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Short communication

Infectability of Human BrainSphere Neurons Suggests Neurotropism of SARS-CoV-2

C. Korin Bullen[1,#], Helena Therese Hogberg[2,#], Asli Bahadirli-Talbott[1], William R. Bishai[1], Thomas Hartung[2,3,4], Casey Keuthan[5], Monika M. Looney[1], Andrew Pekosz[4], July Carolina Romero[2], Fenna C. M. Sillé[2,6], Peter Um[1] and Lena Smirnova[2,#]

[1]Johns Hopkins University, School of Medicine, Department of Medicine, Division of Infectious Diseases, Baltimore, MD, USA; [2]Johns Hopkins University, Bloomberg School of Public Health, Center for Alternatives to Animal Testing (CAAT), Baltimore, MD, USA; [3]CAAT-Europe, University of Konstanz, Konstanz, Germany; [4]Johns Hopkins University, Bloomberg School of Public Health, Department of Molecular Microbiology and Immunology, Baltimore, MD, USA; [5]Johns Hopkins University, School of Medicine, Department of Ophthalmology, Wilmer Eye Institute, Baltimore, MD, USA; [6]Johns Hopkins University, Bloomberg School of Public Health, Department of Environmental Health & Engineering, Baltimore, MD, USA

Abstract

Reports from Wuhan suggest that 36% of COVID-19 patients show neurological symptoms, and cases of viral encephalitis have been reported, suggesting that the virus is neurotropic under unknown circumstances. This is well established for other coronaviruses. In order to understand why some patients develop such symptoms and others do not, we address herein the infectability of the central nervous system (CNS). Reports that the ACE2 receptor - critical for virus entry into lung cells - is found in different neurons supports this expectation. We employ a human induced pluripotent stem cell (iPSC)-derived BrainSphere model, which we used earlier for Zika, Dengue, HIV and John Cunningham virus infection studies. We detected the expression of the ACE2 receptor but not TMPRSS2 in the model. Incubating the BrainSpheres for 6 hours with SARS-CoV-2 at multiplicity of infection (MOI) of 0.1 led to the infection of a fraction of neural cells with replication of the virus evident 72 hours later. Virus particles were found in the neuronal cell body extending into apparent neurite structures. PCR measurements corroborated the replication of the virus, suggesting at least a tenfold increase in virus copies per total RNA. Leveraging state-of-the-art 3D organotypic cell culture, which has been shown to allow both virus infection and (developmental) neurotoxicity but is at the same time simple enough to be transferred and used in a BSL-3 environment, we demonstrate, for the first time, the potential critically important neurotropism of SARS-CoV-2.

1 Introduction

The question whether severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infects brain cells and induces neuropathology or derails neural physiology is of critical importance for the understanding of this disease and its potential long-term sequelae. Several coronavirus species are neurotropic (Nath, 2020), but this has not been clearly established for SARS-CoV-2. Initial reports from Wuhan indicate that 36% of COVID-19 patients have neurological symptoms (Mao et al., 2020), in the meantime confirmed by a European collective (Helms, et al., 2020) and a case of acute hemorrhagic necrotizing encephalopathy, a rare encephalopathy that has been associated with other viral infections, has been reported in a COVID-19 patient (Poyiadji et al., 2020). One reason why some but not all patients show neurological manifestations could be that the blood-brain-barrier (BBB) normally hinders virus entry but is impaired in some by inflammatory conditions. The possible infection of brain endothelial cells was just reported (Paniz-Mondolfi et al., 2020).

[#] authors contributed equally

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Correspondence: Lena Smirnova, PhD
Johns Hopkins Bloomberg School of Public Health
Department of Environmental Health & Engineering
Center for Alternatives to Animal Testing (CAAT)
615 N Wolfe St, W7032
Baltimore, MD, 21205, USA
(lena.smirnova@jhu.edu)

The availability of experimental models to study SARS-CoV-2 is critical not only for drug development (Busquet et al., 2020) but also to understand environmental cofactors for public health measures. Viral infections are the prototypic species-specific diseases. There is no good small laboratory animal disease model for COVID-19. SARS-CoV-2 seems to be most closely related to several coronaviruses in Asian bats and reptiles, but these do not lend themselves easily as lab animals, and as far as we know they also do not display the human pathology (Zhou et al., 2020; Zhang et al., 2020). Recent reports on the infectability of macaques are of limited use with respect to throughput and species differences.

