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Zika Virus Infection In The Developing Mouse Produces Dramatically Different Neuropathology Dependent On Viral Strain

1 **Title:** Zika Virus Infection In The Developing Mouse Produces Dramatically Different
2 Neuropathology Dependent On Viral Strain

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4 **Abbreviated Title:** Zika virus-induced neuropathology in the mouse

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6 **Authors:** Kevin K. Noguchi, Ph.D.[1*], Brant S. Swiney[1], Sasha L. Williams M.A.[1], Jacob N.
7 Huffman M.A.[1], Katherine Lucas[1], Sophie H. Wang[1], Kayla M. Kapral[1], Amber Li[1], Krikor T.
8 Dikranian, M.D., Ph.D.[2]

9
10 [1]Department of Psychiatry, Washington University in St Louis, 63110
11 [2]Department of Neuroscience, Washington University in St Louis, 63110

12

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34 ***To whom correspondence should be addressed:**
35 Kevin K. Noguchi, Ph.D.
36 Assistant Professor of Psychiatry
37 Washington University in St. Louis
38 Department of Psychiatry
39 4559 Scott Avenue
40 St. Louis, MO 63110
41 noguchik@wustl.edu
42 (O) 314-362-7007
43

44 **ABSTRACT**

45 Zika virus (ZIKV) infection during pregnancy has been causally linked to a constellation of

46 neurodevelopmental deformities in the fetus resulting in a disease termed Congenital Zika

47 Syndrome (CZS). Here we detail how ZIKV infection produces extensive neuropathology in the

48 developing mouse brain and spinal cord of both sexes. Surprisingly, neuropathology differs

49 depending on viral strain with a French Polynesian isolate producing primarily excitotoxicity and

50 a Brazilian isolate being almost exclusively apoptotic but occurring over a prolonged period that

51 is more likely to produce severe hypoplasia. We also show exposure can produce a

52 characteristic pattern of infection that mirrors neuropathology and ultimately results in gross

53 morphological deformities strikingly similar to CZS. This research provides a valuable mouse

54 model mirroring the clinical course of disease that can be used to test potential therapies to

55 improve treatment and gain a better understanding of the disabilities associated with CZS.

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64 **SIGNIFICANCE STATEMENT**

65 Zika virus (ZIKV) infection during pregnancy has been causally linked to a constellation of

66 neurodevelopmental deformities in the fetus resulting in a disease termed Congenital Zika

67 Syndrome. Despite its devastating effects, very little is known about how ZIKV infection

68 produces fetal neuropathology. Here we detail the temporal progression of ZIKV infection in the

69 mouse brain and spinal cord resulting in massive neurodegeneration of infected regions. We

70 also report a ZIKV strain from a region of Brazil with high levels of microcephaly (abnormally

71 small head circumference) produces particularly devastating neuropathology.

72

73 **INTRODUCTION**

74 First discovered in the Ugandan Zika forest in 1947, the Zika virus (ZIKV) initially produced

75 sporadic outbreaks throughout Africa and Asia before causing major epidemics in Micronesia

76 and French Polynesia where there was concern human infection was producing Guillain-Barre

77 syndrome (Cao-Lormeau et al., 2016; Lazear et al., 2016). In 2015, interest increased

78 dramatically once ZIKV infection was correlated with a dramatic increase in microcephaly

79 resulting in at least 1950 confirmed cases in Brazil alone. While definitions vary, microcephaly

80 is a medical condition in which a baby is born with an abnormally small head circumference.

81 The Centers for Disease Control has subsequently established a causal relationship between

82 ZIKV infection and microcephaly (Rasmussen et al., 2016) which was followed by their most

83 difficult emergency health response ever undertaken (Beck, 2016). While research initially

84 focused on microcephaly, clinicians now use the term Congenital Zika Syndrome (CZS) to

85 highlight the constellation of symptoms ZIKV infection can produce during neurodevelopment.

86 Symptoms of CZS include microcephaly, calcifications, epileptic seizures, arthrogryposis, and

87 neuromotor deficits (Saad et al., 2018). Additionally, while estimates suggest hundreds of

88 thousands of women were infected during their pregnancy (Adams Waldorf et al., 2018), it is

89 unknown if undetected fetal pathology occurred that will manifest later with age. Since CZS is a

90 newly discovered disease, its long-term effects are unknown but will undeniably lead to a

91 lifetime of severe intellectual disability for many affected. With a lifetime price of care estimated

92 at $10 million per effected individual (Frieden et al., 2016), the total financial cost for these

93 children will likely be enormous.

94 Despite the devastating effects of this disease, we still know little about how infection leads

95 to neuropathology or whether virulence differs between strains. While current research

96 suggests ZIKV infection can destroy neural progenitor cells and reduce neurogenesis, this

97 would not explain why microcephaly can occur following third trimester infection (Brasil et al.,

98 2016) or develop postnatally after normocephalic birth (van der Linden et al., 2016b) when

4

99   almost all cerebral neurogenesis is complete (de Graaf-Peters and Hadders-Algra, 2006).

100 Another mystery is why the ZIKV was only associated with microcephaly following the 2015

101 Brazilian outbreak despite its discovery in 1947. One reason may be an adaptive mutation that

102 increased infectivity in neural progenitor cells (NPCs) resulting in more significant microcephaly

103 (Yuan et al., 2017). This mutation occurred just prior to the 2013 French Polynesian outbreak

104 and has remained unaltered in subsequent epidemics as the virus spread to Brazil and the rest

105 of the Americas.   However, there are differences in microcephaly rates after this time.   The

106 2015 Brazilian outbreak resulted in infections throughout the country, yet 83.3% of all

107 microcephalic cases were concentrated in a small northeastern region of the country (de

108 Oliveira et al., 2017b). As a result, important epidemiological questions remain as to whether

109 differences in neuropathology between strains may be responsible for drastically differing

110 microcephaly rates.   Others have reported infection of juveniles or adults with African strains

111 produce more severe pathology than Asian strains (Duggal et al., 2017; Tripathi et al., 2017).

112 Additionally, direct intracerebral infection of newborns pups with a more contemporary

113 Venezuelan strain has been reported to produce more severe microcephaly than an ancestral

114 Cambodian strain.   However, we are unaware of studies that directly compare differences

115 between the Brazilian strain and others that may explain the high rates of microcephaly

116 following the 2015 Brazilian outbreak.   Based on this information, we examined the

117 neuropathology produced in the developing rodent brain following infection with clinical isolates

118 from French Polynesia or Paraiba - the northeastern Brazilian state where microcephaly rates

119 were highest (Jaenisch et al., 2017).   Our findings reveal that exposure produces a

120 characteristic pattern of infection resulting in extensive neuropathology that result in gross

121 morphological changes strikingly similar to CZS.   We further discovered different strains

122 produce different types of pathology with the Brazilian strain being far more virulent and more

123 likely to produce gross morphological changes consistent with microcephaly.

124

125 **MATERIALS AND METHODS**

126 *Experimental design and statistical analysis:* We examined the ability of two ZIKV strains to

127 induce neuropathology in wild-type C57BL/6J developing mice. All experiments included both

128 male and female mice, but this was not tracked for statistical analysis. Animals were first

129 examined for neuropathology using silver staining (detects all types of degeneration) and

130 activated caspase-3 (AC3; detects only apoptosis). For each strain and days post infection

131 (DPI) timepoint, at least two litters were examined to ensure consistent results. This information

132 was then used to further examine infection pattern and type of neuropathology using a variety of

133 methods. Histology and assessments were done blind to treatment. All procedures were

134 approved by the Washington University in St. Louis Institutional Animal Care and Use

135 Committee in accordance with the Guide for the Care and Use of Laboratory Animals (Anon,

136 2011). Sample sizes were based on experience using similar analysis in previous studies

137 (Noguchi et al., 2008; Cabrera et al., 2016) to gain enough power to detect statistically

138 significant differences if present. Since quantification is performed in several brain regions, it

139 can be argued we need to control for multiple comparisons. We therefore applied a Bonferroni

140 correction for multiple comparisons by dividing the α level (α = 0.05) by the number of

141 comparisons for AC3 (α < 0.0071), silver staining (α < 0.0071), and area measurements (α <

142 0.013). Statistical tests that would become non-significant following correction are indicated in

143 Table 1. It should be noted this correction is very conservative and increases the chance of

144 false negatives (an error in which significant result becomes non-significant)(Lindquist and

145 Mejia, 2016). More detailed descriptions of experimental design and statistical analysis can be

146 found in the subsections below.

147 *Zika Virus:* The H/PF/2013 (French Polynesia, 2013; **FP/2013**) and Paraiba (Brazil, 2015;

148 **Paraiba/2015**) ZIKV strains were a kind gift from the laboratory of Michael Diamond who

149   obtained them from the Arbovirus Branch of the Centers for Disease Control and Prevention or

150   Steve Whitehead (NIH) respectively.   Virus was propagated and maintained as described

151   previously (Miner et al., 2016a) by propagating in Vero cells, titrating using a focus-forming

152   assay, and storing in aliquots at -80 °C. Neonatal mice were intraperitoneally inoculated with

153   10^3 FFU in a volume of 25 uL and immediately returned to dams. Mock infection was carried

154   out by injecting an equal volume of vehicle alone. All neonates within a litter received the same

155   treatment (infected or mock infected) to eliminate the possibility of cross infection between pups.

156   *Silver Staining*: The de Olmos cupric silver staining procedure is a sensitive method for

157   staining degenerating cells (DeOlmos and Ingram, 1971; Noguchi et al., 2005; Creeley et al.,

158   2013).   For silver staining and immunohistochemistry, treated animals were sacrificed at

159   different DPI by deep anesthesia prior to transcardial perfusion with 4% paraformaldehyde in

160   TRIS buffer (pH 7.4) and, after postfixation, brains were sectioned on a vibratome (DSK

161   Microslicer DTK-1000N) at 75 uM in the coronal (cerebrum) or sagittal (cerebellum) plane.

162   Silver staining was performed in free floating sections as described previously (DeOlmos and

163   Ingram, 1971; Noguchi et al., 2005) by washing sections in distilled deionized water,

164   preincubating in a cupric-silver solution overnight after heating to 33° C, washing in acetone,

165   incubating for 35 minutes in a silver diamine solution, reduction using formaldehyde/citric acid,

166   washing with distilled water, bleaching with 0.3% $K_3Fe(CN)_6$, and stabilizing using $Na_2S_2O_3$.

167   *Immunohistochemistry*:   Immunolabeling with polyclonal antibodies was performed by

168   quenching sections in 3% hydrogen peroxide/methanol, blocking in 2% BSA/0.2% milk/0.1%

169   Triton X-100 mixture in PBS, and incubation in primary antibody overnight. The next day,

170   sections were then reacted with ABC reagents (Vectastain ABC Elite Kit) prior to chromogen

171   labeling with VIP (Vector VIP). All primary antibodies used were rabbit polyclonal: Activated

172   caspase-3 (Cell Signaling Technology Inc., Danvers, MA, USA; Cat# 9661L; 1:1000); Iba1

173   (Wako Chemicals, Richmond, VA, USA; Cat# 1919741; 1:15,000; RRID: AB_839504) It should

174   be noted that viral infection can increase endogenous Fc receptor binding resulting in false

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175   positive labeling during immunohistochemistry. We examined this possibility in a pilot study and

176   found ZIKV infection does indeed produce false positive labeling of microglia when mouse

177   monoclonal antibodies were used even in the absence of primary antibodies. Interestingly,

178   rabbit polyclonal antibodies raised against different epitopes labeled unique populations of cells

179   indicating they do not produce false positive labeling. This makes sense in that the mouse Fc

180   receptor would selectively bind to mouse antibodies and not those from other species. Based

181   on this information we avoided using mouse monoclonal antibodies in these studies.

