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Supplementary Materials for

Acute and chronic neurological consequences of early-life Zika virus infection in mice

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:
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Table S2. Raw data of main figures (Excel file provided separately).
Table S3. Raw data of supplementary figures (Excel file provided separately).

Materials and Methods

Virus. Zika virus (ZIKV) was isolated from a febrile patient in the state of Pernambuco, Brazil (gene bank ref. number KX197192). The stocks used in the experiments were produced and tittered as previously described in Coelho et al., 2017 *(45)*. As a control, the same volume of virus-free conditioned medium of C6/36 cells was used (mock). In some experiments, a second control group comprising inactivated virus was used. Inactivated ZIKV (iZIKV) was obtained by direct exposure of virus sample to UV-light for 30 min. Successful inactivation was confirmed by subsequent plaque assay. Dengue virus (DENV) stocks (serotype 2, strain 16681, gene bank ref. number NC_001474) were produced and tittered as previously described *(46)*.

Animals and neonatal infection. All procedures used in the present study followed the "Principles of Laboratory Animal Care" (US National Institutes of Health) and were approved by the Institutional Animal Care and Use Committee of the Federal University of Rio de Janeiro (protocol #052/2017). Naïve 10 week-old female Swiss mice were obtained from our facilities and mated for two weeks (four females per male). After this period, dams were housed individually until delivery. On the third day after birth (P3), each litter was left with ten pups per dam, whenever possible, with equal numbers of males and females. On two occasions, litters with <10 pups were included in the experiments for evaluation of long-term outcomes of ZIKV infection.

Mice (P3) were infected subcutaneously (s.c.) with 30 µL of ZIKV (10^6 plaque forming units; PFU) or the same volume of mock medium. All littermates received the same treatment in order to avoid cross-contamination. Mice showing any signs of reflux or hemorrhage (~ 5% of animals throughout our study) were excluded from further analyses. In some control experiments, pups

were injected either with iZIKV or with 10^5 PFU of DENV. Pups were evaluated daily for mortality, and body weight was measured every three days from the day of infection until 66 days post-infection (dpi). The area under the curve for body weight gain was calculated for each mouse and analyzed between experimental groups to help identify whether ZIKV-induced decrease in weight gain is persistent over time until animals reach adulthood. As it is known that the number of pups per litter significantly affects the size and weight of pups, in experiments designed to measure body weight gain only litters normalized to 8 pups per dam were used. However, since litters were normalized at the day of infection, due to mortality caused by ZIKV, the number of pups per litter was gradually reduced in the infected group, remaining constant in the mock-treated group.

In experiments aimed at evaluating adult behavior, mice were weaned at post-natal day 21 (P21) and housed with same-sex littermates (2-5 mice per cage). Mice were housed in polypropylene cages and maintained at 25^{o}C with controlled humidity, under a 12 h light/dark cycle and ad libitum access to water and chow. Independent groups of mice were used for pre-weaning and post-weaning behavioral assessments.

For determination of viral replication and cytokine mRNA levels, mock-, ZIKV- or iZIKV-injected mice were killed by decapitation at different times post infection and brains were rapidly removed and frozen in liquid nitrogen until RNA isolation for quantitative Polymerase Chain Reaction (qPCR) analyses.

Neonatal reflexes. Temporal development of grasping reflex, negative geotaxis and righting reflex were evaluated in newborn mice from the day of infection to 7 dpi. Impaired/delayed reflexes or their complete absence in newborns usually indicates abnormalities in neurodevelopment. The righting reflex is the ability of mice to rapidly return to ventral decubitus

when placed in dorsal decubitus on a bench. Two randomly chosen pups from each litter were placed on their backs over a flat surface and were given one minute to complete the task. The grasping reflex is assessed by placing a blunt object on the paw of newborn pups. For this procedure, mice were held by the scruff of their necks and each front paw was individually stroked with the blunt end of a small paperclip. Scores were given as such: 2 if the reflex is present in both front paws; 1 if the reflex is present in only one front paw; 0 if the reflex is absent in both front paws. The negative geotaxis test evaluates not only motor coordination but also the quality of vestibular cue for perception of gravity. Mice were placed on a metallic grid inclined 30° with their heads facing downwards and were given one minute to turn 180°, finishing the task with body and head facing upwards. Mice that fell from the apparatus were retested.