We reported in 2016 the first mass-produced standardized 3D human induced pluripotent stem cell (iPSC)-derived organotypic brain model (human mini-brains or BrainSpheres (Pamies et al., 2017; funded by NIH NCATS)), which consists of different types of neurons, astrocytes and oligodendrocytes. The promise of 3D organotypic models or microphysiological systems (MPS) for biomedical research has most recently been confirmed by our stakeholder workshop (Marx et al., 2020). While the battery of *in vitro* (developmental) neurotoxicological tests is broad, the BrainSphere model lends itself to these studies as it (i) is human-derived, a key prerequisite for studying human-specific viruses, (ii) has high cell density, a key characteristic for cell-to-cell infection of occasional neurotropic viruses, and (iii) allows central nervous system (CNS) functional assays. Our earlier studies with Zika and Dengue viruses in BrainSpheres demonstrated their applicability for studying neurotropic viruses (Abreu et al., 2018). In yet unpublished work, we infected these BrainSpheres with human immunodeficiency virus (HIV) and John Cunningham (JC) virus, where the latter could for the first time be cultured in a cell model (manuscript in preparation). Notably, for infectious disease research in BSL-3 facilities there are limits as to the complexity of set-up that can be utilized. Spheroids, pre-manufactured in regular cell culture laboratories, represent a compromise with respect to simplicity versus tissue architecture and functionality.

Here, the critical question of whether human brain cells can be infected with SARS-CoV-2 was addressed.

2 Materials and methods

BrainSphere differentiation

We previously developed a human 3D BrainSphere model derived from induced pluripotent stem cells (iPSC) with funding from NCATS, NIH (U18TR000547) (Pamies et al., 2017). Briefly, the differentiation protocol covers the stages from neural precursors to differentiated neurons (dopaminergic, glutamatergic, and GABAergic neurons etc.) and glial cells (astrocytes and myelinating oligodendrocytes); BrainSpheres show electrophysiological activity. The BrainSpheres have been proven to be relevant to study key cellular processes involved in neurodevelopment and function including proliferation, differentiation, apoptosis, synaptogenesis, intracellular signaling, myelination, and neuronal network function (Pamies et al., 2017, 2018; Zhong et al., 2020).

BrainSpheres from the NIBSC-8 cell line (UK National Institute for Biological Standards and Control (NIBSC), kindly provided by Drs. Orla O'Shea and Elsa Abranches, UK stem cell bank) were differentiated following our in-house two-step protocol (Pamies et al., 2017). The NIBSC-8 iPSC cell line is mycoplasma-free and with normal female karyotype, authenticated by short tandem repeat with 100% match to the donor material -MRC-9 cells (ATCC, CCL-212). Briefly, iPSCs were differentiated in a monolayer to neuroprogenitor cells (NPC) using serum-free, chemically defined neural induction medium (Gibco). NPC were expanded and a single cell suspension was distributed into uncoated 6-well plates and cultured under constant gyratory shaking (80 rpm, 19 mm orbit) to form BrainSpheres. After 48 h, differentiation was induced with serum-free, chemically defined BrainSphere differentiation medium (Neurobasal electro medium supplemented with B27-electro, glutamax, 10 ng/mL GDNF and 10 ng/mL BDNF). BrainSpheres were differentiated for eight weeks prior to infection. By this time, BrainSpheres consist of different types of neurons, astrocytes and oligodendrocytes (Pamies et al., 2017).

Human gene quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Expression of ACE2 and TMPRSS2 in BrainSpheres was assessed using TaqMan gene expression assay on an Applied Biosystem 7500 Fast Real-Time PCR machine. Briefly, total RNA was isolated with Zymo RNA isolation kit, 500 ng RNA was reverse-transcribed with M-MLV-Reverse transcriptase (Promega) and amplified with ACE2 (Hs01085333_m1) and TMPRSS2 (Hs01122322_m1) TaqMan primers (ThermoFisher Scientific) following default Fast TaqMan cycling conditions. PCR experiments for human receptor expression were done in three independent experiments.