182   *Kaplan-Meier curves*: Animal survival was tracked in mice (n = 137) to produce Kaplan-

183   Meier Curves (Goel et al., 2010; Rich et al., 2010) following P1 infection by noting the DPI when

184   death (or sacrifice due to suffering) or censorship (planned sacrifice to perform histology)

185   occurred until 30 DPI. Results were entered into Prism statistical software (version 5.0a) and

186   analyzed using the Gehan-Breslow-Wilcoxon Test and Log-rank (Mantel-Cox) Test for survival

187   analysis.

188   *Semi-Quantitative Analysis*: Stereological analysis was impossible following some

189   treatments because pathology became so intense that heavy degeneration occurred throughout

190   entire regions preventing discrimination of individual cells. We therefore had a rater, blind to

191   treatment, semi-quantitatively rate the amount of degeneration on slides stained with either

192   activated caspase-3 or silver staining at either 7, 13, or 30 DPI following either mock (n = 23),

193   **FP/2013** (n = 16), or **Paraiba/2015** (n=22) infection. For each animal, both stains were

194   performed in three coronal sections corresponding to +0.02 mm bregma, -1.755 mm bregma,

195   and -2.98 mm bregma respectively in addition to one midsagittal section (0.0 mm lateral) of the

196   cerebellum (Lein et al., 2007). The amount of degeneration was then semi-quantitatively

197   evaluated in the hippocampus, motor cortex, visual cortex, striatum, thalamus, cerebellum, and

198   septum based on a five point Likert scale using the following criteria: 1 = slight to no

199   degeneration, 2 = moderate degeneration, 3 = significant degeneration, 4 = high amounts of

8

200 degeneration, and 5 = intense degeneration (continuous regions of degeneration where

201 individual cells are not discernible).

202   *Quantification of brain regions and weight*: Quantification of brain weight and brain regional

203 areas were performed in animals exposed to the **FP/2013** (n=4), **Paraiba/2015** (n=7), or mock

204 (n=11) infection and sacrificed on 30 DPI. Whole brain weights were measured following

205 removal from the skull prior to sectioning. Brains were then vibratome cut at 75 uM and regional

206 area quantified on four Nissl stained sections (0.1% cresyl violet acetate in 0.3% v/v glacial

207 acetic acid) corresponding to the same regions used for semi-quantitative analysis. Areas were

208 calculated by tracing regions using Stereo Investigator Software (Version 2019, MBF

209 Bioscience, Williston, Vermont) connected to a QImaging 200R camera on a Nikon Labophot-2

210 microscope using a 4X objective. The dorsal cortex, hippocampus, and lateral ventricles were

211 traced on coronal sections and the cerebellum from the sagittal section. The dorsal cortex

212 included all cortex dorsal to the piriform/entorhinal cortex and was chosen due to the isolated

213 degeneration in this region. Regions occurring in multiple sections had areas summed prior to

214 statistical analysis. All quantification was analyzed using one- or two-way ANOVAs and

215 Bonferroni posthocs using Prism statistical software (version 5.0a)**.**

216   *In Situ Hybridization*: Animals were perfused with 4% paraformaldehyde in TRIS buffer (pH

217 7.4) and dissected to remove brain and spinal cord. Tissue was post-fixed overnight,

218 dehydrated with graded alcohols, stored in 70% ethanol, embedded in paraffin, cut at 5 uM,

219 dried overnight, and deparaffinized. ZIKV RNA labeling was performed using a Advanced Cell

220 Diagnostics HybEZ II Oven with ZIKV probe (Cat#: 468361) and visualized with a RNAScope

221 2.5 HD Red Assay (Cat#: 322360) and hematoxylin counterstaining. For ISH/immunolabeling

222 combined technique, ISH for ZIKV RNA was performed first followed by immunolabeling with

223 antibodies for neurons (NeuN; 1:100, Millipore; Cat#:ABN78; RRID:AB_10807945), microglia

224 (Iba1; 1:15,000, Wako Chemicals, Cat#:1919741; 1:15,000; RRID: AB_839504), or astrocytes

225 (GFAP; 1:400, Sigma-Aldrich, Cat#: G9269; RRID:AB_477035) prior to coverslipping. The ISH

226 red assay used for bright field images also fluoresces in the red channel without bleeding into

227 the other channels.

228   *Plastic Embedding and Electron Microscopy*: Animals were first transcardially perfused

229 with 1.5% glutaraldehyde/1% paraformaldehyde following deep anesthesia. Brains were then

230 removed, cut into 1 mm slabs in the coronal plane, osmicated with 1% osmium tetroxide

231 overnight, placed in graded alcohol to dehydrate, cleared in toluene, and finally plastic

232 embedded in araldite. For semithin sections, tissue was then cut using a Leica Ultracut UCT

233 ultramicrotome at 1 uM and stained with Azure II/Methylene blue. For electron microscopy,

234 sections were suspended over a formvar coated slot grid prior to staining with uranyl

235 acetate/lead citrate and visualized using a JOEL JEM1400 transmission electron microscope.

236   *Alizarin Staining*: Alizarin is a standard chemical dye for the detection of calcifications in

237 the brain. A 2% Alizarin Red S (Sigma Aldrich, St Louis, USA) solution was prepared by mixing

238 in 0.1M borate buffer, filtering a 0.45 uM, and adjusting pH to 6.7. Slide mounted sections were

239 then immersed in alizarin solution for 1 hour, rinsed by dipping in tap water for 20 dips, and

240 cleared in 70% ethanol for 20 dips. Tissue was then dehydrated for 2 minutes in 95%, 95%,

241 100%, and 100% ethanol baths followed by immersion in three changes of citri-solve for 5

242 minutes before coverslipping.

243   *Imaging*: Bright field and fluorescent photocomposites were taken on Leica DM400B

244 microscope with a Leica DFC310FX camera using Surveyor software V7.0.0.6 MT (Objective

245 Imaging, Cambridge, UK) to photomerge images. For fluorescent images, each channel was

246 imaged individually and merged in Adobe Photoshop (V 12.0) after adjustment of channel

247 levels.

248   *Viral Sequences*: Viral sequences for **FP/2013** (KJ776791.2) and **Paraiba/2015**

249 (KX280026.1) were obtained from the National Institutes of Health's Genbank

250 (https://www.ncbi.nlm.nih.gov/genbank/) for comparison (Baronti et al., 2014; Tsetsarkin et al.,

251 2016). Missense and untranslated region mutations are discussed however, while there were

10

252   numerous synonymous mutations, they were not mentioned since they are more likely to be

253   silent.

254

255   **RESULTS**

256   <u>**Infection and Behavior**</u>

257   To examine the time course of neuropathology following infection, neonatal wild-type C57BL/6

258   mice of both sexes were inoculated with 10^3 focus forming units (FFU) of clinical isolates from

259   the 2013 French Polynesian (H/PF/2013; **FP/2015**) or 2015 Paraiba Brazilian outbreaks

260   (**Paraiba/2015**) as described previously (Miner et al., 2016a, 2016b).   Injections were

261   administered between postnatal days 1-4 as a single intraperitoneal injection (van den Pol et al.,

262   2017). It is important to note that because the mouse is born neurodevelopmentally immature

263   compared to the human, a newborn pup brain is the equivalent of a human fetus in the early

264   second trimester of gestation (Clancy et al., 2007; Workman et al., 2013). Mock infected control

265   mice were intraperitoneally injected with only vehicle at an equal volume. Behaviorally, some

266   animals displayed motor deficits which, if present, resulted in an unsteady gait or falling during

267   ambulation and an inability or difficulty to perform a righting reflex once placed in a supine

268   position around 10 DPI. Ataxia progressively worsened afterwards and was followed by flaccid

269   paralysis of hindlimbs as animals became moribund by around P14.

270

271   <u>**FP/2013 and Paraiba/2015 produce different Kaplan-Meier curves**</u>

272   We first generated Kaplan-Meier curves for the **FP/2013** and **Paraiba/2015** strains

273   following P1 infection until 30 DPI (**Figure 1A**). While both strains were almost equally lethal,

274   most deaths occurred between 10-17 DPI for **FP/2013** but this period was more evenly

275   distributed and expanded between 8-24 DPI for **Paraiba/2015**. Survival curves are typically

276   analyzed by the Gehan-Breslow-Wilcoxon Test or the Log-rank Mantel-Cox Test. The former is

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277   sensitive to deaths early in the survival curve while the latter is more powerful if the chance of

278   death is the same at all time points.   Interestingly, the Gehan-Breslow-Wilcoxon Test was

279   statistically significant (χ^2 = 10.33, p < 0.001) but the Log-rank Mantel-Cox Test was not (χ^2 =

280   2.366, p > 0.05).   This suggests lethality following **FP/2013** exposure is heightened during an

281   early acute period compared to **Paraiba/2015** that is less intense and occurs over a more

282   prolonged period.

283

284   **<u>French Polynesian Strain</u>**

285   We first examined how neuropathology evolves over time by infecting mice with **FP/2013** and

286   sacrificing for histology at varying DPI.   Neuropathology and infection were assessed using

287   silver staining (detects all types of cellular degeneration), activated caspase-3 (AC3)

288   immunolabeling (detects only apoptosis), and *in situ* hybridization (ISH) for ZIKV RNA (to

289   measure the pattern of infection).   Neuropathology was then quantified using a semi-quantitative

290   degeneration scale on 7, 13, and 30 DPI in sections labeled with AC3 (**Figures 1B-H**) and silver

291   staining (**Figures 1I-O**).   Finally, the long-term effects of infection on hypoplasia was quantified

292   by determining brain weight (**Figures 1P**) and outlining specific regions to determine or area at

293   30 DPI (**Figure 1Q-T**).   Data was then analyzed using one- or two- way ANOVAs followed by

294   Bonferroni post-hoc analysis (**Figure 1** and **Table 1**).

295

296   **FP/2013 Infection: Early Stage - 7 to 13 DPI**

297   Neuropathology began as early as 7 DPI but was more consistently seen by 13 DPI. In general,

298   while AC3 and silver staining tended to overlap, only silver staining could detect degeneration at

299   7 DPI suggesting non-apoptotic degeneration was occurring during this early stage (**Figures**

300   **1B-O**).

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301   *Dorsal Cortex*:   Degeneration in the dorsal cortex included the motor, somatosensory,

302   retrosplenial, and visual cortices. By 10-13 DPI, advanced degeneration involved all layers with

303   heavier bilaminar degeneration in layer IV and the ventral portion of layer V that seemed

304   particularly intense in the somatosensory cortex (**Figures 1B-C,I-J** and **2A-D,G-J**). The ventral

305   cortex was relatively spared but degeneration could be seen in some animals at more advanced

306   stages.   ISH for ZIKV RNA revealed cortical infection mirroring argyrophilia (silver positive

307   degeneration) with a similar bilaminar appearance (compare **Figures 2A**,**G** with **2E**,**K**) and often

308   showed cellular profiles of soma that resembled pyramidal cells (**Figure 2F**).   Interestingly,

309   clinical research has found a similar bilaminar pattern of infection in the dorsal cortex of ZIKV

310   infected fetuses (Bhatnagar et al., 2017) suggesting a similar pattern of infection between

311   species. No similar neuropathology was seen in mock infected controls (**Figures 2C-D** and **2I-**

312   **J**).

