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(Article begins on next page)

Zika virus infection causes microcephaly in iPSC-derived human brain organoids

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Microcephaly is a rare developmental disorder, known to be caused by mutations in centrosomal genes. Thus far, it has only affected around 200 patients worldwide. ~~(Refs)~~. However, the recent Zika virus (ZIKV) outbreak in Brazil saw a 25-fold increase of microcephaly cases, leading to high emotional and economic burdens for patients and their families¹⁻³ ~~(Refs)~~. Thus, insights into the tropism and mode of action of ZIKV in the developing human brain are urgently required. Here, we demonstrate that the French Polynesia ZIKV, a strain associated with microcephaly and genetically identical to the Brazilian isolate, indeed efficiently infects and is highly productive in human brain organoids derived from induced pluripotent stem cells (iPSC). ZIKV targets to and concentrates in proliferating ciliated apical progenitors of the ventricular zone (VZ) where neurogenesis begins. Strikingly, ZIKV infection induces massive neuronal cell death at the periphery of the VZ and cortical plate, which was not observed for apical progenitors within the VZ. This shows that ZIKV is neuroinvasive and targets apical neural progenitors for viral replication. Through this mode of action, ZIKV-infection may reduce cortical volume, an observation that is consistently found in microcephaly patients^{4-6,7} ~~(Refs)~~. Thus our study provides a mechanistic explanation for the reduced neurogenesis and brain size in ZIKV-infected newborn babies.

The ongoing outbreak of mosquito-borne ZIKV infection and sudden increase of microcephaly in Brazil has been declared a “Public Health Emergency of International Concern” by the World Health Organization (WHO)⁸. Identification of ZIKV particles in amniotic fluid of infected pregnant women and microcephalic fetus brain tissues provided an initial link between ZIKV and microcephaly which was further strengthened by the recent report of productive in vitro infection of human neural progenitors cells (NPCs) by the primary ZIKV isolate, African MR 766. Notably, none of the MR766 ZIKV infections in humans over the last six decades was linked to microcephaly. MR766 differs in amino acid composition from the French Polynesian and recent Brazilian isolate (**Fig. 1a and S1a**). It can, however, infect NPCs showing the general vulnerability of NPCs to ZIKV infection. Thus, the characterization of a ZIKV strain that can cause microcephaly is mandatory and will be instrumental in establishing pre-clinical models to understand the pathophysiology of ZIKV-induced microcephaly eventually leading to the design of novel therapeutic strategies.

To address this, we adapt iPSC-derived brain organoids that closely parallel complex human neural epithelium and serves as a disease-relevant tissue to model neurodevelopmental disorders and microcephaly. Importantly, we could optimize neuronal differentiation conditions that allow us to successfully generate microcephaly brain organoids from patient-derived fibroblasts. Thus iPSC-derived brain organoids not only serve as a reliable in vitro model system but also overcomes the practical and ethical limitations in dissecting the effects of ZIKV infection on developing fetal brain tissues.

For our experiments, we used the French Polynesia ZIKV (H/PF/2013, hereafter ZIKV), which is linked to the current microcephaly-associated Brazilian ZIKV epidemic and phylogenetically resembles the recent Brazilian isolate more than the MR766 strain (**Fig. 1a and S1a**). ZIKV infection could be observed in human fibroblasts, iPSCs, and iPSC-derived NPCs. ZIKV infectivity was relatively lesser in iPSCs and fibroblasts indicating a low permissiveness of ZIKV in these cell types. However, to our surprise, compared to fibroblasts and iPSCs, we measured a 100-fold increased infectivity in NPCs in the initial days of infection, indicating an acute infection of neural epithelial stem cells that are highly susceptible and target cell types for ZIKV particles. Immunostaining using an antibody against the flavivirus envelope which recognizes ZIKV gives further evidence of intracellular ZIKV production in the tested monolayer cells with NPC infection levels reaching 40% within 4 days of infection.

To visualize intracellular ZIKV particles, we performed transmission electron microscopy (TEM) of infected NPCs. EM analyzes revealed the presence of ~60 nm (56.3±3 nm) viral particles as electron dense spheres that were often associated with actin filaments and microtubules (**Fig. 1e, S2 and supplementary table 1**). Interestingly, dengue virus, which is also a member of the flavivirus group, interacts with actin filaments for its entry, production and release of viral particles¹⁶ ~~(Refs)~~. Taken together, these results indicate that ZIKV is a neurovirulent factor that can cause productive infection of neuronal cells leading to cell-to-cell infection.

