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Research Paper

# Comparative Analysis Between Flaviviruses Reveals Specific Neural Stem Cell Tropism for Zika Virus in the Mouse Developing Neocortex

Jean-Baptiste Brault [a], Cécile Khou [b], Justine Basset [b,1], Laure Coquand [a,1], Vincent Fraisier [a], Marie-Pascale Frenkiel [b], Bruno Goud [a], Jean-Claude Manuguerra [b], Nathalie Pardigon [b,2], Alexandre D. Baffet [a,*,2]

[a] Institut Curie, PSL Research University, CNRS, UMR144, F-75005 Paris, France
[b] Institut Pasteur, ERI/CIBU Arbovirus Group, 25 rue du Dr Roux, 75015 Paris, France

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ABSTRACT

The recent Zika outbreak in South America and French Polynesia was associated with an epidemic of microcephaly, a disease characterized by a reduced size of the cerebral cortex. Other members of the *Flavivirus* genus, including West Nile virus (WNV), can cause encephalitis but were not demonstrated to cause microcephaly. It remains unclear whether Zika virus (ZIKV) and other flaviviruses may infect different cell populations in the developing neocortex and lead to distinct developmental defects. Here, we describe an assay to infect mouse E15 embryonic brain slices with ZIKV, WNV and dengue virus serotype 4 (DENV-4). We show that this tissue is able to support viral replication of ZIKV and WNV, but not DENV-4. Cell fate analysis reveals a remarkable tropism of ZIKV infection for neural stem cells. Closely related WNV displays a very different tropism of infection, with a bias towards neurons. We further show that ZIKV infection, but not WNV infection, impairs cell cycle progression of neural stem cells. Both viruses inhibited apoptosis at early stages of infection. This work establishes a powerful comparative approach to identify ZIKV-specific alterations in the developing neocortex and reveals specific preferential infection of neural stem cells by ZIKV.



## 1. Introduction

First isolated in 1947 from the blood of a Rhesus monkey in the Zika forest, Uganda (Dick et al., 1952), Zika virus (ZIKV) was recently declared a global public health emergency by WHO (Heymann et al., 2016). After decades of confinement in Africa and Asia, the first large outbreak caused by the virus was recorded in French Polynesia in 2013 (Cao-Lormeau et al., 2014), leading to an unusual increase in the number of Guillain-Barré cases (Paixão et al., 2016). The current South-American epidemic which started in 2015 in Brazil revealed a strong correlation between infection with ZIKV and congenital brain malformations, including microcephaly (Oliveira Melo et al., 2016).

Microcephaly is characterized by smaller head circumference, intellectual disability and seizures, and is due to reduced neuronal production or increased cell death (Barkovich et al., 2012). Recent data strongly supports the link between ZIKV and microcephaly, including detection of the virus in the amniotic fluid, placenta and brain of microcephalic fetuses, as well as in the blood of microcephalic newborns (Calvet et al., 2016; Mlakar et al., 2016; Martines et al., 2016). A retrospective study recently revealed a similar association between the French Polynesian 2013 outbreak and increased rates of microcephaly, supporting the implication of ZIKV (Cauchemez et al., 2016).

ZIKV belongs to the *Flavivirus* genus and is closely related to yellow fever virus (YFV), dengue virus (DENV), West Nile virus (WNV) and Japanese encephalitis virus (JEV). Flaviviruses are arthropod-borne, single-stranded positive-sense RNA viruses, that cause infections in humans with a spectrum of clinical syndromes ranging from mild fever to hemorrhagic and encephalitic manifestations. Several infectious agents, belonging to the so-called TORCH complex, are responsible for congenital infections leading to brain developmental disorders, including microcephaly (Neu et al., 2015). However, neurotropic flaviviruses such as WNV and JEV, responsible for post-natal encephalitis, are rarely linked to congenital brain malformations, such as microcephaly (O'Leary et al., 2006; Chaturvedi et al., 1980). Thus, neurovirulence of ZIKV in human fetuses must rely on mechanisms that are different from those involved in WNV or JEV neural infection, for example by infecting a particular set of fetal cells.