313   *Visual Regions*: Consistent with our previous research suggesting the ZIKV can gain entry

314   through visual pathways (Miner et al., 2016b), one of the first regions to show degeneration is

315   the optic tract and lateral geniculate nucleus (LGN; visual relay center in the thalamus) around 7

316   DPI (**Figures 2G-J, Figures 3A-B**). This complements previous research showing neonatal

317   ZIKV inoculation produces infection in the retina first that spreads to the LGN (van den Pol et

318   al., 2017). This is also consistent with the high incidence of retinal/optic nerve degeneration

319   (Miner et al., 2016b) and visual impairment in CZS children (Ventura et al., 2018) suggesting

320   viral infection occurs similarly between species. As infection progressed, other visual areas

321   degenerate including the superior colliculus and visual cortex (**Figures 1C,J** and **2G-J**).

322   Infection mirrored silver staining with early punctate ISH labeling in the optic tract (compare

323   **Figures 3A and 3C**) followed by the LGN and visual cortex (**Figures 2K-L**).

324   *Hippocampal Formation*: Injury in the hippocampal formation was usually absent at 7 DPI

325   but became severe at later stages suggesting this was not the primary site of infection (**Figure**

326   **1D**,**K**). The most consistent degeneration was in the dentate gyrus where argyrophilia was

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327   seen in granule cells and the molecular layer neuropil connecting granule cell dendrites to

328   perforant path axon terminals (**Figures 2G-J, Figures 3D-E**). Degeneration also occurred less

329   frequently in CA3 and CA1 pyramidal cells (**Figures 2G-J**). Interestingly, these structures form

330   a trisynaptic circuit with granule cells sending afferents through the mossy fibers to CA3 and

331   CA3 connecting to CA1 through Scheaffer collaterals (**Figures 3D-E**). This may suggest the

332   virus spreads transynaptically through connected regions. Closer examination revealed a large

333   number of degenerating microglia in the dentate polymorph layer, stratum oriens, and stratum

334   radiatum (**Figures 2G-J** and **Figures 3D-E**). Hippocampal infection also closely mirrored

335   microglial and neuronal death (compare **Figures 2G-H** with **2K-L** and **Figures 3D with 3F**).

336   Clearly ISH labeled granule cell and pyramidal cell neurons could be seen in the dentate gyrus

337   and CA1/CA3 respectively (**Figures 2K-L**). While infection also occurred in regions with

338   microglial degeneration in the polymorph layer, stratum oriens, and stratum radiatum, it was

339   difficult to determine cell type.

340   *Cerebellum*: The cerebellum was one of the most heavily affected regions (**Figures 1E**,**L**).

341   It actually contains more neurons than the cerebrum with the vast majority being granule cell

342   neurons that send axons to the superficial molecular layer where they bifurcate in opposite

343   directions before synapsing with dendrites from multiple Purkinje cells. Extensive argyrophilia of

344   both granule and Purkinje cells were seen along with degenerating neuropil of their synaptic

345   connections in the molecular layer (**Figures 2M-P**). The cerebellum also exhibited high ZIKV

346   RNA in the molecular, Purkinje, and granule cell layers similar to argyrophilia (compare **Figures**

347   **2M-N** with **2Q-R**). ISH labeled Purkinje cells were identifiable due to their characteristic large

348   size and position but it was difficult to definitively identify other infected cell types (**Figure 2R**).

349   *Spinal Cord*: Since layer V somatosensory cortex is injured and sends numerous afferents

350   to the corticospinal tract (even more than the motor cortex in the mouse (Sengul and Watson,

351   2015)) we next examined the spinal cord. Degeneration occurred in the grey matter but was

352   more extensive in the funiculus (white matter). The most conspicuous degeneration was in the

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353   corticospinal tract which is consistent with fetal CZS autopsies showing this pathway is often

354   shrunken or missing (**Figures 2S-T**) (Chimelli and Avvad-Portari, 2018). Activated caspase-3

355   immunolabeling revealed corticospinal toxicity was primarily apoptotic and encompassed large

356   portions of this tract (**Figure 2U**). Infection was also heavy in the funiculus which contains

357   numerous ascending and descending pathways such as the corticospinal, vestibulospinal,

358   reticulospinal, and spinothalamic tracts (**Figure 2V**). The spinal grey also exhibited infection to

359   a lesser degree. The spinal cord was one of the earliest regions to show infection suggesting a

360   secondary route of neuroinvasion. This could be seen as punctate labeling in the cytoplasm

361   surrounding the nucleus of some cells in the spinal lateral horn around 7 DPI (**Figure 3G**).

362   Interestingly, the herpes virus also gains entry to the brain through the visual and spinal

363   (corticospinal) pathways suggesting a similar route of neuroinvasion (Lewis et al., 1989;

364   Kidokoro et al., 2017).

365   *Other Regions*:   Neuropathology could also be seen in other regions including the

366   thalamus, caudate/putamen, septum, and subiculum (**Figures 1F-H, 1M-O, 2A-D** and **2G-J**)

367   which mirrored infection (**Figures 2E-F** and **K-L**).

368

369   **Early neurodegeneration is excitotoxic**

370   *Early neuropathology includes limited apoptosis*:   Once we established the pattern of

371   degeneration/infection, we next examined differences in neuropathology. We first applied both

372   silver staining and AC3 immunolabeling on sections from the same animal and region. AC3

373   was highly correlated with regional silver staining but exhibited far fewer cellular profiles in most

374   regions (**Figure 4**). Silver staining also marked some cell types not seen with AC3 staining

375   such as microglia and Purkinje neurons. Both stains labeled the corticospinal tract that was

376   more distinct with AC3 immunolabeling and often involved large portions of this pathway as it

377   projected from the cortex ventrally as the internal capsule and continued into the spinal cord

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378   (**Figures 4C-D and 4G-J**).   From this comparison, we conclude ZIKV infection produces

379   significant apoptosis but there is also extensive non-apoptotic death occurring.

380   *Plastic embedding reveals vacuolization and apoptosis*:   To examine this non-apoptotic

381   death further, we plastic embedded tissue (which preserves the fine micro- and ultra- structural

382   morphology in greater detail) and stained with the nuclear stain Azure II/Methylene blue.   The

383   hippocampal formation exhibited heavy neuropathology including large regions of neuronal

384   vacuolization (fluid filled cavities within the cytoplasm) and abnormal nuclear chromatin

385   condensation in the granule cells of the dentate gyrus (**Figure 5A**).   Similar vacuolization could

386   be seen in the pyramidal cells of CA3/CA1 intermixed with lower amounts of apoptosis along

387   with dendritic swelling in the stratum oriens dorsal to the CA region (**Figure 5B**).   Beyond the

388   hippocampus, the cortex also exhibited large regions of heavy vacuolization interspersed with

389   pyknotic apoptotic cells (**Figure 5C**).   None of this pathology could be seen in mock infected

390   controls in the same regions (**Figures 5D, E, F** respectively).

391   *Neuronal excitotoxicity and microglial degeneration*:   Since we were approaching

392   magnification limits of the microscope, we next examined plastic embedded tissue using

393   electron microscopy.   In the cortex, neurons in an early phase of degeneration exhibited

394   cytoplasmic condensation, swelling of granulated endoplasmic reticulum, and nuclear

395   margination where clumps of aggregated chromatin localize to the inner edge of the nuclear

396   envelope (called a "clock-face" profile; compare **Figures 5G** with **5H**).   This is often

397   accompanied by condensation of cytoplasm leading to compaction of organelles, vacuolization

398   as the endoplasmic reticulum swells, and further vacuolization of the nuclear envelope (**Figures**

399   **5H-I**).   Similar pathologic changes could be seen in the dentate gyrus granule cells (**Figure 5J**)

400   and CA1 hippocampal pyramidal cells (**Figure 5K**) consistent with Azure II/Methylene blue

401   staining.   Importantly, the dendritic swelling, nuclear margination, and vacuolization we see are

402   all classical hallmarks of excitotoxicity - the pathological process by which neurons are

403   damaged or killed by glutamatergic overstimulation (Ishimaru et al., 1999).   Tissue ultrastructure

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404   also revealed other signs of an injured brain including parenchymal hemorrhage (**Figure 5L**)

405   where leaking red blood cells can be seen in extravascular space similar to clinical reports

406   (Brasil et al., 2016). While lymphocytes are rarely seen in the healthy brain, numerous could be

407   seen in infected brains (**Figure 5M**) suggesting an adaptive immune response following viral

408   infection. Finally, we also found degenerating microglia (**Figure 5N**) suggesting infection is

409   additionally toxic to this cell type consistent with our microscopic examination.

410   *Infection produces microglial activation and fragmentation as microglia degenerate*: Since

411   silver staining and ultrastructural examination revealed microglial pathology, we next

412   immunolabeled for the microglial marker Iba1 at different DPI. Uninfected controls displayed

413   evenly distributed ramified (resting) microglia with thin extended processes (**Figures 6A-B**) that

414   became activated (thickened retracted processes connected to distended soma) following

415   infection by 7 DPI (**Figures 6C-D**). By 10 DPI, regions with high amounts of degeneration either

416   displayed microglia fragmenting into cellular debris or were more sparsely populated (**Figures**

417   **6E-F**) suggesting microglial pathology.

418

419   **FP/2013 Infection: Late Stage: - 13 to 30 DPI**

420   *Neuropathology either clears or transitions to apoptosis around 13 DPI*: After around 13DPI,

421   infection and degeneration will decrease into smaller focal regions before being eliminated

422   completely for most animals. However, a small number of animals will have neuropathology

423   increase dramatically and shift to heavy apoptosis around 13 DPI. When cortical degeneration

424   transitions to apoptosis, it expands to all layers but is still highly restricted dorsally (**Figures 7A-**

425   **D**). Hippocampal apoptosis was primarily restricted to large numbers of neurons in the dentate

426   gyrus, CA1, and CA3 although microglial degeneration can also be seen with silver staining

427   (**Figures 7E-H**). Apoptosis in the cerebellum gradually became more dense until focal regions

428   of degeneration were seen in large sections of the granule cell layer (**Figures 7I-L**). Compared

429   to earlier stages of pathology, degeneration transformed from more granular degeneration of

17

430   individual cells to dense sections of apoptotic tissue dying en masse. This may suggest the

431   virus can more easily spread in these regions to adjacent cells (regardless of cell type) rather

432   than exclusively spreading transsynaptically.   By 30 DPI, animals exhibit no evidence of

433   continuing degeneration (**Figures 1B-O**). Some animals appear to show gross morphological

434   changes including cortical thinning (**Figures 7M-O**), hippocampal/cerebellar hypoplasia

435   (**Figures 7N-P**), and ventriculomegaly (**Figures 7M-O**).   However, brain weight and

436   quantification of regional areas was not significantly decreased suggesting this transition to

437   more severe pathology is uncommon (**Figures 1P-T**).

438

439   **Paraiba/2015 strain primarily produces apoptosis and is much more virulent**

440   Previous research suggests an adaptive mutation first seen in the 2013 French Polynesian

441   outbreak increased infection of NPCs and the ability to produce microcephaly (Yuan et al.,

442   2017).   However, the subsequent Brazilian outbreak resulted in drastically different

443   microcephaly rates with 83.3% restricted to a small northeastern region of Brazil despite ZIKV

444   infections throughout the country.   This may suggest subsequent ZIKV strains evolved to

445   become even more neurotoxic during this outbreak. We therefore examined whether a ZIKV

446   strain isolated from a region with high rates of microcephaly (**Paraiba/2015** isolated from

447   northeastern Brazil) produces neuropathology similar to the **FP/2013**.

448

449   *Paraiba/2015 produces apoptosis and is more likely to produce microencephaly*: Animals were

450   infected at 10^3 FFU with the **Paraiba/2015** and sacrificed at different DPI.   Quantification

451   revealed no increase in degeneration at 7 DPI that increased dramatically by 13 DPI suggesting

452   a more gradual progression to neurotoxicity compared to **FP/2013** (**Figure 1B-O**).

