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In Utero Exposure to Zika Virus Results in sex-Specific Memory Deficits and Neurological Alterations in Adult Mice

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Abstract

Transplacental transmission of Zika virus (ZIKV) during early pregnancy may lead to several neurological alterations, known as congenital Zika syndrome. We have previously shown that intravaginal infection of immunocompetent dams with ZIKV during the early stage of pregnancy triggers neuroinflammation in fetuses, characterized by increased brain expression of inflammatory factors, including IL-6, IL-18, CCL2, CXCL1, and CXCL10. The present study sought to understand the long-term consequences of these early cerebral alterations. For that, pregnant FVB/NJ immunocompetent females were infected intravaginally on the gestational day (GD) 4.5 and experiments were performed when offspring reached between 4 to 5 months of age. The exposure to ZIKV promoted significant neuronal cell loss detected in the CA1 region of the hippocampus of adult mice. However, only females showed reduced expression levels of brain-derived neurotrophic factor (BDNF), postsynaptic density protein 95 (PSD95), and syntaxin-1A, which are important genes related to neuronal function and synaptic plasticity. Moreover, the protein levels of the pre- and postsynaptic markers, SNAP25 and PSD95, respectively, were decreased exclusively in ZIKV-exposed female mice, indicating that ZIKV exposure *in utero* induces synaptic loss. Additionally, only females exposed to ZIKV showed risk-taking behavior and hippocampal-dependent spatial memory deficit. Together, these results demonstrate that intravaginal infection of mice on GD4.5 induces neurological alterations in the offspring detectable in their adulthood and that female mice, rather than male mice, are more susceptible to ZIKV-induced neurobehavioral alterations.

Summary Statement

In utero exposure to ZIKV leads to decreased number of neurons in adult mice. Female mice exposed to ZIKV *in utero* exhibit lower levels of BDNF, a decrease in synaptic markers, memory deficits, and risk-taking behavior during adulthood.

Keywords

ZIKV, hippocampus, memory deficit, BDNF, neurodegeneration, synaptic loss

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Introduction

Zika virus (ZIKV) is an enveloped single-stranded RNA virus of the Flaviviridae family, Flavivirus genus, that was initially isolated from the serum of a Rhesus monkey in the Zika forest of Uganda in 1947 (Dick, 1952; Kuno et al., 2018). In 2015, ZIKV was responsible for a major outbreak that took place first in Brazil and then spread to the Americas (Bogoch et al., 2016; Meaney-Delman et al., 2016). This virus is transmitted primarily by mosquitoes of the Aedes genus, such as *Aedes aegypt* and *Aedes albolpictus* (Diagne et al., 2015;

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Tesla et al., 2017; Stenn et al., 2019). No clinical signs are detected in the majority of the cases and, when symptoms are present, they are generally mild and include fever, arthralgia, and skin rash (Simpson, 1964; Mitchell et al., 2019). During the 2015 outbreak, inter-human transmission through sexual contact and transmission from mother to fetus was documented (D'Ortenzio, 2016; Hills et al., 2016; McCarthy, 2016; Venturi et al., 2016). ZIKV was shown to survive in testicular and vaginal cells for several days after infection, in addition to being able to cross the placental barrier in pregnant women and reach the fetus (Hazin et al., 2016; Matheron et al., 2016; Prisant et al., 2016). Evidences from the 2015 outbreak have clearly demonstrated an association between ZIKV infection and congenital neurological malformations in children born from mothers infected during pregnancy (Brasil et al., 2016; de Araújo et al., 2016; França et al., 2016; Victora et al., 2016). Infection in pregnant women can lead to severe damage to fetal brain development, such as hydrocephalus, multifocal dystrophic calcifications, cortical atrophy, ventriculomegaly, and microcephaly (Carvalho et al., 2016; Mlakar et al., 2016). Fetal brain disorders caused by ZIKV infection during pregnancy are known as congenital ZIKV syndrome (CZS).

Several studies have investigated the neurological alterations induced by maternal ZIKV infection in newborns exhibiting microcephaly. However, little is known about the neurological consequences in adult life resulting from ZIKV exposure during the embryonic period in the case of normocephalic babies at birth. Considering that only 1 to 13% of exposed fetuses exhibit physical malformations at birth (França et al., 2016) and that various clinical manifestations were reported in normocephalic babies exposed to ZIKV during pregnancy (Karwowski et al., 2016; van der Linden et al., 2016), more studies are needed to identify the possible neurological alterations in adult-onset. For instance, motor impairments and seizures were reported in normocephalic babies exposed to ZIKV *in utero* (Karwowski et al., 2016; van der Linden et al., 2016). Moreover, neuropathologies, including intracranial calcifications, polymicrogyria, and ventricular enlargement, have been reported in infants without microcephaly (Melo et al., 2016; Aragao et al., 2017; Chimelli et al., 2017; Honein et al., 2017). These follow-up studies indicate that the absence of microcephaly at birth does not exclude the development of ZIKV-related brain abnormalities later in life, raising the possibility that a large number of individuals exposed to ZIKV *in utero* may present with neurobehavioral deficits as they grow and become adults (Einspieler et al., 2019; Nielsen-Saines et al., 2019).

In this context, the aim of the present study was to evaluate neurological impairment in adult mice born from females infected with ZIKV on gestational day (GD) 4.5 via intravaginal route. In a previous study of our group, employing this same protocol, we found that although the liver and brain of all dams and the placentae of most fetuses were positive for ZIKV, the virus was detected in the brain of only about 42% of embryos by GD17.5 (Khaiboullina et al., 2019). In the present study, experiments were performed when offspring reached between 4 to 5 months of age. ZIKV exposure during the embryonic period led to significant neuronal alterations in the hippocampus of adult mice. Interestingly, we observed sex differences in outcomes of ZIKV infection, as females exhibited risk-taking behavior, spatial memory deficit and synaptic alterations, while such disorders were not observed in males. Thus, our results demonstrate that intravaginal infection of mice with ZIKV during pregnancy induces neurological alterations in the offspring detectable in their adulthood and that female mice are more susceptible to the deleterious effects derived from this exposure.

Materials and Methods

Materials

Alcohol 75% (Sigma Aldrich), Bovine serum albumin (BSA) (Sigma Aldrich), Chloroform (Sigma Aldrich, C2432-500ML), ECL Western blotting detection reagents (CAT # #RPN2232), Entellan (MERCK), Glycogen (Invitrogen, CAT # AM9510), Hydrogen peroxide (Sigma Aldrich, CAT # H3410-1L), Isopropanol (Sigma Aldrich), Mouse anti-NeuN antibody (EMD Millipore, CAT # MAB377), Nuclease-free water (Ambion), Paraformaldehyde (Sigma Aldrich), Power SYBR® green PCR master mix (Thermo Fisher Scientific, CAT # 4367659), Rabbit anti-Iba-1 antibody (Thermo Fisher Scientific, CAT # PA5-21274), Rabbit anti-PSD95 polyclonal antibody (Invitrogen, CAT # 516900), Rabbit anti-S100-β antibody (Abcam, CAT # ab52642), Rabbit anti-Snap25 polyclonal antibody (Abcam, CAT # ab5666), Rabbit anti-Vinculin antibody (Abcam, CAT # ab129002), SG vector peroxidase substrate kit (Vector Labs, SK-4700), Sucrose (Sigma Aldrich), Tissue tek (Sakura), Triton X 100 (Sigma Aldrich), Trizol (Life Technologies, 15596018), Vectastain elite ABC kit (Mouse IgG) (Vector Labs, CAT # PK-6101).

Animals

FVB/NJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were bred and housed in a controlled room at 23°C on a 12 h light/12 h dark cycle. All animal procedures were approved by the Committee on Animal Ethics of the Universidade Federal de Minas Gerais (CEUA/UFMG, permit protocol 400/2018).