SARS-CoV-2 infection of BrainSpheres

SARS-CoV-2/Wuhan-1/2020 virus was provided to Dr Andrew Pekosz by the US Centers for Disease Control and Prevention (CDC). VeroE6 cells were used to grow the virus and determine the infectious virus titers as described earlier (Schaecher et al. 2007a,b). BrainSpheres were distributed into a 24-well plate at 15 spheres per well. BrainSpheres were transferred to the BSL3 facility and infected with SARS-CoV-2/Wuhan-1/2020 (MOI 0.1). At 6 hours post infection (hpi), BrainSpheres were washed by two-step wash and given fresh medium or lysed for RNA extraction. Supernatant samples were taken from the medium immediately after it was added to the BrainSpheres (6 hpi). BrainSpheres were incubated further until 72 hpi. At 72 hpi, supernatant samples were collected for quantification of virus RNA, and BrainSpheres were lysed for RNA extraction or fixed for immunohistochemistry. All infection experiments were conducted in two independent experiments with triplicates with appropriate MOCK controls included.

Viral RNA RT-qPCR

RNA from BrainSphere lysates was extracted using Zymo Quick RNA microPrep kit (Zymo Research) according to the manufacturer's protocol. On average 600 ng of total RNA was extracted per sample. A QIAamp Viral RNA mini kit (Qiagen) was used to isolate RNA from supernatants according to the manufacturer's protocol. cDNA was synthesized from the viral RNA using qScript cDNA SuperMix (Quantabio) following the manufacturer's protocol. The cDNA was quantified by RT-qPCR on a StepOnePlus Real Time PCR system (Applied Biosystems) with TaqMan Fast Advanced Master Mix (Applied Biosystems). SARS-CoV-2 RNA was detected using premixed forward (5'-TTACAAACATTGGCCGCAAA-3') and reverse (5'-GCGCGACATTCCGAAGAA-3') primers and probe (5'-FAM-ACAATTTGCCCCCAGCGCTTCAG-BHQ1-3') designed by the CDC as part of the 2019-nCoV CDC Research Use Only (RUO) kit (Integrated DNA Technologies, Catalog #10006713) to amplify a region of the SARS-CoV-2 nucleocapsid (N) gene. PCR conditions were as follows: 50°C for 2 min, 95°C for 2 min, followed by 45 cycles of 95°C for 3 s and 55°C for 30 s. Serially diluted (10-fold) plasmid containing the complete SARS-CoV-2 N gene (Integrated DNA Technologies, Catalog #10006625) was measured to generate a standard curve for quantification of viral RNA copies. The limit of detection for the assay was 1 x 10^1 RNA copies. For cell lysates, viral copies were normalized to the human RNase P (RP) gene using premixed forward (5'-AGATTTGGACCTGCGAGCG-3') and reverse (5'-GAGCGGCTGTCTCCACAAGT-3') primers and probe (5'-FAM-TTCTGACCTGAAGGCTCTGCGCG-BHQ-1-3') included in the same 2019-nCoV CDC RUO kit.

Immunohistochemistry

At 72 h post infection (hpi), BrainSpheres were fixed with 2% paraformaldehyde and stained as described in (Smirnova et al., 2015) with anti-MAP2(a+b) antibody (Sigma), SARS-CoV-1 M-peptide rabbit antisera (McBride and Machamer, 2007) and mouse anti human-SARS-CoV/SARS-CoV-2 Spike antibody (Sino Biological). Nuclei were stained with Hoechst 33342 (blue).

3 Results

3.1 Expression of ACE2 but not TMPRSS2 in NPCs and mature BrainSpheres

It was shown that SARS-CoV-2 utilizes angiotensin-converting enzyme 2 (ACE2*)* receptors to enter the cells and transmembrane serine protease 2 (TMPRSS2) for S protein priming (Hoffmann et al., 2020). Using quantitative RT-qPCR, we found the *ACE2* gene expressed in relatively low copy numbers in two different iPSC lines [NIBSC-8 (Fig. 1) and CRL-2097 (data not shown)]. Notably, the receptor was already expressed in NPC. During differentiation, expression rates first dropped but then rebounded. Expression of the *TMPRSS2* gene was below the detection limit in the BrainSpheres (data not shown).

