"In the midst of every crisis, lies great opportunity."
Albert Einstein (1879-1955)

"Any sufficiently advanced technology is indistinguishable from magic."
known as Clarke's third law
Arthur C. Clarke (1917-2008), British science fiction writer

Food for Thought ...

COVID-19 – Prime Time for Microphysiological Systems, as Illustrated for the Brain

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Abstract

The development of therapies for and preventions against infectious diseases depends on the availability of disease models. Bioengineering of human organoids and organs-on-chips is one extremely promising avenue of research. These miniature, laboratory-grown organ systems have been broadly used during the ongoing, unprecedented coronavirus 2019 (COVID-19) pandemic to show the many effects of the etiologic agent, severe acute respiratory coronavirus 2 (SARS-CoV-2) on human organs. In contrast, exposure of most animals either did not result in infection or caused mild clinical signs – not the severe course of the infection suffered by many humans. This article illuminates the opportunities of microphysiological systems (MPS) to study COVID-19 *in vitro*, with a focus on brain cell infection and its translational relevance to COVID-19 effects on the human brain. Neurovirulence of SARS-CoV-2 has been reproduced in different types of human brain organoids by 10 groups, consistently showing infection of a small portion of brain cells accompanied by limited viral replication. This mirrors increasingly recognized neurological manifestations in COVID-19 patients (evidence of virus infection and brain-specific antibody formation in brain tissue and cerebrospinal fluid). The pathogenesis of neurological signs, their long-term consequences, and possible interventions remain unclear, but future MPS technologies offer prospects to address these open questions.

1 Introduction

The large family *Coronaviridae* includes viruses that are neuro-invasive, neurotropic, and neurovirulent in numerous animals. Seven coronavirids are pathogenic to humans (Arbour et al., 2000; Lau et al., 2004; Perlman and Netland, 2009; Giraudon and Bernard, 2010; Desforges et al., 2020; Montalvan et al., 2020; Lima et al., 2020). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a betacoronavirus. Since the end of 2019, coronavirus disease 2019 (COVID-19), caused by SARS-CoV-2, has impacted the world. This pandemic, raging for almost two years already, has infected more

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than 200 million individuals, killing more than 4.5 million[1] by October 2021.

Symptoms and signs of COVID-19 include fever, cough, fatigue, loss of taste or smell, shortness of breath, muscle aches, chills, sore throat, runny nose, headache, and chest pain[2]. Only 25% of patients (95% confidence interval: 16-38%) stay asymptomatic throughout the course of infection (Alene et al., 2021), whereas the disease leads to involvement of the lung and various other organs and death in initially up to 14% of cases and now 0.5-2.5%[3]. Neurological effects, now sometimes termed NeuroCOVID, have been reported in up to 80% of patients hospitalized with COVID-19 and are associated with poor prognosis and increased lethality (Chou et al., 2021). Early central nervous system (CNS) signs, and specifically encephalopathy, are associated with risk of severe COVID-19 and are possible markers for an unfavorable prognosis (Marra et al., 2021).

Before the pandemic, in 2016, neurological disorders were the leading cause of disability-adjusted life years (276 million) and the second leading cause of death (9 million) worldwide (GBD 2016 Neurology Collaborators, 2019). The recent contribution of NeuroCOVID and its possible long-term consequences is not clear yet.

Here, we discuss strategies to use complex human cell-based microphysiological systems (MPS) to understand NeuroCOVID, including the collection of data and the identification of treatment strategies.

2 Animal models of COVID-19

The response of the medical community to COVID-19 depends very much on modeling COVID-19, whether traditionally in animal models or using new approach methods (NAMs) (Busquet et al., 2020). When the pandemic began, no "good" animal model of COVID-19 could be established, and such a model is still lacking today. This lack of animal models apparently did not impede the remarkably fast development of COVID-19 vaccines and therapeutics. Indeed, the absence of animal models compelled clinicians to accelerate the preparation and the undertaking of human clinical trials, apparently with enormous success rates well above the average 6% market entry probability for vaccines after entering clinical trials (Pronker et al., 2013). It is important to emphasize that the COVID-19 pandemic enabled acceleration of traditional medical countermeasure development because (a) numbers of patients were extremely high, enabling large clinical trials, (b) mortality was relatively low with placebo groups, hence not prohibiting clinical trials on ethical grounds alone, and (c) some medical countermeasure platform technologies were already available and could be built upon immediately. Therefore, COVID-19-related medical countermeasure research and development is a good example of speed and efficiency under certain circumstances, but it is not necessarily proof that animal experiments are not needed in general for development of medical countermeasures against other diseases (Hartung and Zurlo, 2012).

Yet, animal modeling is associated with numerous challenges. To evaluate the efficacy of a medical countermeasure in an animal model, a suitable animal needs to be found that can be infected with the agent of interest and that upon infection develops a disease that is phenotypically and pathogenetically highly similar to the human disease. Candidate countermeasures should then result in protection from disease and/or death and be associated with mechanistic biomarkers that increase confidence that these results can be translated into the clinic. None of these milestones are trivial, and they continue to challenge the medical community for many important diseases. Nonhuman animals may be immune to infection with a human virus due to genetic restriction factors or other forms of immunity; may replicate the virus in the absence of clinical progression due to replication control of the immune system; may develop a disease vastly different from the human disease because of dissimilar virus receptor distribution or other physiological differences; may have drastically accelerated or decelerated disease courses, thereby modifying the windows of opportunity for countermeasure evaluation; and may metabolize countermeasures differently or faster or slower than humans, thereby complicating the calculation of target doses for human treatment.

In the case of the current COVID-19 pandemic, these issues are exemplified by the attempts to establish small rodent models. Laboratory mice overexpressing human angiotensin-converting enzyme 2 (ACE2), the cellular receptor used by SARS-CoV-2, were utilized to increase susceptibility to SARS-CoV-2 infection (Dinnon et al., 2020; Muñoz-Fontela et al., 2020). Although these animals indeed became susceptible, the observed disease course was not highly reminiscent of human COVID-19.[4] Part of the reason for the disappointing outcome may be that it remains unknown which cells or tissues should express human ACE2, and which ones should not, and how to achieve the appropriate ACE2 distribution to mimic the human distribution and functionality. As summarized by Muñoz-Fontela et al. (2020), golden hamsters and domestic ferrets can be efficiently infected with SARS-CoV-2, but the resulting courses of disease do not mimic the human involvement of organs other than those of the respiratory system (Muñoz-Fontela et al., 2020, and Table 1 therein).

