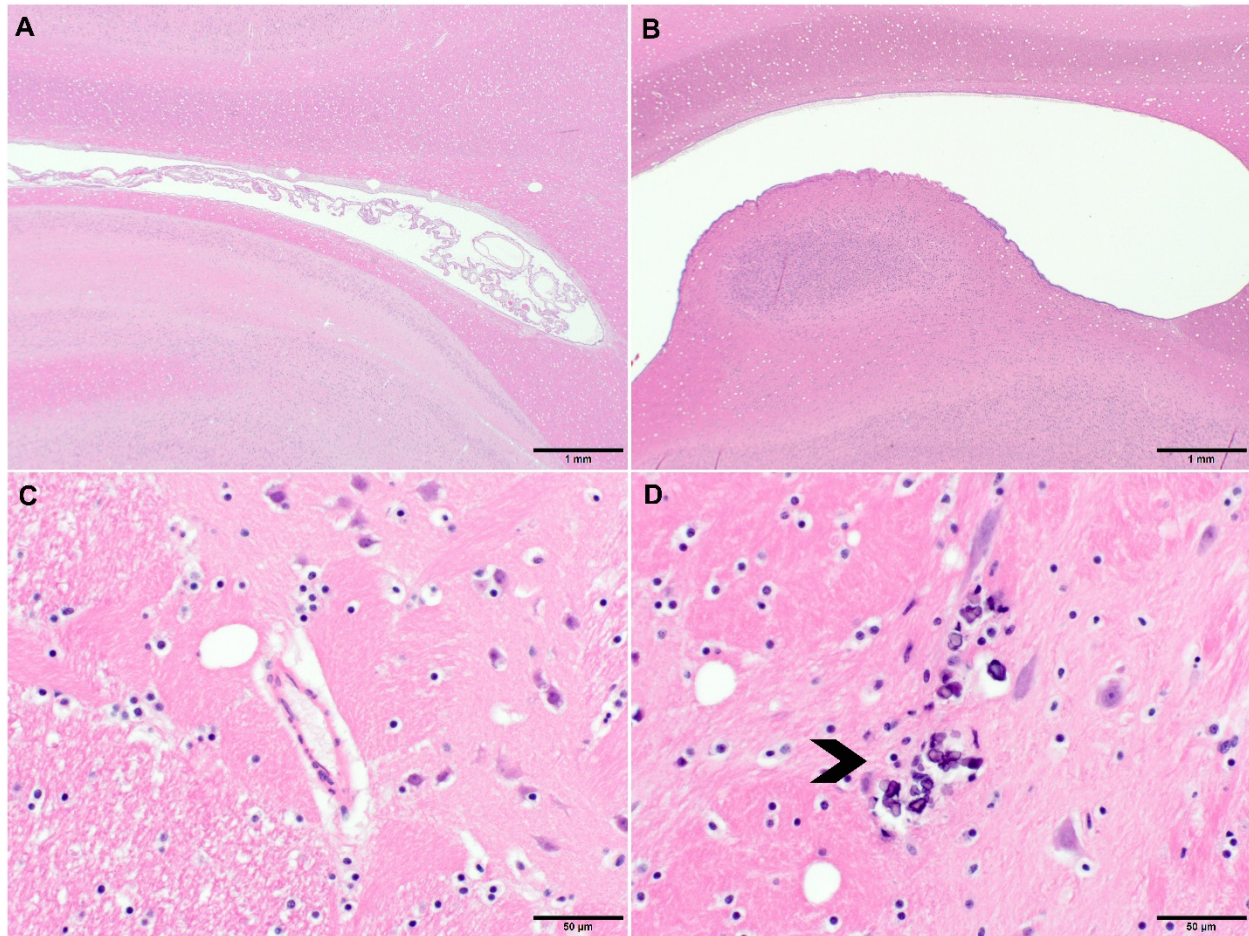


Figure 5. Neurohistopathology at 12 months of age after postnatal ZIKV infection of infant RMs. (A) Hematoxylin and eosin (H&E) staining of occipital cortex of control animal, 20 × magnification; (B) H&E staining showing distention of lateral ventricle in a occipital cortex of ZIKV-infected RM, 20 × magnification; (C) H&E staining of basal ganglia (putamen) of control animal, 400 × magnification; (D) Areas of neuropil calcification (arrowhead), H&E staining, 400 × magnification.