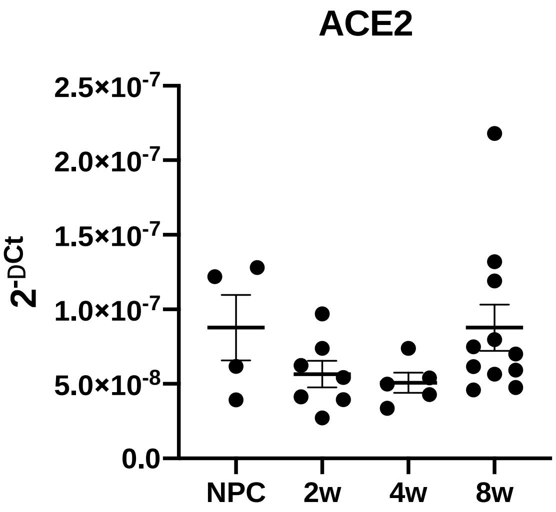

Fig. 1: mRNA expression of angiotensin converting enzyme 2 receptor (ACE2) over time in human NIBSC-8 iPSC differentiated into neuroprogenitor cells (NPC) and BrainSpheres (for 2, 4 and 8 weeks)
Data shown combine three independent experiments with at least two biological replicates per condition; the 4 weeks timepoint was done in two independent experiments.

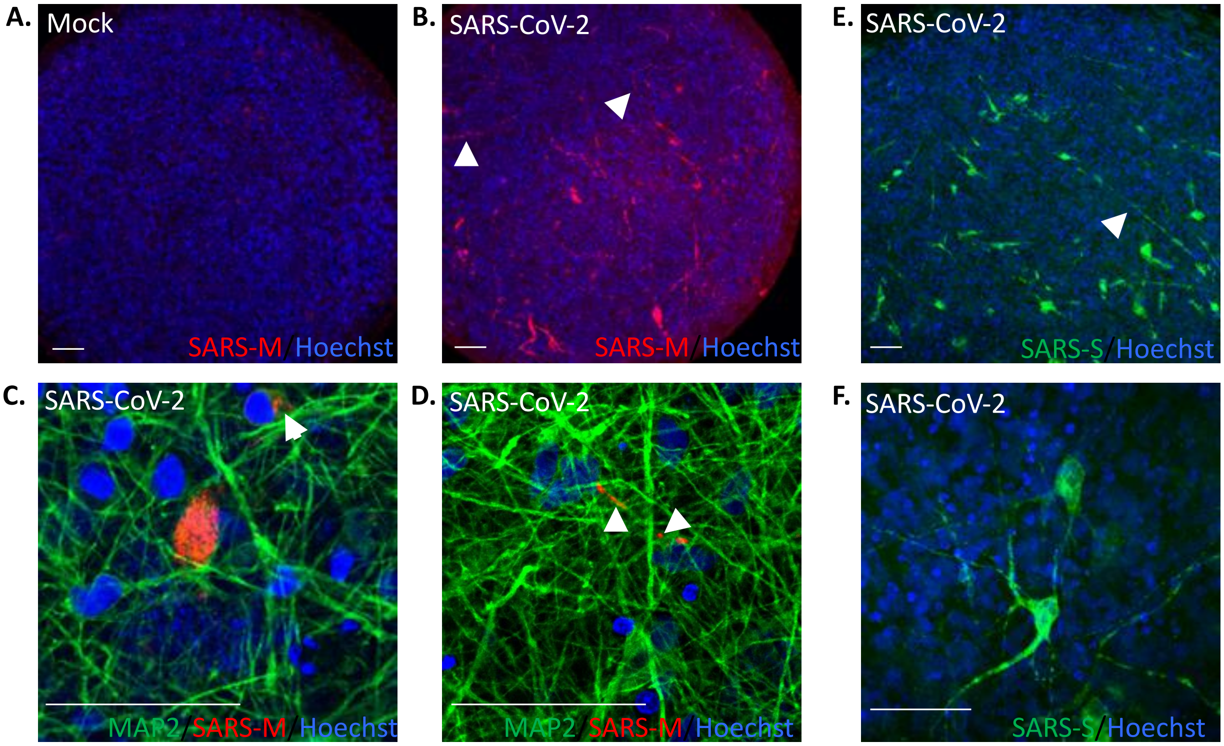

Fig. 2: A small fraction of neuronal cells in BrainSpheres contains virus particles at 72 hpi
BrainSpheres infected with SARS-CoV-2 at MOI of 0.1 were analyzed at 72 hpi for M protein (red) expression by immunofluorescence (A) - (D). Neuronal marker MAP2 (green) was used to stain the neurons. Arrowheads indicate colocalization and presence of the virus in neurites. (E) and (F) BrainSpheres stained for SARS-CoV-2 spike protein (green). Images representative of five BrainSpheres. Nuclei are stained with Hoechst 33342 (blue). Scale bar 50 µm.