Even if a suitable animal model is identified, it needs to be taken into consideration that viral infection outcomes may vary drastically, even within animals of the same species. In humans, for instance, during the infamous Cutter Incident of 1955, 120,000 doses of poliomyelitis vaccine were mistakenly produced with live, rather than inactivated, poliovirus (Nathanson and Langmuir, 1963). Approximately 40,000 children developed

[1] https://coronavirus.jhu.edu

[2] https://www.mayoclinic.org/diseases-conditions/coronavirus/symptoms-causes/syc-20479963

[3] https://ourworldindata.org/mortality-risk-covid

[4] https://www.news-medical.net/news/20210118/Can-transgenic-mice-studies-illuminate-neurological-complications-associated-with-SARS-CoV-2-in-humans.aspx

disease without involvement of the CNS, 56 developed paralytic poliomyelitis, and five died from severe disease. Such diversity in outcomes in a heterogenous population can rarely be modelled in animals due to the much lower number of animals that are used in a given study.

All these shortcomings demonstrate the need for new approaches to study emerging infectious diseases and to evaluate candidate medical countermeasures (Busquet et al., 2020). The U.S. Food and Drug Administration (FDA) demonstrated its commitment to delve into the development of complementary approaches or even alternatives to animal models when it announced in December 2020 the Innovative Science and Technology Approaches for New Drugs (ISTAND) Pilot Program[5] aimed to fast-track the qualification and use of novel drug development tools (DDTs). This announcement explicitly includes the "*[u]se of tissue chips (i.e., microphysiological systems) to assess safety or efficacy questions, development of novel nonclinical pharmacology/toxicology assays [and] …[u]se of artificial intelligence (AI)-based algorithms to evaluate patients, develop novel endpoints, or inform study design*".

3 Societal pressures

Through the combination of stem cell technology and bioengineering, the last decade has provided a broad variety of human organ models. These *in vitro* platforms model organ-level functions using complex human cell-based systems within microenvironments that mimic biochemical and mechanical influences within the body (Marx et al., 2016, 2020; Park et al., 2019; Roth et al., 2021). They lend themselves to research on human pathogens, such as SARS-CoV-2 (Clevers, 2020; Mallapaty, 2020; DeKosky et al., 2021; Deguchi et al., 2021).

In vitro research is largely uncontroversial in the public eye, relatively inexpensive, and amenable to iterative rounds of trial and error using statistically robust study designs. Hence, there is intrinsic opportunity with *in vitro* systems to conduct wide-ranging exploratory research (Hartung, 2013). In contrast, a percentage of the population is adamantly against *in vivo* (i.e., higher animal such as vertebrates) experimentation[6,7,8], both because of ethical but also financial considerations (Meigs et al., 2018). It is the law in many countries that an animal experiment should not be performed if an alternative method is reasonably available (Hartung, 2010), and some countries outright prohibit experimentation on certain animals, such as apes, with increasing pressure to expand such prohibitions to animals of lower species.

Given the societal pressure to reduce animal experimentation (Graham and Prescott, 2015), metrics are needed to demonstrate that fewer animals are being used over time and, ideally, are being replaced with alternative and, ideally, superior approaches. A 2019 U.S. Government Accountability Office report found that federal agencies actively promote the use of alternative methods in a variety of ways, including the incorporation of new methods into guidance, modification of regulations and policies, training on the use of alternative methods, and development of strategic plans to minimize the use of animals, but they have not routinely developed metrics to quantify the impact of these measures on ongoing animal use. In response, a recent whitepaper (February 2021) from the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Metric Workgroup recommended that each U.S. federal agency develop metrics to measure progress toward implementation of alternative methods in toxicity testing[9]. However, such recommendations have not yet been issued for medical countermeasure development in the infectious disease research field.

4 MPS to the front!

The species-specificity of infections has prompted the use of human organoids and organ-on-chip models for COVID-19 research (Busquet et al., 2020). The U.S. National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and collaborators lead the MPS for COVID Research (MPSCoRe) working group[10] to coordinate the use of MPS with the aim to reduce animal use in studies on COVID-19 and future emerging infectious diseases (Kleinstreuer and Holmes, 2021). The working group is operating in partnership with the U.K. National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), the National Institute of Allergy and Infectious Diseases (NIAID) Division of Microbiology and Infectious Diseases (DMID), the U.S. Army Development Command (DEVCOM) Chemical Biological Center, and the National Center for Advancing Translational Sciences (NCATS) with the following objectives:

- provision of a neutral forum to facilitate interaction and engagement between international collaborative research efforts;
- increased awareness of COVID-19-related MPS technologies and support of their application in assessing the safety and efficacy of potential novel therapeutics through building connections between technology developers and end users;
- collaboration with global authorities to understand how MPS model-derived results can be considered in a regulatory context;
- provision of cross-discipline and cross-sector expertise in discussing and characterizing model performance and readiness criteria;

[5] https://www.fda.gov/drugs/drug-development-tool-ddt-qualification-programs/innovative-science-and-technology-approaches-new-drugs-istand-pilot-program

[6] https://www.navs.org/gallup-poll-fewer-americans-support-medical-testing-on-animals/#.X1_VCS05RdA

[7] https://www.navs.org/pew-survey-shows-most-americans-now-oppose-animal-experimentation/#.X1_VWS05RdA

[8] https://www.crueltyfreeinternational.org/what-we-do/latest-news-and-updates/new-poll-reveals-us-united-against-cosmetics-animal-tests

[9] https://ntp.niehs.nih.gov/iccvam/docs/about_docs/iccvam-measuringprogress-feb2021-fd-508.pdf