The cerebral cortex, a layered structure involved in higher cognitive functions, is strongly affected in microcephalic patients (Barkovich et al., 2012). During its normal development, all cortical neurons and most glial cells are generated, directly or indirectly, by the radial glial

* Corresponding author.
*E-mail address:* alexandre.baffet@curie.fr (A.D. Baffet).
[1] The third and fourth authors equally contributed to this study.
[2] Shared last authorship.

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progenitor (RGP) cells (Kriegstein and Alvarez-Buylla, 2009). These cells are highly polarized and elongated, spanning the entire thickness of the developing neocortex. The apical process of RGP cells is in contact with the ventricular surface and the cerebro-spinal fluid (CSF), while their basal process is in contact with the pial surface and serves as a track for neuronal migration (Taverna et al., 2014). Genetic alterations leading to microcephaly are well known to affect RGP cell division, fate or survival (Fernández et al., 2016).

*In vitro* studies using induced Pluripotent Stem Cells (iPSCs)-derived brain cells, neurospheres and brain organoids have shown ZIKV infection of human neural stem and progenitor cells (Tang et al., 2016; Garcez et al., 2016; Qian et al., 2016; Dang et al., 2016). Recently, two different mouse models for ZIKV infection were developed and revealed a range of development defects including placental damage, developmental delay, ocular defects and embryonic death (Cugola et al., 2016; Miner et al., 2016). Another study described RGP cell infection and reduced cortical thickness after ZIKV injection into the lateral ventricle of embryonic brains (Li et al., 2016). It remains unclear if RGP cell infection was merely due to their location at the site of virus injection or if ZIKV has a specific tropism for these cells in the developing neocortex. This question is particularly important as it is still unknown if the virus reaches the developing brain *via* the cerebrospinal fluid (CSF), where ZIKV was detected (Rozé et al., 2016), or *via* blood vessels after crossing the placenta, as recently suggested (Miner et al., 2016). Another outstanding question is whether all flaviviruses share similar characteristics of infection in the developing brain, or if ZIKV, and especially microcephaly-associated ZIKV, exhibits a specific behavior in this tissue. In view of the rare congenital abnormalities associated with other flaviviruses, including following intrauterine WNV infection (O'Leary et al., 2006), a comparative analysis between flaviviruses should provide a framework to identify ZIKV-specific mechanisms leading to microcephaly.

## 2. Materials & Methods

### 2.1. Flavivirus Production

#### 2.1.1. Cells

Rhesus monkey Vero NK kidney cells were maintained at 37 °C in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% FBS. *Aedes albopictus* C6/36 cells were maintained at 28 °C in L15 medium supplemented with 10% FBS.

#### 2.1.2. Virus Stock

DENV-4 strain 63632/76 (Burma) was produced in C6/36 cells. WNV strain IS98 was produced as described in (Alsaleh et al., 2016). ZIKV strain Pf13 was isolated from a viremic patient in French Polynesia in 2013 by the team of V. M. Cao-Lormeau and D. Musso at the Institut Louis Malardé in French Polynesia (Cao-Lormeau et al., 2014). To produce viral stocks, C6/36 cells were infected with ZIKV strain Pf13 at an MOI of 0.1 and supernatants were harvested 5 days post-infection at the onset of cytopathic effect.

#### 2.1.3. Titration

Vero NK cells were seeded in 24-well plates. Tenfold dilutions of virus samples were prepared in DMEM and 200 μL of DMEM supplemented with 2% FBS and 200 μL of each dilution were added to the cells. The plates were incubated for 1 h 30 min at 37 °C. Unadsorbed virus was removed, after which 400 μL of DMEM supplemented with 2% FBS and 400 μL of DMEM supplemented with 1.6% carboxymethyl cellulose (CMC), 10 mM HEPES buffer, 72 mM sodium bicarbonate, and 2% FBS was added to each well, followed by incubation at 37 °C for 72 h for ZIKV and WNV, and for 96 h for DENV-4. The CMC overlay was aspirated, and the cells were washed with PBS and fixed with 4% paraformaldehyde for 20 min, followed by permeabilization with 0.1% Triton X-100 for 5 min. After fixation, the cells were washed with PBS and incubated for 1 h at room temperature with anti-E antibody (4G2), followed by incubation with HRP-conjugated anti-mouse IgG antibody. The assays were developed with the Vector VIP peroxidase substrate kit (Vector Laboratories; catalog no. SK- 4600) according to the manufacturer's instructions. The viral titers were expressed in focus-forming units (FFU) per milliliter.