Hindlimb suspension. At 9 dpi, animals were individually removed from their home cages and placed inside a 50 mL laboratory tube padded with cotton balls, facing the interior of the tube, suspended by the hindlimbs. Time to fall inside the tube was measured as an assessment of muscle strength and general neuromuscular function.

Pole test. Mice at 15 to 21 dpi were placed head-up at the top of a vertical wooden pole (height: 50 cm; diameter: 1.5 cm) covered by rough clothing. The base of the pole was placed in a mouse home cage containing clean sawdust. When placed at the top of the pole, animals orient themselves downwards and descend the length of the pole back into the cage. Animals underwent 2 days of training with five trials each to learn the task. On the third day, a test session was performed, and animals were again placed at the top of the pole and the time to orient downwards (time to turn) as well as the total time to descend the pole (time to descend) were measured; the three best performances of each animal were selected and used for the statistical analysis.

Rotarod. This test was performed in the rotarod apparatus (Insight LTDA) at 85-95 dpi. For habituation, mice were individually placed in the apparatus floor for 3 minutes and then on the aluminum cylinder without rotation for another 2 minutes. Afterwards, animals were submitted to three test sessions (~1 hour intervals) when each mouse was placed on the aluminum cylinder at an initial speed of 5 rotations per minute (RPM) which gradually increased to 35 RPM during 5 minutes. Latency to fall the cylinder was measured, and the mean of the sessions was used. Mice that fell before 15 seconds were retested.

Open field test. Mice at 85-95 dpi were individually placed at the center of an arena (30 x 30 x 45 cm). Total distance traveled in the apparatus was recorded for 5 minutes by ANY-maze software (Stoelting Company). The arena was thoroughly cleaned with 70% ethanol in between trials to eliminate olfactory cues and illuminated with an indirect source of light (~100 lux).

Novel object recognition test. The test was carried out in the same day and in the same box used for the open field test. Test objects were made of plastic and had different shapes, colors, sizes and textures. During behavioral sessions, objects were fixed to the box using tape to prevent displacement caused by exploratory activity of the animals. Preliminary tests showed that none of the objects used in the experiments evoked innate preference. Before training, each animal was submitted to a 5 min-long habituation session, in which it was allowed to freely explore the empty arena. Training consisted in a 5 min-long session during which animals were placed at the center of the arena in the presence of two identical objects. The time spent exploring each object was recorded by a trained researcher. Sniffing and touching the object were considered exploratory behaviors. The arena and objects were cleaned thoroughly with 70% ethanol in between trials to eliminate olfactory cues. Ninety minutes after training, animals were again placed in the arena for the test session, in which one of the objects used in the training session

was replaced by a new one. Again, the time spent exploring familiar and novel objects was measured. The results were expressed as percentage of time exploring each object during the training or test sessions. Animals that successfully learn the task recognize the familiar object andexplore the novel object > 50% of the total time. All animals showed comparable exploration time towards objects used in training and test sessions.

Social approach test. The social approach test was performed at 100-110 dpi in a three-chamber apparatus, which consisted in a rectangular transparent acrylic box (60 cm x 45 cm x 30 cm) with two walls dividing the box in three equal chambers of 20 cm x 45 cm x 30 cm each. Cylindrical aluminum cages of 8 cm of diameter (9.5 cm height) were used to contain a stranger mouse (a mouse with which the test mice had never had any contact before). Sociability was assessed by placing one empty cage in one lateral chamber and one cage containing the stranger mouse in the opposite chamber. The test mouse was kept in the middle chamber for 5 minutes, after which the walls were removed and the mouse was allowed to fully explore the apparatus for 10 minutes. Time exploring each cage was manually evaluated by a trained researcher blind to the experimental condition.

