Supplemental Information

Zika Virus Can Strongly Infect and Disrupt Secondary Organizers in the Ventricular Zone of the Embryonic Chicken Brain

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Figure S1: Quantification of brain regions. (Related to Figure 2) All measurements were obtained from images using ImageJ software as described in the Experimental Procedures and Supplemental Experimental Procedures. A) In 3dpi embryos (n=10 mock controls; 8 ZIKV), area measurements were taken for the eye (#1), MB (#2), and TE (#3). Widths (#4, #5) were defined as largest diameter of each brain compartment. B) In 7dpi dissected brains (n=10 mock controls; 11 ZIKV), three areas were measured: total brain (FB+MB; #6), TE (#7) and MB (#8). Width diameters (#9, #10) were measured for each brain compartment and TE+MB length (#11) was measured as a straight line along the midline joining A-P vertices of the total brain area trace (#6). C) In MRI scans of 7dpi dissected brains (n=5 per condition), a middle slice of the MB from the MRI scan stack was chosen for linear measurements. The thickness (largest measure) of the TE nuclei structures (#12), MB cortical structures (#13) and mesencephalic nuclei (#14) were measured. The lateral ventricles of the TE were insufficiently resolved to ensure accurate delineation, so outlines of the MB ventricles across several sections were used as a measure of MB ventricular volume (#15). Error bars represent mean ± SEM. Abbreviations: TE-telencephalon, MB-midbrain, FB-forebrain, V-ventricle.
Figure S2: ‘Hot-spots’ of ZIKV infection observed in 3dpi brains. (Related to Figure 3) A) Comparable sections stained with dsRNA for multiple embryos show repeatability of the ‘hot-spots’ of infection observed. Strong infection of thalamic roof plate, MB basal plate and MB roof plate is indicated with blue, red and black arrowheads, respectively. Due to slight differences in the angle of sectioning and the range of sections we have stained for each embryo, certain regions of infection indicated in Figure 3A are not obvious in one low power frame we have presented here. For MB basal plate infection particularly, panels for embryos 6 and 9 that are classified as weakly infected, include insets showing modest dsRNA immunolabeling from fluorescent (and color inverted) images captured in the same approximate location in that domain a few sections away. B) Whole mount brain-half of a 3dpi embryo stained for dsRNA to show hot-spots of infection along the ventricular surface (*=mesenchymal infection in the tissue surrounding the brain is not considered as a hot-spot). The basal plate at MB+DE, hypothalamus, isthmus, and HB show robust infection in this example; there is also some infection in pretectum. Anomalous non-specific labeling is visible in TE due to tissue damage during dissection (cyan arrowheads). Dashed black line shows the approximate plane of sections shown in panel A. C) Schematic shows recurrent hot-spots mapped on an E5 (3dpi) brain. Brain segments are labeled based, in part, on evolutionarily-conserved genoarchitecture (Cavodeassi and Houart, 2012; Echevarría et al., 2003; Ferran et al., 2008; Wurst and Bally-Cuif, 2001. D) Schematic shows presumed prosomeres superimposed on an E5 chicken brain (solid grey lines for anterior-posterior prosomeres and dashed lines for dorsal-ventral subdivisions). Also shown are the approximate expression domains of SHH, FGF8B and BMP7- three morphogens we mapped in ZIKV-infected brains. Abbreviations: D, Dorsal; P, Posterior; FB, forebrain; MB, midbrain; HB, hindbrain; TE, telencephalon; Th, thalamus; Pth, prethalamus; PreT, pretectum; MHB, MB-HB boundary; Hypo, hypothalamus; V, ventricle.