[10] https://ntp.niehs.nih.gov/go/mps

Tab. 1: Original research on SARS-CoV-2 neurotropism using human brain organoids

Date of publication	Article	Main findings (novel findings bold)
Accepted and published online 26 June 2020	Bullen et al., 2020	– **ACE2 receptor in all stages of brain organoid development** – **Infection of a small percentage of brain cells** – **500-fold replication within 72 h and virus shedding**
Accepted: 24 July 2020; published online 4 August 2020	Zhang et al., 2020	– ACE2, **TMPRSS2, cathepsin L, and furin were readily detected** in human neural progenitor cells; virus replication and cell death – Brain organoid infection colocalized **with neuronal marker TUJ1 and NPC marker NESTIN**; replication and shedding
Accepted 31 August 2021; published online 23 September 2021	Ramani et al., 2020	– Virus targets neurons – **Altered distribution of tau, hyperphosphorylation** and neuronal cell death
Accepted 7 September 2020; published online 8 September 2020	Yi et al., 2020	– Spike-containing SARS-CoV-2 pseudovirus transduced neural layers within brain organoids (10% of neurons) – ACE2 expression was sustained during the development of brain organoids
Accepted 16 September; published online 21 September, 2020	Jacob et al., 2020	– Neurons and astrocytes were sparsely infected, but **choroid plexus epithelial cells** underwent robust infection
Accepted 7 October 2020; published 3 December 2020	Pellegrini et al., 2020	– ACE2 expression in mature choroid plexus cells – Tropism of virus for choroid plexus epithelial cells but little to no infection of neurons or glia
Accepted December 10, 2020; published 12 January 2021	Song et al., 2021a	– Infection with accompanying **metabolic changes** in infected and neighboring neurons – **No type I interferon response** – **Blocked with ACE2-antibodies** or cerebrospinal fluid from a COVID-19 patient
Accepted 15 June 2021; published online 20 June 2021	Pedrosa et al., 2021	– Non-permissive infection of brainspheres reflecting cortical brain-like tissue – SARS-CoV-2 infection of neural cells triggers an **increased pro-inflammatory cytokine response**
Accepted 23 December 2020; published online 29 December 2020	Wang, C. et al., 2021	– Low-grade infection of neurons and astrocytes that is boosted in neuron-astrocyte co-cultures and organoids – **Increased infection of isogenic ApoE3/3 and ApoE4/4 hiPSCs** – **Remdesivir treatment** inhibits infection
Published online 12 Feb 2021; published 9 Mar 2021	Tiwari et al., 2021	– Astrocytes, and neurons express low levels of ACE2 and TMPRSS2 and correspondingly are not highly permissive to infection
Accepted 26 January 2021; published 11 May 2021	McMahon et al., 2021	– Glial cells and cells of the choroid plexus were preferentially targeted in cortical organoids – ACE2 expression in infected cells – No viral replication and cell death involving DNA fragmentation
Accepted: 15 June 2021; published online 9 July 2021	Wang, L. et al., 2021	– pericyte-like cells (PLCs) integrated into a cortical organoid enhance infection – **virus spreading to astrocytes and mediating inflammatory type I interferon responses**

– support in the assessment of MPS-derived data against *in vivo* preclinical and clinical data; and
– ensure recognition of the 3Rs opportunities provided by MPS platforms.

As the applications of MPS in this critical time have been manifold, replicating SARS-CoV-2 interactions with a wide range of human organs and related immune responses, it is not possible to review all MPS use in COVID-19 research here; we refer the reader to recent literature (Tang et al., 2020; de Melo et al., 2021). Here, we focus on one example of promising COVID-19-related MPS research prompted by early use of brain organoids (Pamies et al., 2017) for virus infections (Abreu et al., 2018) that showed for the first time that SARS-CoV-2 can infect human brain cells (Bullen et al., 2020).

5 Brain organoids pioneered knowledge on SARS-CoV-2 brain infection

We suggested already in April 2020 that MPS might give us the answer, whether SARS-CoV-2 can infect brain cells[11]. In June 2020, Bullen et al. (2020) published the first peer-reviewed report on human brain cell infection by SARS-CoV-2. It is interesting to examine how well this ground-breaking work[12] was validated by other investigations and clinical findings. The original articles listed in Table 1 reproduced and expanded the original work.

Notably, this list does not include self-archived (preprint) studies, e.g.[13], which often do not pass peer review within a year after upload (the exclusion of these studies shall not belittle their contributions, as there can be many reasons why full publication was not pursued).

All these original research papers have in common that they find a relatively low-grade infection of brain cells. The identity of the infectable cell is debated, with evidence for astrocytes and choroid plexus cells, and controversial findings for neurons. However, the central role of the ACE2 receptor for SARS-CoV-2 infection was confirmed several times. These studies also triggered several reviews (Sanclemente-Alaman et al., 2020; Mao and Jin, 2020; Ng et al., 2021; Caporale and Testa, 2021; Willner et al., 2021; Bodnar et al., 2021; Harschnitz and Studer, 2021; Ramani et al., 2021; Chen et al., 2021).

Notably, despite human clinical evidence of neurological impact caused by SARS-CoV-2 infection, no animal studies suggesting brain infection were published prior to these organoid studies. In April 2020, Natoli et al. (2020) compared brain affection in other coronavirid animal models and noted the lack of studies on any type of pathway targeting the CNS or peripheral structures. For rhesus monkeys and SARS-CoV-2, they stated, "*no clinical disease reproducible equivalent in severity to human disease*" and, for murine models, "*brain involvement – not described*". Similarly, Dickinson (2020) suggested such studies in October 2020, referring to other coronavirus models, but also cautioned that "*[d]ifferences in specific coronavirus and host factors are reflected in major variations in incidence and mechanisms of CNS coronaviral infection and pathology between species*."

Notably, Song et al. (2021b) included in their organoid work evidence for CNS infection in laboratory mice transfected with human ACE2. Imai et al. (2020) reported low levels of SARS-CoV-2 in golden hamster brain tissue; however, the samples included the olfactory bulb, which is known to be infectable in humans, contributing to loss of smell. Shan et al. (2020) described that, in rhesus monkeys clinical signs were mild and no viral RNA was detectable by means of real-time reverse transcription polymerase chain reaction (RT-qPCR) in the blood during the course of infection (14 days) that might allow hematogenous spread to the brain. Monchatre-Leroy et al. (2021) detected viral RNA in the brain (olfactory bulb and/or medulla oblongata) of both hamsters and ferrets with overall mild clinical disease.