Seizure evaluation. Seizure evaluation was performed following protocol described by *(47)*. Recordings for all groups were performed simultaneously for one hour daily. In protocol 1, the time of day for recordings was chosen at random to eliminate possible effects of hormones, stress, body temperature and other variables on seizure development, but was always performed between 9 am and 5 pm. In recording protocol 2 (fig. S1D), animals were always recorded between 11 am and midday. For recordings, dams and their respective pups were removed from their home cages and placed in a box measuring 41 cm x 34 cm x 18 cm containing clean sawdust. Male and female pups were individually identified with permanent ink on their backs,

and high-resolution recording was performed using a Nikon D3300 camera fixed on the ceiling of the room. Videos were analyzed by an experienced researcher blind to experimental condition, who identified animals showing any degree of Racine scale seizures *(48)*. The percentage of animals showing seizures per litter during the one-hour session was determined.

Pharmacological treatments. Swiss mice were infected with 10^6 PFU of ZIKV on P3 and randomly assigned into one of the following groups: phosphate buffer saline (PBS); infliximab (20 µg/day) or N-acetylcysteine NAC (50 µg/kg/day) *(49)*. Treatments were given once a day intraperitoneally (i.p.) from the day of infection to 12 dpi. Development of seizures was assessed at 12 dpi in one "one hour-long recording session" as described above.

Electrographic recordings. Mice at 9 dpi were anesthetized (Isoflurane) and implanted with 4 gold plated electrodes (1.0 mm length; 2 in the right and 2 in the left cortical surface; fig. S2A). This allowed right and left cortical activity to be independently analyzed. During recording sessions performed between 10 and 12 dpi, mice were individually placed in a Plexiglas box measuring 41 cm x 34 cm x 18 cm. The external tip of the electrodes was then plugged to a connector linked to isolated wires that could reach the amplifier (FE136 Animal Bio Amp – AD Instruments). Signals were then digitalized (PowerLab 8/35 - ADInstruments) and saved in LabChart 8 (AD Instruments). Each animal was continuously recorded for 2 h. After recordings, animals were returned to their home cages. The same animals were recorded for up to 3 consecutive days.

Pentylenetetrazol (PTZ)-induced seizures. PTZ (Sigma-Aldrich) was dissolved in saline to a final concentration of 1% and frozen until the day of experiment. Adult mice (100-110 dpi) received PTZ (50 mg/kg, i.p.) and were immediately placed in empty 3 L glass beakers. Latency

to first seizure and number of seizures were evaluated by an experienced researcher during the following 20 minutes.

Tissue collection and preparation. Mice were killed by decapitation at 3, 6, 12, 18, 30, 36 or 100 dpi for qPCR and at 23 and 100 dpi for histological and immunohistochemical analyses. For mRNA extraction, brains were rapidly removed, frozen in liquid nitrogen and maintained at -80ºC until the moment of extraction. For histology and immunohistochemistry, mice were deeply anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) before transcardiac perfusion with cold saline solution followed by fresh ice-cold 4% formaldehyde. Brains were removed, post-fixed for 24 h in 4% formaldehyde, and embedded in paraffin after dehydration and diaphanization. Macroscopic atrophy of the brains was assessed by the determination of total dorsal brain area, using the Image J software.

Quantification of reactive oxygen species. Brains of mock and ZIKV-infected mice were dissected at 12 dpi and gently homogenized in cold Dulbecco's Modified Eagle's Medium (DMEM;Invitrogen; 1:10 w/v) using a glass tissue grinder. Reactive oxygen species (ROS) were determined in each sample by adding 2 µM of 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA - Invitrogen) to 200 µl of a 1:1 mixture of homogenate/DMEM, followed by incubation for 30 minutes in the dark at 37°C. DCF fluorescence was quantified using a fluorescence microplate reader (VICTOR™ X3 – PerkinElmer), at 492-495/517-527 nm, as recommended by manufacturers. Baseline fluorescence levels were subtracted from the final fluorescence values for each individual sample.