In the mammalian brain, self-renewing, multipotent NPCs are responsible for neurogenesis¹⁷ ~~(Refs)~~. Neural epithelium development requires rapid expansion of the NPC pool via symmetrical divisions. After reaching a sufficiently large pool, NPCs then asymmetrically divide to generate various neuronal cell types that form the cortical layers of the brain^{4, 17, 18} ~~(Refs)~~. Thus, even a minor disturbance in division can impair the proportion of symmetric divisions leading to NPC pool disruption and reduction of the total number of neurons generated. This is suggested to be a cause of primary microcephaly^{4, 14} ~~(Refs)~~. Studies analysing brain organoids derived from microcephaly patients have indeed identified premature differentiation of NPCs to be a potential cause of microcephaly^{12, 14} ~~(Refs)~~.

Thus, to test if ZIKV infection could trigger NPC differentiation into neurons, we analyzed infected NPCs and found that ZIKV-infected NPCs but not uninfected NPCs differentiated into TUJ1-positive neurons at the expense of proliferating nestin-positive NPCs (**Fig. 2a and b**). Interestingly, we found ZIKV particles as spherical objects localized within the axons of differentiating neurons (**Fig. 2c**). Furthermore, our 24hr ethynyl-deoxyuridine (EdU) pulse labeling experiments revealed that ZIKV at higher doses could inhibit NPC proliferation as measured by reduced EdU incorporation (**Fig. 2d**). Reduced cell proliferation and spontaneous differentiation of ZIKV-infected NPCs is consistent with that of microcephaly NPCs^{12, 14} ~~(Refs)~~. Notably, microcephaly NPCs that exhibited premature differentiation harbored defective centrosomes due to poor recruitment of centrosomal proteins^{12, 14, 19} ~~(Refs)~~. Analyzing the centrosomes of ZIKV-infected NPCs revealed that ZIKV infection indeed reduces the centrosomal recruitment of PCNT and Cep152, proteins that are required for normal centrosomal functions (**Fig. 2e and f**)^{20, 6, 21} ~~(Refs)~~. Together, these studies suggest that the effects caused by ZIKV infection are reminiscent of microcephaly NPCs^{12, 14, 44, 45} ~~(Refs)~~.

To determine if these effects caused by ZIKV infection could underlie microcephaly, we used iPSC-derived 3D brain organoids. We adapted our recently described method to generate brain organoids which display fairly similar ventricular zones (VZ) between organoids¹⁴. These VZs contain nestin-positive proliferating radial glial cells on the apical side facing the lumen and TUJ1-positive neurons at the basal side away from the lumen forming a cortical region (**Fig. S3a**)^{13, 14} ~~(Refs)~~.

We generated nine-day old brain organoids, which were then infected with ZIKV for eleven-days prior to analyses. We also infected organoids with lymphocytic choriomeningitis virus

(LCMV), a rodent-borne virus that is associated with aseptic meningitis and encephalitis (**Fig. S3b**)^{22, 23}. Strikingly, a productive viral infection was identified only with ZIKV as was shown by a rapid increase of infectious ZIKV particles in the culture supernatants of the organoids indicating that neural epithelial tissues are highly susceptible and target tissues for ZIKV particles (**Fig. 3a**). While the uninfected organoids grew up to 3.5 mm in size, ZIKV-infected organoids exhibited an attenuated growth as was observed in microcephaly (**Fig. 3a**). We analyzed localization of ZIKV by immunostaining and found massive viral infection consistent with our infectivity titrations (**Fig. 3c and S4a**). Importantly, ZIKV infection was concentrated at proliferating apical progenitors of the VZ where neurogenesis begins. These cells contain primary cilia emanating from their apical surface extending into the lumen of the VZ indicating that they are ciliated apical progenitors (**Fig. 3c**). Furthermore, ZIKV staining localizing to radial filament-like structures between the ciliated apical progenitors suggest that ZIKV can also target radial glial cells emerging from the VZ (**Fig. 3c and S4a**). Serial section EM analyzes further identified the intracellular localization of ZIKV particles in the apical progenitors of the VZ (**Fig. 3d and S5**).

Recently, the African ZIKV strain was shown to cause cell death of NPCs². ~~(Refs)~~. To test if French Polynesia ZIKV that targets apical progenitors at the VZ could also cause cell death, we performed Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) to identify dying cells. To our surprise, the distribution of dying cells within an organoid differed markedly from the distribution of ZIKV-infected cells. Specifically, we found ~~apical~~ progenitors ~~lining-of~~ the VZ to be free from TUNEL staining. Instead increased levels of TUNEL-positive cells were observed at the periphery of the VZ. This finding suggests that ZIKV-infection possibly induces the death of differentiated neuronal cells that emerge from the VZ to form various brain regions including the cortical plate (**Fig. 3e**).