453   Degeneration began around 10 DPI with apoptosis being much more prevalent but occurring in

454   the same set of regions (**Figures 8A-D**). Apoptosis peaks by 10-13 DPI with AC3 labeling

455   becoming extremely dense in many regions and most brains exhibiting gross morphological

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456 changes (**Figures 1B-O, 8E-H)**. Protracted degeneration frequently led to hippocampal

457 hypoplasia resulting in its dorsal retraction (**Figures 8F-G**) along with the beginning of cortical

458 thinning (**Figures 8E-G**). The cerebellum also revealed greater levels of granule cell apoptosis

459 along with hypoplasia (**Figure 8H**). By 30 DPI, increases in degeneration were frequently seen

460 but occurred in more limited patches in the cortex, hippocampus, and cerebellum (**Figures 1B-**

461 **O, Figures 8I-P**). Unlike **FP/2013**, quantification at 30 DPI revealed significant reductions in

462 brain weight (22%) along with reduced area in the dorsal cortex (10%), hippocampus (37%),

463 and cerebellum (57%) (**Figures 1P-S**). The cerebellar apoptosis was primarily isolated to the

464 granule layer of certain lobes that were selectively atrophied and resulted in hypoplasia so

465 severe it was often noticeable upon brain removal (**Figure 8L**). While lateral ventricular size

466 was over twice the size of controls, this did not reach significance due to high variance (**Figures**

467 **1T**). ISH labeling, like apoptosis, was more intense than **FP/2013** infection but generally

468 involved the same regions and mirrored argyrophilia (**Figure 9**)**.** More specifically, the sparser

469 labeling with **FP/2013** exhibited more granular infection of individual cells whereas

470 **Paraiba/2015** exposure could produce patches of dense ISH labeling that affected all cells

471 within it. To investigate this further, we performed immunohistochemistry for different cell type

472 markers on tissue labeled with ISH for ZIKV RNA at 10 DPI. In areas with high infection, there

473 were a large number of neurons (NeuN) but more limited labeling of microglia (Iba1) and

474 astrocytes (GFAP) (**Figure 10**). This suggest neurons are the primary cell type infected.

475

476 *Plastic embedding, electron microscopy, alizarin staining, and immunolabeling*:

477   We next examined this pathology further by performing plastic embedding and Azure

478 II/Methylene blue staining. Consistent with AC3 labeling, infection with **Paraiba/2015** produced

479 massive amounts of apoptosis with the cortex, dentate gyrus, and CA1 showing shrunken soma

480 with nuclear chromatin condensation (**Figures 11A-C**). Closer examination with electron

481 microscopy confirmed a large number of neurons displaying the classic signs of apoptosis

19

482   (Ishimaru et al., 1999). This included reductions in soma volume along with nuclear chromatin

483   condensation into spherical electron-dense nuclear masses (**Figure 11D**) consistent with Azure

484   II/Methylene blue staining. At later stages, the nuclear envelope lost integrity allowing the

485   nucleoplasmic contents to spill into the cytoplasm as the cell was broken down further (**Figure**

486   **11E**). Other ultrastructural signs of pathology/infection could be seen including parenchymal

487   hemorrhage (**Figure 11E**), microglial phagocytosis (**Figure 11E**), axonal pathology (**Figure**

488   **11F**), and neutrophil diapedesis indicating an adaptive immune response (**Figure 11G**).

489   Interestingly, in the CA3 region, their seemed to be more argyrophilic cells than AC3 positive

490   cells suggesting non-apoptotic death was occurring (**Figures 12A-B**). Subsequent plastic

491   embedding revealed cell pathology typical of both apoptosis and excitotoxicity (**Figure 12C**).

492   Therefore, while apoptosis dominates, limited excitotoxicity is additionally present. We next

493   examined pathology using two other methods. Since cerebellar lobes with high apoptosis

494   developed crystal precipitate, we also performed alizarin staining and confirmed the presence of

495   calcification (**Figure 12D**). We also performed Iba1 staining which showed a reduced density of

496   microglia or large patches devoid of these immune cells altogether (**Figures 12E-F**). This may

497   indicate that continued microglial degeneration can produce immunocompromised regions

498   where virus can flourish.

499

500   **DISCUSSION**

501   Here we provide a detailed description of the neuropathology produced by two ZIKV strains

502   in the developing mouse brain. This revealed that **FP/2013** and **Paraiba/2015** induce pathology

503   in a similar set of structures while producing very different neuropathology. **FP/2013**-induced

504   neuropathology occurs in two stages with early degeneration occurring through excitotoxicity

505   followed by intense apoptosis in a smaller set of animals that resolves by 30 DPI. Alternatively,

506   **Paraiba/2015** progresses more slowly resulting in extensive apoptotic degeneration that

507   reaches significance at 13 DPI and continues until at least 30 DPI. This prolonged, unrelenting

508 degeneration is consistent with the extended lethality seen in our Kaplan-Meier curves and is

509 the likely mechanism through which **Paraiba/2015** can produce severe

510 microencephaly/hypoplasia. Excitotoxicity can be seen following numerous types of insults

511 including seizures, hypoxia/ischemia, traumatic brain injury, or other diseases that produce

512 deficient cellular energy reserves. These conditions ultimately disrupt ionic gradients leading to

513 the release of the excitatory neurotransmitter glutamate which produces excessive stimulation

514 and the postsynaptic influx of calcium ions that activate several enzymes such as proteases,

515 endonucleases, and phospholipases that damage the cell (McDonald and Johnston, 1990). The

516 vacuolization we see occurs when the neuron attempts to protect itself from excessive free

517 intracellular calcium by sequestering it in the endoplasmic reticulum or other organelles

518 (Noguchi, 2001). This, along with dendritic swelling and chromatin margination, are classical

519 signs of excitotoxicity which can produce degeneration distinct from apoptosis (Ishimaru et al.,

520 1999). This suggests that as infected neurons become compromised, they release glutamate

521 following dysregulation of neurotransmitter reserves. This is consistent with important research

522 from two independent labs that found ZIKV infection in neuronal cell cultures results in

523 glutamate release and excitotoxicity (Costa et al., 2017; Olmo et al., 2017; Gaburro et al., 2018).

524 Since seizures primarily produce injury through excitotoxicity, it is also consistent with the high

525 incidence of epilepsy in CZS (van der Linden et al., 2018). There are several potential reasons

526 why ZIKV infection produces apoptosis. One possibility is that apoptosis is an innate immune

527 response whereby cells commit suicide rather than host viral infection and replication. Viruses

528 can also release proteins that inhibit apoptosis which might explain why this type of

529 degeneration is delayed following **FP/2013** infection (Everett and McFadden, 1999). A second

530 possibility is that neurons become so compromised, apoptosis is initiated to efficiently remove

531 the cell using programmed cell death. Finally, apoptosis may also be initiated through

532 transneuronal degeneration - the secondary degeneration in an uninjured neuron due to its

533 disconnection from others (Fornito et al., 2015). Transneuronal degeneration is much more

21

534   likely to arise in the developing brain and occurs because some neurons (such as those

535   projecting through the corticospinal tract) die off following the loss of synaptic connectivity

536   (Torvik, 1956; Trumpy, 1971).

537   We also describe a characteristic pattern of infection/neuropathology that models the

538   clinical course of CZS.   This includes frequently reported CZS abnormalities such as

539   microencephaly, calcifications, parenchymal hemorrhage, and axonal pathology.   The specific

540   pattern of effected regions are also consistent with CZS including: [1] Degeneration in the retina,

541   optic nerve (Miner et al., 2016b) and other visual areas consistent with clinical reports of

542   pathology in these same structures and visual impairment in 84.6% of CZS children (de Paula

543   Freitas et al., 2016; Ventura et al., 2016, 2018).   [2] Infection and pathology in the spinal cord

544   which is consistent with spinal cord atrophy reported in 83.3% of children with CZS (Aragao et

545   al., 2017).   More specifically, we found the corticospinal tract was especially affected which is

546   often atrophied or missing in CZS and may explain why ZIKV infection can produce

547   arthrogryposis - a condition where weakness/paralysis of muscles *in utero* prevents movements

548   that relieve joint pressure leading to joint deformities due to a fixed fetal position (van der Linden

549   et al., 2016a; Aragao et al., 2017; Carod-Artal, 2018; Chimelli and Avvad-Portari, 2018).   [3]

550   Severe cerebellar hypoplasia which occurs in 52% of CZS patients (Melo et al., 2016; Meneses

551   et al., 2017).   [4] A bilaminar pattern of infection in the cortex consistent with CZS autopsies

552   (Bhatnagar et al., 2017).

553   This description of ZIKV-induced neuropathology is important for several reasons.   Firstly,

554   since CZS was first recognized in 2015, current sufferers are still children and it is unknown

555   what behavioral deficits will manifest with age.   As a result, knowing what regions are vulnerable

556   may provide important insight into possible long-term consequences of this disorder.   Secondly,

557   our research suggests it is clinically important to know the viral strain of infection since this may

558   predict the virulence and help answer some epidemiological mysteries surrounding the ZIKV

559   epidemic.   The extensive hypoplasia produced by **Paraiba/2015** is consistent with the high rates

560 of microcephaly in the Brazilian region it was isolated from. Interestingly, others have infected

561 neonatal mice (at 10^6 plaque forming units) with a ZIKV isolate from a region close to Paraiba

562 and shown reduced dorsal brain area, prolonged infection, and long-term behavioral deficits

563 (Nem de Oliveira Souza et al., 2018). This adds further evidence that strains isolated from

564 Northeastern Brazil around 2015 may be particularly likely to produce microencephaly. Why

565 **Paraiba/2015** produces prolonged infection and increased virulence is currently unknown but

566 examination of its sequence reveals two missense (base pair positions 3534 and 8007) and one

567 3'UTR (base pair position 10392) mutations compared to the **FP/2013** strain. Since

568 **Paraiba/2015**-induced neuropathology is prolonged, the flaviviral 3'UTR mutation may be

569 particularly important since this region plays an important role in viral replication and evasion of

570 the host immune response (Ng et al., 2017). Some theories additionally suggest evolution

571 favors a biphasic pathogenic virulence that is initially high but decreases over time as the

572 number of susceptible hosts decreases (Berngruber et al., 2013). This may explain why a

573 second wave of infection in Brazil did not produce dramatic increases in microcephaly (de

574 Oliveira et al., 2017a) and will be the focus of future research. Finally, our mouse model allows

575 us to more fully characterize how the ZIKV induces neuropathology which can be used to more

576 accurately assess potential therapies. For instance, our research shows microencephaly only

577 occurs after prolonged and extensive degeneration. Therefore, animal models that only use this

578 criterion may miss more subtle damage which can have clinical relevance. Consistent with this

579 idea, the Centers for Disease Control originally reported 6% of pregnancies with evidence of

580 infection resulted in ZIKV-associated birth defects (Honein et al., 2017), however, a follow-up

581 study of children above 1 year of age found 14% had a birth defect and/or neurodevelopmental

582 abnormalities possibly associated with CZS (Rice et al., 2018). This percentage may go up as

583 children age since it is difficult to behaviorally test neurodevelopmental deficits in children this

584 young (Davidson et al., 2016).