3.2 Immunohistochemical identification of SARS-CoV-2-infected neurons

In order to analyze their infectability, BrainSpheres were fixed at 72 hpi and stained with fluorescent antibodies against neuronal marker MAP2 and SARS-Cov-2 protein M and spike protein (S). A small fraction of neural cells containing virus particles was detected by confocal microscopy (Fig. 2). Co-staining with neuronal marker MAP2 demonstrated the presence of the SARS-CoV-2 M protein in neuronal soma (Fig. 2C). In certain cells, the particles extended from the cell body into the neurites (Fig. 2 B, D, E arrow heads). Similarly, neural cells were positive for SARS-CoV-2 spike protein (Figure 2E and F). The large number of virus particles in the neurons is suggestive of active virus replication within the infected cell. In addition, some degree of cell bursting was observed in various cells (supplemental video 1[1]), presenting as non-cell-shaped clouds of virus, suggesting that the virus may initiate cell lysis.

3.3 Increasing levels of viral RNA in SARS-CoV-2 infected BrainSpheres indicate viral genome replication

In order to quantify cell-associated viral RNA, BrainSpheres were lysed at 6 and 72 hpi; supernatants were also collected at 6 and 72 hpi. We detected a baseline of cell-associated SARS-CoV-2 genome copies in BrainSphere lysates at 6 hpi, which markedly increased by two log orders at 72 hpi (Fig. 3A). After extensive washing to remove the viral inoculum at 6 hpi, an unexpectedly high number of virus copies remained in the supernatant, which was greatly reduced at 72 hpi (Fig. 3B), possibly due to virus degradation in the supernatant over time and/or possible further penetration of virus from the supernatant into the spheres. This decrease of viral copies in the supernatant (35-fold), was significantly lower than the 107-fold increase of viral copies in the BrainSphere lysate. This suggests that the increase in intracellular viral copies is due to virus replication within the cells rather than continued penetration of the residual virus from the supernatant over time. The detected viral copies in the supernatant after washing the BrainSpheres might be background from the remaining residual viral inoculum, similar to results reported in previous MERS-CoV and SARS-CoV studies (Wang et al., 2020; Chu et al., 2016).

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[1] https://share.getcloudapp.com/NQugZeb9