Virus

A low-passage-number clinical isolate of ZIKV (HS-2015-BA-01), obtained from a viremic patient with a symptomatic infection in Bahia State, Brazil, in 2015, was used. The complete genome of the virus is available at GenBank under the accession no. KX520666. Virus stocks were propagated in C6/36

Aedes albopictus cells and titrated as described previously (Costa et al., 2017).

Intravaginal Infection

Detection of the estrus phase was undertaken by vaginal lavage collected using 20 µL of sterile PBS and transferred to a glass slide to determine the cell morphology by light microscopy. Female mice (2 to 4 months of age) in the estrus phase were placed with one male for a period of 24 h in order to obtain timed pregnant females. Four days after intercourse, another vaginal lavage was performed to verify pregnancy. Females identified as pregnant were randomly assigned and inoculated with 10 µL of ZIKV (1.0×10^6 PFU) or PBS (MOCK infection) on the gestational day (GD) 4.5. After infection, each pregnant female was kept in a separate cage. A total of 5 pregnant female mice were infected with ZIKV, whereas 7 females were MOCK infected. ZIKV infection of dams was confirmed in the spleen by RT-qPCR. ZIKV-infected dams gave birth to 17 females and 15 males and MOCK-infected dams offspring consisted of 11 female and 19 male mice. Offspring was weaned (day 21) and separated between males and females (day 30). All experiments and sample collections were performed in male and female mice, exposed or not to ZIKV during the embryonic period, when they reached adulthood (4 to 5 months).

Open Field Test

Spontaneous locomotor activity was assessed using an automatic open field apparatus (LE 8811 IR Motor Activity Monitors PANLAB, Harvard Apparatus; Spain), with acrylic box dimension of $450 \times 450 \times 200$ mm (width x depth x height). All experiments using open field apparatus were performed during the light cycle. First, mice were habituated to the behavioral testing room for at least 60 min. Then, animals were placed in the open field apparatus and horizontal activity (distance traveled) and center distance (as a % of total distance) was measured during 60 min. Quantification of total activity was calculated using the ACTITRACK program.

Object Location Task

This test is based on mice preferential spontaneous exploration of objects placed at a novel location. The apparatus used was an open box made of PVC 50 cm × 35 cm × 25 cm surmounted by a video camera and a light. Two identical objects made of glass or plastic were used. Objects weight was such that they could not be displaced by mice. As far as we could ascertain, they had no natural significance for mice and they had never been associated with reinforcement. Initial tests showed that mice did not have any preference for the objects used. The general procedure consisted of three different phases: a familiarization phase, a training phase and a test phase. On the 1st day, mice were individually submitted to a single familiarization session of 10 min, during which they were introduced to the empty arena. Twenty hours later, animals were submitted to a single 10 min training session during which two identical objects were placed in symmetrical positions from the center of the arena and each object was 15 cm from the side walls. After a 90 min delay, during which mice returned to their homecage, animals were reintroduced into the arena for 10 min (test phase) and exposed to the same objects, but one of the objects was displaced to a novel position. To control odor cues, the apparatus was cleaned with 70% ethanol and ventilated between each session and animal. All sessions were performed during the first part of the light cycle and mice were acclimated to the room for at least 15 min before the beginning of each session. Exploration time was defined as sniffing or touching the object with the nose. Data are expressed as recognition index, calculated according to the following formulae: 1) Training phase: time exploring object 1 × 100/(time exploring object 1 + time exploring object 2); 2) Test phase: time exploring the object 1 placed at the new location x 100/(time exploring the object 2 placed at the familiar + time exploring the object 1 placed at the new location), as previously described (Doria et al., 2018).

Y Maze Test

The apparatus consists of a Y-shaped maze with three identical arms (30 cm x 6 cm x 20 cm) identified as A, B, and C. This test assesses working memory based on mice preference to investigate a new arm of the maze rather than returning to one that was previously visited. Mice were placed at the end of one of the arms and allowed to walk freely through the maze for 8 min. It was defined as correct spontaneous alternation when mouse enters different arms three times in a row (ex: ABCABCABC…). Correct spontaneous alternation index was calculated as: (total number of alternations/total number of arms traversed - 2) x 100 (Miedel et al., 2017). It was considered that animals that reached an alternation index greater than 50% had preserved working memory.

Immunohistochemistry (IHC)

Mice, at 4 to 5 months of age, were anesthetized (ketamine 80 mg/kg/xylazine 8 mg/kg) and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Mouse brains were dissected out and stored in 4% PFA for 72 h. Prior to sectioning, brains were put into 30% sucrose in PBS overnight at 4°C. Brains were coronally sectioned in cryostat and 30 µm slices were stored in cryoprotect solution. Coronal slices containing hippocampus were selected for immunohistochemistry analysis using a peroxidase-based immunostaining protocol to label free-floating sections. In brief, endogenous peroxidase activity was quenched using 0.3% hydrogen peroxide and washed 2 times per 5 min with PBS. Then, membranes were permeabilized using 1% Triton X-100 for 10 min. Non-specific binding was blocked for 30 min using 1.5% horse serum in the case of NeuN

immunostaining or goat serum in the case of S100-β and IBA-1 immunostaining. Slices were incubated with either mouse anti-NeuN (1:1000), rabbit anti-S100-β (1:1000) or rabbit anti-IBA-1 (1:500) primary antibodies, in 2% normal horse or goat serum (from Vector Elite Kit) and 3% BSA in PBS overnight at 4°C. Sections were washed in PBS and then incubated in secondary antibody, biotinylated horse anti-mouse (1:200, Vector Elite ABC kit mouse) or biotinylated goat anti-rabbit (1:200, Vector Elite ABC kit rabbit) for 90 min at 4°C. Finally, sections were incubated in avidin-biotin enzyme reagent complex (from Vector Elite Kit) for 90 min at 4°C, according to the manufacturer's instructions. Immunostaining was visualized using a chromogen (Vector SG substrate). Sections were mounted on slides and visualized using an Axio Imager A2-Carl Zeiss Microscope with a Zeiss 20 × lens, representative 220 µm × 150 µm areas of CA1 hippocampus or 250 µm × 250 µm areas of CA3 were imaged for analyses. The number of NeuN, S100-β or IBA-1-positive puncta per image was counted by a blinded observer using the cell counter tool from ImageJ (NIH, USA, RRID:nif-0000-30467), as previously described (Doria et al., 2015).