585   There are also at least two major limitations to this model. Firstly, neonatal infection

586 occurred during a rodent period equivalent to the human second/third trimester when infection

587 can still produce CZS but most cerebral neurogenesis is complete (de Graaf-Peters and

588 Hadders-Algra, 2006; Brasil et al., 2016; van der Linden et al., 2016b). Therefore, the limited

589 population of cerebral NPCs during this time prevents us from examining their role in disease

590 progression. Our findings may therefore only provide insight into exposures occurring after the

591 first trimester or first trimester infections that persist into later gestational periods. Interestingly,

592 we found no pathology in the large population of postnatal cerebellar NPCs suggesting they

593 play no significant role in its subsequent hypoplasia. A second limitation of our model is that our

594 infection during the postnatal period prevents us from modeling vertical transmission that may

595 lead to different effects on neuropathology. For instance, this may lead to different routes of

596 neuroinvasion or allow fetal infection with ZIKV strains that may not ordinarily cross the

597 placenta. Regardless, it is clear the prolonged degeneration we found results in severe

598 microencephaly that closely models CZS.

599   In summary, CZS is a devastating disease that produces a unique constellation of severe

600 neurodevelopmental disabilities effecting thousands of children. Unfortunately, due to its recent

601 discovery, many basic questions regarding this disease and its ultimate consequences still

602 remain unanswered. Using the developing mouse animal model, we detail for the first time how

603 the ZIKV can produce a characteristic pattern of infection that mirrors neuropathology resulting

604 in gross morphological abnormalities striking similar to CZS. This toxicity is more complicated

605 than originally thought with the type and degree of neuropathology dependent on viral strain. It

606 is hoped this information can be used to provide insight into mechanisms of strain-dependent

607 neurotoxicity, a better understanding of the disabilities associated with CZS, and an animal

608 model to test potential treatment regimens that more accurately models disease progression.

609

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783

784 **FIGURE AND TABLE LEGENDS**

785 **Fig. 1. Quantitative analysis reveals Paraiba/2015 infection results in prolonged**

786 **lethality/degeneration and more severe hypoplasia.** [**A**] Kaplain-Meier curves reveal

787 **FP/2013** infection produces acute lethality occurring primarily between 10-17 DPI but

788 **Paraiba/2015** infection results in almost equal lethality with deaths occurring during an

789 expanded period between 8-24 DPI. [**B-H**] AC3 immunolabeled sections rated on our

790 degeneration scale reveal no effect at 7 DPI, both viruses increasing apoptosis at 13 DPI, and

791 only **Paraiba/2015** increasing apoptosis at 30 DPI. [**I-O**] Silver stained sections rated with the

792 same scale shows infection with **FP/2013** increases degeneration between 7-13 DPI that as

793 absent by 30 DPI.   Alternatively, **Paraiba/2015** infection results in delayed argyrophilia

794 beginning at 13 DPI that leads to prolonged degeneration extending to at least 30 DPI in several

795 regions. [**P**] At 30 DPI, brains weights were significantly lighter for **Paraiba/2015** only. [**P-S**]

796 Nissl stained sections had regions outlined revealing significant reductions in area for the dorsal

797 cortex, hippocampus, and cerebellum following **Paraiba/2015** infection only.   Group key and

798 Bonferroni post-hocs values located in lower-right box. For ANOVAs and F-values see Table 1.

799

800   **Fig. 2. Histology reveals extensive degeneration that mirrors infection in the early stage**

801   **(7-13 DPI).** Frontal Cortex [A-F]: [**A-B**] Silver staining reveals ZIKV exposure produces heavy

802   degeneration 10 DPI in the frontal cortex, somatosensory (SS), caudate putamen (C/P), and

803   septum (SE) but [**C-D**] absent in mock (MOCK) infected controls. [**E-F**] In situ hybridization

804   (ISH) for ZIKV RNA (red) reveals infection mirrors degeneration in which pyramidal shaped cells

805   can be distinguished (yellow arrows). Hippocampus [G-L]: [**G-H**] Infection produces

806   degeneration in visual cortex (VIS), lateral geniculate nucleus (LGN) of the thalamus, superior

807   colliculus (SC), and hippocampal formation including CA1, CA3, and dentate gyrus (DG).

808   Degenerating microglia can also be seen in the dentate polymorph layer (PL), stratum oriens

809   (SO), and stratum radiatum (SR) but is absent in controls [**I-J**] which have regions diagrammed.

810   [**K-L**] ISH labeling reveals infection mirrors degeneration including heavy infection in granule

811   cells (blue arrows) and pyramidal cells in CA3/CA1 (yellow arrows) but less distinct staining in

812   the PL, SO, and SR. Cerebellum [M-R]: [**M-N**] ZIKV inoculation produces heavy argyrophilia in

813   the granule cell layer (GCL), Purkinje cell layer (PCL; red arrows), and their connections in the

814   molecular layer (ML) but little toxicity in the white matter (WM). [**O-P**] Mock infected controls

815   exhibit no signs of toxicity which has regions diagrammed. [**Q-R**] ZIKV RNA was also detected

816   in the cerebellar granule cell, Purkinje, and molecular layers mirroring toxicity in which large

817   infected Purkinje cells (yellow arrows) could be seen. Spinal Cord [S-V]: [**S**] Degeneration was

818   lighter in the grey matter but particularly heavy in the funiculus (white matter) including the

819   corticospinal tract (red arrows). [**T**] No similar toxicity was seen in mock infected controls. [**U**]

820   Activated caspase-3 (AC3) staining reveals intense apoptosis in the corticospinal tract (red

821   arrows) along with lower levels in white and grey matter. [**V**] ZIKV infection is especially heavy

822   in the funiculus with lower levels in the spinal grey. Outlined regions in A, C, E, G, I, K, M, O, &

823   Q are magnified in B, D, F, H, J, L, N, P, & R respectively. Scale bars: [A-R] = 500 uM; [S-V] =

824   100 uM.

34

825 **Fig. 3. Magnified views of silver staining and ISH labeling in the dentate gyrus, optic**

826 **tract, and spinal cord.** [**A-C**] Argyrophilic fibers of the optic tract (arrows) could be seen

827 degenerating in infected animals 7 DPI [**A**] but not in mock infected controls [**B**]. [**C**] ISH

828 labeling for ZIKV RNA reveled punctate labeling of the optic tract (red arrows) resembling

829 argyrophilia. [**D-E**] At 10 DPI, [**D**] degeneration can be seen in the dentate gyrus granule cell

830 layer (GCL), molecular layer (ML) neuropil, and microglia in the polymorph layer (PL). [**E**] Mock

831 infected controls exhibit no degeneration. Diagram of synaptic connections is overlaid showing

832 axons of the perforant path synapsing with dendrites of granule cells which, in turn, send

833 efferents through mossy fibers (MF) to CA3. [**F**] ISH labeling for ZIKV RNA reveals a pattern of

834 infection that mirrors degeneration. [**G**] The spinal cord exhibited early infection 7 DPI that

835 exhibited punctate labeling in the cytoplasm surrounding the nucleus in the spinal lateral horn.

836 Scale bars = 100 uM.

837

838 **Fig. 4. A comparison of activated caspase-3 and silver staining reveals both apoptotic**

839 **and non-apoptotic degeneration is occurring.** Silver and activated caspase-3 (AC3) staining

840 was performed in the same animal and region for comparison. While there was a strong

841 correlation between the two, silver staining revealed more extensive pathology. [**A**] AC3

842 labeling in the cortex shows apoptotic pyramidal cells (arrows) extending their apical dendrites

843 to more superficial layers along with debris from less distinct cells 9 DPI. [**B**] Silver staining in

844 the same region shows more extensive degeneration although it is difficult to distinguish cellular

845 profiles. [**C**] Apoptosis was seen in the pyramidal and granule cells in the hippocampus along

846 with axons of the internal capsule (a part of the corticospinal tract) projecting ventrally to the

847 spinal cord (arrows). [**D**] Silver staining revealed neuronal along with heavy microglial

848 degeneration in the hippocampus. Some fibers from the corticospinal tract can be seen in

849 addition to degeneration in the LGN below the hippocampus. [**E**] AC3 immunolabeling shows

850 numerous apoptotic granule cells in the cerebellum but far more are present with silver staining

851 [**F**] along with large Purkinje cells (arrows) and argyrophilic neuropil in the molecular layer. [**G-**

852 **H**] The corticospinal tract exhibited heavy and more distinct AC3 labeling compared to silver

853 staining [**I-J**], however, more argyrophilic profiles were seen in other areas. Outlined regions in

854 G and I are magnified in H and J respectively. Scale bar: A, B, C, D = 500 uM; G, I = 250 uM;

855 E, F = 100 uM.

856

857

858

859 **Fig. 5. ZIKV infection produces excitotoxicity, apoptosis, and other signs of pathology.**

860 Plastic sections [A-F]: At 13 DPI, brains from ZIKV [**A-C**] or mock [**D-F**] infected animals were

861 plastic embedded and stained with Azure II/Methylene blue. [**A**] ZIKV infection produced heavy

862 vacuolization (formation of fluid filled cavities within the cytoplasm) of dentate gyrus granule

863 cells (yellow arrows) along with abnormal nuclear chromatin condensation (red arrows). [**B**]

864 Vacuolization (yellow arrows) could also be seen in the CA1 along with both dendritic swelling in

865 the stratum oriens (red arrows) and apoptosis (black arrows). [**C**] Similar vacuolization (yellow

866 arrows) and apoptosis (black arrows) was seen in the dorsal cortex (retrosplenial pictured).

867 Control animals displayed no pathology in the dentate gyrus [**D**], CA1 [**E**], or retrosplenial cortex

868 [**F**]. Electron Microscopy [G-N]: [**G**] Image of a normal cortical neuron exhibiting homogeneous

869 nuclear chromatin (*), a single nucleolus (yellow arrow), and thin endoplasmic reticulum (ER)

870 (red arrow) within the cytoplasm. [**H**] Image taken 13 DPI shows cortical neuron displaying

871 cytoplasmic condensation and vacuolated segments of ER (yellow arrow). Nuclear margination

872 (end clumping) of nuclear chromatin (white arrows) is seen in a "clock-face profile" along with

873 the nuclear envelope expanding into a large vacuolar space (*). [**I**] Cortical neuron in a late

874 stage of degeneration exhibiting terminal nuclear degeneration and large cytoplasmic vacuoles

875 (*). [**J**] A healthy granule cell (red arrow) among four undergoing excitotoxicity (yellow arrows)

876 in the dentate gyrus of the hippocampus. Degenerating neurons exhibit vacuolar swelling of ER

877 (*) and terminal nuclear degeneration. [**K**] CA1 pyramidal hippocampal neuron exhibiting signs

878 of excitotoxicity including nuclear chromatin margination forming a clock-face profile and

879 swelling of ER undergoing vacuolization (red arrow). [**L**] Parenchymal hemorrhage could be

880 seen in multiple areas of the brain with numerous red blood cells (R) lacking a nucleus

881 interspersed among myelinated axons (yellow arrows). [**M**] A single lymphocyte (L) can be seen

882 next to a heavily vacuolated (V) degenerating neuron (N). [**N**] A degenerating microglia (M) in

883 the hippocampus presenting with a very condensed and shrunken cytoplasm, thin vacuolated

884 processes, and nuclear dystrophy. Scale Bars: [A-F] = 10 uM; [G-N] = 2 uM.

37

885   **Fig. 6. Iba1 immunolabeling reveals microglia undergo initial activation followed by**

886   **fragmentation.** [**A-B**] Microglia in the visual cortex (left) and hippocampus (right) after mock

887   infection exhibit ramified microglia with thin elongated extensions with small soma indicative of

888   an inactivated macrophage. [**C-D**] At 7 DPI, microglia become activated with thickened

889   retracted processes and distended cell bodies. [**E-F**] By 10 DPI, microglia become less dense

890   and begin to fragment in regions with high amounts of degeneration. Scale Bars = 100 uM.

