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Dissecting the molecular mechanisms related to the onset of neurological complications in three cellular models of SARS-CoV-2 infection: iPSC-derived motor neurons, iPSC-derived dopaminergic neurons, iPSC-derived human cortical organoids.

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INDEX

1. SUMMARY

Although COVID-19 typically leads to respiratory disorders, recent evidence show that acute and sub-acute neurological complications are reported in patients not only with severe disease, but also in otherwise minimally symptomatic or asymptomatic people [1–3]. Notably, up to 65% of COVID-19 affected patients reported a wide range of neurological conditions, such as decreased sense of smell or hyposmia, dizziness, headache, nausea, vomiting, impaired consciousness, as well as cases of encephalopathy, but also peripheral disorders including Guillain-Barre syndrome and myositis-like muscle injury [2, 4–8], suggesting detrimental effects of SARS-CoV-2 on both the central and peripheral nervous system (CNS, PNS). Multiple studies have shown neuroinflammation in patients with COVID-19, which may underlie these neurological and neuropsychiatric symptoms [7, 9–17]. In addition, as with other viruses, the hypothesis that SARS-CoV-2 infection may accelerate neurodegeneration and the risk of neurodegenerative diseases is gaining increasing attention. [18–21]. However, the molecular mechanisms responsible for these disfunctions, as well as the potential neurotropism of SARS-CoV-2, are still under investigation. To date it remains unclear whether the neurological symptoms are a consequence of direct neural infection, para-infectious or post-infectious immune-mediated disease, or sequalae of systemic disease [1, 22]. It has been hypothesized that SARS-CoV-2 might affect the dopamine pathway [23–28] which in turn would interfere with neuronal activities.

In this frame, it has been demonstrated both *in-vivo* and *in-vitro* that SARS-CoV-2 is able to infect different neuronal cell types with different degrees of success [29–37]. Further, considering that human brain tissue is difficult to access, particularly from patients with a contagious pathogen due to safety concerns [38], 2D and 3D *in vitro* models can provide a viable and safe alternative and represent a suitable model system to test the neurotoxic effects of SARS-CoV-2 [32, 34–37, 39].

In this context, during my PhD training I've been involved in different projects in collaboration with different research groups, whose general purpose was to assess the relationship existing between SARS-CoV-2 infection and the alteration of the nervous system. Thus, I was given the chance to work with three different neuronal models, including dopaminergic neurons, iPSC derived motor neurons, and human cortical organoids, in order to investigate this extremely current and concerning condition. Each of these projects, addressed below in the text, has independently contributed to broadening knowledge about the relationship between SARS-CoV-2 and the nervous system.

From this scenario, using human iPSC differentiated to dopaminergic neurons (DA neurons) we found that infection with EU and Delta variants results in a reduced intracellular content and

extracellular release of dopamine, while tyrosine hydroxylase was upregulated at the mRNA level and downregulated at the protein level. DOPA-decarboxylase and dopamine transporter were downregulated at both mRNA and protein levels, and in addition, *in vitro* SARS-CoV-2 infection was associated with altered MAP2 and TAU expression and increased neuronal stress markers. These results suggest that SARS-CoV-2 affects dopamine metabolism and production, partially explaining the neurological symptoms.

In parallel, for the very first time, we found that SARS-CoV-2 can productively infect human iPSC-derived MNs probably by binding CD147 and NRP1 receptors. Furthermore, SARS-CoV-2 infection in iPSC-MNs significantly altered the expression of genes (IL-6, ANG, S1PR1, BCL2, BAX, Casp8, HLA-A, ERAP1, CD147, MX1) associated with cell survival and metabolism, as well as antiviral and inflammatory response. Such information will be important to unveil the biological bases of neuromuscular disorders characterizing SARS-CoV-2 infection and the so called long-COVID symptoms.

At the same time, we observed that iPSC-Human Cortical Organoids (HCO) were productively infected by SARS-CoV-2, probably by binding CD147 receptor. Furthermore, SARS-CoV-2 infection as well as Spike exposure of MNs were accompanied by the activation of apoptotic and stress pathways (caspase 3, caspase8, Bcl2) (S100B), inflammatory process (CCL2, NLRP3) and induce expression of both Interferon Stimulated Genes (IFITM1, IFITM3, STAT1, NFkB) and the antigen presentation pathway (ERAP1, ERAP2, HLA-A and TAP). Overall, the results obtained suggest that HCO organoids may be infected by SARS-CoV-2 and their homeostasis may be significantly altered even following S-exposure.

These three independent projects contributed to partially explain some of the neurological symptoms, characterizing the Long COVID symptomatology. Further analyses are ongoing to unveil the molecular mechanisms responsible for the neuronal disorders characterizing SARS-CoV-2 infection and the so called long-COVID symptoms in these neuronal models.

2. INTRODUCTION

2.1 SARS-CoV-2 emergence and COVID-19 pandemic

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) belongs to a highly diverse family of enveloped, positive-sense single-stranded RNA viruses. These viruses are able to infect animals and humans causing infections, which result in respiratory and enteric diseases with different degrees of severity.

In the last 20 years three coronaviruses that cause severe respiratory pathologies in humans have emerged. This is the case of SARS-CoV (Severe Acute Respiratory Syndrome-Coronavirus) outbreak in 2002 in Guangdong, China and MERS-CoV (Middle East Respiratory Syndrome Coronavirus) in 2012 in Middle East. The severe acute respiratory syndrome coronavirus 2 causing coronavirus disease 2019 (COVID-19) was first reported in Wuhan, China in December 2019 [40, 41]. Several local health facilities reported clusters of patients with pneumonia of unknown cause that were epidemiologically linked to a seafood and wet animal wholesale market in Wuhan, Hubei Province, China (http://wjw.wuhan.gov.cn/front/web/showDetail/2019123108989). These patients were showing symptoms highly similar to patients infected with SARS and MERS, such as cough, fever, chest pain, shortness of breath and bilateral lung infiltration. On 31 December, Wuhan Municipal Health Commission notified the public of a pneumonia outbreak of unidentified cause and informed the World Health Organization (WHO)[42, 43]. Investigations done by Chinese scientists, who isolated the virus from infected patients and subsequently sequenced the viral RNA, led to the discovery of a novel beta-CoV, which was publicly announced on January 9th. The number of cases grew each passing day and patients without exposure to Huanan Seafood Wholesale Market were identified, underlining the human-to-human transmission of this virus, which was named SARS-CoV-2 (Severe Acute Respiratory Syndrome-Coronavirus-2) on February 11th 2020.

WHO named the disease COVID-19 (Coronavirus Disease-19) and by March 2020, considering the impressive spread of this disease, it was defined as a pandemic (Fig.1) [42]. As of today, more than 6.9 million people have died as a result of the COVID-19 pandemic (https://covid19.who.int/). Considering the fact that vaccinations have been shown to be extremely effective in reducing severe disease presentation and mortality [44], the introduction of novel virus variations highlights the need for a better understanding of SARS-CoV-2 pathogenic mechanisms in order to improve prevention and treatment [45].

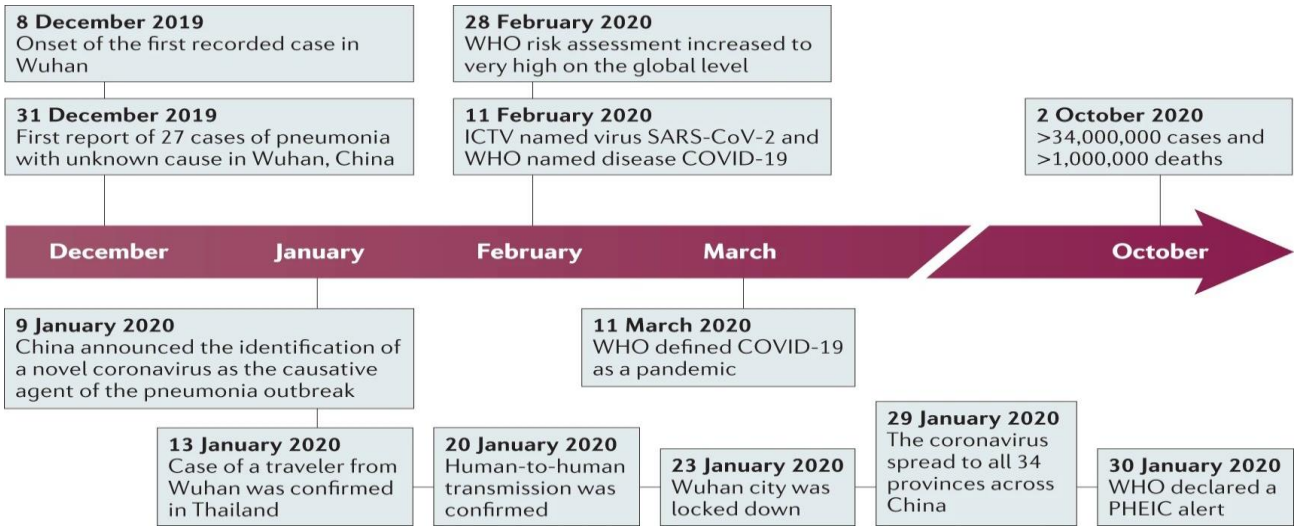


Fig.1 *Timeline of the key events of the COVID-19 outbreak* [42].

2.1.1. SARS-CoV-2 origin

SARS-CoV-2 belongs to the *Nidovirales* order, *Coronaviridae* family. This family of viruses is classified into four main genera: *alpha-coronavirus* (α-CoV), *beta-coronavirus* (β-CoV), *gamma-coronavirus* (γ-CoV), and *delta-coronavirus* (δCoV), with SARS-CoV-2 belonging to the β-CoV one. Coronaviruses are enveloped viruses with a single-stranded, positive-sense RNA genome of 29–30 kb in size and are able to infect different animal species, humans included [46, 47]. Although the specific origin of the SARS-CoV-2 is currently unknown, it is thought that zoonotic transmission is the cause of all human coronaviruses. These viruses can spread to humans either directly or by using a host as an intermediary, a process known as "spillover" [48]. The spread of zoonotic diseases between different species is aggravated by the loss of ecosystem biodiversity caused by human activities such as agricultural practices, unrestrained urbanization, hunting and wildlife trafficking. Bats are the mammals that mostly host zoonotic viruses. They are excellent reservoirs because they are able to properly adapt to these environmental changes, and are less vulnerable to these pathogenic CoVs as a result of evolution [49]. The idea that bats serve as CoVs reservoirs is supported by the fact that SARS-CoV-2 shares 96.2% and 93.3% of its sequence with the bat CoVs identified in Rhinolophus affinis (RaTG13) and Rhinolophus malayanus (RmYN02), respectively [50, 51]. Despite the high sequence identity, there are important differences which concern the receptor-binding domain (RBD) of SARS-CoV-2 that configures bats as precursors of SARS-CoV-2 rather than direct progenitors. The CoVs discovered in Malayan pangolins, however, exhibit strong similarities to the RBD of SARS-CoV-2, indicating they may represent hosts. In contrast to bats, which are not impacted by the pathogenicity of the CoVs, pangolins showed clinical signs, indicating that they are unlikely to be reservoirs, but rather

intermediate hosts [42, 52] (Fig.2). However, it is likely that other intermediate hosts exist, and several studies are ongoing to verify this possibility [53].

Fig.2 *Possible origin of SARS-CoV-2* [52].

2.1.2. SARS-CoV-2 genome and structure

Coronaviruses have long and complex genomes, a feature thought to contribute to their ability to adapt to specific hosts and facilitate cross-species transmission [54, 55]. The genome sequencing of SARS-CoV-2 began in Wuhan during the early phases of the pandemic. Using bronchoalveolar lavages from the first infected patients', scientists tried to identify the virus. By next-generation sequencing technology (NGS) Zhu *et al.* [40] reported the early genome sequence of SARS-CoV-2 of approximate 29,891 bp size [50, 56].

The genome is packed by viral nucleocapsid (N) proteins as a large ribonucleoprotein (RNP) complex and enclosed in an envelope membrane with lipids and viral S (spike), M (membrane) and E (envelope) proteins. The S protein crosses the whole virion surface, conferring a particular shape, that of a crown, hence the name Coronavirus (corona=crown).

The positive, single-stranded RNA of this virus is packed in an envelope, capped at 5' and contains the poly-A tail at 3', thus it is recognized as mRNA by the ribosomes of the host cell. Also, the poly-A tail at 3' confers the genome stability and protection from cellular exoribonuclease digestion [48]. Genomic RNA (gRNA) has a very low GC content, accounting approximately 38% of other coronaviruses, making it particularly unstable to heat [56]. Furthermore, it has a small number of CpG islands. Given that host cells promote viral RNA breakdown via the Zinc-finger Antiviral Protein (ZAP), which can detect and bind CpG islands on viral RNA, this could provide an advantage for SARS-CoV-2 survival [57].