RT-qPCR

RNA from hippocampal samples of mice between 4 to 5 months of age (Postnatal day 120, P120) was isolated using TRIzol™ reagent as per manufacturer's instructions (Thermo Scientific). RNA was resuspended in 11 µL of nuclease-free water, and its concentration was analyzed by spectrophotometer (NanoDrop™, Thermo Scientific). cDNAs were prepared from 2 µg of total RNA extracted in a 20 µL final reverse transcription reaction. RT-qPCR was performed from 10 × diluted cDNA and using Power SYBR™ Green PCR Master Mix in the QuantStudio™ 7 Flex real-time PCR system platform (Applied Biosystems). RT-qPCR assays were performed to quantify mRNA levels of the following *Mus musculus* genes: Tumor necrosis factor alpha (TNF-α); Interleukin 1 beta (IL-1β); IL-6; C-C Motif Chemokine Ligand 2 (CCL2); Brain-derived neurotrophic factor (BDNF); Syntaxin-1A (STX1A); Postsynaptic density protein 95 (PSD95); Hypoxanthine Phosphoribosyltransferase 1 (HPRT1) and 60S ribosomal protein L32 (RPL32). Primers were designed using Primer3Plus Program (26): TNF-α (forward: 5′-GCTGAGCTCAAACCCTGGTA-3′; reverse: 5′-CGGACTC CGCAAAGTCTAAG-3′); IL-1β (forward: 5′-GGGCCT CAA AGGAAAGAATC-3′; reverse: 5′-TACCAGTTGG GGAACT CTGC-3′); IL-6 (forward: 5′-GATGGATG CTACCAAAC TGGA-3′; reverse: 5′-GATGGATGCTACCA AACTGGA-3′), CCL2 (forward: 5′-AACTGCATC TGCCCTAAGGT-3′; reverse: 5′-CTGTCACACTGGTCA CTCCT-3′); BDNF (forward: 5′-GAGAAGAGTGA TGACCATCCT-3′; reverse: 5′-TCACGTGCTCAAAAGTG TCAG-3′); STX1A (forward: 5′-GAGCAGATTGCCA TGTCTGA-3′; reverse: 5′-CACTGC GCAGATGTCAAGTC-3′); PSD95 (forward: 5′-TCTGTG CGAGAGGTAGCAGA-3′; reverse: 5′-AAGCACTCCGTG AACTCCTG-3′); HPRT1 (forward: 5′-GGGCCTCAAA GGAAAGAATC-3′; reverse: 5′-TACCAGTTGGGGAACTC TGC-3′); RPL32 (forward: 5′-GCTGCCATCTGTTTTAC GG-3′; reverse: 5′-TGACTGG TGCCTGATGAACT-3′). Quantification of ZIKV mRNA levels was also performed using the following primers: forward: 5'-TCAAACGAATGGCAGTCAGTG-3' and reverse: 5'-GCTTGTTGAAGTGGTGGGAG-3′. Previous verification of undesired secondary formations or dimers between primers were performed using OligoAnalyser 3.1 tool (Integrated DNA Technologies©), available at https://www.idtdna.com/calc/analyzer. All primers used in this work were validated by serial dilution assay and the reaction efficiency was calculated, comprising 90–110% (data not shown). All RT-qPCRs showed good quality of amplification and changes in gene expression were determined with the $2^{-\Delta\Delta Ct}$ method using HPRT1 and/or RPL32 for normalization.

Immunoblotting

The hippocampus from mice at 4 to 5 months of age were dissected and lysed in RIPA buffer containing SigmaFast™ Protease Inhibitor Cocktail Tablets. Total cellular protein (40 µg) from each sample was subjected to SDS-PAGE, followed by immunoblotting onto nitrocellulose membranes. Membranes were blocked with 5% BSA in wash buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.0, and 0.05% Tween 20) for 1 h and then incubated with either rabbit anti-SNAP25 (1:2000) or mouse anti-PSD95 (1:1000) antibodies in wash buffer containing 3% BSA at 4°C overnight. Membranes were rinsed three times with wash buffer and then incubated with secondary peroxidase-conjugated anti-rabbit IgG (SNAP25) or anti-mouse IgG (PSD95) antibody diluted 1:5000 in wash buffer containing 3% skim milk for 1 h. Membranes were rinsed three times with wash buffer and incubated with ECL western blotting detection reagents. Antibodies were then stripped and membranes were incubated with rabbit anti-vinculin (1:2000) antibody for 2 h and probed with secondary antibody anti-rabbit IgG diluted 1:5000. Non-saturated, immunoreactive SNAP25 and PSD95 bands were quantified by scanning densitometry. Immunoband intensity was calculated using ImageJ (NIH, USA, RRID:nif-0000-30467) to determine the number of pixels of SNAP25 and PSD95 bands.

Statistical Analyses

Means ± SEM are shown for the number of mice indicated in each graph. GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California, USA) was used to analyze data for statistical significance. Data were submitted to the Kolmogorov-Smirnov test for normality evaluation. Statistical significance ($p < 0.05$) was determined by either two-way repeated measures ANOVA followed by Sidewalk's multiple comparisons test or unpaired Student *t*-test.

Results

In our previous study (Khaiboullina et al., 2019), we showed that intravaginal infection of FVB/NJ pregnant female mice with ZIKV on GD4.5 resulted in fetuses exhibiting massive neuroinflammation on GD17.5 (Khaiboullina et al., 2019). To determine the consequences of ZIKV *in utero* exposure for adult offspring, pregnant FVB/NJ mice were challenged with ZIKV (1.0×10^6 PFU) or PBS (MOCK infection) intravaginally, on GD4.5, and offspring at the age of 4 to 5 months were submitted to a series of behavior and cellular/molecular analysis (Figure 1A). Adult female and male mice exposed to ZIKV during the embryonic period did not show any overt alterations in body weight (Sup. Figure 1) and ZIKV was not detected in the brain of these mice by RT-qPCR at 5 months of age (data not shown).

To test whether exposure to ZIKV during the embryonic period would lead to memory deficits in adulthood, female and male adult mice were evaluated through behavioral tests dependent on hippocampal memory, such as the object location task and the Y-maze test. The object location task, which assesses spatial memory, consists of letting mice explore a rectangular apparatus containing two objects for 10 min and, 90 min later, returning them to the apparatus for another 10 min where one of the objects is placed at a new location. It is considered preserved memory when mice explore the object at the new location for longer than the object kept in its original position (Jablonski et al., 2013). ZIKV-exposed females were unable to discriminate between the objects placed at the novel and familiar location, while MOCK-exposed females spent more time exploring the object at the new location (Figure 1B), indicating that ZIKV-exposed female animals exhibit a deficit in spatial memory. ZIKV-exposed males had intact memory, as they were able to distinguish between the object placed at the novel position and the object remaining at the familiar location (Figure 1D). Regarding the Y-maze test, MOCK and ZIKV-exposed male and female mice exhibited preserved working memory, as the index of correct spontaneous alternation exhibited by these experimental groups was higher than chance (50%) (Figure 1C, 1E). The open field test evaluates spontaneous locomotor activity, as well as anxiety/risk-taking behavior by assessing the percentage of time animals spend in the center of the apparatus (Stanford, 2007; Cui et al., 2018). There was no difference in the total distance traveled when comparing MOCK- and ZIKV-exposed female (Figure 1F, 1G) and male (Figure 1H, 1I) mice. However, ZIKV-exposed female mice appeared not to habituate to the open field apparatus, as these animals remained hyperactive in the last 15 min of the task, whereas MOCK-exposed females exhibited a decrease in their spontaneous locomotor activity (Figure 1F). ZIKV-exposed female mice also spent more time in the center of the apparatus than MOCK-exposed mice (Figure 1J, 1K), suggesting risk-taking behavior. No difference in time spent in the center of the apparatus was observed in the case of male mice (Figure 1L, 1M).

It has been shown previously that ZIKV infection increases the number of astrocytes, as well as astrocytic reactivity (Lossia et al., 2018; Ledur et al., 2020). Thus, we evaluated astrogliosis in hippocampal brain slices of adult male and female mice exposed to ZIKV *in utero* by counting the number of cells positive for the astrocytic marker, S100-β. However, the number of astrocytes in the CA1 region of the hippocampus was not different when comparing MOCK- and ZIKV-exposed female (Figure 2A, 2B and 2C) and male (2D, 2E and 2F) mice. To investigate whether other hippocampal regions could exhibit astrogliosis due to ZIKV exposure *in utero*, we determined the number of S100-β positive cells in the CA3 and dentate gyrus (DG) of the hippocampus of the same mice. Again, we found no difference when comparing the number of astrocytes in the CA3 and DG of MOCK- and ZIKV-exposed female and male mice (Sup. Figure 2). We and others have shown that ZIKV infection leads to microglia activation and proliferation (Costa et al., 2017; Figueiredo et al., 2019). Thus, our next step was to determine whether ZIKV-exposure *in utero* could lead to microgliosis during adult life by counting the number of cells positive for the microglia marker, IBA-1. The number of microglia was not changed in the CA1 region of the hippocampus of female (2G, 2H and 2I) and male (2J, 2K and 2L) adult animals exposed to ZIKV during the embryonic period. When the CA3 and DG were analyzed, again, no difference was observed in the number of microglia when comparing MOCK- and ZIKV-exposed animals (Sup. Figure 3). Moreover, no overt morphological alterations were observed when comparing microglia and astrocytes from MOCK- and ZIKV-exposed animals. As we observed evident neuroinflammation in our previous study employing the same infection protocol but investigating embryos on GD17.5, we decided to measure the expression levels of the inflammatory factors we found altered the most (Khaiboullina et al., 2019). Expression of IL-1β, TNF-α, IL-6, and CCL2 was not modified by ZIKV exposure during the embryonic period in female (Figure 2M, 2N, 2O and 2P) and male (Figure 2Q, 2R, 2S and 2T) hippocampus during adulthood. Together, these results indicate that ZIKV-induced neuroinflammation does not persist during adulthood.