Figure S3: Morphogen expression is reduced and cell death is increased in regions of heavy ZIKV infection. A) (Related to Figure 3) Transcripts for 3 morphogens present in secondary organizers were mapped: SHH (MB floor plate), BMP7 (Hypo floor plate) and FGF8B (MHB). PAX6 and dsRNA are shown by immunohistochemical labeling. Each morphogen was reduced in heavily ZIKV-infected regions (black arrowheads). SHH pixels (cyan), PAX6 pixels and distance of the lateral edge of the PAX6 domain from the midline (bracket) were quantified to evaluate domain sizes and neural patterning. B) (Related to Figure 4) Exemplar TUNEL quantification images for a mock-infected control and a ZIKV-injected embryo show the 160µm x 160µm area (blue box) selected approximately 75µm away from the midline. The TUNEL signal was split from the multi-channel fluorescent image, as shown in the flanking magnified quantification fields, and the number of TUNEL-positive pixels was measured after thresholding pixel intensity. Robust ZIKV-infection was determined by dsRNA-labeling of adjacent sections (data not shown).
Figure S4: ZIKV infection in 7dpi embryos. (Related to Figure 4) A) Antibodies to dsRNA and Sox2 show the locations of ZIKV-infected cells and neural progenitors, respectively. Infection hot-spots in the periventricular epithelium are diminished by this age (7dpi), as the neural progenitors are migrating into the MB lobes and showing sparse infection (i-iii). Infected cells in the retina (ii) and extraocular muscles (iii) are often observed. Heavily-infected foci in the retina (ii) showed a blister-like retinopathic phenotype (asterisk) beneath a region with disrupted stratification of the retinal cell layers. White arrowhead points to infected extraocular muscle fibers (iii). B) Dorsal view of exemplar 7dpi embryo heads with a yellow arrowhead pointing to an eye atrophic defect. C) A summary of repeatedly observed regions of infection at 7dpi (n=7). Abbreviations: TE, telencephalon; Th, thalamus; Pth, pretalamus; PreT, pretectum; MB, midbrain; MHB, midbrain-hindbrain boundary or isthmus; HB, hindbrain; Hypo, hypothalamus; V, ventricle.
| Magnification                          | #Embryos with infection (n=15) | #Embryos with robust infection (n=15) |
|--------------------------------------|-------------------------------|--------------------------------------|
| Diencephalon (basal plate)           | 93%                           | 80%                                  |
| Midbrain (basal plate)               | 87%                           | 73%                                  |
| Hypothalamus                         | 80%                           | 73%                                  |
| Retina                               | 60%                           | 53%                                  |
| Pretectum                            | 80%                           | 47%                                  |
| Midbrain-hindbrain boundary          | 53%                           | 47%                                  |
| Hindbrain                            | 80%                           | 33%                                  |
| Thalamus (roof plate)                | 40%                           | 33%                                  |
| Prethalamus (roof plate)             | 47%                           | 27%                                  |
| Midbrain (roof plate)                | 27%                           | 27%                                  |
| Telencephalon                        | 47%                           | 20%                                  |
| Midbrain                             | 20%                           | 20%                                  |
| Telencephalon (roof plate)           | 20%                           | 13%                                  |
| Thalamus                             | 33%                           | 7%                                   |
| Prethalamus                          | 27%                           | 0%                                   |