891

892

893 **Fig. 7. Pathology produced by French Polynesian ZIKV infection can transitions to**
894 **pervasive apoptosis after around 13 DPI.** A small number of animals infected with **FP/2013**
895 had degeneration transition to pervasive apoptosis. <u>Frontal Cortex [A-D]</u>: [**A-B**] Silver staining in
896 the anterior cerebrum around 14 DPI shows degeneration in the caudate/putamen with intense
897 pathology restricted to a thinning dorsal cortex. [**C-D**] AC3 immunolabeling in the same animal
898 reveals an identical pattern of extensive apoptosis. <u>Anterior Hippocampus [E-H]</u>: [**E**] Silver
899 staining reveals heavy degeneration in the cortex with slight hippocampal atrophy and
900 ventriculomegaly (*). [**F**] Closer examination reveals degeneration is particularly heavy in the
901 CA3 region of hippocampus. [**G-H**] AC3 labeling mirrors silver staining indicating these cells die
902 primarily through apoptosis. <u>Cerebellum [I-L]</u>: [**I-J**] Silver staining reveals heavy regions of
903 degeneration isolated to the granule cell layer which is mirrored by AC3 labeling [**K-L**]. 30 DPI
904 [**M-P**]: At 30 DPI, degeneration has ceased but gross morphological changes can be seen
905 including ventriculomegaly (*) [**M-O**] and hippocampal/cerebellar atrophy [**N-P**]. Outlined
906 regions in A, C, E, G, I, K, M, and O are magnified in B, D, F, H, J, and L respectively. Scale
907 Bars = 1 mm.

908

909 **Fig. 8. Pathology produced by Paraiba/2015 infection is more virulent and almost**

910 **exclusively apoptotic.** AC3 immunolabeling was performed at different DPI in the frontal

911 cortex, anterior hippocampus (Ant. Hippo), posterior hippocampus (Post. Hippo), and

912 cerebellum following infection with 10^3 FFU of the Brazilian ZIKV strain. [**A-D**] Apoptosis first

913 appeared approximately 7-10 DPI with heavy degeneration in the dorsal cortex, hippocampus

914 (CA1, CA3, and dentate gyrus), and cerebellar granule cell layer. [**E-H**] Around 10-13 DPI,

915 apoptosis peaks often producing gross morphological changes including, dorsal cortex atrophy,

916 hippocampal retraction dorsally, and thinning of cerebellar lobes. [**I-L**] At 20-30 DPI continuing

917 degeneration produces microencephaly, ventriculomegaly, and atrophy of the dorsal cortex,

918 hippocampus, and cerebellum. [**M-P**] An age matched control brain (P30) is pictured for

919 comparison to images I-L. All images in each column are at the same magnification for

920 comparison. Scale Bars = 1 mm.

921

922 **Fig. 9. ISH labeling for ZIKV and silver staining 10-16 after Paraiba/2015 inoculation**
923 **reveal intense infection and degeneration mirroring apoptosis.** Frontal Cortex [A-D]: [**A**]
924 ISH labeling for ZIKV RNA in the frontal cerebrum reveals intense infection in the dorsal
925 somatosensory/motor cortex and caudate/putamen. [**B**] A close up of outlined region shows
926 almost continuous labeling throughout suggesting infection in all cell types. [**C-D**] Silver staining
927 reveals degeneration matching infection and AC3 labeling. [**E-F**] The hippocampus was also
928 heavily infected throughout the dentate gyrus, CA1, and CA3 regions with silver staining [**G-H**]
929 mirroring the pattern of infection. [**I-J**] ISH labeling in the cerebellum revealed dense infection
930 within entire lobes but less severe in others. [**K-L**] Silver staining was more severe and
931 particularly intense in the granule cell layer of the cerebellum. Scale bars = 1 mm.
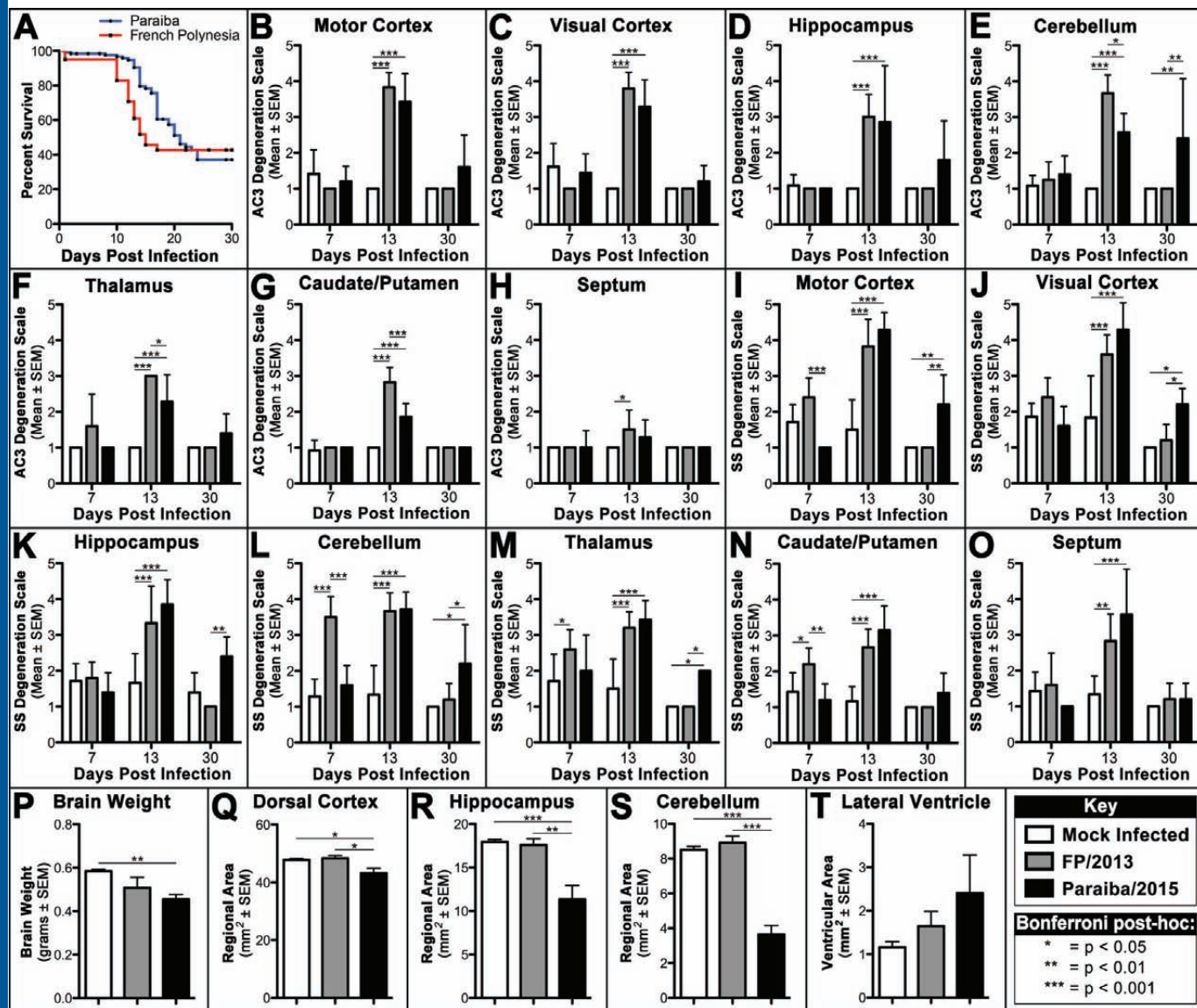
932

933 **Fig. 10. Labeling reveals regions with high ZIKV infection contain numerous neurons but**

934 **more limited microglia and astrocytes at 10 days post-infection**.   [**A-D**]   Tissue was

935 immunolabeled with the neuronal nuclear marker NeuN [**A**], stained with the nuclear stain DAPI

936 [**B**], and ISH labeled for ZIKV RNA. A merged image [**D**] revealed a large number of neurons

937 located in infected regions. [**E-G**] Similar staining with the microglial marker (Iba1, DAPI, and

938 ISH for ZIKV RNA) or [**I-L**] astroglial marker (GFAP, DAPI, and ISH for ZIKV RNA) revealed

939 more limited co-labeling. Scale bar = 100 uM.

940

941 **Fig. 11. Plastic embedding and electron microscopy following infection with Paraiba/2015**

942 **reveals massive apoptosis, parenchymal hemorrhage, axonal pathology, and an adaptive**

943 **immune response.** Plastic Sections [A-C]: Mouse pups were infected with **Paraiba/2015** at 10^3

944 FFU and sacrificed 13 DPI for plastic embedding and staining with Azure II/Methylene blue.

945 Histology revealed a large number of cells displaying characteristic signs of apoptosis with

946 shrunken soma and nuclear chromatin condensation (red arrows) in the [**A**] cortex, [**B**] dentate

947 gyrus, and [**C**] CA1 of the hippocampus. Electron microscopy [D-G]: [**D**] Apoptotic neuron

948 undergoing somal shrinkage and condensation of chromatin in the nucleus (N). **[E]** Perivascular

949 area with hemorrhaged red blood cell (R) located outside the vascular lumen (V) is adjacent to

950 an activated microglia (M). An additional apoptotic cell (A) in the late stage of apoptosis can

951 also be seen. Its bilaminar nuclear envelope is primarily intact except for one portion where

952 disruption allows the nucleoplasmic contents to spill into the surrounding dystrophic cytoplasm

953 (arrows). **[F]** Cross section of an axon undergoing massive swelling and organelle compaction

954 following the breakdown of axoplasmic transport. Part of this swollen axon still retains its myelin

955 sheath (arrows) while the rest is demyelinated and has expanded into the surrounding interstitial

956 space. **[G]** Neutrophils were frequently seen in post-capillary venules in preparation for

957 diapedesis or in the extravascular space following extravasation. Neutrophils were observed

958 adjacent to the endothelium (N1 and N2), adhered to the endothelial wall (N3), or in the

959 extravascular space (N4). Monocytes were also seen undergoing similar transmigration (M1

960 and M2) or in the extravascular space (M3 and M4). This reveals an adaptive immune