### 2.2. Mouse Brain Slices Culture and Infection

CD1 IGS mice were purchased from Charles River. The care and use of these mice were strictly controlled according to european and national regulations for the protection of vertebrate animals used for experimental and other scientific purposes. Coronal slices from E15 embryos were sectioned at 300 μm on a Vibratome (Leica Microsystems) in artificial cerebrospinal fluid (ACSF) solution and then cultured in modified cortical culture medium (M-CCM) containing 71% basal MEM, 25% Hanks balanced salt solution, 2% FBS, 1% N-2 supplement (Thermo Scientific), 1× penicillin/streptomycin/glutamine (GIBCO BRL), and 0.66% glucose. Slices remained incubated in 5% $CO_2$ at 37 °C. Mice embryonic brain slices were infected with $6.10^5$ FFU in 200 μL medium. After 2 h at 37 °C, the inoculum was replaced with 200 μL of fresh M-CCM. The slices were then incubated at 37 °C and fixed with 400 μL of 4% paraformaldehyde for 2 h at 4 °C.

### 2.3. Immuno-staining and Imaging

Brain slices were washed with PBS and stained in PBS, 0.3% Triton X-100 supplemented with donkey serum. Primary antibodies were incubated overnight at 4 °C and secondary antibodies were incubated for 2 h at room temperature. Brain slices were mounted with a small piece of double-sided tape on either side of the slide to act as a small scaffold for the coverslip. Imaging was performed using a spinning disk microscope equipped with a Yokogawa CSU-W1 scanner unit. Antibodies used in this study were: mouse anti-E glycoprotein (4G2) (RD-Biotech); rabbit anti-NeuN (Abcam); rabbit anti-Pax6 (Biolegend); rabbit anti-cleaved caspase 3 (Cell signaling) and goat anti-phospho-Histone 3 (Santa Cruz).

## 3. Results

To better understand how ZIKV infection may lead to microcephaly, we developed an assay to infect cultured mouse embryonic brain slices and tested the ability of ZIKV and other flaviviruses to infect the neocortex (Fig. 1a). We selected an isolate of DENV serotype 4 (DENV-4), responsible for a lethal fulminant hepatitis (Kudelko et al., 2012) and the encephalitic West Nile virus (WNV) Israel 1998 strain (IS98) (Lucas et al., 2004). Importantly, ZIKV infection was performed using the microcephaly-associated French Polynesian 2013 strain (Pf13) which is over 97% identical at the nucleotide level to the Brazilian strain but phylogenetically more distant from the African lineage (Zhu et al., 2016) (Fig. 1b). E15 mouse embryonic brains were sliced and cultured in modified cortical culture medium (M-CCM) (Baffet et al., 2016) and brain slices were infected for 2 h with either virus produced on C6/36 mosquito cells ($6.10^5$ FFU). Viral input was then removed and brain slices cultivated for 24 to 48 h at 37 °C (Fig. 1a). Viral titration revealed productive infection of ZIKV and WNV after 24 and 48 h, but not of DENV-4, indicating that the mouse developing neocortex is able to support replication of these two viruses (Fig. 1c). Immuno-staining experiments confirmed that ZIKV and WNV efficiently infect E15 brain slices whereas no cells infected by DENV-4 were detected (Fig. 1d). Interestingly ZIKV and WNV showed very different distributions. While WNV was distributed throughout the developing neocortex with enrichment in the intermediate zone (IZ) and cortical plate (CP), ZIKV was strongly concentrated in the ventricular zone (VZ) (Fig. 1d). Moreover, ZIKV-infected cells exhibited a characteristic RGP cell morphology, with an apical and a basal process running all the way up to the pial surface (Fig. 1e,