Two-thirds of the SARS-CoV-2 genome are occupied by two large open reading frames (ORFs) ORF1a and ORF1b which are translated into 2 polyproteins, pp1a and pp1b (Fig.3). These are

processed to generate 15–16 non-structural proteins (nsp), 15 of which intervene in the viral replication and transcription complex (RTC), and immune evasion. RTC includes, among others, RNA-processing and RNA-modifying enzymes and a RNA proofreading function necessary for maintaining the integrity of the >30 kb coronavirus genome [51, 58]. The nsps display several functions in the virus's life cycle, which may overlap. Kadam *et al*. [56] divide the population into three categories: the nsps regulating host immunity, the nsps with proteolytic activity and the nsps involved in RNA synthesis, correction, and modification. In detail:

- Nsp1: blocks the antiviral protein synthesis in infected cells by blocking the synthesis of the host proteins. It binds to the 40S ribosome subunit, inhibiting mRNA entry tunnel.
- Nsp2: interacts with prohibitin 1 and 2, two human proteins important for cell proliferation and for mitochondrial function and morphology; thus, it interferes with the host cell cycle.
- Nsp3: a multifunctional, deubiquitinase protein containing several domains, is able to disrupt host translation and to suppress the host innate immune response.
- Nsp4: is involved in the assembly of the viral double-membrane vesicles.
- Nsp5: shows proteolytic activity.
- Nsp6: induces the autophagy process to foster viral replication within membranous organelles.
- Nsp7, 8, 12: nsp7-nsp8 dimerize in order to form a heterotetrameric primase complex. Together with nsp12 they play a role in viral RNA replication.
- Nsp9: is able to bind to single-stranded RNA and it is important for virus amplification.
- Nsp10, 14: nsp10 dimerizes with nsp14, activating it. The N-terminal domain of nsp14 has proofreading exoribonuclease activity, while the C-terminal is involved in the synthesis of the mRNA cap.
- Nsp11: is a short protein composed by only 13 amino acids, it is considered as a disordered protein. Its role is still unknown [59].
- Nsp12: beyond RNA replication, it is involved in mRNA capping.
- Nsp13: it has helicase activity and is involved in RNA synthesis.
- Nsp15: has nuclease activity.
- Nsp16: the most conserved nsp, it forms a complex with nsp10, playing a role in mRNA capping.

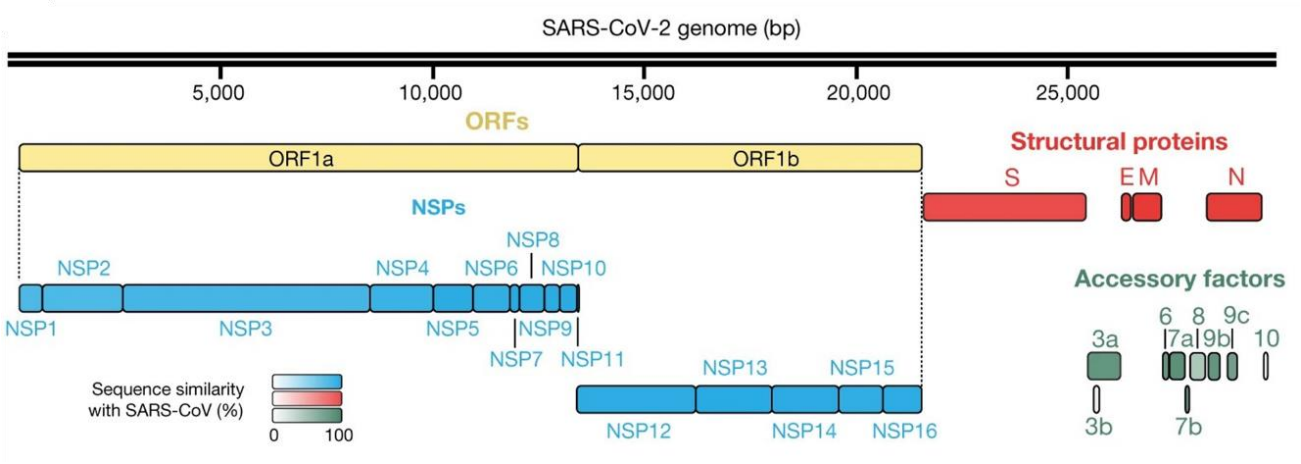


Fig.3 *SARS-CoV-2 genome* [51].

The remaining part of the genome includes ORFs that are transcribed to form a nested set of subgenomic mRNAs (sg mRNAs) encoding the accessory proteins and structural proteins.

Coronavirus accessory proteins are a highly variable set of virus-specific proteins that display limited conservation even within individual species, but they are principally thought to contribute to modulating host responses to infection and are determinants of viral pathogenicity [60]. SARS-CoV-2 genome encodes six annotated accessory proteins (ORF3a, 6, 7a, 7b, 8, and 10; reference GenBank: NC_045512.2). Also, studies aimed at evaluating the coding capacity of SARS-CoV-2 identified several unannotated accessory ORFs, including numerous alternative open reading frames within ORFs S (ORF2d), N (ORF9b, ORF9c), and ORF3a (ORF3b, ORF3c, ORF3d) [61, 62]. Nevertheless, the molecular functions of many accessory proteins remain largely unknown because of the lack of homologies to accessory proteins of other coronaviruses or to other already described proteins [63]. These proteins often contribute to host adaptation and modulation of host immune responses by acting as ion channels, IFN antagonists, suppressors of innate immunity, and altering mitochondrial metabolism, among others, as well as virulence, and represent promising targets for antiviral molecules. Studies are ongoing to better figure out the role displayed by these accessory proteins on SARS-CoV-2 infectivity and pathogenicity [56, 62, 64].

The ORFs encoding the four structural proteins (Spike, Envelope, Membrane and Nucleocapsid protein) are located in the 3′ one-third of coronavirus genomes.

The **Spike (S)** protein is considered the most important one, as it interacts with host cell receptors, mediating virus entry. In detail, S is a trimeric protein and each monomer is composed by three regions: a large ectodomain region, a transmembrane region and an intracellular region which shows a short intracellular tail. The ectodomain region contains two subdomains: S1 that recognizes and

binds the host receptor and S2 that mediates the fusion with the cellular membrane [65]. In turn, S1 contains two subunits: N-terminal domain (NTD) and C-terminal subunit (CTD). The latter contains the receptor binding domain (RBD) capable of specifically binding ACE2. S protein is highly immunogenic, though its surface is dominated by host-derived glycan moieties hindering its recognition from the immune system [66]. The S2 domain is composed by the fusion peptide (FP), heptad repeat 1 (HR1), central helix (CH), connector domain (CD), heptad repeat 2 (HR2), and transmembrane domain (TM). The S protein contains two cleavage sites, the first one is a furin (PRRAR) cleavage site located at the S1/S2 boundary, while the second one is whitin the S2′. Extracellular proteases cleave the S1/S2 cleavage site, so that the S1 can bind to the receptor through the RBD. Next, another proteolytic cleavage occurs in S2' site, mediating the fusion between the viral and host membrane, thus allowing the entry of the viral RNA inside the host cell (Fig.4) [56].

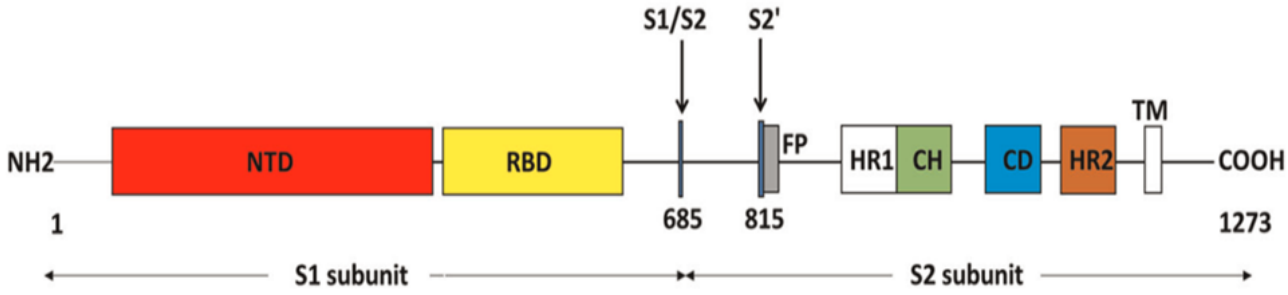


Fig.4 *Spike Protein* [56].

Envelope (E) protein is a conserved, small protein of 76–109 amino acids. It is composed of a short hydrophilic N-terminal domain, a hydrophobic transmembrane domain, and a long hydrophilic C-terminal domain. In the hydrophobic transmembrane domain, there is an amphipathic α-helix capable of oligomerization, which leads to the formation of viroporins [67]. Beside transporting ions such as Ca2+ these viroporins are involved in the assembly and final release of the virus particles from the host cells [56]. E protein is also associated with pathogenesis. By adding to the protein folding load on the endoplasmic reticulum, it leads to ER stress and unfolded-protein response (UPR) leaing to cell apoptosis [68]. Moreover, E protein is involved in the release of the mature virus by assisting the formation of ER-Golgi intermediate compartment (ERGIC). It is precisely here that the virus gets the membrane envelope and then crosses the host secretory pathway in order to be released [69].

Membrane (M) protein is an abundant protein, composed of an extracellular N-terminal domain, three transmembrane domains and an intracellular, large C-terminal domain [70]. It is the M protein which shapes to the viral envelope, and it interacts with the other structural S, E, and N proteins through the transmembrane and intracellular domain [56]. By interacting with the S protein, it holds it in the ERGIC, fostering its incorporation into new virions. By interacting with the N protein, it

stabilizes the nucleocapsid and the virions' internal core while stimulating the completion of viral assembly. Interaction of M and E proteins on the viral envelope also promotes the production and the release of the virus.

Lastly, the **Nucleocapsid (N)** protein, a conserved protein of 422 amino acids, is composed by three domains. The N-terminal domain contains positively charged amino acids involved in RNA binding while the self-association C-terminal domain contains the nuclear localization signal (NLS). There is another disordered region found between these two domains, known as Central Linker (CL), which is able to bind the M protein. In fact, this protein interacts with the E and M structural proteins to facilitate virus envelope formation and particle assembly [71]. By binding to the RNA and by self-association, it packs the gRNA into a protective capsid [43, 56]. Also, the N protein plays a role in replication and transcription of the viral RNA, as well as in the formation of the ribonucleoprotein (RNP) complex. Furthermore, it assists the multiplication and spreading of the virus, by regulating the host cell cycle [56]. According to recent studies, this protein probably regulates the host innate immune response by inhibiting interferon β (IFN-β) production [72].

2.1.3 SARS-CoV-2 Human receptors

The presence of receptors is a necessary condition for the virus to enter target cells. Viral particles bind to a number of receptors in order to enter cells or to stimulate specific host responses such as cytokine production, immune responses, and virus replication. As a result, host cell receptors have emerged as a critical focus for therapeutic and vaccine research. It has been demonstrated that the S protein is responsible for the ability of SARS-CoV-2 to interact with receptors expressed on the membrane of host cells [73, 74]. Entry depends on binding of the surface unit, S1, of the Spike protein to a cellular receptor, which facilitates viral attachment to the surface of target cells. Furthermore, Spike protein priming by cellular proteases is required for entrance, which involves Spike protein cleavage at the S1/S2 and S2' sites and facilitates fusion of viral and cellular membranes, a process driven by the S2 subunit [75]. Different receptors have been identified as interactors of the SARS-CoV-2 Spike protein, including Angiotensin-converting enzyme 2 (ACE2) [76], Neuropilin 1 (NRP1) [77, 78], and Basigin2/EMMPRIN/CD147 (CD147) [79].

ACE2 is one of the major receptor for SARS-CoV-2, which is widely expressed in the cells of the lung, intestine, liver, heart, vascular endothelium, testis, and kidney [80], and potentially contribute to the viral tropism and pathogenicity [60, 81]. This receptor is a component of the renin-angiotensin system (RAS). Renin cleaves angiotensinogen to generate angiotensin I, which is subsequently converted by ACE into angiotensin II and binds to the AT1R receptor, causing vasoconstriction and

inflammation. Angiotensin II is then transformed to angiotensins 1-7, which causes vasodilation and anti-inflammatory effects. Because SARS-CoV-2 promotes ACE2 internalization, it inhibits the conversion of angiotensin II into angiotensin 1-7, which may enhance rather than limit pro-inflammatory reactions [82].