It is possible that the neuroinflammation that was observed on GD17.5 in embryos exposed to ZIKV on GD4.5 could result in the loss of neurons and neuronal precursor cells and, if this is the case, this neuronal loss could still be observable in adult animals. To test that, hippocampal slices from adult mice exposed to ZIKV during the embryonic period were stained with the neuronal marker, NeuN. It is possible to observe a striking reduction in the number of NeuN positive cells in the CA1 of female (Figure 3A, 3B and 3C) and male (Figure 3D, 3E and 3F) mice exposed to ZIKV *in utero* when compared to their respective controls. A decrease in the number of neurons in the CA3 region is also noticeable both in female (Figure 3G, 3H and 3I) and male (Figure 3J,

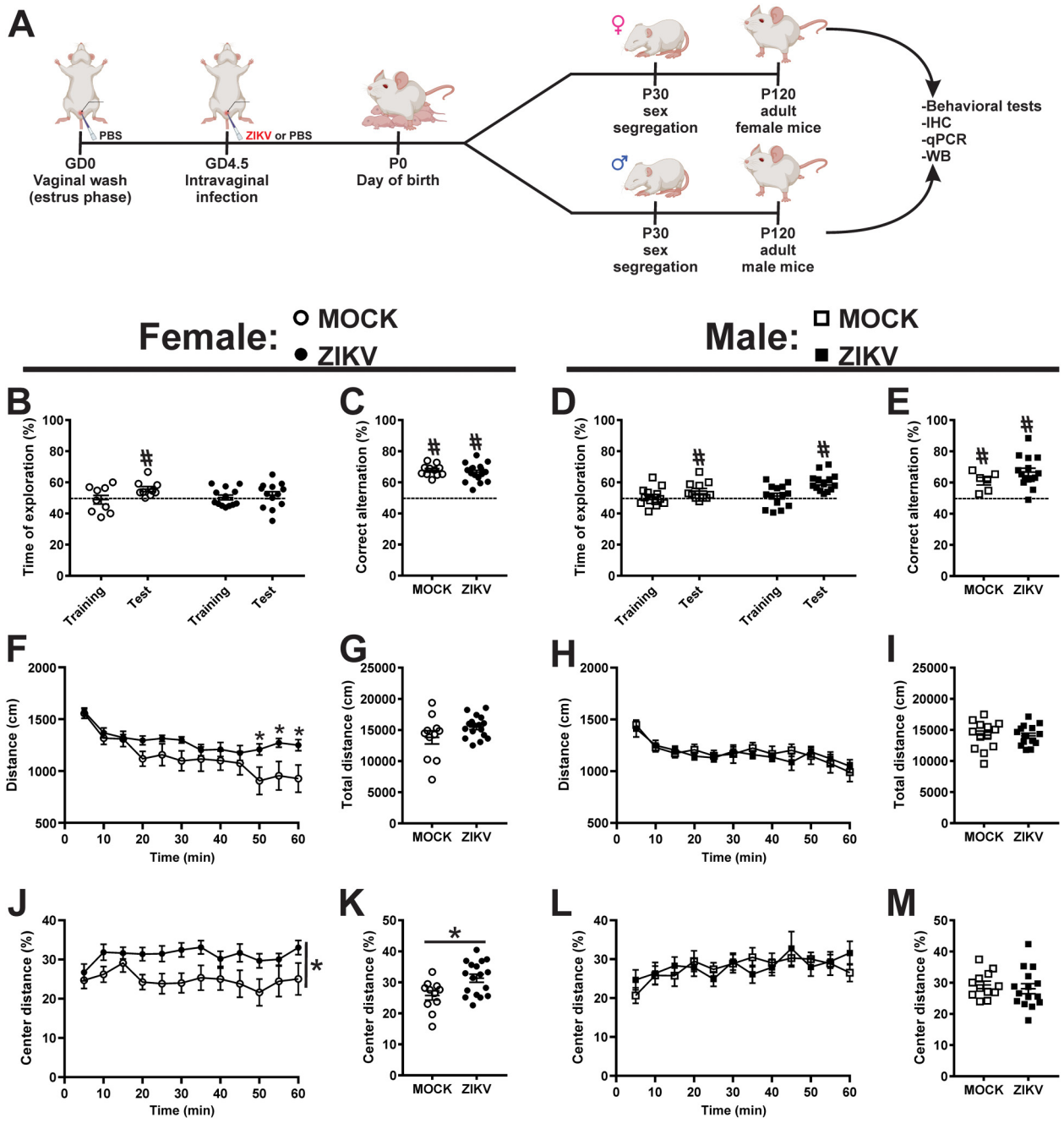

Figure 1. *ZIKV exposure during the embryonic period affects female behavior*. (A) Female FVB/NJ mice were submitted to vaginal lavage to determine the estrus phase and then placed with one male for a period of 24 h to obtain timed pregnant females. They were infected intravaginally with 10 µl of ZIKV (1.0×10^6 PFU) or PBS (MOCK infection) on the gestational day (GD) 4.5. Offspring was separated between males and females at day 40. All experiments were performed in male and female mice, exposed or not to ZIKV during the embryonic period, when they reached adulthood (4 to 5 months). Graphs show time of object exploration by MOCK and ZIKV-exposed female (B) and male (D) mice, calculated as the recognition index for the training and test phases. A score of 50% indicates no preference. Graphs show the spontaneous alternation rate during the Y-maze test by MOCK and ZIKV-exposed female (C) and male (E) mice. A score greater than 50% indicates preserved working memory. Graphs show total distance traveled (F, H) and the percentage of distance traveled in the center of the apparatus (J, L) by MOCK and ZIKV-exposed female and male mice measured at 5 min intervals. Graphs show total distance traveled (G, I) and the percentage of distance traveled in the center of the apparatus (K, M) by MOCK and ZIKV-exposed female and male mice. Each animal was monitored for 60 min. Data represent the means ± SEM. # ($p < 0.05$) indicates significant difference from chance (50%). * ($p < 0.05$) indicates a significant difference as compared to MOCK mice.