Indeed, we noticed a massive reduction of TUJ1 and doublecortin (DCX) protein localizations, which specify neurons and early cortical progenitors of the developing brain (**Fig. 3e-f**). Thus, the reduced or defective cortical region observed in ZIKV-infected organoids is to some extent reminiscent of the neurodevelopmental defects in microcephaly. The finding that ZIKV does not seem to majorly cause apical progenitors cell death, instead causing apoptosis of early neurons emerging from the VZ raises the possibility that ZIKV resides in apical progenitors for its own replication.

To directly test if ZIKV could infect neurons and cause cell death, we adapted a protocol to differentiate NPCs into cortical neurons²⁴. ~~(Refs)~~. We noticed ZIKV infection reaching up to 50% as deduced by ZIKV immunostaining (**Fig. 4a**). Strikingly, as we expected, ZIKV infection resulted in massive cell death as determined by TUNEL assay and a drastic reduction of cell numbers after four-days of infection (**Fig. 4b-c**). From these results, it appears that the ZIKV strain that is associated with the microcephaly epidemic has selectivity in cell types and preferably affects differentiated neuronal cells.

In this study, we demonstrate that the French Polynesia ZIKV, which is closely related to the strain of the recent ZIKV outbreak in Latin America, is neuroinvasive and productively infects human brain organoids (**Fig. 3**). Despite ZIKV targeting proliferating ciliated apical neural progenitors of the VZ, these cells are not driven to die. Instead, ZIKV infection results in spontaneous differentiation of NPCs into neurons (**Fig. 2**). This could potentially lead to depletion of the NPC pool required for proper brain development. On the other hand, the conspicuous cell death response of differentiating neuronal cells from ZIKV-infected apical progenitors could lead to massive direct loss of neuronal cells. Both these mechanisms could impair growth and structural organization of the cortical plate in a developing brain, a hallmark of microcephaly (**Fig. 4d**). Identification of specific neuronal cell types that are vulnerable to ZIKV and the mechanisms by which ZIKV could cause neuronal apoptosis warrants future mechanistic studies. In addition to providing direct evidence that ZIKV can cause microcephaly in disease-relevant neural epithelial tissues, our study also establishes human brain organoids as a pre-clinical model to understand the pathophysiology of ZIKV-induced microcephaly. Thus, brain organoids may serve as a reliable in vitro model system to design therapeutic strategies particularly to prevent ZIKV replication in the apical progenitors where neurogenesis begins.

Fig. 1 ZIKV infects human iPSC-derived NPCs ~~and brain organoids~~ and produces ZIKV particles.

(A) Positions of the amino acid and percentages of differences in the polyprotein of the French Polynesia ZIKV strain in comparison to African (top side) and Brazilian (bottom side) ZIKV strains. Small vertical lines represent amino acid exchanges and small circle represents a deletion. Percentage values indicate the proportion of sequence changes of amino acids in individual viral protein. C: capsid, M: membrane protein including propeptide, Env: envelope protein, NS: non-structural protein.

~~Percentages of differences in the amino acid sequence of the polyprotein of French polynesian H/PPF/2013 ZIKV strain in comparison to the African (outer circle) and Brazilian Zika virus strains (inner circle). C: capsid, M: membrane protein including propeptide, Env: envelope protein, NS: non-structural protein.~~

(B) **OLAF**

(C) Representative images showing ZIKV infection in NPCs. At least two multiplicity of infection (MOI 0.1 and 1.0) was used. Four-days post infected (4 dpi) cells. After 72 hrs of inoculation, cells were immunostained for an antibody that recognizes ZIKV (green). Scale bar 5 μ m.

(D) Percentages of ZIKV-infected fibroblasts, iPSC and NPCs. Error bars are s. e. m *** $p < 0.0001$, ANOVA; $n = 4$ independent experiments.

(E) Electron micrographs showing the presence of uniform-sized spherical electron dense ZIKV particles within the infected NPCs. Note that ZIKV particles are lined up with actin filament-like structure. At least 25 cells were analysed. This figure is to be combined with Fig. 3, S2 and S5 that display ures Sxx, showing ZIKV localizations brain organoids and its association in-with subcellular structures.

Fig. 2 ZIKV infection causes NPCs differentiation and reduces centrosomal proteins recruitment.

(A) ZIKV-infected NPCs differentiated into TUJ1-positive neurons under non-differentiating conditions. Scale bar 10 μ m.