The table is rank-ordered by the percentage of embryos with domains of robust infection in each region. Grey rows represent regions overlapping with presumed secondary organizers at this stage of development.
Table S2: Qualitative blinded analysis for *in situ* hybridization signal. (Related to Figures 3 and S3)

| Transcript | Control (weak or no infection) | ZIKV (strong infection) | Total agreement |
|------------|-------------------------------|-------------------------|-----------------|
| *PTCH1*    | 50% (n=5)                     | 80% (n=5)               | 100% (n=3)      | 75%              |
| *BMP7*     | 100% (n=5)                    | 100% (n=1)              | 100% (n=4)      | 100%             |
| *FGF8*     | 100% (n=2)                    | 75% (n=4)               | 60% (n=5)       | 70%              |

Images of transcript expression from a single control embryo were provided as templates to an observer blinded to treatment condition. Sampling regions included the MB basal plate (*PTCH1*), the hypothalamus (*BMP7*) and the MB-HB boundary (*FGF8*); see Figure S3A for exemplar images. The observer was asked to judge whether the *in situ* hybridization signal was knocked down in comparison to the template. Another observer (not blinded) independently judged whether each ZIKV-infected sample region showed little to no infection in the MHB, or robust infection. Correct responses were those where an assignment of gene knockdown was matched to a region with strong ZIKV infection, or an assignment of normal expression was matched to a control or a sample with little ZIKV infection.
Supplemental Experimental Procedures

Animal injections

The ZIKV stock used for the experiments was of the Asian lineage (H/PF/2013) isolated from a patient in French Polynesia. This strain was grown for a passage 2 (P2) stock from Mike Diamond’s group who obtained it from X. de Lamballerie (Emergence des Pathologies Virales, Aix-Marseille Université, Marseille, France) and the European Virus Archive Goes Global (EVAG) as a P3 stock from Vero cells. The virus was further passaged twice in the Kuhn lab in Vero cells, titered on Vero cells and used at 9.6x10^9 PFU/ml for in vivo injections. The Dengue-2 strain, obtained by Kuhn lab from CDC approximately 15 years ago, was a 5x10^9 PFU/ml stock collected from passage 4 in C6/36 mosquito cells.

Fresh fertilized chicken eggs (Animal Sciences Research and Education Center, Purdue University) were incubated at 37-38°C, windowed on E2, and injected into the MB ventricle with a virus stock to which 0.25% fast green dye was added 1/10 by volume. 10-20nl virus plus dye suspension was delivered by compressed air pressure through a pulled glass micropipette (thin-walled with filament, tip broken to ~10-12µm outer diameter) connected to a picospritzer. Eggs were sealed with packing tape and incubated at high humidity. At the time of injection and harvesting, embryos were staged using Hamburger and Hamilton staging system (Hamburger and Hamilton, 1951) and whole embryos, heads or tissues were collected.

External brain size quantification

Embryos injected with ZIKV suspension at E2 were harvested at 3dpi and 7dpi. For 3dpi harvests, left and right sides of the fixed whole embryo were imaged on a Leica MZFLIII stereomicroscope with a Spot digital camera. At such early ages, the embryo sizes differ significantly from one stage to the next; therefore, the analysis was restricted to HH stage 26 to 27. One stage 26 embryo from a ZIKV-injected group was removed from the analysis because it presented with one eye significantly smaller than the other and similar defects are occasionally observed in control embryos. For 7dpi harvests, fixed whole brains were removed and imaged from a dorsal view. The total dimensions (left and right sides) were combined. To conduct brain dimension measurements, the investigator was blinded to the experimental condition of each sample. Projected areas and widths (Figure S1) were calculated in ImageJ with appropriate scaling calibrations. Only for 7dpi wet weight measurements, the investigator was not blinded.

MRI scanning and quantification

MRI scanning was done using the 7T Bruker Biospec 70/30 USR small animal scanner with a true fast imaging with steady-state precession (TrueFISP). Fixed 7dpi brains were embedded in 4% gelatin in PBS and scanned in a 16mm x 16mm frame using a surface coil receiver with 0.275mm slice thickness. ImageJ was used for image processing and ventricle volume measurements as explained in Experimental Procedures.