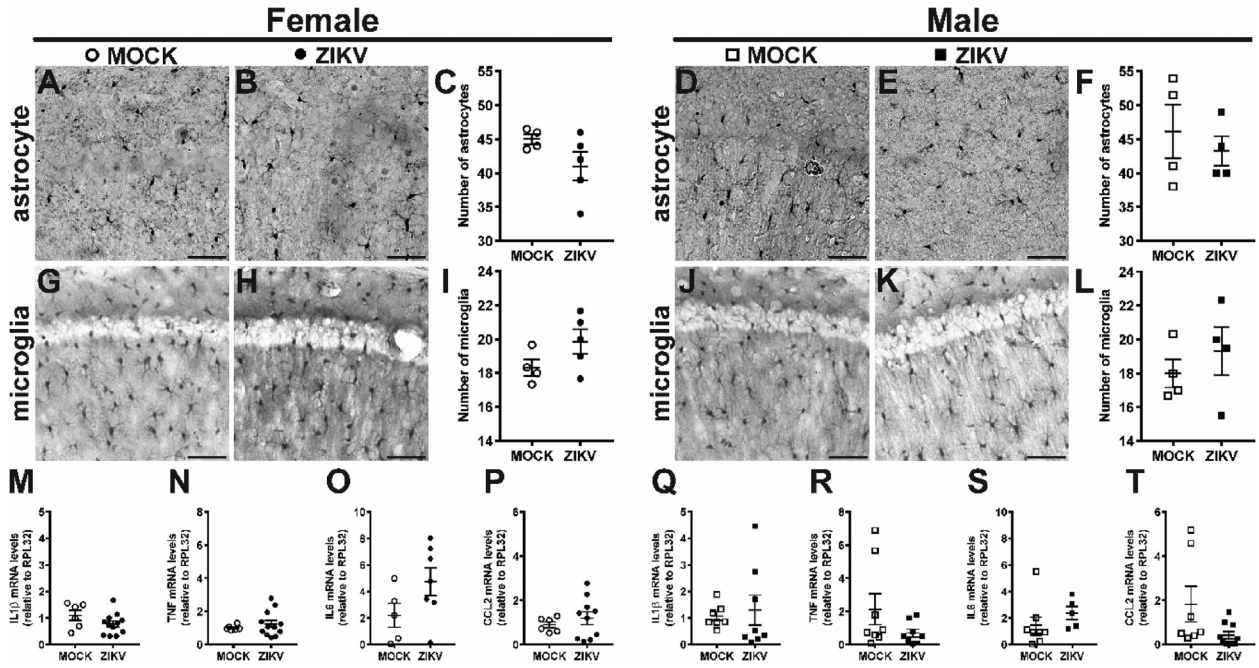

Figure 2. *ZIKV exposure during the embryonic period does not lead to an inflammation that persists until adulthood.* Shown are representative images for S100-β immunostaining of the hippocampal CA1 region from MOCK female (A), ZIKV female (B), MOCK male (D), and ZIKV male (E) mice. Scale bar = 50 μm. Graphs show quantification of S100-β-labeled hippocampal CA1 astrocytes per 62500 μm^2 in MOCK and ZIKV-exposed female (C) and male (F) mice. Data represent the means ± SEM obtained from 3 images taken from 3 histological slices per mouse. Shown are representative images for IBA-1 immunostaining of the hippocampal CA1 region from MOCK female (G), ZIKV female (H), MOCK male (J), and ZIKV male (K) mice. Scale bar = 50 μm. Graphs show quantification of IBA-1-labeled hippocampal CA1 microglia per 62500 μm^2 in MOCK and ZIKV-exposed female (I) and male (L) mice. Data represent the means ± SEM obtained from 3 images taken from 3 histological slices per mouse. Graphs show mRNA levels of IL-1β (M, Q), TNF-α (N, R), IL-6 (O, S) and CCL2 (P, T) in hippocampal samples from MOCK and ZIKV-exposed female and male mice. mRNA levels were assessed by quantitative RT-PCR, which was performed in duplicate and normalized to RPL32 mRNA levels.

3K and 3L) mice. However, in the case of the CA3 region of the hippocampus, these changes did not reach statistical significance. No difference in the number of NeuN positive cells was observed when comparing the DG of MOCK and ZIKV-exposed animals (Figure 3M, 3N, 3O, 3P, 3Q and 3R).

Despite the neuronal cell loss triggered by ZIKV, male mice showed no alteration in the behavioral tests performed in this study, which led us to hypothesize that other factors could be underlying the behavioral deficits shown by females. The brain-derived neurotrophic factor (BDNF) is a protein of the neurotrophin family that is known to act as a neuronal survival factor, in addition to participating in neurogenesis and synaptogenesis (Castrén and Kojima, 2017). Therefore, we decided to evaluate whether ZIKV exposure during the embryonic period could alter the expression of BDNF in the hippocampus of the adult offspring. BDNF mRNA levels were decreased in the hippocampus of adult female (Figure 4A), but not male mice (Figure 4B), exposed to ZIKV during the embryonic period, as compared to control animals. As it has been proposed that memory deficits more closely correlate with synaptic loss than with neurodegeneration (Figueiredo et al., 2019; Moodley and Chan, 2014), we decided to evaluate the expression of the pre- and postsynaptic markers, syntaxin-1A and PSD95, respectively. ZIKV-exposed female animals showed a statistically significant reduction in the expression levels of syntaxin-1A (Figure 4C) and PSD95 (Figure 4E) in the hippocampus, although males showed no difference (Figure 4D and 4F).

As PSD95 and syntaxin-1A mRNA levels indicated that ZIKV-exposed animals could exhibit decreased number of synaptic terminals, we performed western blot experiments to confirm this reduction in the expression of synaptic terminal markers at the protein level. Corroborating the results obtained in the RT-qPCR experiments, there was a decline in the protein levels of the presynaptic marker, SNAP25 (Figure 5A and C), and of the postsynaptic marker PSD95 (Figure 5D and F), in ZIKV-exposed females, as compared to uninfected animals. Male mice exposed to ZIKV exhibited no changes in SNAP25 (Figure 5B and C) and PSD95 (Figure 5E and F) protein levels. Thus, female exposure to ZIKV during the embryonic period leads to a decrease in the number of pre- and postsynaptic markers, which could be an underlying mechanism leading to the memory alterations exhibited by these animals.