In addition to receptor binding, protease activators for SARS-CoV-2 entry have been examined. It has been shown that the cellular transmembrane serine protease 2 (TMPRSS2) and lysosomal proteases are both important for SARS-CoV-2 entry [75, 83]. TMPRSS2 which colocalizes with ACE2 at the cell membrane [84] has been identified as the dominant proteolytic driver of Spike protein activation and SARS-CoV-2 infection of the aerodigestive tract [75, 85–87]. TMPRSS2 is a serine protease enzyme on cell membranes of the epithelial cells of the respiratory system, like alveolar type II cells, gastrointestinal systems, kidney, epididymis, prostate, pancreas, and parathyroid glands [88, 89]. Normally it takes part in pericellular proteolytic cascades for degradative remodelling of the extracellular matrix [90] and proteolytic activation of membrane proteins [91], among other key epithelial homeostasis roles [87, 92], but its role in cells is not definitively known. However, it has been demonstrated that it plays a key role in the entering mechanism of SARS-CoV-2 into the cells. As mentioned before, the primary role of TMPRSS2 in SARS-CoV-2 biology is to prime the virus for membrane fusion via S protein proteolytic cleavage. In fact, TMPRSS2 cleaves the S2 subunit, responsible for fusogenic activity at S2' site [85]. It has been hypothesized that the second cleavage causes some protein rearrangements leading to the fusion of the viral and host membranes, thus the entry of the virus and the release of the viral genome [93].

Because of that, TMPRSS2 inhibitors can be used as prophylactic measures against SARS-CoV-2 infections [91] and the administration of TMPRSS2 inhibitors with ACE2 inhibitors can decrease virus cell entry [94]. Given the above mechanism of entry, co-expression of ACE2 and TMPRSS2 and possibly other host proteases on the same cell is considered essential for SARS-CoV-2 infection. TMPRSS2 is required for the priming of the virus Spike protein, while virus entry in the cell may also depend on the endosomal/lysosomal cysteine proteases cathepsin B and L (CTSB, CTSL) although their activity is likely not essential [75, 95, 96]. More recently, it was found that Furin protease is also involved in the infection process since SARS-CoV-2 contains a furin cleavage site in the Spike protein which is unusual for coronaviruses [96]. As previously mentioned, Spike protein has two cleavage sites S1/S2 and S2'. Furin, a pro-protein convertase, cleaves at the S1/S2 site, generating S1 and S2 subunits of the Spike protein. S1 subunit contains the RBD which is able to bind the ACE2 receptor. In particular, previous studies have shown that SARS-CoV-2 has a unique

four-amino acid insertion (681-PRRA-684) in the Spike protein at nucleotide position 23619-23632 which represents a potential furin cleavage site (682-RRAR-685) [97].

In addition to ACE2, several molecules have been suggested to serve as alternative receptors for SARS-CoV-2. Indeed, SARS-CoV-2 may also exploit the putative alternative receptor CD147 (basigin), a transmembrane glycoprotein expressed ubiquitously in epithelial and immune cells [79, 98]. In particular, CD147 is abundantly expressed on the surface of various cell types throughout the body, including activated lymphocytes, red blood cells, neural tissues [99, 100], epithelial cells, myeloid cells, and skin tissues [101]. Besides being the foundation of the blood grouping system, it can interact with several extracellular and intracellular molecules [102]. Also, CD147, which is involved in matrix metalloproteinases (MMPs) production, interacts with integrins, cyclophilins, monocarboxylate transporter proteins, and caveolin-1 [103]. It has been demonstrated that this receptor regulates many cellular processes including cell proliferation, apoptosis, tumor cell migration, metastasis and differentiation. Therefore, its expression has been correlated to the onset of various diseases such as inflammatory disorders, microbial infections, and some cancers as a consequence of MMPs and cytokines release [103–105]. Even though a modest increase in viral entry was correlated to CD147 higher levels, and its upregulation was observed in obesity and diabetes [106], which are risk factors for developing severe COVID-19, the role of CD147 in SARS-CoV-2 infection is still debated [74, 107].

Other host receptors and/or co-receptors that promote of SARS-CoV-2 cellular entry have been reported. The cellular receptor neuropilin-1 (NRP1, binds furin-cleaved substrates) is a transmembrane glycoprotein with non-tyrosine kinase activity, expressed as dimeric receptors, forming a protective cortex for various molecules such as vascular endothelial growth factors (VEGF) in vertebrates[78]. NRP1 is abundantly expressed in the respiratory and olfactory epithelium [77], with highest expression in endothelial and in the epithelial cells facing the nasal cavity. NRP1 plays a vital role in different physiological processes, including angiogenesis, vascular permeability, immune cell functions, neuronal growth, cellular proliferation and axon control [108–110]. Different groups identified NRP1 as a host factor for SARS-CoV-2 [77, 78]. Indeed, it was shown to bind S1 through the multibasic furin-cleavage site, to promote S1 shedding and to expose the S2′ site to TMPRSS2 [111], thus fostering SARS-CoV-2 infectivity and providing a gateway into the central nervous system [77].

2.1.4. Mechanism of infection and SARS-CoV-2 life cycle

As previously reported, viral infection occurs by the binding of SARS-CoV-2's Spike protein to ACE2 receptors and fusion of the viral envelope with host cellular membranes. Once in the cytosol, the genomic RNA, being positive-sensed, 5' capped and 3'polyadenylated, can be translated by the host ribosomes. Firstly, as shown in figure 5, the ORF1a and ORF1b translation produces the pp1a and pp1a/b polyproteins [112]. These polyproteins undergo proteolytic cleavage by the virus-encoded papain-like protease (PLpro) from nsp3 and the main protease (Mpro) from nsp5, generating the non-structural proteins such as the RNA dependent RNA polymerase (RdRP), the RNA proofreading exonuclease and proteins capable of rearrange the intracellular membranes where the RNA synthesis takes place. These proteins are required for the Replication-Transcription Complex (RTC) formation. This complex, by using the genomic RNA as template, produces an anti-genome and minus-strand sgRNAs (subgenomic RNA). The anti-genome, in turn, is used as template to generate new genomic RNA, which can serve for further RNA synthesis, to produce non-structural proteins or to be packed in the newly formed virions. Instead, the minus-strand sgRNAs controls the synthesis of nested subgenomic mRNAs (sg-mRNAs) [113], which are produced thanks to the presence of Transcription Regulatory Sites (TRS). Throughout the anti-genome synthesis, the RTC reaches these sites, and at TRS elements called TRS body (TRS-B), it stops. The synthesis is once again initiated at the TRS adjacent to a leader sequence (TRS-L).

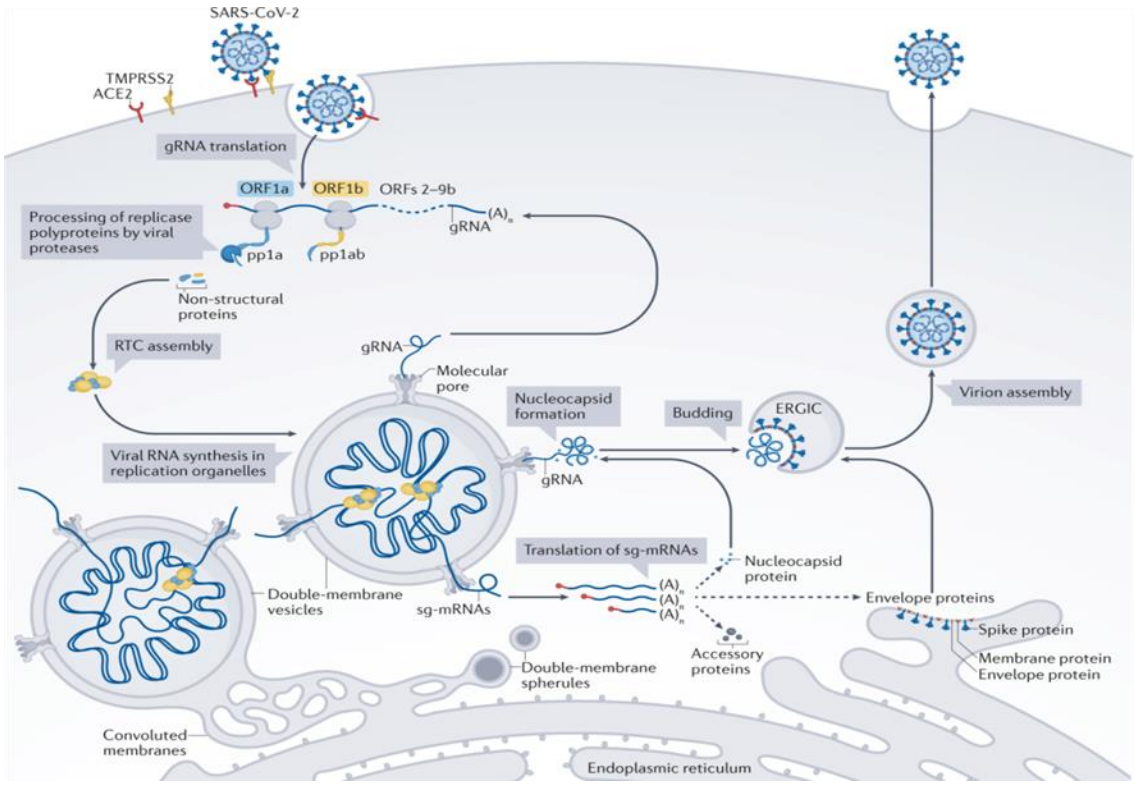


Fig.5 *SARS-CoV-2 infection cycle* [113].

This process results in the generation of a negative-strand sgRNA corresponding to each TRS, which can be employed to produce positive-sense sg-mRNA. Subsequently, these sg-mRNAs are utilized for the synthesis of structural and accessory proteins [60]. This process occurs in “replication organelles” such as the Double-Membrane Vesicles (DMV), the Convoluted Membranes (CM) and Small Open Double-Membrane Spherules (DMS), in order to shield the genome from innate immunity anti-viral mechanisms [114].

After being produced, the structural proteins enter the ER to Golgi intermediate compartment (ERGIC), where they interact with the recently synthesized genomic RNA, leading to the assembly of the viral particles. The final step of the viral cycle consists in the release of the newly produced virions the infected cells. Thise step may be achieved by exocytosis through two different strategies: via the Golgi compartment or through the deacidified lysosomes fusing with cellular membrane [112].

2.1.5 SARS-CoV-2 variants

SARS-CoV-2, like other RNA viruses, is prone to genetic evolution as it adapts to its new human hosts [115]. This is due to a number of factors, including the large size of its RNA genome (approximately 30,000 nucleotides), which is replicated by RNA-dependent RNA polymerase (RdRP) with an associated proofreading enzyme exoribonuclease (ExoN), and the discontinuous nature of transcription [116]. This results in coronaviruses having high rates of recombination, insertions, deletions, and point mutations [117]. Adaptive mutations in the viral genome can alter the virus's pathogenicity, and even a single amino acid exchange can drastically affect its ability to evade the immune system and complicate vaccine development [118]. SARS-CoV-2 has rapidly developed gene mutations, particularly nonsynonymous ones or deletions in the spike protein, as a result of its transmission and adaption in different populations across the world. This has led to the formation and dominance of several lineages in less than two years [115, 119]. Indeed, since being declared a global pandemic by the World Health Organization (WHO) on 11 March 2020, new genetic SARS-CoV-2 variants have emerged, spreading around the world [120, 121]. As mentioned before these variants were characterized by a greater number of non-synonymous mutations, primarily in the Spike protein, especially in the case of Omicron [116]. Notably, each of these variants exhibited unique characteristics in terms of transmissibility, disease severity and immunogenicity[117, 122].
Considering the ongoing concern surrounding SARS-CoV-2 variants and their hasty spreading [115, 122, 123], the WHO has taken measures to categorize these variants into two distinct groups: variants of concern (VOC) and variants of interest (VOI) [124]. VOCs represent variants with significant implications for public health. These variants exhibit increased transmissibility, detrimental changes in the epidemiology of COVID-19, heightened virulence, alterations in clinical disease presentation,

or reduced effectiveness of public health measures, diagnostics, vaccines, and therapeutics. Noteworthy VOCs include: Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), and Delta (B.1.617.2), and Omicron(B.1.1.529) which have become major variants that have affected over 90 countries since May 2021 [115]. On the other hand, VOIs are variants characterized by genetic changes that are either predicted or known to affect viral characteristics. VOIs include C.37 (Lambda) and B.1.621 (Mu), according to a WHO report from September 2021[115].

VOCs have been selected due to their significantly increased transmissibility or immune escape capabilities, requiring close monitoring. Each of these VOCs exhibited advantages in transmission over their predecessors, ultimately becoming dominant in specific regions, such as Alpha in Europe, Beta in southern Africa, and Gamma in South America, or globally, as reported for the Delta and the various Omicron sublineages [116, 125, 126]. Based on the WHO's epidemiological update as of December 11, 2021, five SARS-CoV-2 Variants of Concern (VOCs) have been identified since the onset of the pandemic:

- Alpha (B.1.1.7): The first variant of concern, which was initially detected in the United Kingdom in late December 2020.
- Beta (B.1.351): First reported in South Africa in December 2020.
- Gamma (P.1): First reported in Brazil in early January 2021.
- Delta (B.1.617.2): First reported in India in December 2020.
- Omicron (B.1.1.529): First reported in South Africa in November 2021.