961 response as leukocytes migrate to sites of injury/infection. Scale Bars: [A-C] = 50 uM; [D-G] = 2
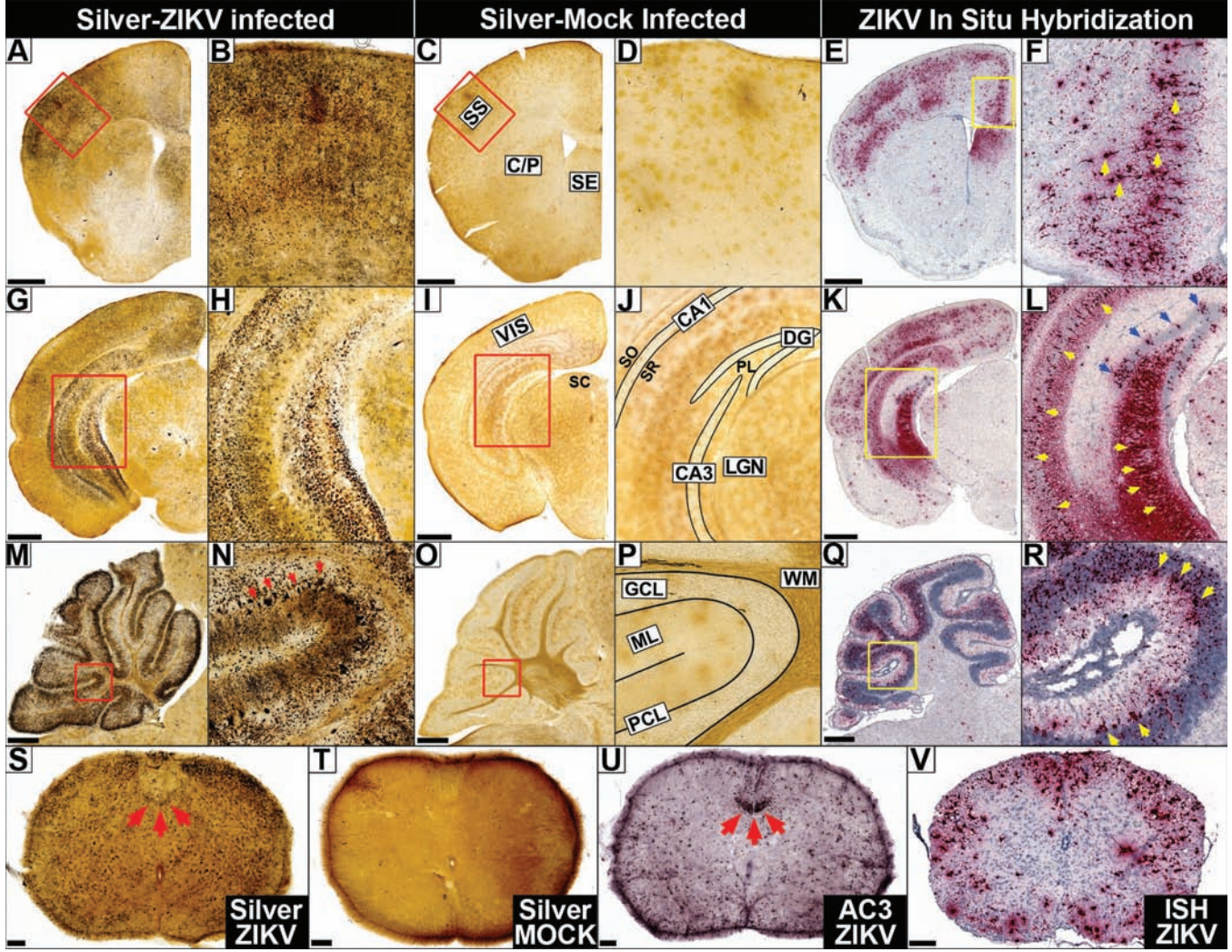
962 uM.

963

964

43

965   **Fig. 12. Infection with Paraiba/2015 produces CA3 excitotoxicity, cerebellar calcification,**

966   **and reductions in microglia.** While AC3 labeling reveals little apoptosis in the hippocampal

967   CA3 region [**A**], silver staining shows much more argyrophilia [**B**] suggesting non-apoptotic

968   death is occurring. [**C**] Semithin plastic sections with Azure II/Methylene blue staining in CA3

969   show the classical signs of both apoptosis (black arrows) and excitotoxicity which includes

970   vacuolization (yellow arrows) and nuclear margination (red arrows). [**D**] Alizarin staining shows

971   calcification is localized to the granule cell layer and present in atrophied lobes. [**E**] Iba1

972   labeling shows microglia are present at a much lower density or are almost entirely missing from

973   a cerebellar lobe (red arrow). Note the adjacent brain stem contains a normal density of

974   microglia. [**F**] Iba1 labeling in an age matched control shows a normal density and distribution

975   of microglia. Note severe cerebellar hypoplasia in images D-E when compared to F which is at

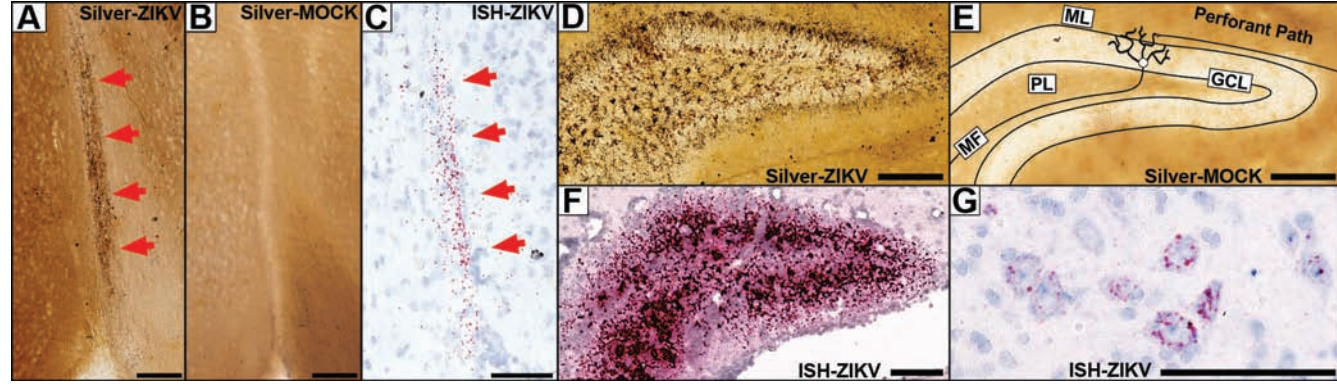976   the same magnification scale. Scale bars: [A-C] = 50 uM; [D-F] = 1 mm.

977

978 **Table 1. Statistical results from ANOVAs in Figure 1.** Main effects, interactions, F-values,

979 and p-values for one- and two-ANOVAs graphed in Figure 1. *indicates value would be non-