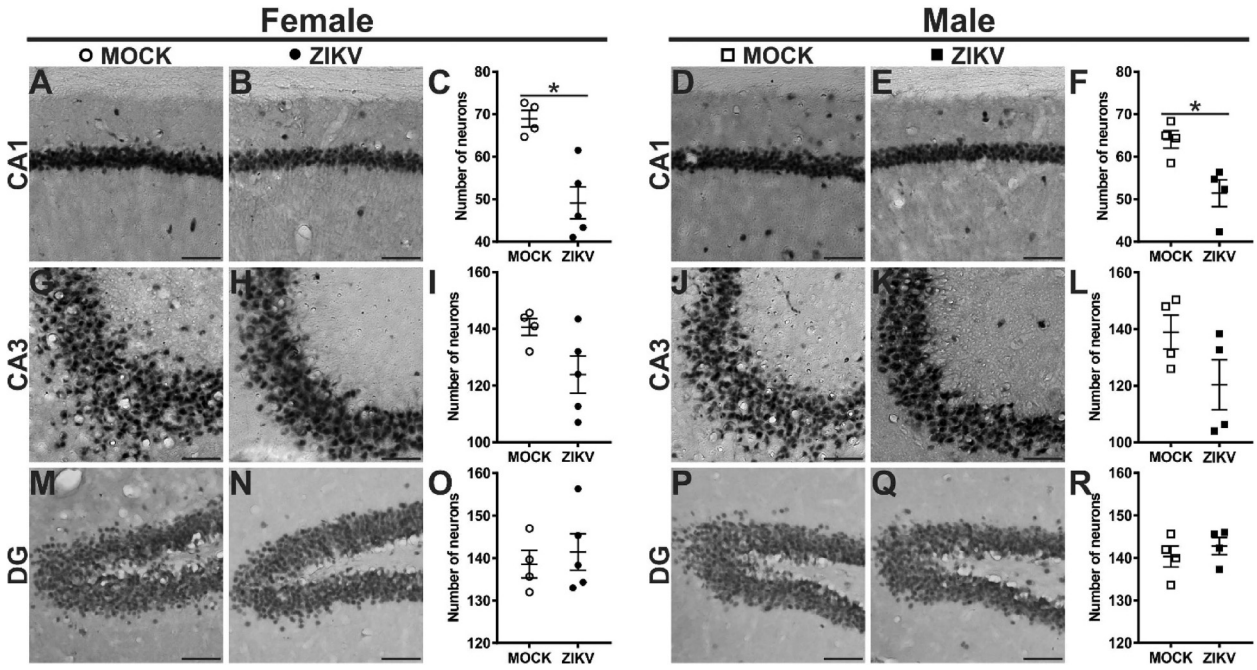

Figure 3. *ZIKV exposure during the embryonic period leads to a decrease in the number of neurons in the hippocampus of adult mice.* Shown are representative images for NeuN immunostaining of the hippocampal CA1 region from MOCK female (A), ZIKV female (B), MOCK male (D), and ZIKV male (E) mice. Scale bar = 50 μm. Graphs show quantification of NeuN-labeled hippocampal CA1 neurons per 22000 μm^2 in MOCK and ZIKV-exposed female (C) and male (F) mice. Shown are representative images for NeuN immunostaining of the hippocampal CA3 region from MOCK female (G), ZIKV female (H), MOCK male (J), and ZIKV male (K) mice. Scale bar = 500 μm. Graphs show quantification of NeuN-labeled hippocampal CA1 neurons per 62500 μm^2 in MOCK and ZIKV-exposed female (I) and male (L) mice. Shown are representative images for NeuN immunostaining of the hippocampal DG region from MOCK female (M), ZIKV female (N), MOCK male (P), and ZIKV male (Q) mice. Scale bar = 50 μm. Graphs show quantification of NeuN-labeled hippocampal CA1 neurons per 62500 μm^2 in MOCK and ZIKV-exposed female (O) and male (R) mice. Data represent the means ± SEM obtained from 3 images taken from 3 histological slices per mouse. * ($p < 0.05$) indicates a significant difference as compared to MOCK mice.

Discussion

The results shown in this study demonstrate that *in utero* exposure to ZIKV leads to decreased number of hippocampal neurons in adult mice. Interestingly, we observed sex differences in outcomes of ZIKV infection, as females exhibited risk-taking behavior, spatial memory deficit and synaptic alterations, while such disorders were not observed in males. It has been shown that transplacental transmission of ZIKV during early pregnancy may lead to several neurological alterations, known as CZS, which is characterized by severe microcephaly, a partially collapsed skull, decreased brain tissue with a specific pattern of brain damage and calcifications, eye alterations, congenital contractures, hypertonia, and restricted body movements (Costello et al., 2016; Hazin et al., 2016; Khaiboullina et al., 2019). When ZIKV reaches fetal brain, it primarily infects NPCs in the cortical subventricular region and the dentate gyrus, promoting cell death and causing deficits in neurogenesis (Li et al., 2016). In addition to directly causing neuronal cell death, ZIKV may also induce neuroinflammation via overactivation of microglia and astrocytes, which further exacerbate neurodegeneration (Hamel et al., 2017; Figueiredo et al., 2019; Khaiboullina et al., 2019). By compromising neurogenesis, these damages to the CNS during the neurodevelopmental stage can generate long-term consequences, such as learning and memory deficits (Raper et al., 2020). The majority of fetuses exposed to ZIKV *in utero* do not display physical malformations at birth (Franca et al., 2016). However, it is now clear that some infants exposed to ZIKV *in utero* with no obvious CZS at birth develop neurodevelopmental abnormalities such as hydrocephalus and severe hearing and ocular defects later in life (Karwowski et al., 2016; van der Linden et al., 2016; Aragao et al., 2017). These evidences raised concerns that infants exposed to ZIKV *in utero*, that are now reaching school age, even if apparently asymptomatic, may present with subtle neurological alterations later in life resulting from changes in neuronal maturation and neuroplasticity, which could lead to motor, behavioral, learning, or psychiatric difficulties. The data shown here shed some light on this issue, indicating that *in utero* exposed mice exhibit behavioral alterations and decreased number of neurons and synapses during adult life and that females appear to be more prone to ZIKV-induced injuries.

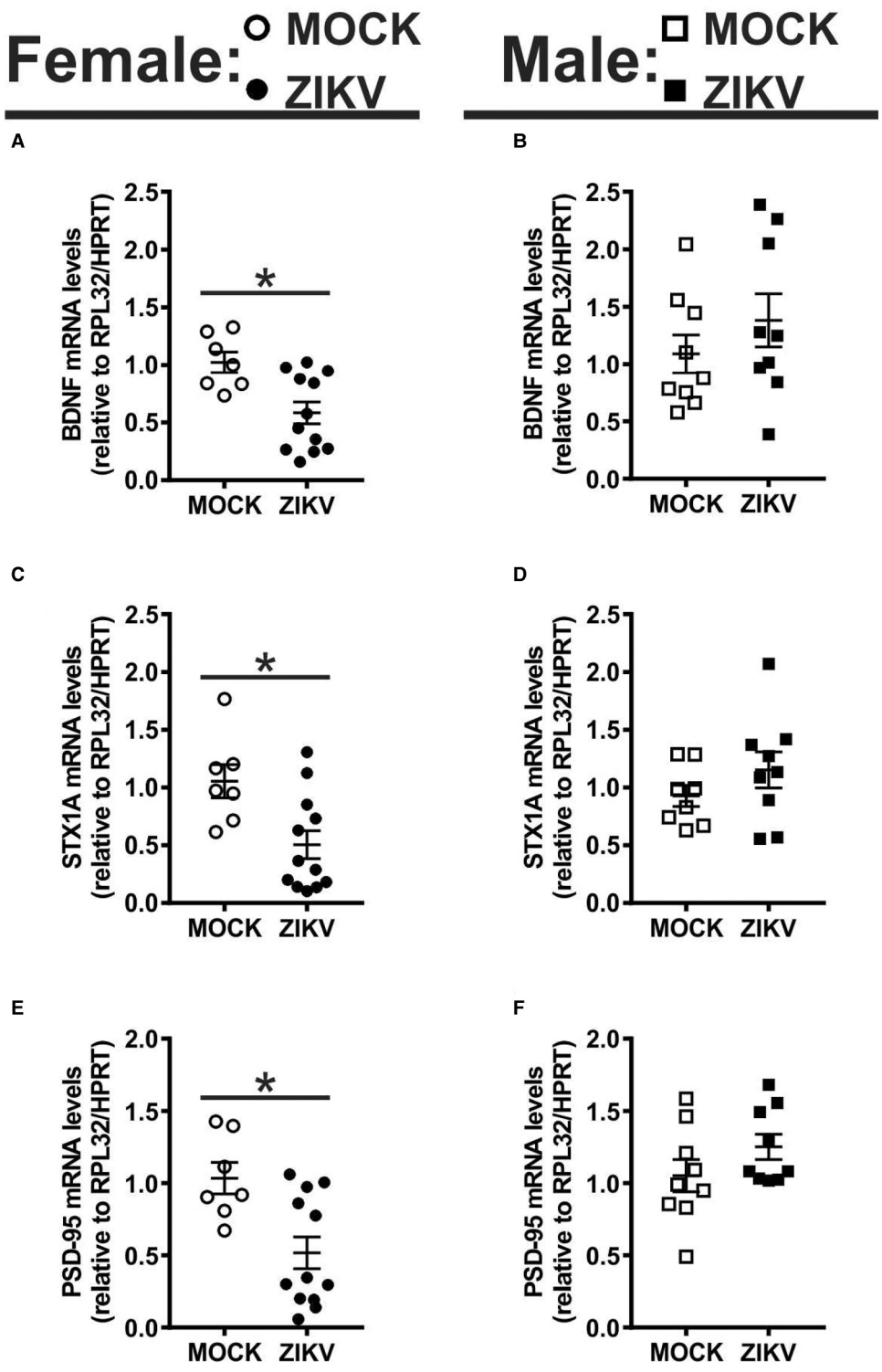

Figure 4. *ZIKV-exposed females show decreased expression of BDNF and the synaptic markers, syntaxin-1A and PSD95 in the hippocampus.* Graphs show mRNA levels of BDNF (A, B), syntaxin-1A (STX1A) (C, D) and PSD95 (E, F) in the hippocampus of MOCK and ZIKV-exposed female and male mice. mRNA levels were assessed by quantitative RT-PCR, which was performed in duplicates and normalized to RPL32 and HPRT mRNA levels. Data represents the means ± SEM. * ($p < 0.05$) indicates a significant difference as compared to MOCK mice.