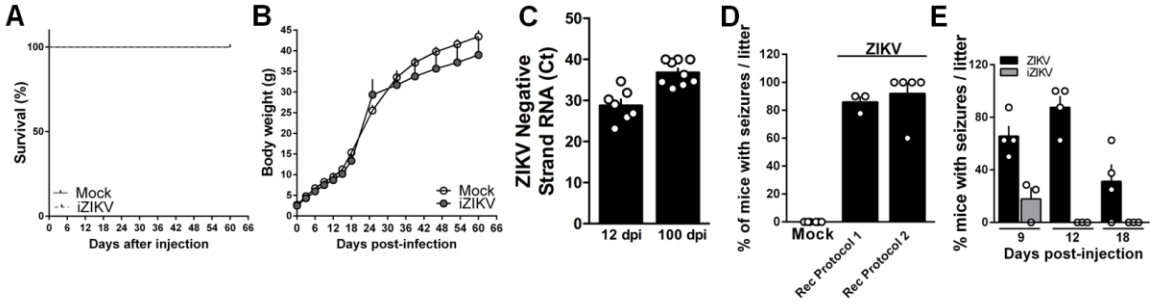
RNA extraction and qPCR. Brains were homogenized to a concentration of 0.5 mg tissue/mL in DMEM, and 200 µL of the homogenate were used for RNA extraction with Trizol (Invitrogen)

according the manufacturer's instructions. Purity and integrity of RNA were determined by the 260/280 and 260/230 nm absorbance ratios. Only preparations with ratios >1.7 and no signs of RNA degradation were used. One μg of total RNA was treated with DNAse I (ThermoFisher Scientific Inc) according to manufacturer's recommendations before complementary DNA (cDNA) synthesis using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific Inc). For negative strand quantification cDNA was synthesized using 2 pM of ZIKV 835 forward primer, 5'-TTGGTCATGATACTGCTGATTGC-3'*(50)* instead of random primers. Analyses were carried out on an Applied Biosystems 7500 reverse transcriptase PCR (RT–PCR) system using the TaqMan Mix (ThermoFisher Scientific Inc). Primers used for ZIKV detection by qPCR coded for sequence adjacent to the envelope-154 glycosilation site, as previously described *(50,51)*: forward, 5'-CCGCTGCCCAACACAAG-30; reverse, 5'-CCACTAACGTTCTTTTGCAGACAT-3'; probe, 5'-/56-FAM/AGCCTACCT/ZEN/TGACAAGCAATCAGACACTCAA/3IABkFQ/-3' (Integrated DNA Technologies). Cycle threshold (Ct) values were used to calculate the equivalence of log10 PFU/mg tissue after conversion using a standard-curve with serial 10-fold dilutions of a ZIKV stock sample. For cytokines and inducible nitric oxide synthase (iNOS) expression, qPCR analyses were performed using the Power SYBR kit (Applied Biosystems; Foster City, CA). Actin was used as an endogenous control. Primer sequences were the following: interleukin 6 (IL-6) Forward: 5'-TTC TTG GGA CTG ATG CTG GTG-3' and Reverse: 5'-CAG AAT TGC CAT TGC ACA ACT C-3'; tumor necrosis factor α (TNF-α) Forward: 5'-CCC TCA CAC TCA GAT CAT CTT CT-3' and Reverse: 5'-GCT ACG ACG TGG GCT ACA G-3'; interleukin 1β (IL-1β): GTA ATG AAA GAC GGC ACA CC and Reverse: ATT AGA AAC AGT CCA GCC CA; iNOS Forward: GTT CTC AGC CCA ACA ATA CAA GA and Reverse: GTG GAC GGG