All five reported VOCs, exhibit mutations in the Receptor-Binding Domain (RBD) and the N-Terminal Domain (NTD). The N501Y mutation, located in the RBD, is a common feature shared by all these variants, except for the Delta one. This mutation enhances the spike protein's affinity for ACE 2 receptors, thereby facilitating viral attachment and subsequent entry into host cells [127]. The RBD, along with the NTD, serves as the primary target for neutralization and plays a crucial role in antibody production in response to antisera or vaccines [128]. Hence, it's crucial to comprehend the biological traits of amino acid alterations, the epidemiological attributes, and vaccine responsiveness in novel SARS-CoV-2 strains for COVID-19 monitoring, mitigation, and management.

However, circulating variants including Delta have posed enormous challenges to the effectiveness of vaccines. The high infectivity and viral load of the Delta variant has contributed to the continuity of the global COVID-19 pandemic. Indeed, this variant, with a strong immune escape ability, was considered as the most contagious variant known so far [129].

2.2 COVID-19

2.2.1 COVID-19 transmission, symptomatology, and pathogenesis

COVID-19 is responsible for thousands of cases worldwide, making it the fifth pandemic since the 1918 flu. Indeed, especially at the beginning, the management of COVID-19 has been really challenging as a consequence of the high infectivity rate and rapid spreading of the infection [130]. After more than three years of SARS-CoV-2 pandemic, a number of scientific and medical successes have been achieved, including the ongoing vaccine campaign.

Despite these undeniable successes, important questions concerning its transmissibility and pathogenesis are still unanswered. Human-to-human transmission is the main route of virus spreading, mainly through direct contact, respiratory droplets, aerosols and through contaminated hard surfaces (fomites) [131–133]. Direct transmission by respiratory droplets released by human-to-human close contacts coughing has been exhaustively demonstrated [134–138]. This results into productive SARS-CoV-2 replication in both the upper respiratory tract (URT) and lower respiratory tracts (LRT). The discovery of the virus's genetic material in body fluids such as blood, urine, feces, sperm, and vaginal fluid has also raised the possibility of blood and sexual transmission, although no confirmed cases of these transmissions have been documented, so far [139].

Numerous studies have also evaluated the possibility of a vertical transmission from mother to child due to their strong expression of the main SARS-CoV-2 receptors in specific cell types of the maternal-foetal barrier [56]. Moreover, the observation that the placental expression of ACE2 and TMPRSS2 is higher during the first trimester and decreases over time suggest that such transmission could be higher in this temporal window [140, 141]. Supporting this hypothesis both SARS-CoV-2-specific IgG, and IgM have been detected in placenta, breast milk and umbilical cord blood of new-borns from infected mothers. As IgM cannot pass through the placenta barrier, it has been assumed that the virus itself, in some specific occasions, is able to enter the foetal circulation triggering an immune response. [142–144].

The most common COVID-19 symptoms tend to appear in about 2 to14 days after virus exposure and the clinical presentation ranges from asymptomatic infection to severe respiratory failure, with fever, cough, muscle pain, headache, loss of taste or smell and malaise in the majority of cases [145–147]. More severe cases of COVID-19 may also develop acute respiratory distress syndrome (ARDS) and acute lung injury, leading to morbidity and mortality caused by damage to the alveolar lumen leading to inflammation and pneumonia [148, 149].

The pathophysiology of SARS-CoV-2 infection is complex and is now known to involve activation of the immune and hematologic systems. The lung is the primary site of infection and transmission of the virus. However, COVID-19 is not limited to local pneumonia, but rather represents a multisystem illness with involvement of different organs and systemic complications [150–152]. In fact SARS-CoV-2 is able to spread and disseminate to new districts, resulting in a systemic disease that may affect the gastrointestinal system, heart, circulatory system, kidneys, liver, and brain [152].

It's unclear how the virus spreads through so many tissues and organs; it could occur through systemic blood circulation, but this dissemination is only transitory and hard to notice. It has been proposed that SARS-CoV-2 can exploit the digestive system [96], the Central and Peripheral Nervous System [153], the lymphatic drainage system [130, 154, 155], or the dissemination through nearby tissues, the so-called viral shedding, which entails the expulsion and release of the viral offspring after a successful reproduction within the host cells [156].

Notably, following SARS-CoV-2 infection the host immune system can trigger a fatal inflammatory condition known as Cytokine Release Syndrome (CRS) in COVID-19 patients [157, 158]. This phenomenon is due to an excessive inflammatory response, in which inflammatory cytokines are swiftly and massively released in response to infective stimuli. Dysregulated proinflammatory cytokine cascades caused by a strong, quick innate immune response are the hallmark of the systemic inflammatory syndrome. In fact, patients needing admission to the intensive care unit (ICU) exhibit this dysregulated inflammatory cytokine storm as a severe condition [159], characterized by an increased serum inflammatory cytokine levels including IL-1β, IL-6 [160, 161], granulocyte-colony stimulating factor (G-CSF), interferon-γ inducible protein 10 (IP-10), and tumor necrosis factor-α (TNF-α) [135, 162–167] (Fig.6). IL-6 cytokine, in particular, is predictive of COVID-19 fatality [163].
When the immune system is overactive, it triggers a massive immune cell and tissue response that causes inflammation, fibrosis, an increase in reactive oxygen species (ROS), a decrease in nitric oxide, an increase in permeability, and lung edema [152].

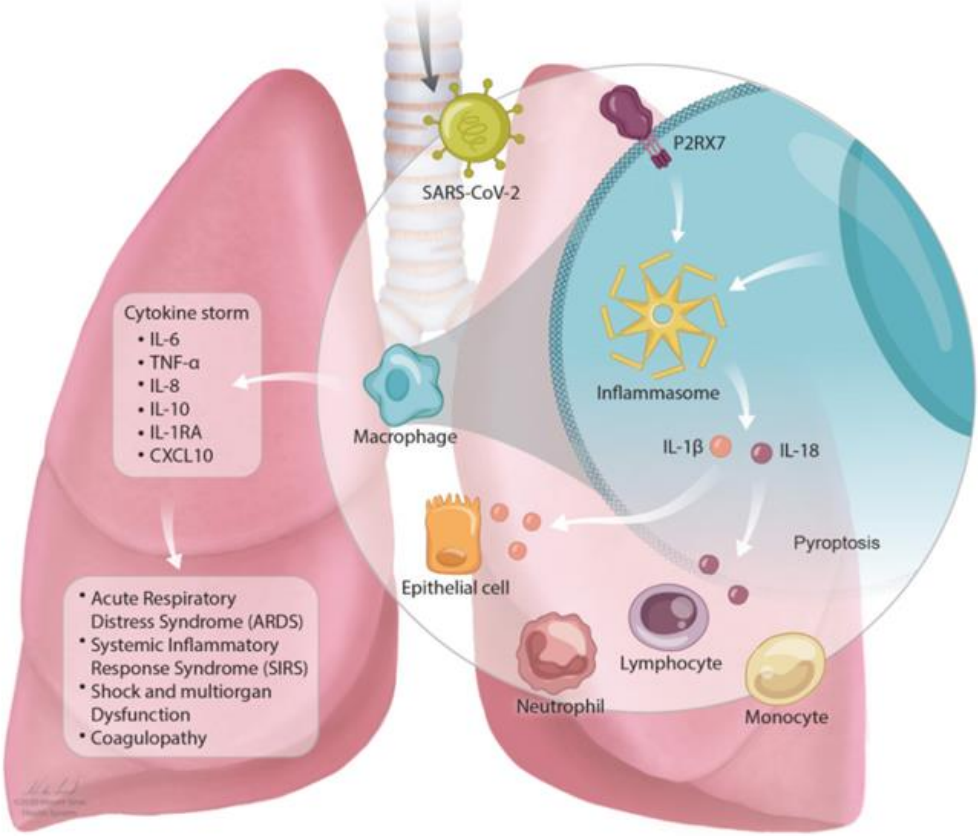


Fig.6 *Lung inflammation in SARS-Cov-2* [162].

At cellular level, the host's immune response, including cytokine expression, leads to the activation and rapid expansion of cytotoxic CD8+ T cells, which affect and kill infected host cells. Similarly, CD4+ T cells are activated, and in turn stimulate plasma cell B cell differentiation to produce antibodies specific to viral proteins, mainly IgM and IgG. Whereas the former rises within few days and declines within several weeks, the latter rises 2-3 weeks post infections but persist much longer. Antibodies together with memory T cells, confer host immunity, albeit temporary, to reinfection [168].

SARS-CoV-2 infection also triggers a process in the respiratory epithelium called pyroptosis, which is a programmed cell death closely related to a high inflammatory state [169]. Hypoxia, widespread alveolar damage, and inflammatory processes therefore affect the symptoms of patients, resulting in dry cough, dyspnea, fatigue, and, in the most severe cases, pneumonia and/or respiratory failure [170].

2.2.2. Molecular mechanisms of innate immune response to SARS-CoV-2 infection

Innate immunity is the first arm of the immune response elicited by viral infections. The SARS-CoV-2 viral RNA released in the cytoplasm is detected by intracellular pattern recognition receptors

(PRRs), such as Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs). The detection of the viral genome in the process of non-self-recognition is controlled by the localization and amount of the viral genome, as well as the localization of the receptors. After activation, these receptors, through a signalling cascade that activates and translocates transcription factors including IFN regulatory factor 3 (IRF3) and NF-kB, trigger the production of cytokines like Type I and III IFNs, TNFα, IL-1, IL-6, and IL-18, limiting infection [171]. Once produced, IFNs interact with their receptors, initiating a signalling cascade, which leads to the transcription of Interferon Stimulated Genes (ISGs) orchestrating the antiviral innate and adaptive immune responses. In detail, when Type I IFN and its dimer receptor (IFNAR) interact, the JAK-STAT signal transduction cascade is triggered, and STAT1 and STAT2 are phosphorylated by JAK1 and TYK2 kinases before forming a complex with IRF9. These complexes enter the nucleus and induce ISG transcription [172]. Studies in animal models and cell-based assays following SARS-CoV-2 infection, as well as serum and transcriptional profiling of COVID-19 patients, revealed an exaggerated abnormal inflammatory response characterized by decreased levels of type I and III IFNs, as well as increased chemokines and IL-6 expression [173]. However, it is now widely accepted, that SARS-CoV-2 has developed some mechanisms that inhibit or delay the production and signalling of Type I IFNs which might occur by evasion of PRR signalling. Indeed, as previously described, viral replication takes place inside "replication organelles" induced by the activity of nsp6, 3 and 4, which hinder their recognition by PRRs. Moreover, nsp10, 12, 14 and 16 by inducing the 5' capping of the ssRNA, promote its recognition as host mRNA and subsequent translation[174]. Additional nsps encoded by SARS-CoV-2 genome are involved in antagonizing IFN responses. Considering the numerous strategies that SARS-CoV-2 has evolved to prevent IFN production, it is conceivable that dysregulation of IFN response is correlated to the pathogenicity of COVID-19. Supporting this hypothesis, SARS-CoV-2 induced antagonism/delay of the IFN signalling goes along with a massive induction of pro-inflammatory cytokines.

As mentioned before, SARS-CoV-2 infection may be related to cell pyroptosis. SARS-CoV-2 N protein promotes the assembly of NLRP3 (NOD-like receptors protein 3) inflammasome needed for the conversion of prointerleukin-1β to mature IL-1β, which through a signalling pathway leads to a large amount of cytokine release [162, 175]. In detail, NLRP3 inflammasome consists of a sensor protein (NLR family PYRIN domain containing-3, NLRP3), an adaptor protein (apoptosis-associated speck-like protein containing a caspase recruitment domain, ASC), and an effector protein (Caspase-1)[176] and has important functions in RNA virus infection [177, 178]. During activation of the NLRP3 inflammasome, NLRP3 PYD interacts with ASC PYD, promotes ASC oligomer formation,

and provides a platform for caspase-1 activation [179]. Activated caspase-1 recruits and cleaves members of the Gasdermin family such as GSDMD for polymerization in the downstream pathway [180] and simultaneously cleaves the precursors of IL-1β and IL-18 to form active IL-1β and IL-18, which are released into the extracellular environment to recruit more inflammatory cells intensifying the inflammatory response [161, 181]. Reduced cell counts and increased IL-1β in the serum of COVID-19 patients may indicate the activation of cell pyroptosis. In addition, the presence of fatal NLRP3 inflammasome aggregates of COVID-19-induced pneumonia in lungs supports the existence of biological relations between viral infection and cytokine release syndrome [182, 183].

High levels of the pro-inflammatory cytokine HMGB1 (High-Mobility Group Box 1) were also found, possibly as a result of SARS-CoV-2-induced nuclear damage [97]. Furthermore, activated pathogenic Th1 cells secrete pro-inflammatory cytokines such as interleukin 6 (IL-6) and granulocyte-macrophage colony-stimulating factor (GM-CSF), which leads to an increase in the production of TNF and other pro-inflammatory cytokines, eventually fostering the cytokine storm. Additionally, increased levels of several chemokines (CCL2, CCL8 CXCL2, CXCL8, CXCL9, and CXCL16), were detected in COVID-19 patients [173].