In summary, to the best of our knowledge no unambiguous CNS infection has been shown in any animal model – except in human ACE2-transgenic mice, in which CNS infection appears to be the leading cause of death (Yinda et al., 2021). This suggests that animal models reflect human CNS involvement in COVID-19 much less than organoid work.

6 Clinical findings on CNS involvement in COVID-19

Data are inconsistent concerning the extent of general neurological signs during COVID-19. Pezzini and Padovani (2020) reported such signs in barely 5% of patients, whereas Chou et al. (2021) found among 3,055 COVID-19 patients that 53% presented with signs and 80% reported symptoms. Other reports indicated that 10% (n = 902) of patients had encephalopathies (Ellul et al., 2020), 25% had CNS manifestations by systematic review including six studies with 765 patients (Asadi-Pooya and Simani, 2020), 18% had neurological symptoms and complications (n = 765) (Yachou et al., 2020), up to 73% had neurological symptoms (n = 580) (Maury et al., 2021), and 36% had neurological symptoms (n = 2533) (Li et al., 2020). Signs and symptoms possibly due to effects on the CNS include confusion, loss of consciousness, seizures, headaches, trouble focusing, and behavioral changes, in addition to the more common symptom of loss of taste and smell (Stevens, 2020). This heterogeneity is even more pronounced when the different effects on the nervous system in SARS-CoV-2-infected patients are distinguished (Tab. 2).

It is crucial to study, understand, and counter the neurological effects of COVID-19 (Lou et al., 2021). A closer look at the studies summarized in Table 2 clarifies that data were collected based on different patient parameters, e.g., inclusion criteria, stage of disease, and different definitions and subcategories of neurological manifestations. Because health care systems were frequently overwhelmed during the pandemic, likely not all neurological manifestations were assessed and documented with the desired scrutiny, possibly leading to underestimation of prevalence in some studies.

These discrepancies are most likely also a result of lack of circumvention of invasive imaging and evaluation techniques (e.g., functional magnetic resonance imaging [fMRI] or electromyography [EMG]) required for adequate diagnosis of CNS involvement during a study's duration to improve the definition of COVID-19-related/-specific neurological signs.

Other studies have found neurological conditions stemming from either the immediate presence of SARS-CoV-2 or an immune response to it; stroke, anosmia, paralysis, cranial nerve deficits, encephalopathy, seizures, meningitis, and delirium were all reported in confirmed COVID-19 patients across the globe

11 https://www.youtube.com/watch?v=viNuW6_M9aA

12 Financial Times 15 June 2020 "Hopkins Researchers Show COVID-19 Could Infect the Brain and Replicate", (behind paywall). https://www.ft.com/content/e5f20455-4422-4eea-9c51-b083040a0878

13 https://www.biorxiv.org/content/10.1101/2020.05.30.125856v1

Tab. 2: Symptoms of COVID-19 patients associated with central nervous system (CNS)

	Chou et al., 2021 (n = 3,055)	Sheraton et al., 2020 (n = 3,308)	Rogers et al., 2021 (n = 99,905)	Vitalakumar et al., 2021 (n = 190,785)	Bodnar et al., 2021 (n = div.)
Acute encephalopathy (psychosis, confusion, memory loss, trouble focusing, behavioral changes, fatigue and 'brain fog')	49%	7%		23% encephalopathy; 34% fatigue; 14% confusion	7-32% encephalopathy; 8-30% dizziness; 1-4% confusion
Loss of consciousness, coma	17%	5%			4-9%
Seizures	1%			4%	< 1%
Syncope	5%				
Headaches	37%	20%	21%	15%	7-70%
Loss of taste and smell	26%	51 / 59%	37 / 43%	27 / 26%	5-70%
Stroke	6%				1.4-5%
Paralysis, Guillain-Barré syndrome	3%			7%	
Meningitis or encephalitis	0.5%			0.6%	
Myelopathy	< 2%				
Aphasia (loss of ability to understand or express speech)	5%				
New movement abnormalities	3%			5%	
Abnormal tone, weakness	4%		41%		
Abnormal brainstem reflexes	8%				
Vomiting, nausea			7-10%		
Sensory abnormalities	2%				
Sleep disorder				15%	37-42%
Depression			23%		33%
Anxiety			16%		36%
Altered mental status			8%	17%	2-28%

Bodnar et al. (2021) report on studies with very diverse group sizes indicated as "n = div".

(Fotuhi et al., 2020). In addition to the aforementioned adverse conditions, vision impairment and ataxia were also found (Mao et al., 2020). In some patients, pathological lesions were detected, including hemorrhagic white-matter lesions and other lesions resembling vascular and demyelinated etiologies; these were confirmed via brain MRI scans and other imaging techniques (Pezzini and Padovani, 2020).

Kanberg et al. (2020) showed that patients with severe COVID-19 had higher plasma concentrations of glial fibrillary acidic protein (GFAP; $p = 0.001$) and neurofilament light (NfL; $p < 0.001$), both biomarkers of brain damage, than controls, whereas GFAP was also increased in patients with moderate disease ($p = 0.03$).

Neurological involvement does not only occur in adults: In children under 18 in England, there were 51 cases of neurological involvement among 1,334 hospitalized patients, giving an estimated prevalence of 3.8 cases per 100 pediatric patients (Ray et al., 2021). Diagnoses included status epilepticus (n = 7), encephalitis (n = 5), Guillain-Barré syndrome (n = 5), acute demyelinating syndrome (n = 3), chorea (n = 2), psychosis (n = 2), isolated encephalopathy (n = 2), and transient ischemic attack (n = 1). This lower prevalence compared to adult cohorts is in line with the overall lower severity of COVID-19 in children. Histopathological analyses detected SARS-CoV-2 by immunofluorescence in several tissues including brain from an infant and a fetus deceased from COVID-19 (Gomes et al., 2020; Marinho et al., 2021).