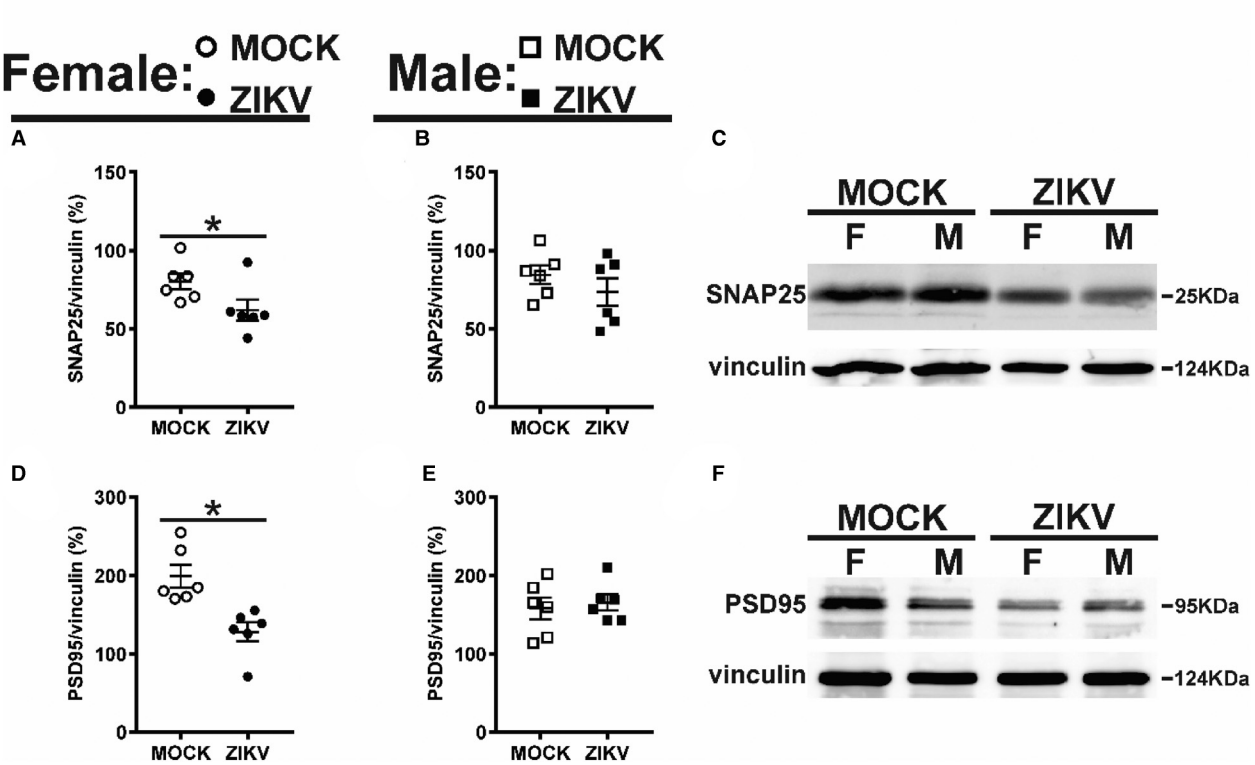

Figure 5. *ZIKV-exposed females exhibit synaptic loss in the hippocampus.* Graphs show the densitometric analysis of SNAP25 protein expression normalized to vinculin in the hippocampus of MOCK and ZIKV-exposed female (A) and male (B) mice. Data represents the means ± SEM. * ($p < 0.05$) indicates a significant difference as compared to MOCK mice. (C) Shown are representative immunoblots for SNAP25 (upper panel) and vinculin (lower panel) expression in the hippocampus of MOCK and ZIKV-exposed female and male mice. 40 μg of total cell lysate was used for each sample. Graphs show the densitometric analysis of PSD95 protein expression normalized to vinculin in the hippocampus of MOCK and ZIKV-exposed female (D) and male (E) mice. Data represents the means ± SEM. * ($p < 0.05$) indicates a significant difference as compared to MOCK mice. (F) Shown are representative immunoblots for PSD95 (upper panel) and vinculin (lower panel) expression in the hippocampus of MOCK and ZIKV-exposed female and male mice. 80 μg of total cell lysate was used for each sample.

Previously, our research group investigated the effects of maternal ZIKV infection on embryos (GD4.5) using an immunocompetent murine model of intravaginal infection (Khaiboullina et al., 2019). The results show that ZIKV triggers neuroinflammation in fetuses (GD17.5), characterized by increased brain expression of inflammatory factors, including IL-6, IL-18, CCL2, CXCL1, and CXCL10 (Khaiboullina et al., 2019). The present study sought to understand the long-term consequences (4-5 months) of these early cerebral alterations induced by the exposure of embryos to ZIKV. Although all dams enrolled in this study were positive for ZIKV, we were unable to detect ZIKV in the brain of adult offspring. Previous studies indicate that replicating ZIKV persists in the brain of mice injected subcutaneously with the virus on the postnatal day one, even one-year post-infection (Ireland et al., 2020). However, there are many differences when comparing this study to ours, including the mouse strain employed (C57BL/6 versus FVB/NJ), the route of infection (subcutaneous versus intravaginal), the day of infection (P1 versus GD4.5), and the brain region analyzed (cerebellum versus hippocampus). Even though we could not detect ZIKV in the brain of adult mice, we assume that approximately half of the offspring had their brains infected by the virus, as we observed an infection rate of about 42% when dams were infected with ZIKV on GD4.5 and embryo brains were analyzed on GD17.5 (Khaiboullina et al., 2019). Moreover, all embryos were exposed to an inflammatory environment, as most placentae (93%) and all dams (liver and brain) were infected by ZIKV (Khaiboullina et al., 2019). Importantly, the robust phenotype exhibited by ZIKV-exposed females, despite the absence of persistent infection, indicates that clearing the virus is not enough to avoid the long-lasting deleterious outcomes of *in utero* exposure to ZIKV.