Such chemokines are presumably responsible for aberrant recruitment of inflammatory macrophages and infiltration of T cells, including cytotoxic T cells, as well as neutrophil in the lung [60, 184].

2.2.3 SARS-CoV-2 and Nervous System

2.2.3.1 Symptomatology

Several studies demonstrate that although COVID-19 typically leads to respiratory disorders, acute and sub-acute neurological complications may affect severe as well as minimally symptomatic or asymptomatic patients [144–150] whose profile and timeline is uncertain [185]. Indeed, up to 65% of COVID-19 affected patients reported a wide range of neurological conditions suggesting detrimental effects of SARS-CoV-2 on the central nervous system (CNS) Fig.7 [186]. Such signs span from the highly frequent anosmia or ageusia (26%) to headache (37%) and encephalitis (0.5%) [187, 188], but also brain fog, neurological sensations, headaches, memory and attention deficit, insomnia, dizziness and balance issues, speech issues, sleep disturbance, anxiety and depression [189–191]; a dysexecutive syndrome consisting of inattention, disorientation, and poor movement coordination [192–194], ataxia, muscle aches and joint pains have been reported as well [195–197].

Moreover, it has been observed that the majority of recovered hospitalized patients (>80%) presents at least one persistent symptom sixty days after symptomatology's onset, which commonly involves

fatigue and dyspnea [198] as well as cerebrovascular disease, seizures, meningitis, encephalitis, loss of smell (anosmia), taste (ageusia) and myositis [199, 200]. Such collection of symptoms, which develops during or following SARS-CoV-2 infection and which continues for more than 12 weeks, is currently named Long-COVID or Post-COVID condition [189, 201, 202] (www.cdc.gov), and might occur as a consequence of direct infection/exposure of neuronal cells to SARS-CoV-2 [170]. Further reinforcing this assumption a great reduction in the grey matter thickness, an increase of tissue damage-related markers in region functionally-connected to the olfactory cortex, and an overall brain size reduction were reported as distinctive traits displayed after SARS-CoV-2 infection [203].

Besides the Central Nervous System, the documented symptoms also imply an alteration of the peripheral nervous system (PNS) following SARS-CoV-2 infection, which could trigger or worsen neurodegenerative disorders, as recently reported by Serrano-Castro *et al.* [204]. Supporting this hypothesis, peripheral nerve damage and a more generalised acute polyneuropathy, known as the Guillain-Barré syndrome (GBS) [199, 205–209], have been reported in SARS-CoV-2-infected patients. In three COVID-19 patients, a possible association with a new diagnosis of myasthenia gravis was documented [210, 211]. Furthermore, peripheral motor neuropathy before the onset of the typical flu-like symptoms of COVID-19 [212, 213] and diagnostic criteria for acute polyradiculoneuropathy have been described as well [214]. Further supporting the detrimental effect of coronaviruses on PNS, both GBS and acute motor axonal neuropathy (AMAN) have been associated to SARS and MERS infections [215, 216].

Multiple studies have shown neuroinflammation in patients with COVID-19, which may trigger these neurological and neuropsychiatric symptoms distinguishing some affected patients [7, 9–17]. In addition, as with other viruses, the presence of pre-existing neurological disorders has been associated with an increased risk of developing COVID-19-related neurological signs [18–21, 217].

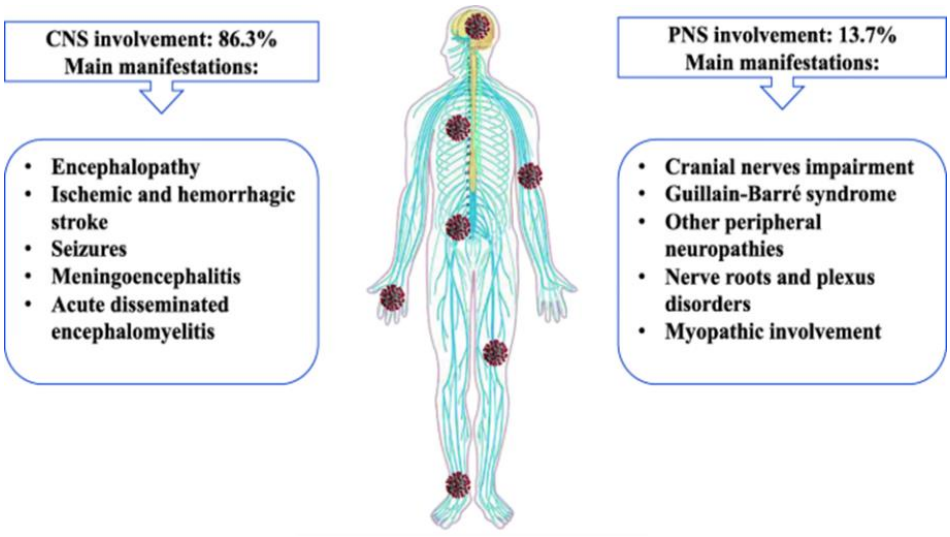


***Fig.** 7 Summary of central and peripheral nervous system involvement by SARS CoV-2* [186].

2.2.3.2 Neuroinvasiveness of SARS-CoV-2

The tendency of SARS-CoV-2 to enter the nervous system and its ability to infect and replicate in CNS cells have been studied extensively, with sometimes seemingly contradicting findings. To date it is unclear whether the neurological symptoms are a consequence of direct neural infection, para-infectious or post-infectious immune-mediated disease, or sequalae of systemic disease [1, 22]. Our understanding of the mechanisms through which SARS-CoV-2 can enter the nervous system and infect nerve cells is still not completely clear. Studies from both post-mortem COVID-19 patients and animal models suggest that SARS-CoV-2 exploits different neuroinvasive strategies and accession routes, all of which seem likewise possible and have been previously associated to other coronaviruses like SARS-CoV [218–220] (Fig.8). These include: i) hematogenous transport of infected immune cells via the circulatory system of the brain tissue; ii) infection of the nasal olfactory epithelium to reach the brain by axonal transport along the olfactory nerve; iii) retrograde virus spread from the lungs to the CNS through the vagus nerve; iv) entry from the ocular epithelium; v) virus invasion through impairment of the blood-brain barrier (BBB).

Lessons learnt from other neurotropic viruses can be useful to understand the actual stratagem adopted by SARS-CoV-2 to invade the CNS. Some viruses reach the CNS by infecting peripheral neurons and then using the axonal transport system [221–224]. This may indicate a potential route for SARS-CoV-2 entry into the cranial nerve. The human respiratory system, which serves as main area for SARS-CoV-2 replication, is innervated by a number of cranial nerves (CNs). The nasal cavity is specifically innervated by the trigeminal and olfactory nerves (CNV and CNI), the upper respiratory

tract by the facial and glossopharyngeal nerves (CNVII and CNIX), and the lower respiratory tract by the vagus nerve (CNX). The olfactory bulb, vagus, or trigeminal nerves may serve as SARS-CoV-2 retrograde pathways from the cribriform plate to the brain, according to recent research [225–228].

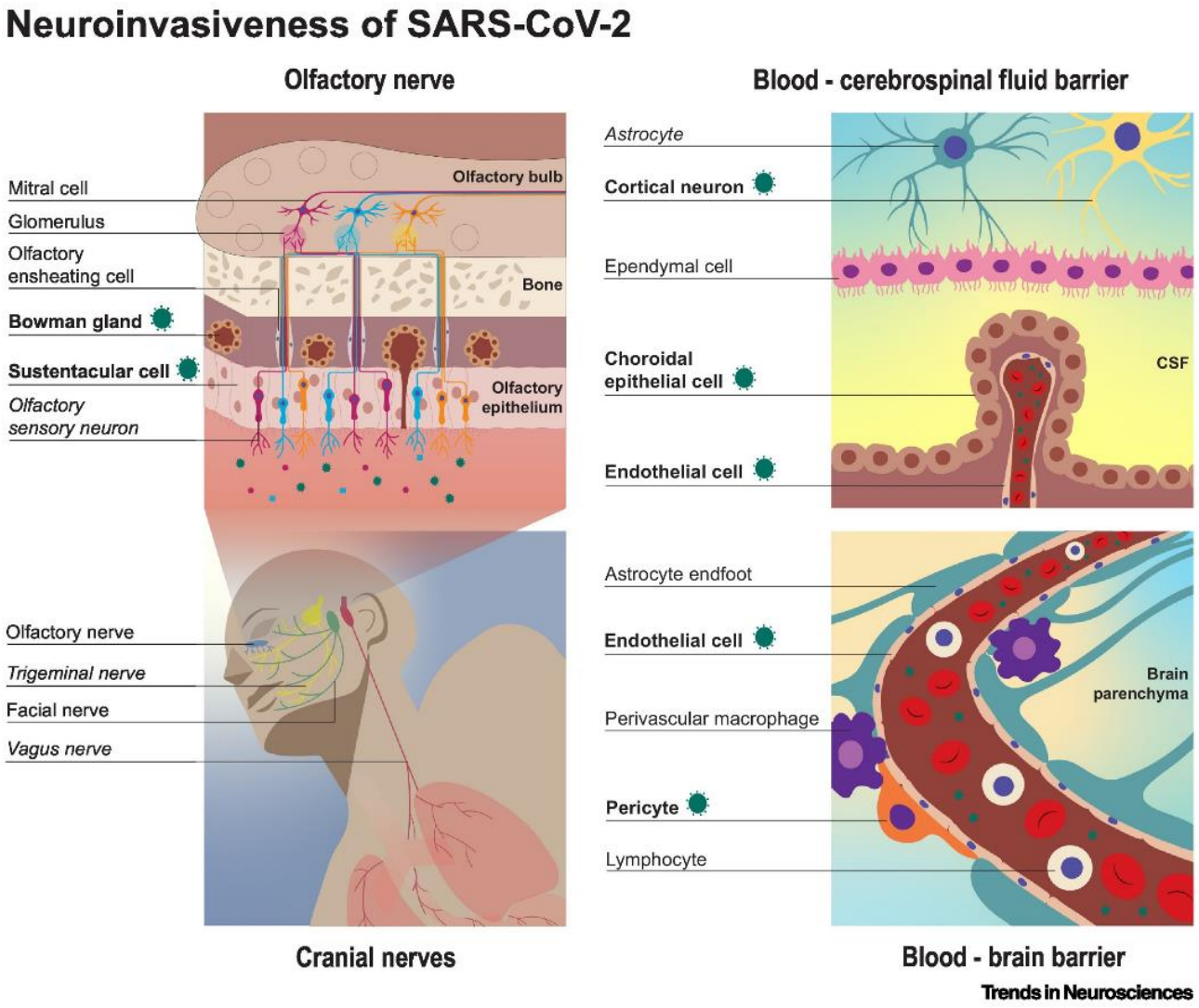


***Figure 8**. Possible neuroinvasive routes of SARS-CoV-2* [229].

Isolated anosmia and ageusia with or without respiratory symptoms have been largely observed during the COVID-19 outbreak. As mentioned before, one possible route for SARS-CoV-2 entrance into the central nervous system, as for many other viruses, CoVs included, is the direct penetration along the olfactory nerve [224, 226, 230–232]. This possibility is corroborated by the broad expression of ACE2 by oral mucosa epithelial cells [233] and the detection of viral RNA or viral protein in sustentacular cells in the olfactory mucosa and, to a lesser extent, in olfactory sensory neurons in humans and experimental animal models [226, 227, 229, 234–237].

Dendrites from olfactory receptor neurons are projected into the nasal cavity, and axons are extended via the cribriform plate into the brain's olfactory bulb [232]. SARS-CoV-2 may use retrograde pathways to spread from the olfactory nerves to other parts of the brain after damaging the olfactory mucosa and generating symptoms like headaches, consciousness problems, and seizures [224].
Due to the identification of viral RNA in the trigeminal ganglion, which suggests that SARS-CoV-2 spreads by nerve terminals to the soma of sensory neurons in humans [226, 238–241], and the discovery of SARS-CoV-2 proteins in vagus nerve fibers by immunohistochemistry in humans [240, 242], CNS invasion via the trigeminal nerve and the vagus nerve has been proposed, as well. To the best of our knowledge, no evidence of viral invasion through the glossopharyngeal or facial nerves has been documented [229].
There is increasing evidence that human CoV reversibly invade peripheral nerve terminals, spread retrograde along nerve synapses, and gain access to the CNS via neuronal active transport [230, 243, 244]. Trans-synaptic transfer of virus has been demonstrated for several CoV, including HCoV-OC43, hemagglutinating encephalomyelitis virus 67(HEV67), and avian bronchitis virus. In particular, OC43 uses axon transport to spread between neurons [245, 246]. SARS-CoV-2 may invade and diffuse in neurons via the same propagation strategy, including endocytosis and exocytosis, to spread within the synaptic cleft and undergo fast axonal transport to move along microtubules toward the neuron cell body [225].