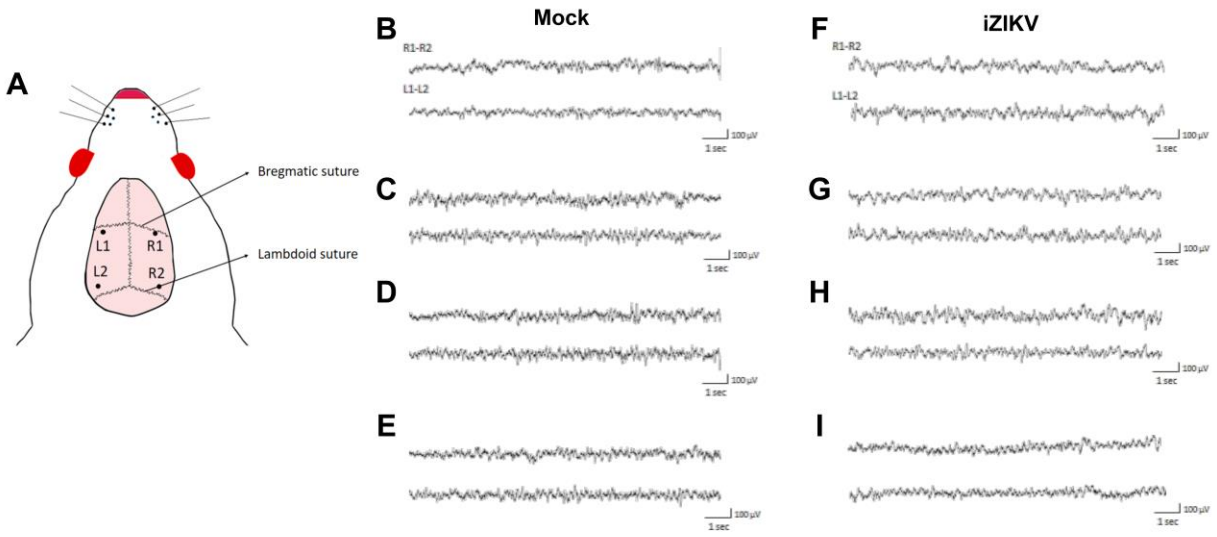
TCG ATG TCA C; KC: Fwd: CAC CTC AAG AAC ATC CAG AGC and Reverse: AGG TGC CAT CAG AGC AGT CT; Actin Forward: 5'-TGT GAC GTT GAC ATC CGT AAA-3' and Reverse: 5'-GTA CTT GCG CTC AGG AGG AG-3'.

Histological and immunohistochemistry analyses. Paraffin-embedded brain tissue sections (3-5 µm) were immersed in xylene for 10 minutes, rehydrated by incubation in absolute ethanol followed by 95% and 70% solutions of ethanol in water. For general histology, sections were stained by haematoxylin-eosin and imaged by light microscopy. For immunohistochemistry, slides were incubated with 3% hydrogen peroxide (H_2O_2) in methanol for inactivation of endogenous peroxidase. Antigens were reactivated by treatment with 0.01 M citrate buffer for 40 min at 95°C. Slides were washed in PBS and incubated with primary antibodies (Rabbit anti-Iba-1, Abcam, 1:200; rabbit anti-GFAP, DAKO, 1:500) for 12-16 hours at 2-8°C. After washing with PBS, slides were incubated with biotinylated secondary antibodies (Vector, 1:500) for 1 hour at room temperature, washed twice with PBS and incubated with streptavidin-biotin-peroxidase (Vector) for 30 min. The peroxidase reaction was visualized with 3,3'-diaminobenzidine (DAB) substrate for 1 to 5 min or until a brown precipitate could be observed. Identical conditions and reaction times were used for slides from different animals (run in parallel) to allow comparison between immunoreactivity densities. Reaction was stopped by immersion of slides in distilled water. Counter-staining was performed with Harris hematoxylin. Slides were washed in running water, dehydrated in alcohol, cleared in xylene, mounted in resinous medium and examined with light microscopy using a Sight DS-5M-L1 digital camera (Nikon) connected to an Eclipse 50i light microscope (Nikon) at different magnifications.

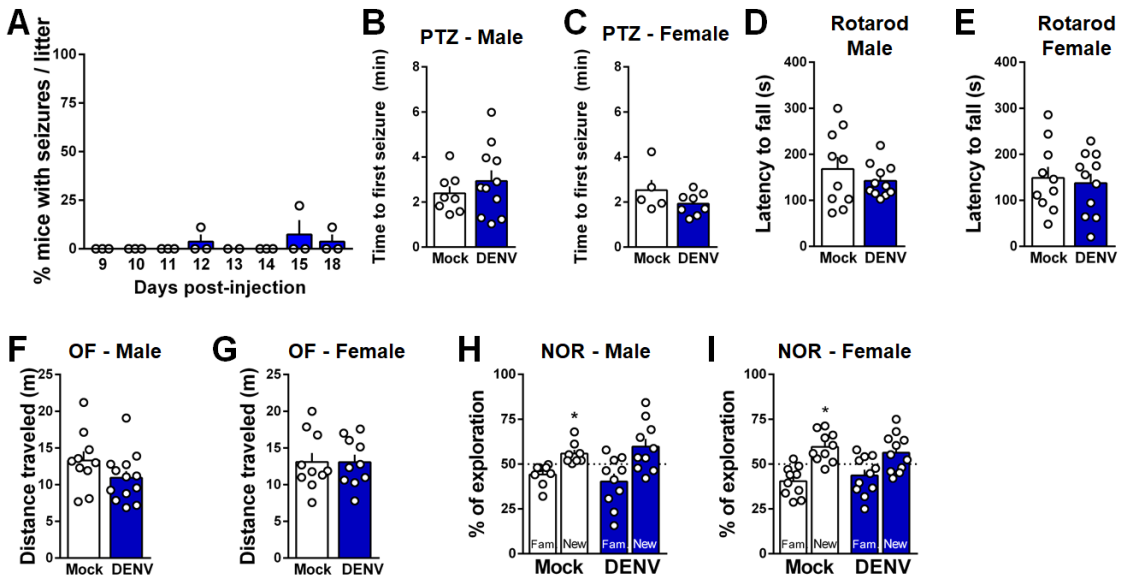
Supplementary Figures and Legends:



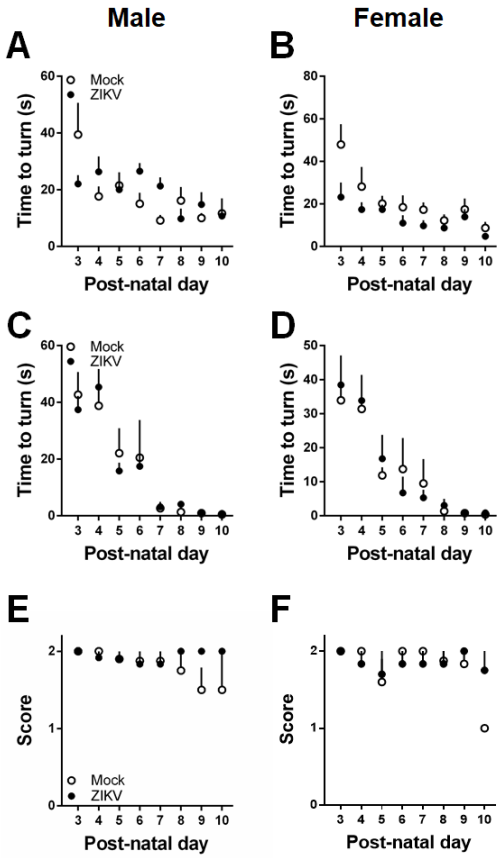
Supplementary Figure 1. Late viral brain replication and seizure evaluation in UV-inactivated ZIKV-injected mice. Mice were injected subcutaneously with 10^6 PFU ZIKV, UV-inactivated ZIKV (iZIKV) or mock at post-natal day 3. (**A**) Survival curve for mock- and iZIKV-infected groups. (n = 3 mock litters, 2 iZIKV litters). **(B)** Body weight measurement in mock and iZIKV-infected mice (n = 3 mock litters, 2 iZIKV litters). **(C)** Brains of ZIKV-infected mice were dissected at 12 or 100 dpi and processed for determination of ZIKV negative strand by qPCR (n = 7 for 12 dpi and 9 for 100 dpi). **(D)** Percentage of mice with seizures in ZIKV-infected litters using two different protocols (see Methods). (n = 4 mock, 3 ZIKV litters in protocol 1 and 5 litters in protocol 2). **(E)** Percentage of mice with seizures at 9, 12 and 18 dpi in ZIKV- and iZIKV-infected litters (n = 4 ZIKV litters, 3 iZIKV litters).Data are expressed as mean ± S.E.M.