7 Neurological pathomechanisms

NeuroCOVID pathomechanisms have been summarized by several authors (Koralnik and Tyler, 2020; Troyer et al., 2020; Steardo et al., 2020; Stevens, 2020; Pezzini and Padovani, 2020; Za-

nin et al., 2020; Domingues et al., 2020; DosSantos et al., 2020; Valiuddin et al., 2020; Hascup and Hascup, 2020; Murta et al., 2020; Pallanti et al., 2020; Marshall, 2021; Pouga, 2021; Mahalakshmi et al., 2021).

A number of hypotheses attempt explanations of neurological pathomechanisms (Fig. 1). The severe infection hypothesis, based on many COVID-19 cases with SARS-CoV-2 nucleic acids present in spinal fluid and in brain cells, states that the virus enters the brain and causes an acute infection (Szcześniak et al., 2020). The main pathway for brain invasion is believed to be the olfactory route or the bloodstream. A hypothesis concerning chaos in the body holds that a systemic immune system overdrive situation entails an overcompensating inflammatory response to SARS-CoV-2 (known colloquially as a "cytokine storm"), which results in tissue damage in the body – often worse than what the virus could have accomplished alone, including brain dysfuntion. This appears to also include the generation of auto-antibodies against neurons found in the cerebrospinal fluid (Franke et al., 2020). The blood-clotting abnormality hypothesis pertains to the occurrence of strokes in patients caused by impairment of their blood clotting system.

Several viruses are known to activate the coagulation cascade, leading to brain ischemia (which causes strokes), and it appears that SARS-CoV-2 does this as well (Koralnik and Tyler, 2020). The coagulation is caused by an infection-induced inflammatory response. The hypoxic conditions that occur because of the ischemia also again lead to increased thrombus formation, resulting in further increased clotting activity. An increase in pro-coagulant molecules was also found in some COVID-19 patients with brain ischemia.

SARS-CoV-2 is also able to infect endothelial cells in the brain (Zhang et al., 2021) and replicates in the cerebral arterial walls, triggering inflammation (Pezzini and Mandovani, 2020). The inflammatory cells that build up in the triggered region create a cytokine storm, and they may increase blood-brain barrier (BBB) permeability, resulting in cerebral circulation dysfunction. This dysfunction can increase the risk of brain ischemia and, in tandem, the occurrence of strokes. In a similar way, the inflammatory response in the brain can cause encephalitis; the virus triggers an inflammatory response and causes the brain to become inflamed in return. These findings indicate that NeuroCOVID is a complex situation resulting from both direct and indirect effects of virus infection.

The neurological consequences most likely linked to a direct invasion of the nervous system by SARS-CoV-2 are encephalitis, anosmia, and dysgeusia and possibly blood clotting resulting in stroke. Tissue samples from deceased confirmed COVID-19 patients contained viral particles in frontal lobe neurons, which indicates that the virus was indeed physically present in the brain (see Section 9). Anosmia and dysgeusia also seem to occur as a direct result of the presence of virus: Olfactory epithelial cells express ACE2, enabling direct infection with resulting cellular damage. This observation is countered by at least one study that concluded that non-neuronal cells, rather than neurons, in the olfactory mucosa are infected by SARS-CoV-2 (Brann et al., 2020).

With accumulating patient data, research is still asking the following questions surrounding neurological effects of COVID-19: (1) Does the virus invade the brain and infect neurons, and what are its effects? (2) What are the mechanisms that SARS-CoV-2 uses to invade the brain, and does it infect brain endothelial cells and choroid epithelial cells to cause the adverse neurological effects? (3) Is the cytokine storm (resulting from systemic inflammation) the cause of post-infection neurological symptoms, persistent infection, or autoimmunity induced by the infection? As animal models of COVID-19 appear ill-suited to address these questions, insights are being increasingly gained from a combination of human clinical evidence and brain organoid systems.

Neurological symptoms are not only common during the acute infection: Patients with long COVID, a term describing the long-term consequences following the acute infection, often report neurological long-term side effects (e.g., anxiety, depression, memory lapses, inability to concentrate, sleep difficulties, dizziness, and fatigue) (Lopez-Leon et al., 2021). According to a Lancet editorial, "*[i]n the UK, for example, an estimated 945 000 people (1.5% of the population) had self-reported long COVID on July 4, 2021, according to the UK Office for National Statistics, including 34 000 children aged 2-16 years.*"[14] The post-infectious immune-mediated neurological effects of SARS-CoV-2 have suggested similarities to the autoimmune disease Guillain-Barré syndrome (GBS) and inflammatory diseases, including acute hemorrhagic necrotizing encephalitis (ANE) and acute dissemination encephalomyelitis (ADEM) (Pezzini and Padovani, 2020). GBS occurs in COVID-19 patients at much higher rates than it does in the general population; SARS-CoV-2 may cause nerve damage, which manifests in GBS, ANE, and ADEM. It seems that, after a latent period, the virus induces a secondary process that causes these neurological effects. ANE seems to also be tied to the aforementioned cytokine storm, with its tell-tale lesions in the brainstem, white matter, and cerebellum presenting in some patients (Poyiadji et al., 2020). However, more recent studies have provided conflicting evidence; one study found no link with GBS (Keddie et al., 2021). Another study found that patients infected with COVID-19 and diagnosed with GBS later typically had better prognoses than those infected and not diagnosed with GBS (Fotuhi et al., 2020). These aspects will require clinical studies or possibly long-term MPS cultures to understand their etiology.

Virus infections and the resulting inflammatory responses are known risk factors for autism spectrum disorders (ASD) (Shuid et al., 2021; Lins, 2021) and, with definitive diagnoses only two to three years after birth, time will tell whether COVID-19 infection fuels ASD or other neurodevelopmental disorders in children born to SARS-CoV-2-infected mothers. Stem cell-derived organoid models could address such possible neurodevelopmental hazards. Noteworthy, the brain organoid models were used earlier to identify chemical, pesticide and drug-associated derangements of