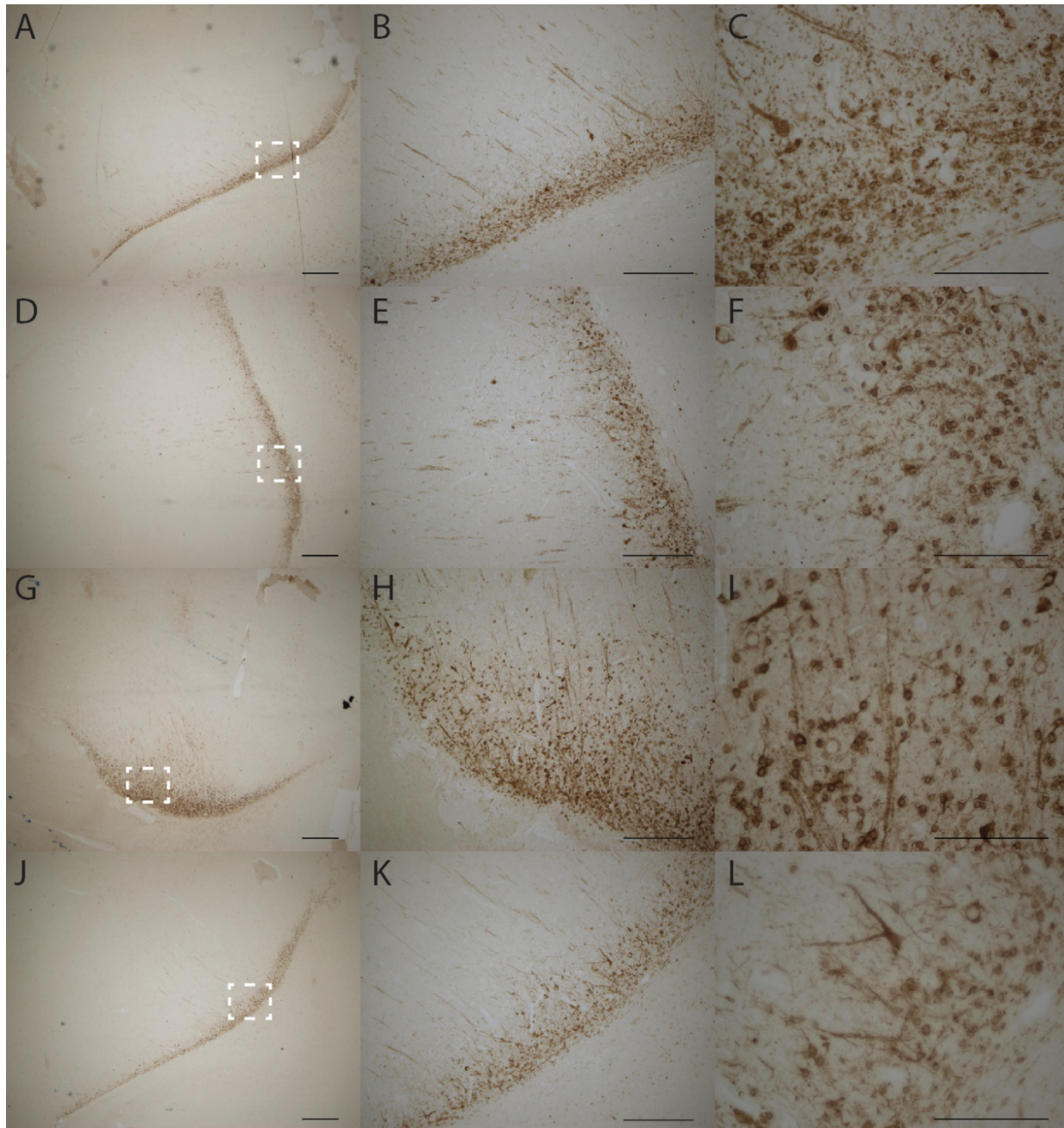
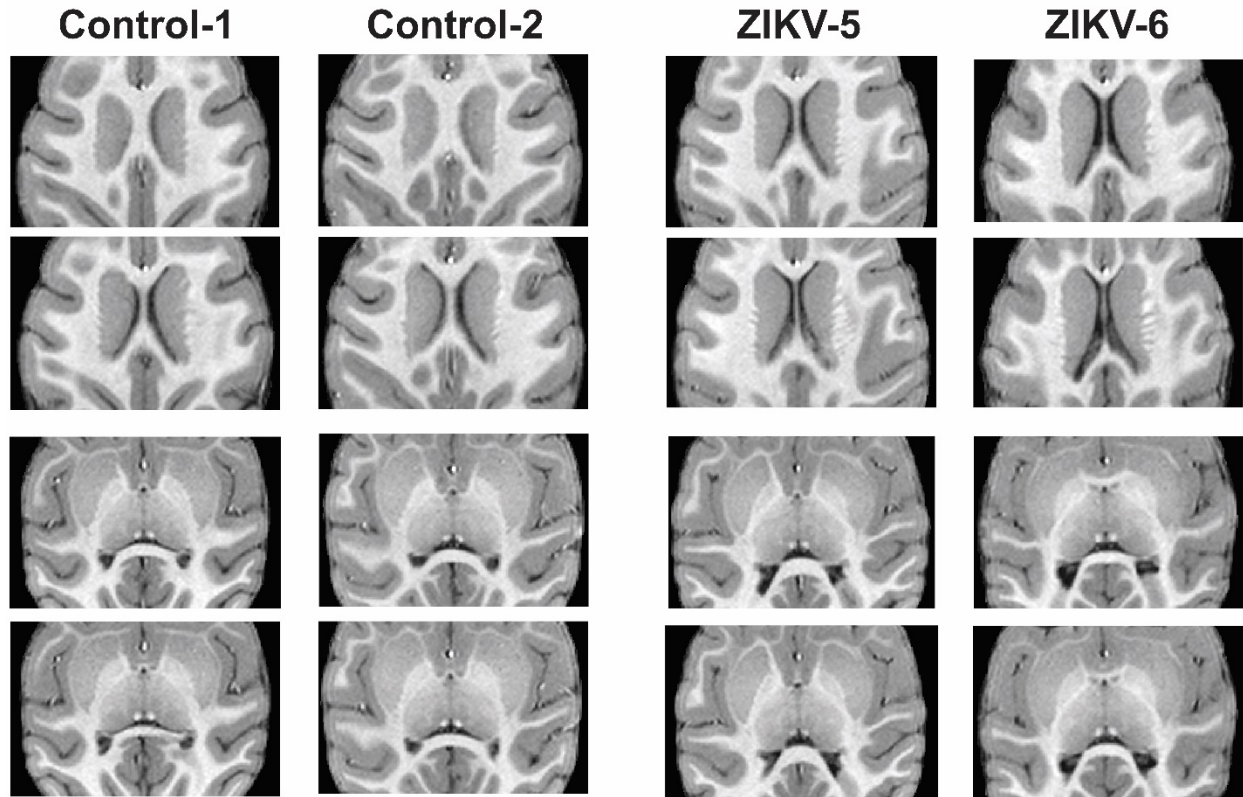


Figure 6. Postnatal ZIKV infected infant RMs exhibit increased immature neurons in the amygdala at 12 months of age. Doublecortin staining in the paralamina nucleus of the amygdala representative sections shown for ZIKV-5 (A-C), ZIKV-6(D-F), Control-1 (G-I) and Control-2 (J-L). Left panels (A, D, G, J) are taken at 2.5x magnification and the white dotted box is the location of the higher magnification images. Middle panels (B, E, H, K) are taken at 10x magnification. Right panels (C, F, I, L) are taken at 40x magnification. Scale bars 2.5x=500 μ m; 10x=250 μ m; 40x=100 μ m.



Supplemental Figure 1. Series of horizontal T1-weighted structural MRI images through the level of the lateral ventricle at 12 months of age in control and ZIKV-infected RMs.