(B) Bar diagram quantifies percentages of NPC's differentiation after four days of post infection (4 dpi). Error bars show \pm s. e. m. *** $p < 0.001$, ANOVA. $n = 3$ independent experiments. At least 400 cells were analysed in each condition. Scale bar 10 μ m.

(C) ZIKV localization (green) in the axons (magenta, TUJ1) of differentiated neurons. Scale bar 5 μ m. Inset at the right shows series of ZIKV spheres (green) on axons (magenta). Phase image shows the presence of ZIKV particles within the axon. Scale bar 1 μ m.

(D) ZIKV-infection at MOI 1.0 causes ~~a~~ reduced cell proliferation of NPCs. Error bars are \pm s. e. m. * $p < 0.05$ (MOI 1.0), t-test. At least 500 cells were analysed in each condition.

(E-F) ZIKV-infected NPCs (green) fail to efficiently recruit centrosomal proteins PCNT and Cep152 (red). Ordinary Two-way ANOVA followed by Sidak's multiple comparisons test. n = 3 independent experiments with at least 100 cells were analysed at each condition. Error bars are s. e. m. Scale bar, 5 μ m (for over view) and 1 μ m (for single cells).

Fig. 3 ZIKV infection targets ciliated apical progenitors of human brain organoids and perturbs neurogenesis. infected brain organoids display microcephaly-like phenotypes.

(A) OLAF/Martin. In this experiment, each organoid was infected with 10^5 pfu (plaque forming units) of ZIKV.

(BA) Age-matched ZIKV-infected human brain organoids are smaller than the control organoids (n=645 in each). Nine-day old organoids were infected with ZIKV and further grown for subsequent eleven days. Scale bar, 1 mm.

(CB) ZIKV immunostaining (green) shows that ZIKV targets apical progenitors of the VZ. Arrows at the magnified image at right mark ZIKV staining (green) the radial glial cells at the apical side towards the lumen. VZ marks ventricular zone and CP defines (dotted lines) the approximate location of cortical plate area which is at the basal side away from the lumen. Cortical plate is defined by TUJ1 immunostaining (magenta). Note a drastic reduction of TUJ1-positive neurons (magenta) at the cortical area of ZIKV-infected organoids. Bar diagram at right quantifies the percentage of ZIKV-infected cells at the VZ. Error bars are \pm s. e. m. ***p<0.0001, ANOVA. At least 12 VZs from four independent organoids were analysed in each condition.

(D) ZIKV targeted cells at the VZ are ciliated apical progenitors that emanates cilia (Arl13b, magenta) extending into the ventricular lumen. Dotted lines at the magnified image at right define the ciliated apical progenitors that are infected with ZIKV (green). Cartoon at right depicts ciliated apical progenitors forming the VZ and a ZIKV particle (green spiky sphere).

(f)

(EC) ZIKV-infected organoid (ii) not control organoid (i) contains ZIKV particles as revealed by EM. Magnified electron dense ZIKV spheres at the right (red arrows). Typically, these ZIKV particles seem to have a rough surface with minute spikes. This figure is to be combined with Fig. 3, S2 and S5.

(F)

(G)

Figures SXXX.

Fig. 4 ZIKV infects cortical neurons and induces cell death.

(A) Percentage of ZIKV infection in neurons differentiated from iPSC. Error bars show \pm s. e. m. *** $p < 0.0001$, t -test. $n = 3$ independent experiments. At least 200 cells were analysed in each condition.

(B) Representative images of ZIKV-infected neurons displaying TUNEL-positive cells. After 96-hrs of 72-hrs of inoculation (4 dpi), cells were immunostained for TUJ1 antibody that recognizes neurons (magenta). ~~and~~ TUNEL staining is (green). Scale bar 5 μ m.

(C) Quantification of TUNEL-positive neurons and NPCs after 9672-hrs of ZIKV infection. Error bars show \pm s. e. m. *** $p < 0.0001$, ANOVA. $n = 3$ independent experiments containing at least 200 cells in each condition **(i)**. Number of neurons at cells-within 200 μ m² area after ZIKV-infection **(ii)**. Note a drastic reduction in neuronal ~~al~~ but not NPC number suggestive of s cell death.

(D) Hypothetical model of ZIKV infection causing microcephaly. Left panel, normal brain development showing various processes containing healthy cells and cortical plate. Right panel, ZIKV infected microcephalic brain showing concentrated ZIKV at the apical progenitors of the VZ. ZIKV-infected differentiated neurons and cells emerging from the VZ are dying resulting in reduced cortical volume and microcephaly. Different regions of developing brain and cell types are given.

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