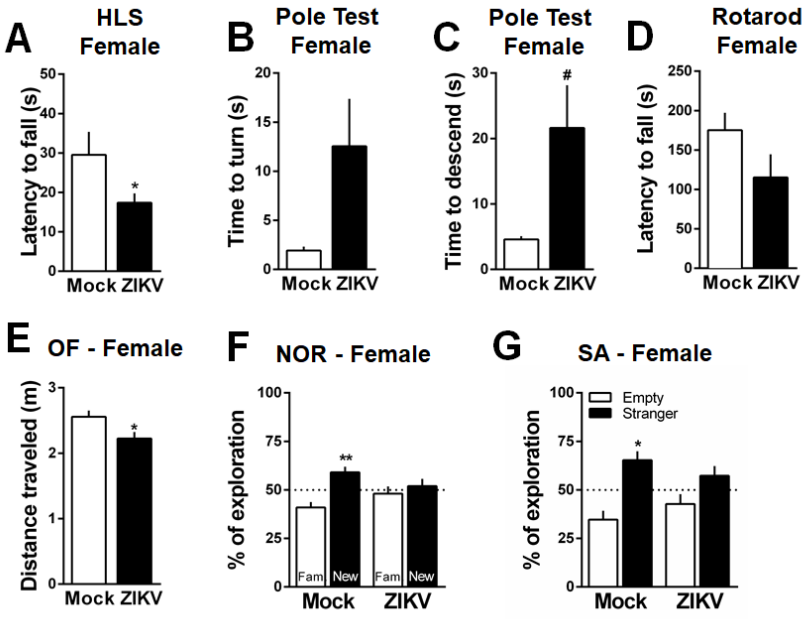
Supplementary Figure 2. Electrode implantation and electrographic recordings from mock- and UV-inactivated ZIKV-injected mice. Mice were injected subcutaneously with 10^6 PFU of ZIKV, UV-inactivated ZIKV (iZIKV) or equal volume of mock medium at post-natal day 3. **(A)** Schematic drawing of electrodes implanted in the right (R1-R2) and left (L1-L2) cortex of mice at 9dpi. Electrographic recordings obtained in the right and left cortex from mock- **(B-E)** and UV-inactivated ZIKV-injected **(F-I)** mice (10 to 12 dpi). No sign of epileptiform activity is seen in neither group. N = 2 mice/group. For recordings in ZIKV-infected mice, see Figure 2.



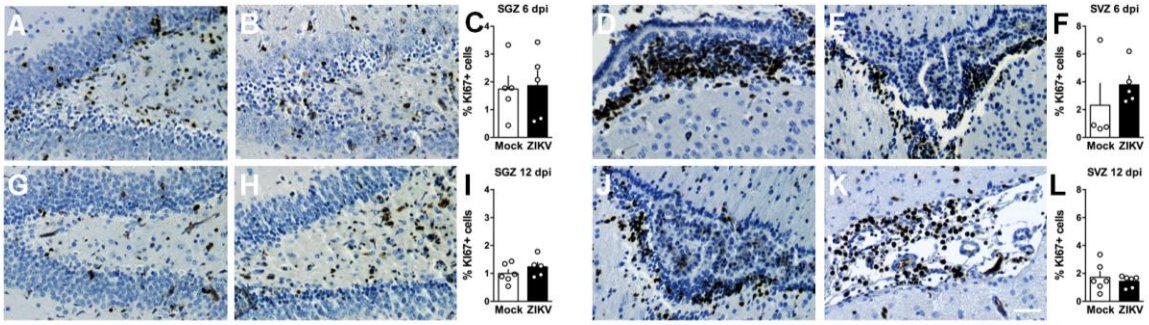
Supplementary Figure 3. DENV does not induce seizures or long-term behavioral impairment in Swiss mice. Mice were injected subcutaneously with 10^5 PFU of dengue virus (DENV) or equal volume of mock medium at post-natal day 3. (**A**) Percentage of mice with seizures in DENV-infected litters in one hour-long recording sessions performed from 9 to 15 dpi, and at 18 dpi. (n = 3 DENV litters) (**B, C**) Time to first seizure for male (**B**) (n = 8 mock, 11 DENV) and female (**C)** (n = 5 mock, 8 DENV) mice after an i.p. injection of pentylenetetrazol at 60-65 dpi. (**D, E**) Latency to fall in the rotarod for male (**D)** (n = 10 mock, 11 DENV) and female **(C)** (n = 10 mock, 11 DENV) mice at 60-65 dpi. **(F, G)** Distance traveled in the open field test by male **(F)** (n = 10 mock, 13 DENV) and female **(E)** (n = 10 mock, 10 DENV) mice at 60-65 dpi. **(H, I)** Percentage of exploration towards familiar (Fam.) and novel (New) objects in the novel object recognition test performed in male **(H)** (n = 8 mock, 10 DENV) and female **(I)** (n = 10 mock, 11 DENV) mice at 60-65 dpi. Data are expressed as mean ± S.E.M. In H: *p = 0.0289 and #p = 0.0531, in I: *p = 0.0054 and #p=0.077, one-sample Student's t test compared to fixed value 50.