Immunohistochemistry

For cryosections, tissue samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), dehydrated with graded sucrose (10%, 20%, 30% sucrose in PBS), embedded in 7.5% gelatin/15% sucrose in PBS or TFM® (General Data Company) and 20µm sections were collected. For whole mounts, live tissue was dissected to isolate intact brains that were then fixed, dehydrated by graded methanol solutions and stored at -20°C in methanol. The primary antibodies used were dsRNA (1:500, J2, mouse IgG2a, SCICONS), Sox2 (1:500, goat IgG[H+L], Santa Cruz), PAX6 (1:50, mouse IgG1, deposited to the Developmental Studies Hybridoma Bank [DSHB] by Kawakami, A.; DSHB Hybridoma Product PAX6), NNX2.2 (1:50, mouse IgG2b, deposited to DSHB by Jessell, T.M./Brenner-Morton S.; DSHB Hybridoma Product 74.5A5) and pH3 (1:200, rabbit IgG[H+L], Calbiochem). Fluorophore-tagged secondary antibodies (Life Technologies, 1:250 to 1:500 dilution), and nuclear label TO-PRO®-3 Iodide (642/661) (1:2000, Life Technologies) were added to the secondary antibody solution as needed. TUNEL labeling was performed prior to immunohistochemistry using Roche In Situ Cell Death Detection Kit as per manufacturer instructions. For dianinobenzidine (DAB) colorimetric stains on cryosections, after the primary antibody incubation, endogenous peroxidase activity was saturated with 0.3% H2O2 in methanol at -20°C for 30 minutes. Biotinylated secondary antibody (1:250, horse anti-mouse IgG[H+L], Vector labs) was applied and the labelling was further amplified by avidin-biotin complex reaction with horseradish peroxidase (HRP) tagged to biotin (Vectastain ABC kit, Vector labs). Slides were developed with 0.5mg/ml dianinobenzidine (DAB) with 0.06% H2O2 in 50mM Tris buffer. For whole mount DAB staining, HRP-tagged secondary antibody (1:200, goat anti-mouse IgG[H+L]), Thermofisher Scientific) was used, followed by DAB oxidation reaction (Kawakami et al., 1997).

In situ hybridization

The slides with cryosections were incubated with RNA-probe designed against chicken genes for SHH, FGF8B, BMP7 or PTC1H1 overnight at 72°C. Anti-DIG Fab fragments (1:3500, Roche) conjugated with alkaline phosphatase were used to amplify the signal. Nitro-blue tetrazolium dye solution at pH9.5 (SigmaFast™ BCIP/NBT tablet, Sigma-Aldrich) was used as a colorimetric detection method for signal development. PAX6-DAB labeling was executed subsequently on SHH-labeled sections and NNX2.2-DAB labeling on PTC1H1-labeled sections. For PTC1H1, BMP7 and FGF8B, an observer blinded to
treatment condition was provided with a set of template images from a control embryo. They then classified each sample region from additional embryos as showing signal intensity that was similar to, or weaker than, the control templates.

**SHH, PAX6 and NKX2.2 morphometry**

ImageJ (NIH) was used to quantify SHH, PAX6 and NKX2.2 domains. 20X (numerical aperture=0.75) images of colorimetric SHH-PAX6 stains were channel-separated by deconvolution. One or two middle sections of the MB basal plate were imaged for left and right sides separately. The same data collection method was used for adjacent set of sections co-labeled with PTCH1-NKX2.2. Pixels numbers for SHH, PAX6 and NKX2.2 were quantified using auto local thresholding. The distances from the midline of the lateral boundaries of PAX6 and NKX2.2 domains were measured in ImageJ using a calibrated line tool.

**TUNEL quantification**

TUNEL quantification was performed using ImageJ (NIH). High resolution images were captured using a 20X lens (numerical aperture=0.75) on a confocal microscope. The MB basal plate was analyzed by quantifying 3 to 4 middle sections of the domain. ZIKV-infected embryos were selected for the analysis only if the MB basal plate was considered well infected. Approximately 75µm from the midline a box of 160µm x 160µm was cropped with one edge of the box aligned with the ventricular surface. TUNEL-positive pixels in the field of interest were measured by thresholding using a pre-determined pixel intensity cut-off value. The measure was averaged over the 3 to 4 sections and represented as the percentage of the total area evaluated.

**Supplemental References**

Kawakami, A., Kimura-Kawakami, M., Nomura, T., and Fujisawa, H. (1997). Distributions of PAX6 and PAX7 proteins suggest their involvement in both early and late phases of chick brain development. Mech Dev. 66, 119–130.