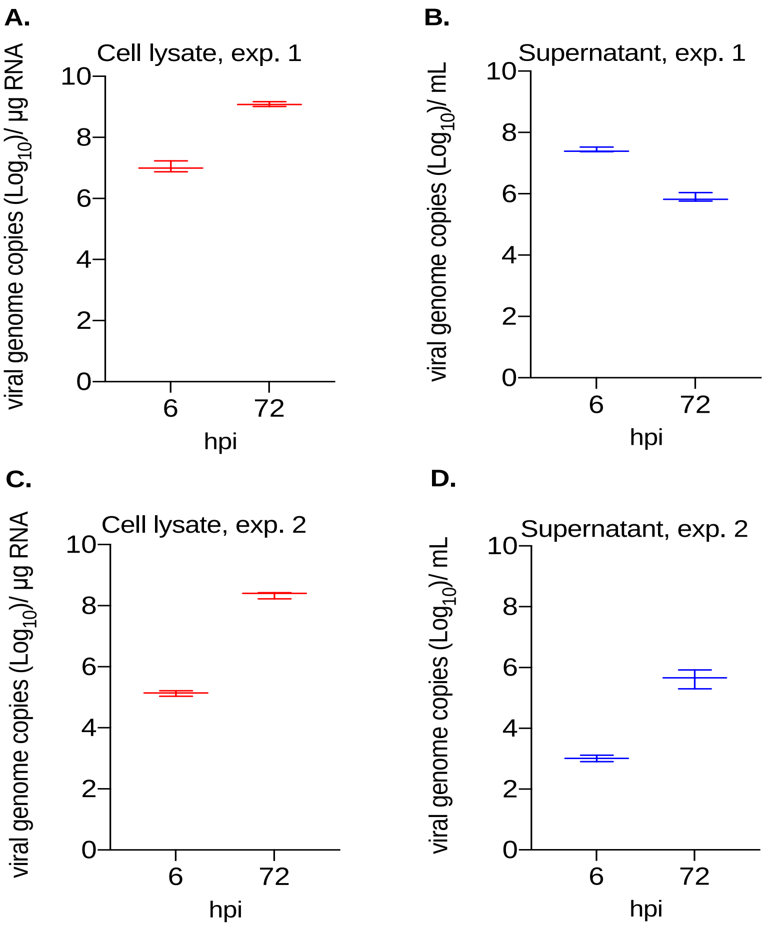

Fig. 3: SARS-CoV-2 replicates in infected human BrainSpheres
(A) and (B) – experiment 1. (C) and (D) – experiment 2. Cell lysate associated SARS-CoV-2 genome levels presented as viral genome copies per µg total RNA (A and C) and SARS-CoV-2 genome copies per mL (600 µl per well) in the culture supernatant (B and D) from 15 BrainSpheres (representing about 600 ng RNA) infected with SARS-CoV-2 at MOI of 0.1 at 6 hpi and 72 hpi. Data are shown as box and whiskers plots displaying the data distribution through their quartiles at each time point from two experiment done in triplicate technical replicates (n=3).

In order to overcome the issue of high virus background in the supernatant, we made use of the fact that BrainSpheres are cultured in suspension, which allows more rigorous washing steps. In the repeat experiment, 6 hours hpi, BrainSpheres were transferred into Eppendorf tubes, rigorously washed and plated into a new plate for 72 hour-post infection incubation. RNA was collected in triplicates from the supernatant and cell lysate after washing (6 hpi) and 72 hpi from SARS-CoV-2 infected cells and corresponding MOCK control. In this experiment, the rigorous washing steps strongly decreased the number of viral particles in the supernatant 6 hpi (Figure 3D), but still resulted in a similar increase in virus copy numbers to about 10^8 virus equivalents in cell lysates (Figure 3C). This confirms that the intracellular increase in both experiments was due to virus replication and not continued penetration of the residual virus from the supernatant over time. Surprisingly, elimination of the high background at 6 hpi in the second experiment, led even to an increase in viral copy numbers in the medium (Figure 3D), suggesting virus shedding, which might be hidden in the first experiment due to high level of residual viral inoculum. This further confirmed the viral replication in the BrainSpheres and suggests a productive infection.

4 Discussion

The question whether SARS-CoV-2 infects brain cells and induces pathology or derails neural physiology is of critical importance for the understanding of this disease and its potential long-term sequelae. For this reason, we decided to report with this short communication our initial findings, at a moment when tens of thousands of patients are hospitalized. This might increase attention to neurological symptoms and add to the considerations in developing treatments.

The relatively frequent neurological symptoms in COVID-19 patients (Mao et al., 2020, Helms et al., 2020) prompted us to investigate whether SARS-CoV-2 can directly infect BrainSpheres. Notably, one of the key symptoms of COVID-19 appears to be a loss of the sense of smell, i.e. an olfactory dysfunction (Bagheri et al., 2020; Giacomelli et al., 2020), which was thought also to indicate infection of the cranial nerve; however, most recently, it was suggested to be due to infection of non-neuronal cells in the olfactory system (Brann et al., 2020). More reports support the presence of virus in CNS, e.g. the presence of viral particles in CSF biofluid (Zhou et al., 2020), which suggests rather a direct infection and not a secondary immune response.

Neural damage biomarkers were also found in the plasma of patients with COVID-19 (Kanberg, et al., 2020). The infectability of neural cells is not unexpected (Baig et al., 2020), as expression of the *ACE2* receptor gene, which was found to be critical for virus entry (Hoffmann et al., 2020), was detected in the BrainSpheres and has previously been shown by others in certain neurons (Xu et al., 2011, Chen et al., 2020). SARS-CoV-2 has been reported to use its spike (S) proteins to facilitate viral entry through the ACE2 receptor and to use the serine protease TMPRSS2 for S protein priming (Hoffmann et al., 2020). We found no detectable expression of *TMPRSS2* in the BrainSpheres, suggesting alternative processing.