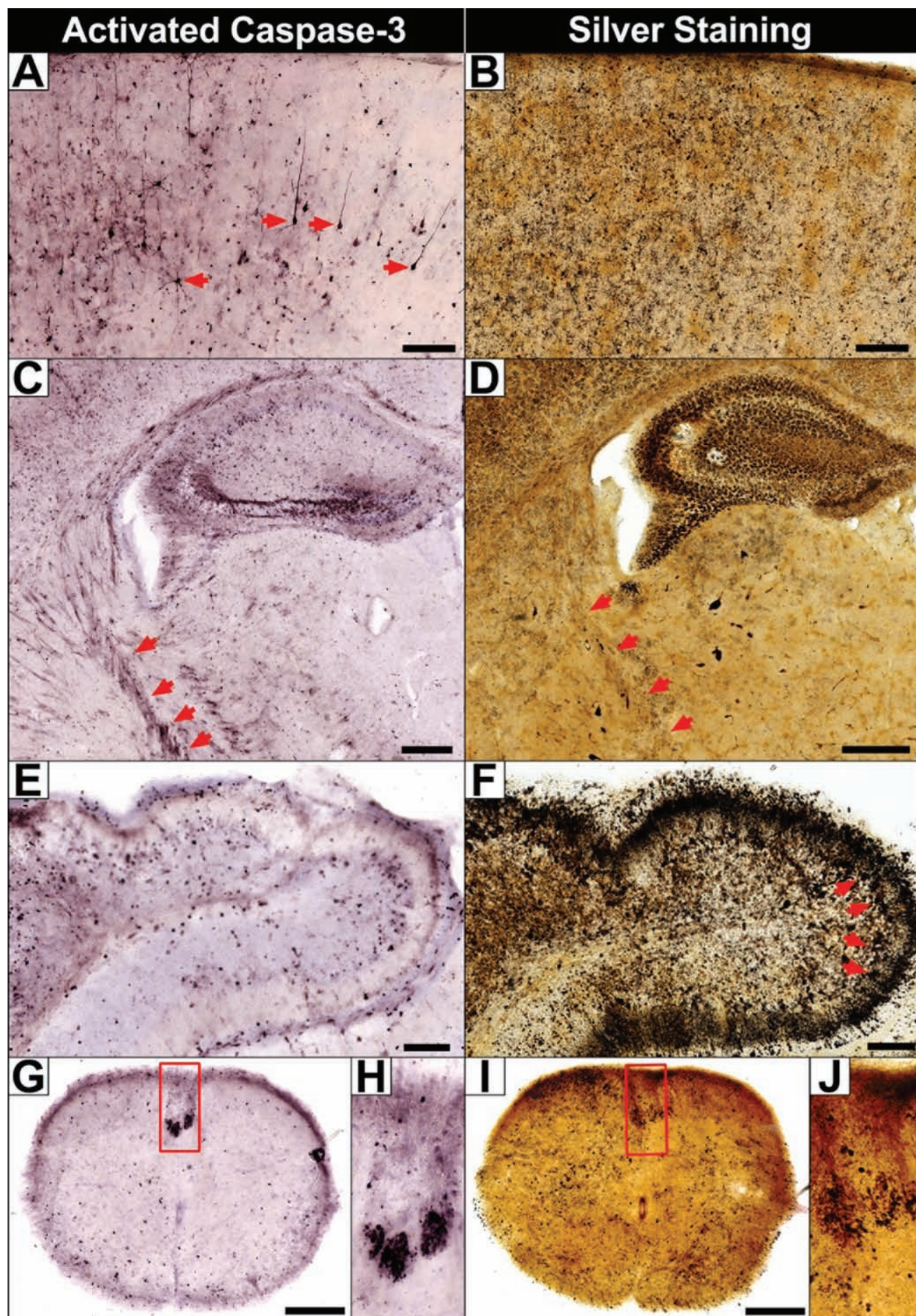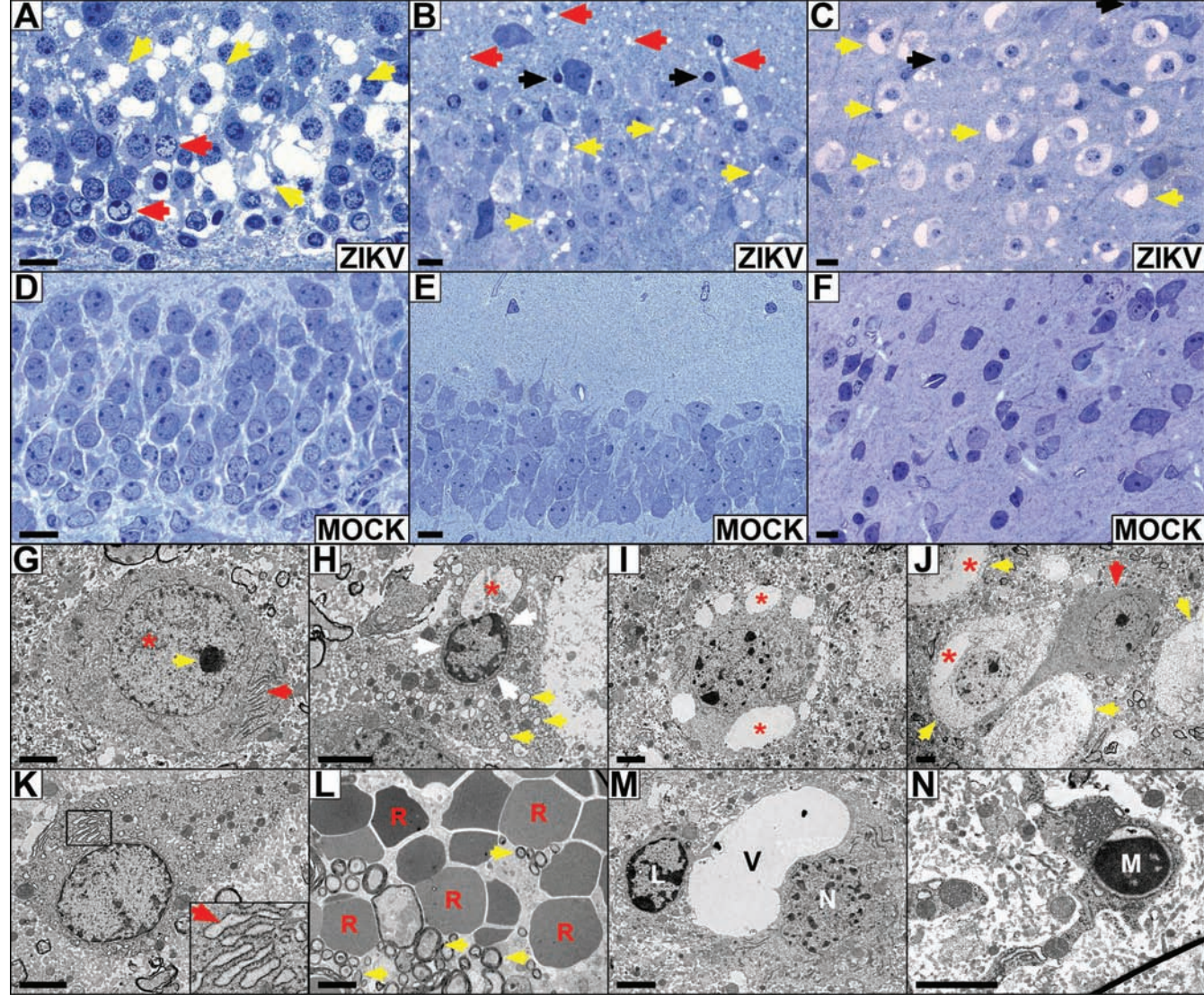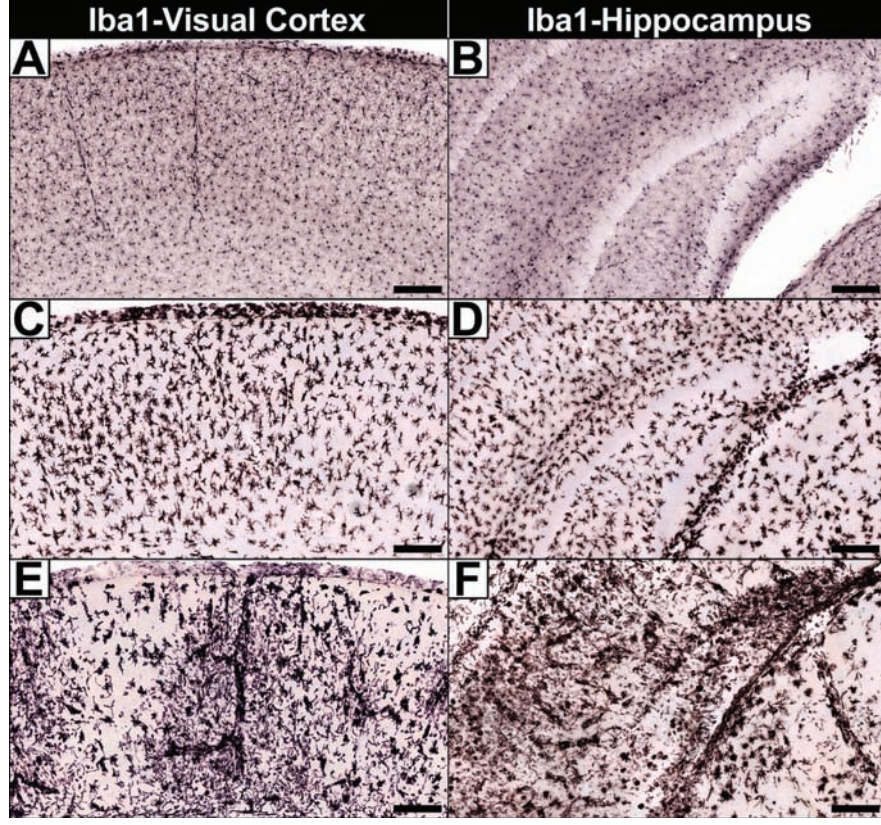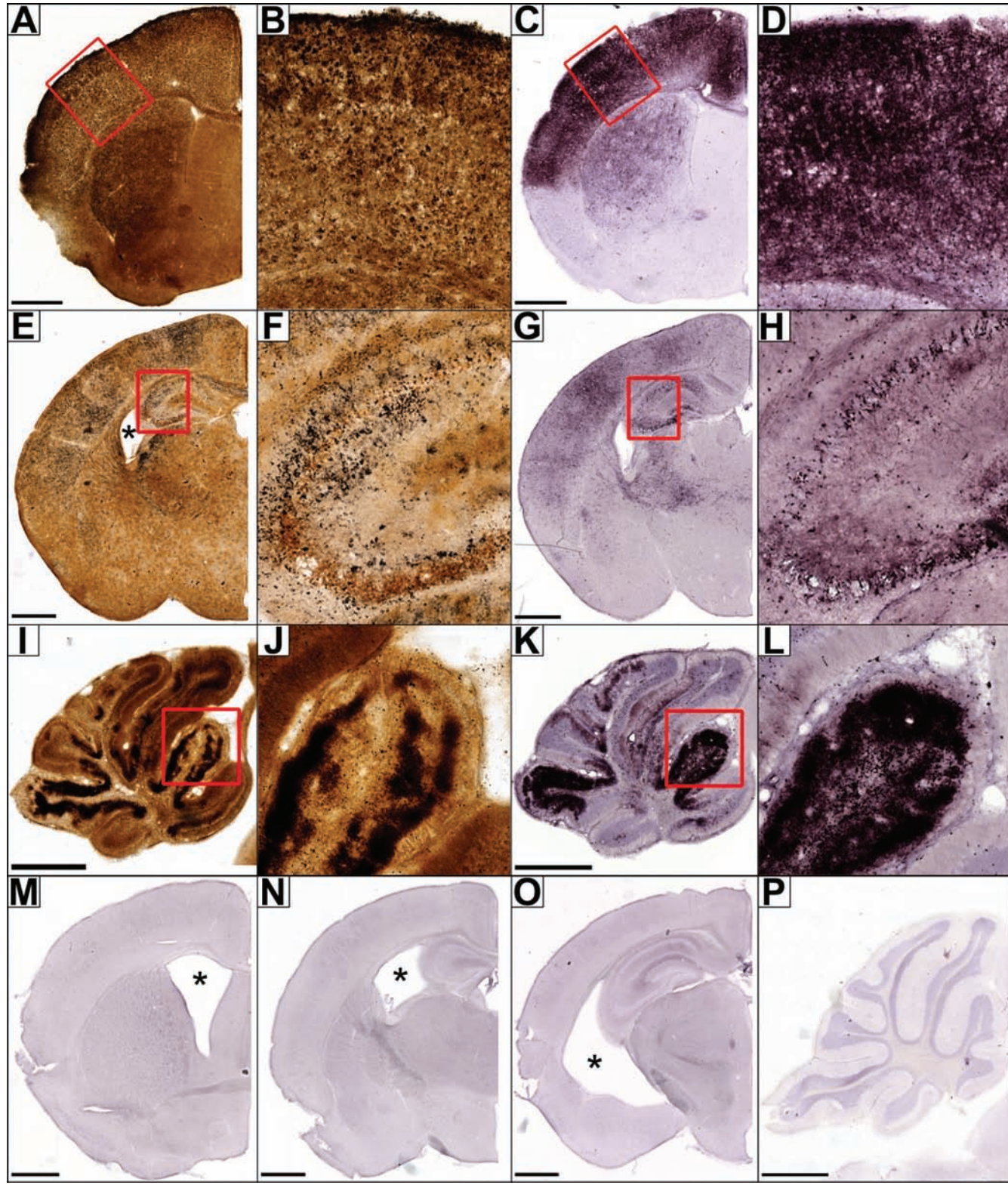980 significant following Bonferroni correction for multiple comparisons.

981

Silver-ZIKV infected	Silver-Mock Infected	ZIKV In Situ Hybridization

A, B, C (SS, C/P, SE), D, E, F, G, H, I (VIS, SC), J (CA1, SO, SR, DG, PL, CA3, LGN), K, L, M, N, O, P (GCL, ML, PCL, WM), Q, R, S (Silver ZIKV), T (Silver MOCK), U (AC3 ZIKV), V (ISH ZIKV)

A Silver-ZIKV B Silver-MOCK C ISH-ZIKV D Silver-ZIKV E ML Perforant Path PL GCL MF Silver-MOCK F ISH-ZIKV G ISH-ZIKV

Activated Caspase-3

Silver Staining

Iba1-Visual Cortex | Iba1-Hippocampus

Frontal Cortex	Ant. Hippo	Post. Hippo	Cerebellum

ZIKV
7-10 DPI

ZIKV
10-13 DPI

ZIKV
20-30 DPI

MOCK
P30

In Situ Hybridization	Silver Staining
A B	C D
E F	G H
I J	K L

Neurons — A: NeuN; B: DAPI; C: ZIKV; D: MERGE
Microglia — E: Iba1; F: DAPI; G: ZIKV; H: MERGE
Astrocytes — I: GFAP; J: DAPI; K: ZIKV; L: MERGE

A AC3-CA3
B Silver-CA3
C Plastic-CA3
D Alizarin
E Iba1
F Iba1

Region	Marker	Treatment	Days Post-Infection	Interaction
Hippocampus	AC3	F[2,40] = 05.30, p = 0.0091*	F[2,40] = 12.40, p < 0.0001	F[4,40] = 04.36, p = 0.0051
Motor Cortex	AC3	F[2,51] = 16.65, p < 0.0001	F[2,51] = 51.87, p < 0.0001	F[4,51] = 18.74, p < 0.0001
Visual Cortex	AC3	F[2,50] = 13.44, p < 0.0001	F[2,50] = 54.07, p < 0.0001	F[4,50] = 21.13, p < 0.0001
Striatum	AC3	F[2,51] = 36.11, p < 0.0001	F[2,51] = 97.88, p < 0.0001	F[4,51] = 31.70, p < 0.0001
Thalamus	AC3	F[2,40] = 15.91, p < 0.0001	F[2,40] = 23.75, p < 0.0001	F[4,40] = 08.51, p < 0.0001
Cerebellum	AC3	F[2,39] = 13.32, p < 0.0001	F[2,39] = 16.67, p < 0.0001	F[4,39] = 09.22, p < 0.0001
Septum	AC3	F[2,40] = 01.54, p = 0.2227	F[2,40] = 05.03, p = 0.0113*	F[4,40] = 01.54, p = 0.2096
Hippocampus	Silver Stain	F[2,42] = 09.29, p = 0.0005	F[2,42] = 23.93, p < 0.0001	F[4,42] = 07.96, p < 0.0001
Motor Cortex	Silver Stain	F[2,42] = 19.55, p < 0.0001	F[2,42] = 49.65, p < 0.0001	F[4,42] = 18.59, p < 0.0001
Visual Cortex	Silver Stain	F[2,41] = 14.15, p < 0.0001	F[2,41] = 34.46, p < 0.0001	F[4,41] = 07.85, p < 0.0001
Striatum	Silver Stain	F[2,42] = 13.31, p < 0.0001	F[2,42] = 26.44, p < 0.0001	F[4,42] = 10.38, p < 0.0001
Thalamus	Silver Stain	F[2,41] = 14.75, p < 0.0001	F[2,41] = 21.77, p < 0.0001	F[4,41] = 05.19, p = 0.0018
Cerebellum	Silver Stain	F[2,41] = 30.81, p < 0.0001	F[2,41] = 22.43, p < 0.0001	F[4,41] = 11.29, p < 0.0001
Septum	Silver Stain	F[2,42] = 04.81, p = 0.0132*	F[2,42] = 20.97, p < 0.0001	F[4,42] = 05.67, p = 0.0010
Dorsal Cortex	Nissl Stain	F[2,21] = 06.48, p = 0.0064	N/A	N/A
Hippocampus	Nissl Stain	F[2,19] = 15.41, p < 0.0001	N/A	N/A
Cerebellum	Nissl Stain	F[2,19] = 62.60, p < 0.0001	N/A	N/A
Lateral Ventricle	Nissl Stain	F[2,19] = 01.78, p = 0.1965	N/A	N/A
Brain Weight	N/A	F[2,19] = 20.58, p < 0.0001	N/A	N/A