The maintenance of homeostasis is crucial for the CNS's functionality. Blood-brain and blood-CSF barriers both play a crucial part in preventing the free circulation of undesirable molecules, pathogens, and cells into the brain. In particular, the BBB is the first line of defence and it is composed by cerebral microvascular endothelium, astrocytes, pericytes and extracellular matrix [222, 247, 248]. In hematogenous entry, there are 2 ways for the virus to cross the BBB and disseminate into the CNS: the vascular endothelial cell pathway, where the virus can infect endothelial cells of the BBB to gain access, and the immune cell pathway, where the virus can infect leukocytes [199, 221, 222, 249]. After primary infection, SARS-CoV-2 may be able to spread into the circulation, resulting in subsequent virus propagation through the BBB or B-CSF into the CNS. SARS-CoV-2 viral RNA and viral particles have been found in the blood or serum of COVID-19 patients[250–252]. SARS-CoV-2 may bind to ACE2 expressed in the capillary endothelium of BBB to gain access into the CNS [253]. According to electron microscope images, SARS-CoV-2 binds to the ACE2 receptor and then enters vascular endothelial cells by endocytosis and exocytosis to spread from one cell to another [254, 255]. Indeed, SARS-CoV-2 virus-like particles were found in astrocytes, pericytes, and brain

capillary endothelial cells, indicating that astrocytes are a possible point of entry for the virus into the central nervous system [225].

On the other hand, Although the neuroinvasivity of SARS-CoV-2 has not yet been confirmed, multiple lines of evidence suggest that coronaviruses can infect leukocytes, including lymphocytes, granulocytes, monocytes and monocyte derivatives [256] and, once activated these leukocytes disseminate towards other tissues, and cross the BBB to access the CNS in a process known as a Trojan horse mechanism [221].

Although there is no conclusive evidence on whether the virus can cross the BBB, SARS-CoV-2 antigens have been detected in small vessel endothelial cells in humans and animal studies imply that such invasion may occur, at least in a minority of patients [229].

2.2.3.3 Neuroinflammation and Neurovirulence of SARS-CoV-2

Although the exact mechanism by which SARS-CoV-2 enters the CNS is unknown, it has been widely documented that when the virus gets into the brain tissue, it causes a series of inflammatory events. These include swelling of brain tissue, obstruction of blood flow, interstitial edema, and cerebral vasodilation, supporting the idea that inflammation is one of the main causes of CNS problems caused by the virus [153]. Neuroinflammation is a complex innate immune response evoked by the brain tissue in order to repair cell damage, limit infection, or eliminate pathogens. Although acute neuroinflammation leading to glial and endothelial cell activation, is typically considered neuroprotective, chronic stimulation may damage the CNS [257, 258]. Indeed, Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS) are all linked to persistent neuroinflammation [259].

Microglia consist of myeloid cells that reside in the CNS and are the main responsible for neuroinflammation regulation. Indeed, they display a significant role in maintaining the tissue homeostasis and orchestrating the immunological responses to damage or infection, among other roles [257, 260, 261]. Studies from both post-mortem COVID-19 patients [7, 9–17, 218, 262] and animal models [14–16] showed extensive microglial activation with pronounced neuroinflammation in the brainstem [263], which may exacerbate neurological and neuropsychiatric symptoms.

The presence of activated parenchymal myeloid cells, defined by an increase in the number of CD68+ or IBA-1+ cells, indicators of phagocytosis, is a typical finding in post-mortem brain tissue analysis from COVID-19 patients[15, 17, 264–266].

Several studies have found evidence for CNS inflammation after SARS-CoV-2 infection. Activated microglia were found in the olfactory bulb, in the substantia nigra, hindbrain, dorsal motor nucleus of the vagus nerve, and in the medulla[229, 267]. In addition, Single nucleus RNA-sequencing analyses of the CNS confirmed that that SARS-CoV-2 infection alters microglial transcriptome in COVID-19 patients, and in particular the expression of genes related to innate immune signalling, such as proinflammatory cytokines, chemokines, and reactive oxygen species, interferon regulator factor 8 (IRF8) and complement protein C1QC, or cell stress pathways, such as ATF5, which regulates transcriptional responses to stress and RIPK1, which promotes cell death [17, 266, 268].

Astrocytes, the most prevalent glial cells in the CNS, are also critical in the regulation of innate and adaptive immune responses during an infection, as well as in the preservation and permeability of the BBB [258, 269].

SARS-CoV-2 may direct neuroinvasion to mediate inflammatory responses and microglia and astrocytes activation has been identified as a significant feature of the neuroinflammatory response generated by SARS-CoV-2 infection [270]. It has been hypothesized that leukocytes in the CNS release pro-inflammatory cytokines like TNF, which can harm oligodendrocytes and/or neurons, as well as chemokines like CCL5, CXCL10, and CXCL11, which induce chemoattraction of activated T cells and/or other leukocytes [199, 221]. Following infection, astrocytes can release chemokines such as CCL2, CCL5, and CXCL12, which recruit other leukocytes. SARS-CoV-2 may thereby trigger an abnormal neuroinflammatory cycle, resulting in neuropathology [199].

Microglial activation can also occur in response to danger-associated molecular patterns (DAMPs), such as the production of adenosine triphosphate (ATP) or reactive oxygen species [268]. ATP, a key damage-associated molecular pattern, is released in response to tissue damage activating its surface receptors, namely P2X7. This receptor, a P2X ligand-gated ion channel receptor, is typically expressed on microglia and oligodendrocytes in the CNS [271, 272]. Its signalling has been linked to the cytokine storm's development as it efficiently activates NLRP3 inflammasomes, leading to caspase-1 activation and IL-1β and IL-18 release [225, 273]. P2X7/NLRP3 axis activation not only generates an inflammatory microenvironment but also amplify chemotaxis signalling and controls cell orientation.

However, COVID-19 neuroinflammation is a highly complex phenomenon and may be caused by simple direct infection of SARS-CoV-2 to the CNS or a combination of direct infection and indirect systemic responses [153]. The systemic increase in inflammatory mediators could explain the multi-organ damage affecting some COVID-19 patients, as well as the impact of SARS-CoV-2 on the CNS [274]. Proinflammatory cytokine expression in the peripheral blood not only increases vascular

permeability, abnormal blood coagulation, and multiple-organ failure, but it may also disrupt the BBB, increasing microvascular permeability and facilitating SARS-CoV-2 entry into the brain [199, 255, 275]. External cytokines can affect the brain by crossing the BBB, enabling other substances to traverse [274, 276]. In this condition, the infected brain parenchyma may recruit external innate immune cells to enter the CNS and mediate the inflammatory response.
Intracranial cytokine storms, which could result in BBB breakdown without direct viral invasion, could be responsible for the development of acute necrotizing encephalopathy or Guillain-Barré syndrome, and the coagulopathy seen in COVID-19, when combined with a cytokine storm, could make patients vulnerable to thrombotic and/or haemorrhagic cerebrovascular events [212, 224, 277, 278]. Otherwise, viremia could cause the transport of viral particles or Spike proteins to the BBB even in the absence of productive CNS infection. This might trigger endothelial innate immune responses, BBB damage, and cause neuroinflammation and microglia activation [268].

The possibility that SARS-CoV-2 proteins serve as PAMPs independently from a productive infection of the CNS, is supported by several scientific evidence. As a matter of fact, SARS-CoV-2 Spike protein was demonstrated to trigger a pro-inflammatory response on brain endothelial cells that, in turn, may alter the BBB properties and functions [279]. Likewise, SARS-CoV-2 spike protein was proven to induce phenotypic changes in hematopoietic cells [280]; while Albornoz *et al.* have shown that SARS-CoV-2 isolates, as well as Spike protein alone, can both prime and activate the NLRP3 inflammasome in the human microglia through NF-κB and ACE2 [263]. Furthermore, the addition of Spike proteins to human microglia *in vitro* promoted cytokine production and metabolic alterations, confirming the idea that this is able to cross the BBB triggering a prolonged microglial inflammatory response during COVID-19 [281]. Additionally, the use of an advanced 3D microfluid model of the human BBB and post-mortem studies on the cerebral pathology of COVID-19 patients provide further evidence that Spike protein can directly damage and alter the function of the BBB as well as trigger an inflammatory response in microvascular endothelial cells, where ACE2 receptor is widely expressed [199, 255, 282].

Direct viral infection and/or indirect systemic responses correlate to the activation of the immune system and inflammatory mediators, including cytokines, are the likely causes of the acute symptoms of COVID-19 and may explain the long-term sequelae of SARS-CoV-2 infection.

2.2.3.4 Neurotropism of SARS-CoV-2

The ability of SARS-CoV-2 to infect and replicate in CNS cells has been intensively investigated, with sometimes seemingly contradictory results. In fact, the majority of research on SARS-CoV-2 infection of CNS cell types have revealed that infection is limited to a subset of cells and that viral replication is frequently inefficient or even abortive.

However, *ex vivo, in vivo,* and *in vitro* studies suggest that SARS-CoV-2 is able to infect different kinds of neuronal populations, with different degrees of success [29–35, 283–292]. Indeed, it is now clear that being susceptible to SARS-CoV-2 is not always related to the virus's ability to replicate and to produce progeny virus. Dopaminergic neurons, cortical neurons, cerebral microvascular endothelial cells, and chorionic epithelial cells are all susceptible to infection but, among these different cell types, there are differences in the permissiveness to SARS-CoV-2 [31, 32, 34–36, 229, 288, 293–295]. Additionally, animal studies propose that dopaminergic neurons and, to a lesser extent, cortical neurons, microglia, and astrocytes are susceptible to SARS-CoV-2 infection [23, 37, 229]. Studies in choroid plexus organoids showed that choroidal epithelial cells are permissive for SARS-CoV-2 infection, as well [31, 34–36, 229, 295, 296].

Anyway, the molecular mechanisms sustaining SARS-CoV-2 infection of nerve cells are yet to be defined. For instance, ACE2, the major actor involved in multi-organ SARS-CoV-2 infection, shows low expression levels in the human brain [150]. Thus, despite the initial concept of ACE2 distribution as the major determinant of SARS-CoV-2 infectivity and spread, other factors have been called-on as responsible for SARS-CoV-2 neurovirulence. In fact, despite ACE2 is expressed in neurons, astrocytes, and oligodendrocytes throughout the brain [297], NRP1 [77, 78], CD147 [7], TMPRSS [100], and Furin [83, 298], show higher and broader patterns of expression in neuronal cells compared to ACE2 (www.proteinatlas.org). The highest expression of CD147 is in cranial endothelial cells and pericytes. NRP1 is highly expressed in excitatory neurons and Cranial endothelial cells [299]. It was also suggested that SARS-CoV-2 might use the dopamine receptor as an additional entry receptor [300]. More generally, in light of the COVID-19-related symptomatology, it has been hypothesized that SARS-CoV-2 might affect the dopamine pathway [24–27, 301, 302]. However, concerning this issue, no scientific evidence has been produced so far.

2.3 Dopamine (DA)

2.3.1 Dopamine (DA) and SARS-CoV-2

As stated above, severe cases of COVID-19 show respiratory distress, although some patients show neurologic signs, ranging from headache, nausea, and vomiting to movement and psychiatric manifestations. Increasing evidence highlights the capability of CoVs to infect the CNS, causing neurological diseases. Responsible for the tropisms of viruses may be the tissue distributions of the host receptors. In case of SARS-CoV-2, ACE2 the main virus entry receptor, is expressed in the brain beyond human airway epithelia, lung parenchyma, vascular endothelia, kidney cells and small intestine. It is also possible that CoVs may first invade peripheral nerve terminals, and gain access to the CNS via a synapse-connected route[303]. In this frame, it is remarkable that an alteration of the DA synthetic pathway was recently documented to be involved in the pathophysiology of COVID-19. A bioinformatics approach detected a coexpression link of ACE2 with DDC, the enzyme that converts L-DOPA into DA. Both, ACE2 and DDC, are expressed in intestinal epithelial cells, where L-DOPA is converted into DA, increasing the levels of blood-circulating DA. This suggests that abnormal expression of ACE2 and DDC may lead into altered blood levels of AD in COVID-19 patients[28]. Another stream of interpretation suggests that apart from ACE2, SARS-CoV-2 can exploit DA receptors for its entry into the CNS. These receptors also regulate local immunity in the brain[304]. Additionally, it has been proposed that DA can inhibit lymphocytic function, probably by inducing apoptosis, in a concentration-dependent manner[305]. Mechanistically, this might occur through increased levels of D1-like DA receptors, which stimulate cAMP, decreasing the innate immune response; at the same time, enhanced expression of D2-like DA receptors might lead to the cytokine storm, fostering a reduction of the adaptive immune response (Figure 12)[304]. However, this remains a hypothesis, and specific studies investigating the role of DA in the frame of SARS-CoV-2 specifically, are still missing.