Supplementary Figure 4. Neonatal ZIKV infection had no effect on the development of reflexes in newborn mice. Mice were injected subcutaneously with 10^6 PFU ZIKV or equal volume of mock medium at post-natal day 3, and negative geotaxis **(A-B),** righting reflex **(C-D)** and grasping reflex **(E-F)** were measured in male and female mice, respectively, until 7 dpi (n = 2-7 litters per group per day).



Supplementary Figure 5. Neonatal ZIKV infection induces motor and cognitive dysfunction in female mice. Mice were injected subcutaneously with 10^6 PFU ZIKV or equal volume of mock medium at post-natal day 3. **(A)** Latency to fall in the hindlimb suspension test performed in female mice (n = 4 mock, 8 ZIKV) at 9 dpi. **(B-C)** Time to turn and time to descend in the pole test performed in female mice (n = 6 mock, 10 ZIKV) at 15-21 dpi. **(D)** Latency to fall in the rotarod for female mice (n = 12 mock, 7 ZIKV) at 85-95 dpi. **(E)** Distance traveled in the open field test by female mice (n = 20 mock, 11 ZIKV) at 85-95 dpi. **(F)** Percentage of exploration towards familiar (Fam.) and novel (New) objects in the novel object recognition test performed female mice (n = 19 mock, 12 ZIKV) at 85-95 dpi. **(G)** Percentage of exploration of empty cage or cage containing a stranger mouse in the social approach task for female (n = 12 mock, 10 ZIKV) mice at 90-100 dpi. Data are expressed as mean ± S.E.M. HLS = hindlimb suspension test; OF = open field test; NOR = novel object recognition test; SA = social approach test. In A: *p = 0.0421, in C: #p = 0.0662, in E: *p = 0.0322, Student's t test. In F: **p = 0.0049; in G: *p = 0.0064, one-sample Student's t test compared to fixed value 50.



Supplementary Figure 6. Neonatal ZIKV infection does not affect cellular proliferation in the hippocampus. Immunohistochemistry was performed in brains of mock- and ZIKV-infected mice at 6 or 12 dpi. **(A-F)** Representative images and quantification of percentage of Ki67 positive cells evaluated in the subgranular zone (SGZ) of the dentate gyrus of the hippocampus (**A-C**) (n = 5 mock, 5 ZIKV) and subventricular zone (SVZ) (**D-F**) (n = 4 mock, 5 ZIKV) of mock- and ZIKV-infected mice at 6 dpi. **(G-L)** Representative images and percentage of Ki67 positive cells evaluated in the SGZ **(G-I)** (n = 6 mock, 5 ZIKV) and SVZ **(J-L)** (n = 6 mock, 6 ZIKV) of mock- and ZIKV-infected mice at 12 dpi. Bar graphs represent mean ± S.E.M. Scale bar: 50 μm.

Supplementary Tables:

Supplementary Table 1. Seizure analysis in ZIKV-injected mice.

Mice were injected subcutaneously with 10^6 PFU of ZIKV at post-natal day 3, electrodes were implanted at 9 dpi and electrographic recordings were acquired. Total number of seizures and seizure duration were obtained for each mice (n = 4 ZIKV).

		10 dpi			11 dpi			12 dpi		
Experimental Group	**Identification**	**Total Number of Seizures**	**Total recording time (min)**	**Seizure duration (min)**	**Total Number of Seizures**	**Total recording time (min)**	**Seizures duration (min)**	**Total Number of Seizures**	**Total recording time (min)**	**Seizure duration (min)**
ZIKV	Mouse 01	4	120	5.50	2	120	5.94	2	120	7.24
ZIKV	Mouse 02	4	120	5.29	4	120	7.57	4	120	6.19
ZIKV	Mouse 03	2	120	5.48	2	120	6.45	4	120	5.51
ZIKV	Mouse 04	4	120	6.78	2	120	8.76	4	120	9.07

Seizures Duration (min)	6.65 ± 1.28
Events rate (nº seizures/h)	1.58 ± 0.52
Total nº of Seizures recorded	38