[14] https://www.thelancet.com/action/showPdf?pii=S0140-6736%2821%2901900-0

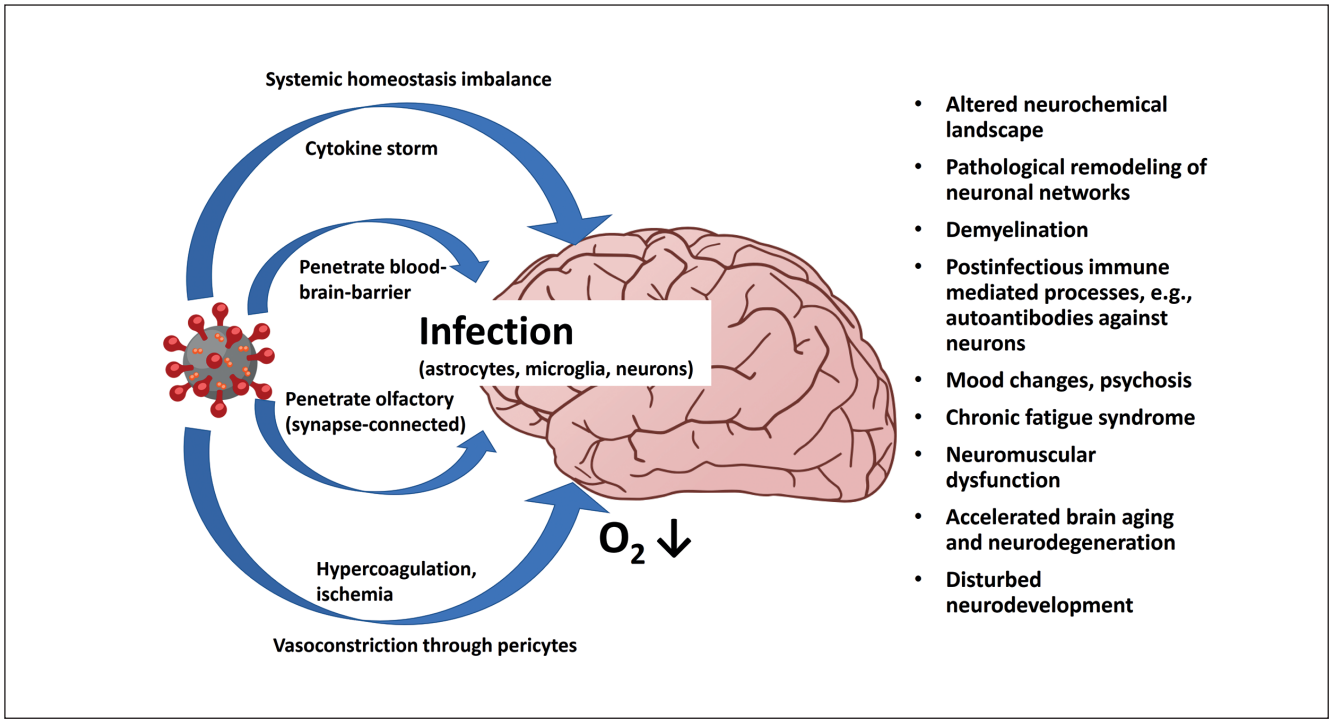

Fig. 1: Suggested key mechanisms of NeuroCOVID
Images: brain (https://freesvg.org/human-brain), SARS-CoV-2 (https://freesvg.org/sars-cov-2-coronavirus-cartoon)

neurodevelopment (Pamies et al., 2018; Zhong et al., 2020; Hogberg et al., 2021) including disruption of myelination (Chesnut et al., 2021a,b) and interindividual differences (Modafferi et al., 2021).

Similarly, virus infections are known risk factors for neurodegeneration (Abbott, 2020), raising concerns for COVID-19 (Miners et al., 2020). New research reported at the Alzheimer's Association International Conference (AAIC) 2021[15] found associations of COVID-19 and persistent cognitive deficits, including the acceleration of Alzheimer's disease pathology and presentation. These notably not peer-reviewed findings include[16]: "*Scientific leaders, including the Alzheimer's Association and representatives from nearly 40 countries – with technical guidance from the World Health Organization (WHO) – are part of an international, multidisciplinary consortium*[17] *to collect and evaluate the long-term consequences of COVID-19 on the central nervous system, as well as the differences across countries. Initial findings from this consortium presented at AAIC 2021 from Greece and Argentina suggest older adults frequently suffer persistent cognitive impairment, including persistent lack of smell, after recovery from SARS-CoV-2 infection.*" Other key results reported at AAIC 2021 include:

– "*Biological markers of brain injury, neuroinflammation and Alzheimer's correlate strongly with the presence of neurological symptoms in COVID-19 patients.*
– *Individuals experiencing cognitive decline post-COVID-19 infection were more likely to have low blood oxygen following brief physical exertion as well as poor overall physical condition.*"

The consequences of COVID-19 on neurodevelopment of the fetus or young infant as well as the possible impact on neurodegeneration later in life are challenges to which MPS might contribute.

8 Virus pathways to the brain

Viruses typically use either hematogenous dissemination or neuronal retrograde dissemination to enter the brain. Virions can travel throughout the body via the bloodstream and eventually cross the BBB and enter the brain (hematogenous), or they can infect peripheral neurons and hijack them to enter the brain (neuronal). SARS-CoV-2 appears to use both mechanisms (Pezzini and Padovani, 2020; Szcześniak et al., 2021):

Using the hematogenous route, SARS-CoV-2 can pass through the epithelial barrier after infecting a person's airways into the blood and enter the CNS by infecting endothelial cells in the

[15] https://www.alz.org/aaic/overview.asp
[16] https://www.technologynetworks.com/neuroscience/news/covid-19-linked-to-increased-levels-of-alzheimers-biomarkers-351476
[17] https://www.alz.org/research/for_researchers/partnerships/sars-cov2-global-brain-study

BBB. The virus can also infect the blood-cerebral spinal fluid (CSF) barrier of the choroid plexus, i.e., the epithelial layer in the brain that secretes and maintains CSF and prevents pathogens, immune cells, and cytokines from leaking into the CSF and the brain. The infection damages the choroid plexus and causes it to leak, leading to virion entry into the CSF and the brain (Pellegrini et al., 2020). SARS-CoV-2 could also enter the brain at circumventricular organ locations (areas without BBB in which hormones are secreted into the blood), meaning that the virus does not travel through the CSF initially (Chigr et al., 2020). Other viruses closely related to SARS-CoV-2 also infect leukocytes, which then cross the BBB to the brain; these afflicted leukocytes then produce cytokines and induce production of chemokines in astrocytes, causing inflammation to occur and yielding subsequent neurological effects (Desforges et al., 2020).