2.3.2 Dopamine (DA) metabolism

Dopamine (DA) (3-hydroxytyramine) is a neurotransmitter, synthesized in the periphery and mostly in the Central Nervous System (CNS), specifically in *substantia nigra*, ventral tegmental area (VTA) and hypothalamus. It is a key player in body functions such as memory, movement, reward and motivation[306]. Belonging to the catecholamines group, together with norepinephrine and epinephrine, it is not able to cross the blood-brain barrier, it binds to metabotropic, G-linked receptors. Dopamine (DA) derives from tyrosine, an essential amino acid that comes from the diet. However, since L-phenylalanine is converted to tyrosine by the phenylalanine hydroxylase, DA can be obtained

in an indirect way also from phenylalanine. Its synthesis occurs in the cytosol, whereby tyrosine is converted to levodopa (L-DOPA) by the rate-limiting enzyme tyrosine hydroxylase (TH), which uses as cofactors oxygen (O_2), iron (Fe^{2+}) and tetrahydrobiopterin. Next, DOPA decarboxylase (DDC), using pyridoxal phosphate as cofactor, converts L-DOPA to DA. Once synthesized, the vesicular monoamine transporter 2 (VMAT2) transports dopamine into synaptic vesicles, where it is stored in presynaptic terminals until released in the synaptic cleft [307–309] (Fig.9). There are two mechanisms that regulate DA release: phasic and tonic transmission. In the case of phasic transmission, action potentials trigger the fast and transient release of DA in the synaptic cleft. Intrasynaptic DA concentrations are in the millimolar range, able to stimulate postsynaptic receptors. Before it can diffuse into the extracellular space DA is rapidly removed from the synaptic cleft and taken-up by the DA transporter (DAT). Instead, tonic transmission occurs in the absence of presynaptic action potentials. In this case, the released DA escapes from the synaptic cleft and diffuses within the extracellular space[310].

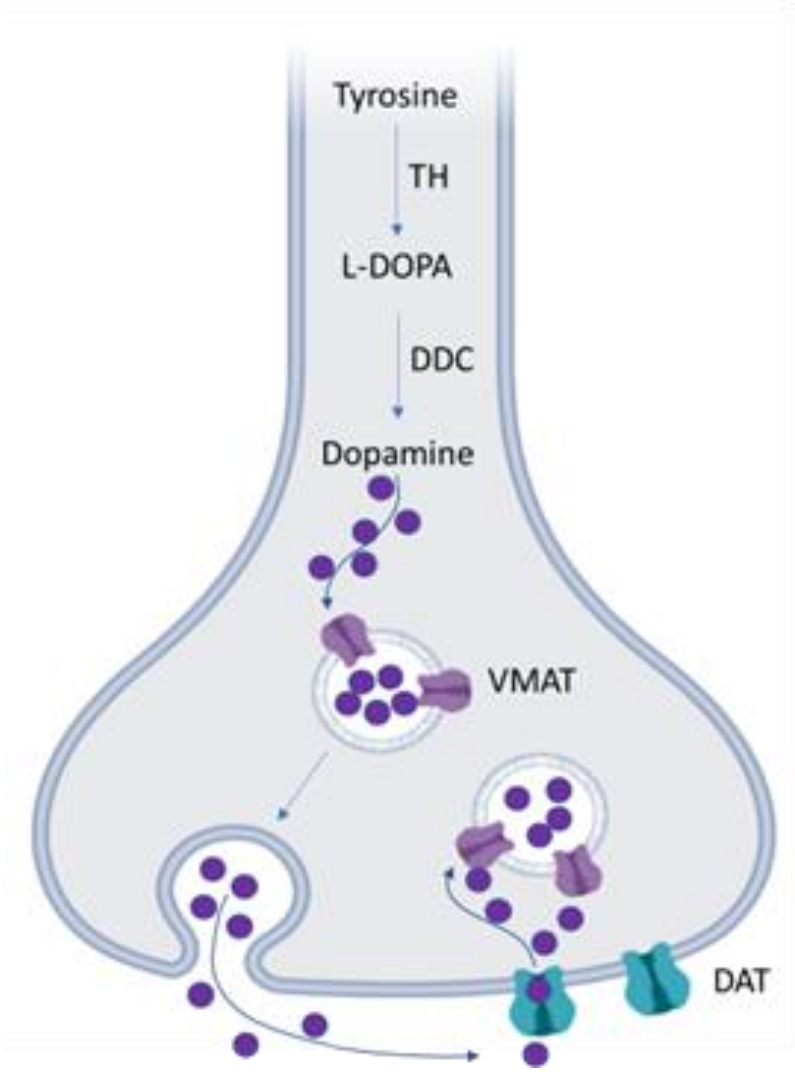


Fig.9 *Synthesis of DA* [309]

Once in the extracellular space, DA can bind to postsynaptic receptors or presynaptic autoreceptors on the presynaptic neuron. Following the action potential, elicited by the postsynaptic neuron, DA is released and then taken-up by presynaptic neurons, a process mediated by DAT [308]. The retaken DA is sequestrated in synaptic vesicles by VMAT2. Free cytosolic DA is degraded by monoamine oxidase (MAO) into 3,4-Dihydroxyphenylacetaldehyde (DOPAL), which in turn, is converted by aldehyde dehydrogenase (ALDH) into 3, 4-dihydroxyphenylacetic acid (DOPAC). Degradation of DOPAC by catechol-O-Methyltransferase (COMT) results in one of the main dopamine degradation products, homovanillic acid (HVA)[311]. Non sequestrated or degraded DA undergoes oxidation in quinones, and DA-quinones can internally cyclise and form aminochrome by the addition of an amine group. All this leads to the formation of a dark pigment, called neuromelanin, found in *substantia nigra* [308, 312].

The physiological actions of DA are mediated by G protein-coupled receptors (GPCRs) that are divided into two main groups: D1-like (including D1 and D5) and D2-like (including D2, D3 and D4). These receptors are not only present in CNS, but also at peripheral levels, including heart, blood vessels, retina and kidney.

- D1-like DA receptors couple to $G\alpha_s$ activating adenylyl cyclase, which in turn will lead to the production of the second messenger cAMP, stimulating the activity of cAMP-dependent protein kinase A (PKA)[313, 314]. PKA phosphorylates specific proteins like the cAMP response-element binding-protein (CREB) transcription factor, involved in regulation of gene transcription[315].
- D2-like DA receptors couple to $G\alpha_i$ inhibiting adenylyl cyclase, reducing in this way the levels of intracellular cAMP, inhibiting the activity of PKA [313, 314].

However, DA signaling involves a plethora of downstream pathways. D1 DA receptor (D1DR) is engaged in ERK1/2 phosphorylation [316]. Instead, D2 DA receptors (D2DR) can take part in Akt/GSK3 signaling cascade by involving the multifunctional adaptor protein β-arrestin 2 (βArr2). Once activated by phosphorylation, Akt will inactivate GSK3 isoforms [317].

DA is more than just a neurotransmitter, because depending on the type of the receptor and second messenger, it is involved in different signaling pathways.

2.3 *In vitro* cellular models

Despite numerous reports of neurological findings in patients with COVID-19, the molecular mechanisms responsible for these dysfunctions, as well as the potential neurotropism of SARS-CoV-

2, are still under investigation. Human pluripotent stem cell (hPSC) based models are ideal tools for investigating the interaction between SARS-CoV-2 and cells of the central nervous system (CNS) and peripheral nervous system (PNS). These models, whether in a two-dimensional (2D) or three-dimensional (3D) format, enable the exploration of neural and non-neural cells from various CNS regions. It is well known that these cells exhibit significant heterogeneity in their gene expression profiles, functionality, and immunological status. Considering that human brain tissue is difficult to access, particularly from patients with a contagious pathogen due to safety concerns [38], induced pluripotent stem cells (iPSCs)-derived human cortical organoids can provide a viable and safe alternative and represent a suitable model system to test the neurotoxic effects of SARS-CoV-2.

In fact, 3D *in vitro* models have proved to be very useful to investigate the human brain development and different neurological disorders [32, 318–323] and, even in response to the COVID-19 pandemic, hiPSC-derived organoids have been used to model SARS-CoV-2 infection in different organs, including the intestine [324, 325], kidney [326], liver [296], lung [327, 328], and brain [32, 34–37, 39]. It has become evident that SARS-CoV-2 may affect several organs and tissues, including the brain [329, 330] and several studies have shown that this virus can infect and replicate in cells of multiple organs, resulting in transcriptional changes indicating altered inflammatory responses and cellular functions.

3. AIM OF THE STUDY

To date, SARS-CoV-2 has infected millions and affected billions of human beings and several studies have been struggling to find suitable therapeutic approaches to contain the infection/replication of this virus. Since the emergence of COVID-19 and related neurological manifestations, the ability of SARS-CoV-2 to enter and productively infect CNS cells has been extensively studied. Indeed, although several observations suggest that SARS-CoV-2 can trigger both brief and long-term (long-COVID) neuropathic effect, the molecular mechanisms responsible for such alterations are mostly unknow and need extensive and exhaustive studies. Albeit no conclusive evidence on whether the observed neurological sequelae are attributable to direct CNS invasion by the virus, or Spike exposure, a plethora of studies converge in documenting heterogeneous manifestations which support its neuropathological potential. Useful insights for SARS-CoV-2-induced CNS alterations have been provided by studies in human iPSC-derived neurons or brain organoids, that are *in vitro* scaled-down, 3D models recapitulating molecular, cellular, and functional aspects of the developing human brain. In this context, during my PhD I've been involved in different projects in collaboration with different research groups, whose purpose was to assess the relationship existing between SARS-CoV-2 infection and the alteration of the nervous system. In this frame, I was given the chance to work with three different neuronal models, including dopaminergic neurons, iPSC derived motor neurons, and human cortical organoids, in order to investigate this extremely current and concerning condition. Each of these independent projects has provided attractive results described in as many research papers, which will be referenced in the text using the following nomenclature: **I.** SARS-CoV-2 hampers dopamine production in iPSC-derived dopaminergic neurons. **II.** Human motor neurons derived from induced pluripotent stem cells are susceptible to SARS-CoV-2 infection. **III.** iPSC-derived Human Cortical Organoids display profound alterations of cellular homeostasis following SARS-CoV-2 infection and Spike protein exposure. All of these three studies have already been published as indicated below [309, 331, 332].

I. It has been demonstrated *in-vivo* and *in-vitro* that SARS-CoV-2 is able to infect different neuronal cell types with different degrees of success [29–37]. It was suggested that SARS-CoV-2 might use the dopamine receptor as an additional entry receptor [304]. More generally, it has been hypothesized that SARS-CoV-2 might affect the dopamine pathway [23–28]. From this scenario, we exploited an *in-vitro* model represented by human iPSC differentiated to dopaminergic neurons (DA neurons) and assess their infectability by three SARS-CoV-2 variants (EU, Delta, Omicron) and the effect of

SARS-CoV-2 exposure on the expression of both mRNA and proteins involved in dopamine metabolism.

II. The documented neuromuscular dysfunctions related with COVID-19 suggest the occurrence of widespread alterations possibly affecting all the motor unit components. Nonetheless, the impact of SARS-CoV-2 exposure on motor neuronal cells specifically has not been investigated so far. Therefore, by using an *in vitro* model of human motor neurons differentiated from induced pluripotent stem cells (iPSC-MNs), we aimed to assess, for the first time i) the expression of SARS-CoV-2 main receptors; ii) iPSC-MNs infectability by SARS-CoV-2; and iii) the effect of SARS-CoV-2 exposure on iPSC-MN transcriptome.

III. SARS-CoV-2 infection can impact various organs and tissues, including the brain [329, 330]. The molecular processes initiated in response to SARS-CoV-2 and/or spike protein exposure, as well as the potential affinity of SARS-CoV-2 for neuronal cells, remain only partially understood. Furthermore, several evidence indicate that SARS-CoV-2 spike protein triggers a pro- inflammatory response on brain endothelial cells that, in turn, may alter the blood brain barrier (BBB) functions [279]. To investigate this issue, by using iPSC-derived- human cortical organoids (HCO) we assessed: i) the expression of SARS-CoV-2 main receptors; ii) their infectability by SARS-CoV-2; iii) their alteration following Spike protein exposure; and iv) the effect of SARS-CoV-2 on human cortical organoids transcriptome.

Overall, the gathering of all the information, acquired through these three independent experimental models, will be pivotal to clarify the cellular and molecular mechanisms triggered by SARS-CoV-2 interaction with different elements of the Central and Peripheral nervous system. Indeed, such evidence could at least partially explain the neurological symptomatology displayed by some SARS-CoV-2 infected subjects or patients suffering from Long-COVID manifestations and could possibly suggest new intervention strategy to be exploited in the setting up of alternative therapeutic interventions.