The observation of bursting cells, irregular, non-cell-shaped clouds of virus (see supplemental video[1]) and the increase of viral particles in the supernatant over time, indicate that infected cells shed virus particles, but it is unclear whether the infection is sustained by infecting other cells in our model. Future experiments with longer incubation times, longer hpi and/or higher MOI will be needed to answer these questions. It will be key to identify whether the infected cells represent a specific subpopulation of neurons and/or glia cells —for example, those expressing ACE2, as the rather low level of gene expression would suggest that only a subpopulation of neural cells expresses ACE2. Due to the low MOI used here, it could, however, also just be a probabilistic event where a few brain cells begin to be infected. The large number of virus particles in these cells in both experiments at 72 hpi suggests replication as corroborated by the PCR findings in the lysed BrainSpheres. Ongoing experiments aim to identify the neural cell type(s) that are infected.

These experiments require repetition, including varied initial number of virus (MOI), analysis of neural pathology and functionality, inclusion of different donors of iPSC of different genders, and extended time courses, etc. Virus replication, as evidenced by RT-qPCR and the accumulation of large numbers of virus particles in individual cells in this experiment can only occur when the virus infects living cells. Therefore, at least the fundamental possibility of CNS infection has been established in the experiments reported here. The aforementioned experiments will provide more insight into the mechanistic processes underlying CNS infection. It is important to note that no major damage to the BrainSpheres was noted. However, this might change when incubation periods are prolonged and higher virus titers are used or when functional assays address more subtle changes.

The majority of patients might, however, be well protected by a functioning BBB and this should be included in future research. A shortcoming of the BrainSphere model is the absence of immune cells, which in the brain are the microglia, which do not derive from neural precursor cells but from the mesoderm germ layer and invade the developing brain from the blood. Our studies with Dengue, Zika (Abreu et al., 2018), HIV and JC virus (manuscript in preparation) already investigated the effects of adding microglia to BrainSpheres, which resulted in cytokine release and neuronal damage. It will be important to follow up on this initial SARS-CoV-2 research with immunocompetent BrainSpheres.

The BrainSpheres represent a model of the developing brain and previously has been used to demonstrate the developmental neurotoxicity of pesticides (Pamies et al., 2018) and drugs (Zhong et al., 2020). It will be of critical importance to show whether neurodevelopment is affected by SARS-CoV-2 infection. Importantly, transplacental transmission of SARS-CoV-2 has been reported in a neonate born to a mother infected in the last trimester (Vivanti et al., 2020), which caused placental inflammation and neurological manifestations in the neonate consistent with those observed in infected adults. As the BBB is not functional in the most critical early months of brain development (Serlin et al., 2015), there is limited protection against exogenous infections. Clinical evidence cannot yet be expected, however, as most children at critical phases of embryo and fetal development are yet to be born and more subtle neurodevelopmental disorders often take time to manifest and diagnose after birth. It will be important to study whether brain infection occurs also in otherwise asymptomatic patients, especially children with possible long-term consequences.

This study provides evidence that SARS-CoV-2 can infect neural cells, likely contributing to neurological outcomes and possibly to neurodevelopmental disorders. This means we have to face yet another potentially devastating pathomechanism of COVID-19. The observed infectability and replication warrants further research to help treat this disease and protect especially vulnerable populations, such as those exposed to neurotoxicants, pregnant women, and possibly those with pre-existing neurological conditions. In the future, it is of fundamental importance to understand whether SARS-CoV-2 impacts not only the mature but also the developing brain. This model uniquely lends itself to addressing these questions. Overall, this study stresses the versatility of microphysiological systems (MPS) (Marx et al., 2016, 2020) as enabling technologies beyond the replacement of animal tests.

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Conflict of interest

TH and HH are named inventors on a patent by Johns Hopkins University on the production of mini-brains (also called BrainSpheres), which is licensed to AxoSim, New Orleans, LA, USA. TH, LS and HH consult AxoSim, and TH is a shareholder.

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