The neuronal route would entail the invasion of nerve terminals by SARS-CoV-2 and its spreading retrogradely across olfactory mucosa and nerve synapses, with the virus eventually reaching the CNS and spreading to other brain structures (Solomon and Normandin, 2020). A study found that the olfactory mucosa of deceased patients had the highest viral load compared to other neuroanatomical structures suggesting that the virus does indeed use the olfactory nerve to reach the brain (Meinhardt et al., 2021). ACE2, which plays a pivotal role in virus invasion, is found throughout the CNS – including in neurons, astrocytes, and, notably, the olfactory bulb (Baig et al., 2020). The choroid plexus expresses the highest levels of ACE2 compared to all other brain regions (Pellegrini et al., 2020). Choroid plexus cells that produce lipoprotein express ACE2 at higher levels than other choroid plexus cells. Follow-ups found that choroid plexus cells producing lipoprotein are found in abundance in the choroid plexus of mature adults and less in children, which could explain the higher prevalence and/or severity of neurological COVID-19 manifestations in adults compared to children (Pellegrini et al., 2020).

Although COVID-19 cases during pregnancy are typically mild, reports on transmission of virus through the placenta, although still controversial, have been published (Marinho et al., 2021; Komine-Aizawa et al., 2020; Taglauer et al., 2020; Fenizia et al., 2020); noteworthy, SARS-CoV-2 was detected in fetus brain (Marinho et al., 2021). Nevertheless, the question whether virus could spread to the developing brain and disrupt development remains. Neuroimmune and endocrine changes at the maternal-fetal interface may lead to neuropathological outcomes (Granja et al., 2021).

9 Evidence of SARS-CoV-2 infecting brain cells in patients

The critical evidence of infection of the CNS could come only from two principal sources, i.e., evidence of the virus in the CNS (direct virus evidence in brain tissue from autopsies) or specific antibodies formed in the CNS in response to virus in the brain (and released into the cerebrospinal fluid). This has been most comprehensively addressed by Li et al. (2021), identifying 97 relevant papers that reported on a total of 468 COVID-19 patients who underwent PCR testing for SARS-CoV-2 of CSF samples, with 30 positive cases (6.4%) from 25 papers. Li et al. (2021) also identified 28 autopsy studies that reported structural CNS abnormalities in 134 (66%) of 202 patients who died from COVID-19.

Aghagoli et al. (2021) reviewed studies analyzing human postmortem brain tissue, concluding that human coronavirus variants and SARS-CoV-2 can infect neurons and glia, implying that SARS-CoV-2 may have similar neurovirulence. Among 202 patients (Li et al., 2021), brain tissue samples from 108 patients were tested for SARS-CoV-2; SARS-CoV-2 RNA and viral proteins were detected in 33% and 25% of tested patients, respectively, and 21% showed positive results for both. Among all patients who underwent viral detection, 52% were PCR-positive, whereas viral proteins were detected in 29%. Li et al. (2021) explained this discrepancy: "*It is reported that some COVID-19 patients had a detectable level of SARS-CoV-2 in the blood. ... Therefore, viral PCR detection may give false-positive results due to the blood contained in the neural tissue. On the other hand, because neurotropic viruses usually infect some neurons only in specific brain regions, a sample homogenate containing uninfected neuronal and glial cells may have extremely low viral RNA, leading to false-negative results by PCR assays. Moreover, PCR detection cannot distinguish the types of cells which are infected in the neural tissue. Probably for these reasons, not all the results of PCR assays can be confirmed with* in situ *hybridization or immunohistochemistry*."

Najt et al. (2021) identified 27 studies on neuroimaging abnormalities (including five case series, eight cohort studies, and 14 case control studies) and noted alterations in olfactory areas, along with neighboring brain regions, including prefrontal and limbic regions, which showed SARS-CoV-2. A number of case studies showing proof of virus included two cases of CNS involvement with positive RT-PCR tests in CSF (Luis et al., 2021) as well as autopsy reports documenting the presence of SARS-CoV-2 in brain tissues (Benameur et al., 2020; Wu et al., 2020; Gasmi et al., 2021) or CSF (Moriguchi et al., 2020; Zhou et al., 2020; Finsterer and Scorza, 2021). Pouga (2021) identified four publications, which provided evidence of the presence of SARS-CoV-2 within the CNS. Matschke et al. (2020) analyzed 43 deceased patients: Activation of microglia and infiltration by cytotoxic T lymphocytes was most pronounced in the brainstem and cerebellum, and meningeal cytotoxic T lymphocyte infiltration was seen in 34 of the 43 (79%) patients. SARS-CoV-2 could be detected in the brains of 21 (53%) of 40 patients, with SARS-CoV-2 viral proteins found in cranial nerves originating from the lower brainstem and in isolated cells of the brainstem. However, although virus was present, the authors concluded that there was no evidence for CNS damage directly caused by SARS-CoV-2. Despite these studies, some authors still consider neuro-invasion to be rare (Najjar et al., 2020). Espindola et al. (2021) reported detection of SARS-CoV-2 RNA in CSF in two of 58 cases. Liu et al. (2021) concluded that "*only 1.28% COVID-19 patients who underwent cerebrospinal fluid (CSF) tests were positive for SARS-CoV-2 in CSF. However, this does not mean the absence of CNS infection in most COVID-19 patients because postmortem studies revealed that some patients with CNS infection showed negative results in CSF tests for SARS-CoV-2. Among 20 neuro-*

pathological studies reported so far, SARS-CoV-2 was detected in the brain of 58 cases in nine studies, and three studies have provided sufficient details on the CNS infection in COVID-19 patients." However, Alexopoulos et al. (2020) found high-titer anti-SARS-CoV-2 antibodies in the CSF of all eight comatose or encephalopathic patients.

In conclusion, the clinical presence of brain infection is without doubt, however, the proportion of patients affected is not clear.