To assess whether the exposure of embryos to ZIKV during gestation induces memory deficits, behavioral experiments focusing on hippocampal-dependent memory were performed (Zhang et al., 2016; Costa et al., 2017; Rosa-Fernandes et al., 2019; Zhang et al., 2019). Surprisingly, female, but not male mice, exposed to ZIKV during the embryonic period exhibited compromised spatial memory. Allied to this memory deficit,

female mice exposed to ZIKV exhibited risk-taking behavior, as they spent a greater percentage of time in the center of the open field apparatus, while male mice did not show such behavioral alteration in comparison to their control group. These results differ from what has been published previously, showing that ZIKV infection leads either to no change or to anxious behavior, as ZIKV-infected animals spend less time in the center of the open field apparatus (Cui et al., 2018; De Sousa et al., 2020). However, a recently published study indicates that ZIKV-infected animals (P1) spend more time on the open arm of the elevated plus maze, which is indicative of risk-taking behavior (Ireland et al., 2020). Interestingly, among mice exposed to ZIKV, only females showed cognitive impairment, lack of habituation, and increased time in the center of the open field apparatus. Thus, it is possible that these changes have not been observed in previous models because most studies only assess males. However, we cannot rule out the possibility that the alterations observed in this study are specifically related to the paradigm we employed: FVB/NJ mice exposed to ZIKV during the embryonic period (GD4.5) through intravaginal infection of pregnant females. Few studies have compared male and female immunocompetent animals exposed to ZIKV during the gestational period. Although employing subcutaneous ZIKV infection one day after birth, it is worth mentioning one study that assessed the neural and behavioral alterations of males and females using four different mouse strains, C57BL/6, AG129S1, FVB/NJ, and DBA (Snyder-Keller et al., 2019). AG129S1 female mice, 14 days after infection, exhibited higher levels of neurodegeneration when compared to males. When mice were evaluated by the open field and elevated plus maze tests, AG129S1 infected females showed hyperactive and impulsive behavior compared to males (Snyder-Keller et al., 2019). In the case of FVB mice, although infected females tended to behave abnormally, the results were not statistically different as compared to those of MOCK-infected animals (Snyder-Keller et al., 2019). In this context, we hypothesize that we found different results because in our study dams were infected intravaginally with ZIKV on GD4.5, which may have affected the developing CNS during a very vulnerable period for neurodevelopment. Moreover, this study corroborates the data shown here indicating that female mice exposed to ZIKV during gestation are more prone to neurological alterations. Another study employing Swiss mice infected with ZIKV on postnatal day three also shows that females, but not males, exhibit impaired social behavior (Nem de Oliveira Souza et al., 2018). During the ZIKV epidemic of 2015 and 2016, many children developed microcephaly, but an even greater number were exposed to ZIKV during pregnancy (Victora et al., 2016). We are still unaware of the cognitive consequences of this exposure as most of these children have not reached school age. However, a few studies have shown that young children exposed to ZIKV during pregnancy, even without microcephaly, exhibit cognitive impairment (Karwowski et al., 2016; Aguilar Ticona et al., 2021). Future studies evaluating cognitive alterations in children and young adults exposed to ZIKV during gestation will be important to determine whether females are more susceptible to ZIKV-induced neurological alterations. Furthermore, the mechanisms underlying CNS sex-specific responses to *in utero* ZIKV exposure should be further investigated.

Embryonic exposure to ZIKV caused massive neuronal cell loss in the CA1 region of the hippocampus in both male and female mice. Although the CA3 region also appeared to be affected, the difference between ZIKV and MOCK-infected animals did not reach statistical difference. The connections between neurons in the CA1 and CA3 areas are crucial for short- and long-term memory (Malenka, 2003). Therefore, the memory deficits presented by females exposed to ZIKV could result from this neuronal loss. However, the neuronal loss presented by males exposed to ZIKV during the embryonic period does not seem to have affected their performance in the Y maze and object recognition tests. Several studies indicate that memory deficit is better correlated with the loss of synaptic terminals (Abraham et al., 2019). The hippocampal gene expression analysis indicated that the expression of the pre- and post-synaptic markers, syntaxin-1A and PSD95, respectively, were reduced in females exposed to ZIKV, although this alteration was not observed in males. Importantly, the decrease in synaptic markers at the mRNA level was mirrored by a decrease in SNAP25 and PSD95 protein amounts exclusively in females exposed to ZIKV *in utero*. Furthermore, BDNF expression was decreased in female animals exposed to ZIKV, even though BDNF levels were shown to be unchanged in males. BDNF is important for synaptogenesis, being able to regulate the translation of proteins in synapses (Santos et al., 2010). BDNF up-regulates the expression of PSD95, a protein of great importance for memory (Hu et al., 2011). For instance, it has been shown that the expression of PSD95 was decreased in a murine model of Alzheimer's disease (Hong et al., 2016) and that the memory and learning deficits presented by these animals were attenuated when the expression of PSD95 was increased through the use of a gene editing tool (Bustos et al., 2017). Thus, we hypothesize that the memory deficit observed in females may be related to a decrease in the number of synaptic terminals.

The results presented herein show that embryonic exposure to ZIKV mainly affects females. It is well known that females and males have different types of microglia depending on age. Although the number of microglia between female and male mice is not different until GD18.5, the brain of males has a greater number of amoeboid-type microglia, known to promote greater inflammation during neurodevelopment, while microglia of females have a higher inflammatory profile during adulthood (Hanamsagar and Bilbo, 2016). However, we did not observe differences in the number and overall morphology of microglia when comparing MOCK versus ZIKV-exposed male and female mice. Estradiol is also pointed as a key factor contributing to sex differences

in brain homeostasis. 17β-estradiol (E2), the main female steroid hormone, has a neuroprotective effect and it is important for synaptogenesis (Srivastava et al., 2010). In mice, ovarian E2 synthesis occurs only after birth and, until then, the only source of this hormone is through maternal synthesis (Varshney and Nalvarte, 2017). However, in female fetuses the plasma protein α-fetoprotein binds and reduces the bioavailability of E2, as this hormone has a masculinizing effect during neurodevelopment (Varshney and Nalvarte, 2017). In cultured cells, it has been shown that ERβ activation by estrogen hormones decreases the neuroinflammation promoted by LPS (Baker et al., 2004). In addition, positive modulators of estrogen receptors have been shown to reduce microglia activity following LPS challenge in adult animals (Tapia-Gonzalez et al., 2008), indicating that this decrease in neuroinflammation is also relevant in vivo. Female ERβ knockout mice showed reduced levels of BDNF in the hippocampus region and deficits in spatial memory (Rissman et al., 2002; Chhibber et al., 2017). Furthermore, ERβ modulation promotes increased neuronal plasticity in the hippocampus and improves performance in hippocampal-dependent cognitive tests (Fan et al., 2010). During the neurodevelopmental stage, males have not only E2, but also other androgen hormones, such as dihydrotestosterone and testosterone, which promote activation of synaptogenesis (Hatanaka et al., 2015; Guo et al., 2020). Therefore, we hypothesize that maternal exposure to ZIKV may have a more striking effect in female mice because they cannot count on the protective effects of E2 and androgen hormones. The result is increased neuroinflammation, which may increase synaptic pruning, and decreased levels of BDNF and synaptogenesis, leading to memory deficits later in life. To test this hypothesis, it would be interesting to block E2 and androgen actions in male fetuses and evaluate neuroinflammation and synaptic pruning.

Conclusion

Using an immunocompetent murine model of intravaginal infection, we demonstrated that embryonic exposure to ZIKV caused massive neuronal cell loss in the CA1 region of the hippocampus in adult mice. Interestingly, the expression of pre- and postsynaptic markers at the mRNA and protein levels were reduced in the hippocampus of females exposed to ZIKV, although these alterations were not observed in males. Expression of the neuronal survival factor BDNF was also decreased in the hippocampus of female animals exposed to ZIKV. In agreement with these molecular alterations, when evaluated in behavioral tests focusing on hippocampal-dependent memory, female, but not male mice, exhibited compromised spatial memory and risk-taking behavior. Taken together, our results indicate that exposure to ZIKV during the embryonic stage causes damage to neurons and hippocampal synapses and induces consequent memory deficit in adult mice, mainly affecting females. Even though male mice appear not affected in the behavioral tests employed in our study, the massive neuronal loss observed in the CA1 hippocampal region suggests that these animals could also be compromised and that more behavioral tests should be performed. The results shown here should bring awareness to the importance of follow up studies aiming at evaluating the cognitive performance of children exposed to ZIKV *in utero* and born without obvious CZS and special attention should be given to girls, as, according to our results, females are more prone to ZIKV-induced neurological disorders.

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Declaration of Conflicting Interests

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Supplemental material

Supplemental material for this article is available online.

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