10 NeuroCOVID complexity and adverse outcome pathways

Initiated by the Joint Research Center (JRC) at the European Commission, more than 60 scientists from more than 40 organizations are working on the "*Modelling the Pathogenesis of COVID-19 Using the Adverse Outcome Pathway (CIAO) framework*"[18]. This project aims to create a network of adverse outcome pathways (AOPs) to describe the complexity of COVID-19. Previously, the AOP framework has mainly been applied to understand how chemical exposure can trigger a molecular initiating event that activates different key events in a specific order, ultimately leading to an adverse outcome. The difference between hypothetical mechanisms and AOPs is that the key event relationships need to be well-established and described. In the CIAO project, the AOP approach is used to provide a mechanistic understanding of the physiopathology of a human disease (Nymark et al., 2021). The expectation is that the AOPs can enhance diagnostics and drug development. It has been well-recognized that SARS-CoV-2 can infect many different cell types and organs, but it is not clear if the early key events in each organ are the same or take place in the same order.

The CIAO working group that focuses on the nervous system is currently working on several AOPs including anosmia, encephalitis, seizure, and stroke (Wittwehr et al., 2021). Similar to our summaries above, the working group concluded that short-term anosmia is one of the best-described neurological adverse outcomes in COVID-19; key events proposed to be involved are ACE2 receptor binding in olfactory epithelium, regeneration of sustentacular cells, and association to the neuro-olfactory bulb. Furthermore, the disruption of the blood-brain barrier and inflammation are common key events, which potentially are linked to many neurological adverse outcomes in COVID-19, such as encephalitis, stroke, multiple sclerosis, epilepsy, and seizures (see above).

However, not all patients infected with SARS-CoV-2 experience the same symptoms, and it is believed that several modulating factors – such as chemical exposure, disease, diet, age, genetics, and sex – can influence the severity of the disease (Wittwehr et al., 2021). The CIAO project intends to also incorporate major modulating factors when evidence exists for their interaction with the developed AOPs. The AOP framework will not only identify key events and mechanisms but also knowledge gaps, which can provide guidance in designing the next experiments, preferably using human relevant methods.

11 Conclusions

By impacting humans in every way possible – including viral invasion, economic downturn, behavioral change, and altered lifestyle – the profound effect that COVID-19 has had on the world is undeniable. In response to the COVID-19 pandemic, we have witnessed nine medical miracles, as nine vaccines have been developed, tested, and accepted within a year. The extraordinary speed of vaccine development was possible because typical development phases were accelerated or skipped, and vaccine candidates were moved into clinical trials with minimal animal testing. Safety tests, including a small number of nonhuman primate studies, were part of this development, but the traditional larger studies with animal models could not take place within these time constraints. Based on this experience, strategies for vaccine and therapeutic development should be revisited, at least for some viruses.

In terms of the mechanisms of infection and entry into the brain, research for clarification and confirmation is needed. Future studies should focus on these post-infection effects, as these can alter the life course of individuals irreversibly. What seems concrete is the neuronal and hematogenous routes that SARS-CoV-2 takes to invade the nervous system. Future studies should also consider researching medications that can effectively target the virus passage through the blood-brain barrier or drugs which can pass it themselves to target virus in the CNS to be taken by patients with severe disease.

One note concerning the studies used to analyze ACE2 receptor activity in neurons and choroid plexus cells is the use of living patients, deceased patients, and *in vitro* models. There have been some discrepancies, so the question is what reflects best human pathophysiology and intervention options. Living patients and deceased patients diagnosed with COVID-19 represent the most accurate hosts, but each individual has a different set of health outcomes pertaining to COVID-19, no matter the commonalities (Jacob et al., 2020). There is a limitation to the number of patients able to provide data and information (Calina et al., 2020). Animal models are controversial and currently do not adequately mimic severe human disease (Pellegrini et al., 2020).

In light of these limitations, the use of organoids to simulate the effects of COVID-19 seem particularly promising. MPS have emerged as one of the most promising approaches to continue driving human-relevant research with minimal animal use. The imminent completion of Good Cell Culture Practice (GCCP) 2.0 extending to MPS will also further their quality and usefulness (Pamies et al., 2020); this promises quality guidance for *in vitro* work embracing latest technologies. MPS have started to make an impact, and groups applying these are increasingly coordinating and collaborating. "*While the field of organoid technology is still at an infant stage of development, brain organoid models*

[18] https://www.ciao-covid.net/

have provided several crucial pieces of evidence on the effects of SARS-CoV-2 on the brain." (Ng et al., 2021). Projects including MPSCoRe and the COVID-19 AOP development in the CIAO project, the upcoming 1st MPS World Summit[19] in 2022, and the creation of an international MPS society will further data collection and awareness of the part MPS can play in providing understanding of SARS-CoV-2 and its pathways.

Specifically, the use of MPS may illuminate SARS-CoV-2 entry into the brain and subsequent neurological pathomechanisms that lead to NeuroCOVID. Challenges for MPS for NeuroCOVID ahead include (see also Ng et al., 2021):

– identification of the conditions and cell types under which infection, replication and shedding of SARS-CoV-2 occurs (leading to better protocols and hypotheses for the clinic);
– improvement of brain organoids for COVID-19 studies (maturity, immune competence, blood-brain-barrier, regional differentiation, long-term culture);
– combination of brain organoids with models of, for instance, the olfactory nerve;
– comparison of relevant genetic backgrounds through iPSCs to understand interindividual differences;
– establishment of models of SARS-CoV-2 blood-brain barrier crossing;
– study of brain immune activation as consequence of infection;
– study the possible disruption of neurodevelopment;
– study the neurodegenerative effect of infection;
– study neuropsychiatric effects of infection including cross-talk of brain regions;
– probe pathways from infection to disease manifestation;
– efficacy evaluation of medical countermeasures; and
– study factors improving resolution of infection.

The contribution of MPS to the challenge of COVID-19 has just begun.

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Conflict of interest

T.H. and H.T.H. are named inventors on a patent by Johns Hopkins University on the production of BrainSpheres, which is licensed to AxoSim, New Orleans, LA, USA, and they receive royalty shares. T.H. is Consulting Vice-President of Scientific Affairs of AxoSim and H.T.H. and L.S. consult them. T.H. also consults AstraZeneca, American Type Culture Collection (ATCC), InSphero, and Apellis Pharmaceuticals on MPS.

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