4. MATERIALS AND METHODS, RESULTS, DISCUSSION

4.1 I. SARS-CoV-2 hampers dopamine production in iPSC-derived dopaminergic neurons.

Cappelletti G, Carsana EV, Lunghi G, Breviario S, Vanetti C, Di Fonzo AB, Frattini E, Magni M, Zecchini S, Clerici M, Aureli M, Fenizia C. SARS-CoV-2 hampers dopamine production in iPSC-derived dopaminergic neurons. Exp Mol Pathol. 2023 Sep 30;134:104874. doi: 10.1016/j.yexmp.2023.104874. Epub ahead of print. PMID: 37775022.

4.1.1 Materials & Methods

4.1.1.1 iPSC culture

Human iPSC clonal line obtained from fibroblasts of a healthy subject was purchased from Coriell Institute (AICS-0022-037). Parental hiPSC line (WTC/AICS-0 at passage 33) derived from fibroblasts was reprogrammed using episomal vectors (OCT3/4, shp53, SOX2, KLF4, LMYC, and LIN28). iPSCs were grown in geltrex-coated (1% for 1h at 37°C) 6-well plates and cultured in complete Essential 8 Medium. At 80–90% confluence, cells were passaged using Accutase (3 min 37°C) and plated at a density of 10^4 cells/cm^2 in complete Essential 8 Medium supplemented with 10 μM Rock inhibitor for 24h.

4.1.1.2 Differentiation of iPSC to dopaminergic neurons

iPSCs were differentiated to DA neurons according to the protocol described by Zhang *et al*.[285]. Cells at 70% confluence were cultured in proper media as follows:

- day 0: KSR medium (81% DMEM, 15% KSR, 100x 1% non-essential amino acids, 100x 1% 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin) supplemented with 10 μM SB431542 and 100 μM LDN-193189;

- days 1 and 2: KSR medium supplemented with 10 μM SB431542, 100 nM LDN-193189, 0.25 μM SAG, 2 μM purmorphamine, and 50 ng/mL FGF8b;

- days 3 and 4: KSR medium supplemented with 10 μM SB431542, 100 nM LDN-193189, 0.25 μM SAG, 2 μM purmorphamine, 50 ng/mL FGF8b, and 3 μM CHIR99021;

- days 5 and 6: 75% KSR medium and 25% N2 medium (97% DMEM, 100x 1% N2 supplement, 100 U/ml penicillin, and 100 µg/ml streptomycin) supplemented with 100 nM LDN-193189, 0.25 µM SAG, 2 µM purmorphamine, 50 ng/mL FGF8b, and 3 µM CHIR99021;

- days 7 and 8: 50% KSR medium and 50% N2 medium supplemented with 100 nM LDN-193189 and 3 µM CHIR99021;

- days 9 and 10: 25% KSR medium and 75% N2 medium supplemented with 100 nM LDN-193189 and 3 µM CHIR99021;

- days 11 and 12: B27 medium (95% Neurobasal medium, 50x 2% B27 supplement, 1% Glutamax, 100x, 100 U/ml penicillin, and 100 µg/ml streptomycin) supplemented with 3 µM CHIR99021, 10 ng/mL BDNF, 10 ng/mL GDNF, 1 ng/mL TGF-b3, 0.2 mM ascorbic acid, and 0.1 mM cyclic AMP;

- from day 13 to the end of differentiation: B27 medium supplemented with 10 ng/mL BDNF,10 ng/mL GDNF, 1 ng/mL TGF-β3, 0.2 mM ascorbic acid, and 0.1 mM cyclic AMP.

After 20 days of differentiation, cells were split using Accutase (3 min 37°C) and plated on geltrex-coated plates at a density of 2×10^5 cells/cm^2. At day 26 of differentiation cells were plated at a density of $6x10^4$ cells/cm^2 in 12 wells plates and at day 30 of differentiation experiments were performed.

4.1.1.3 SARS-CoV-2 infection

The European (EU - B.1), the Delta (B.617.2) and the Omicron (BA.1) SARS-CoV-2 lineages were a kind gift of Dr. Davide Mileto, Clinical Microbiology, Virology and Bio-emergence Diagnosis, ASST Fatebenefratelli-Sacco, Department of Biomedical and Clinical Sciences, University of Milan, Milan, Italy. Viruses were isolated from positive nasopharyngeal swabs, propagated, and titrated using the permissive cell line Vero E6 (ATCC, VA, USA). All SARS-CoV-2 strains were identified by means of whole genome sequencing and the sequences were submitted to GISAID (EU EPI_ISL_41297], Delta EPI_ISL_1970729, and Omicron EPI_ISL_1649798). All the experiments with SARS-CoV-2 virus were performed in BSL3 facility; virus was inactivated according to institutional safety guidelines, before samples analyses outside BSL3 area. In order to assess infectious viral particles concentration, TCID50, was performed as elsewhere described [333]. Briefly, Vero E6 were seeded at 2×10^4 cells per well in a 96-well plate. Eleven 1:10, or 1:3 when needed, serial dilutions of the viral stock were performed in 2% FBS medium. For each dilution, eight wells were infected (n=8). Eight wells were left uninfected as control. 1 -hour post infection (hpi), each well was thoroughly washed three times with pre-warmed PBS and the culture media replaced

with 10% FBS DMEM. At 72 hpi, supernatants were removed, cells fixed by paraformaldehyde (PFA - Sigma-Aldrich, MO, USA) 4% for 1h at room temperature, then stained by 0.2% crystal violet solution (Sigma-Aldrich, MO, USA). By applying the Reed-Muench method with the correction for the proportional distance (PD)[334], we were able to assess the TCID50 and to calculate the MOI in our experiments. Then, DA neurons were challenged with 5, 0.5 or 0.05 MOI of SARS-CoV-2. After an o.n. incubation, cells were thoroughly washed three times with pre-warmed PBS and replenished with the complete growth medium. Upon media refill, at 0, 48 and 96 hpi, supernatants were collected to monitor infection. At 48, 72 and 96 hpi, cells were lysed for RNA or protein extraction, whereas supernatants were harvested and appropriately stored.

4.1.1.4 Other stimuli

DA neurons were challenged with 1 µg/ml LPS, with 5 MOI of respiratory syncytial virus (RSV) or with 5 MOI of heat-inactivated EU SARS-CoV-2 (iSARS). iSARS was obtained by heating the virus for 20' at 70°C [335].

4.1.1.5 MTT

Cytotoxic effect was evaluated by means of an MTT assay: cells were seeded in 96-well plates (2×10^4 per well) infected with SARS-CoV-2 viruses at different concentrations (from 10^2 MOI down to 10^{-8} MOI, applying serial 10^1 dilution; n=8) or mock infected (CTRL). At 96 hpi, cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Briefly, 30 µl of MTT (final concentration, 0.5 mg/mL) was added to each well under sterile conditions, and the 96-well plates were incubated for 4 h at 37 °C. Supernatants were removed, and dimethyl sulfoxide (100 µl/well) was added. The plates were then agitated on a plate shaker for 5 min. The absorbance of each well was measured at 490 nm with a Bio-Rad automated EIA analyzer (Bio-Rad Laboratories, Hercules, CA, USA). The viability of CTRL cells was considered 100%, while the other conditions were expressed as percentages of CTRL.

4.1.1.6 mRNA extraction and quantification

Culture supernatants were collected, and Maxwell RSC Viral Total Nucleic Acid purification kit was used to extract RNA from 250µl of cell culture supernatants employing the Maxwell RSC Instrument (Promega, WI, USA). Each well was then thoroughly washed three times with pre-warmed PBS. Cells were lysed and collected in 100 µl of RNAzol (TEL-TEST Inc., TX, USA). RNA extraction was performed employing the acid guanidium-phenol-chloroform (AGPC) extraction method, as

elsewhere described [74]. Finally, RNA was reverse-transcribed and amplified by OneStep MMix (Promega, WI, USA) on a CFX Opus real-time thermocycler (Bio-rad, CA, USA). cDNA quantification for IFITM1 (- F 5'-TCTTGAACTGGTGCTGTCTGG-3'; R 5'-ACTTGGCGGTGGAGGCATAG-3'), IFITM3 (F 5'-ACTGGGATGACGATGAGCA-3'; R 5'-AGCATTCGCCTACTCCGTGA-3'), MxA (F 5'- CCAGAGGCAGGAGACAATCAG-3'; R 5'-TCTTCGGTGGAACACGAGGT-3'), TH (F 5'- CGACCCTGACCTGGACTTGGA-3'; R 5'-GGCAATCTCCTCGGCGGTGT-3'), VMAT2 (F 5'- CCATTGCGGATGTGGCATTT-3'; R 5'-TCTTCTTTGGCAGGTGGACTT-3') (Sigma Aldrich, MI, USA), S100B (Assay ID: qHsaCED0045890), DDC (Assay ID: qHsaCED0037636), DAT (Assay ID: qHsaCID0006207) (Bio-rad, CA, USA), SARS-CoV-2 N1 (F 5'-CAATGCTGCAATCGTGCTAC-3'; R 5'-GTTGCGACTACGTGARGAGG-3') and N2 (F 5'-GCTGCAACTGTGCTACAACT-3'; R 5' TGAACTGTTGCGACTACGTG-3') (IDT, IA USA) was analyzed as ΔΔCt and presented as relative ratio between the target gene and the GAPDH housekeeping mRNA.

4.1.1.7 Protein determination

Protein concentration of samples was assessed with the DC™ protein assay kit according to manufacturer's instructions, using bovine serum albumin at different concentrations as standard.

4.1.1.8 Immunoblotting

Immunoblotting for DA neurons total cell lysates were performed using standard protocols. Aliquots of proteins were mixed with Laemmli buffer (0.15 M DTT, 94 mM Tris-HCl pH 6.8, 15% glycerol, 3% w/v SDS, 0.015% blue bromophenol) and heated for 5 min at 95°C. Proteins were separated on 4–20% polyacrylamide gradient gels and transferred to PVDF membranes by electroblotting. PVDF membranes were incubated in blocking solution (5% non-fat dry milk (w/v) in TBS-0.1% tween-20 (v/v)) at 23 °C for 1 h under gentle shaking. Subsequently, PVDF membranes were incubated overnight at 4 °C with primary antibodies diluted in blocking solution. The day after, PVDF membranes were incubated for 1 h at 23 °C with secondary HRP-conjugated antibodies diluted in blocking solution. PVDF were scanned using the chemiluminescence system Alliance Mini HD9 (Uvitec, Cambridge, United Kingdom) and band intensity was quantified using ImageJ software (v2.1.0/1.53c). The following primary antibodies were used for immunoblotting: monoclonal mouse anti-TH (dilution: 1:2500; RRID: AB_628422), polyclonal rabbit anti-MAP2 (dilution: 1:1000; RRID: AB_10693782), monoclonal mouse anti-Tau (dilution: 1:1000; RRID: AB_10695394), polyclonal rabbit anti-GAPDH (dilution: 1:10000; RRID: AB_796208), monoclonal mouse anti-calnexin (dilution:1:1000; RRID: AB_397884). The following secondary antibodies were used: Goat-

anti-rabbit HRP-conjugated (1: 2 000; RRID: AB_2099233) and Goat-anti-mouse HRP conjugated (dilution: 1: 2 000; RRID: AB_228307).

4.1.1.9 Immunocytochemistry

Cells were seeded on coverslips in a 24-well. 48 h post infection assay, SARS-CoV-2 infected and not infected cells were fixed in PBS containing 4% paraformaldehyde (PFA) at RT for 10 min, followed by permeabilization with 0,1% TritonX-100 in PBS for 10 min. Cells were treated with PBS 1% BSA for blocking at RT for 1h, and incubated with primary antibodies: anti-N Nucleocapsid SARS-CoV-2 antibody (cell signalling, #33717), 1:400; MAP2 (cell signalling, #4542), 1:1000; GLUK2 (abcam, ab66440), 1:200; Pax6 (DSHB, AB_528427), 1:250; Sox2 (Millipore, AB5603), 1:500; TH (R&D, MAB7566), 1:100; Nestin (Cell signalling, #33475), 1:100; GABA (Sigma, A2129), 1:500, at 4°C o.n. and stained with secondary antibodies (Alexa Fluor 488, 586 or 647, 1:500, abcam, Cambridge, UK) for 1h at RT. Coverslips were mounted using a mounting medium with DAPI (Enzo Life Sciences, Milan, Italy). Confocal imaging was performed with a Leica TCS SP8 System equipped with a DMi8 inverted microscope and a HC PL AP0 40x/1.30 Oil CS2 (Leica Microsystems, Wetzlar, Germany) at a resolution of 1024x1024 pixels.

4.1.1.10 Dopamine measurement

Total dopamine levels in the cell lysates and in the culture medium were quantified using a direct competitive chemiluminescent enzyme-linked immunosorbent assay (ELISA) dopamine kit (Catalog number: EU0392, Fine Test®, Wuhan, China) according to the manufacturer's instructions. Absolute values were obtained based on a standard curve and expressed as ng of dopamine/ ml/ mg proteins.

4.1.1.11 Statistical analyses, graphs and images

For the study variables, medians and ranges were reported for quantitative variables. T test and ANOVA were used with a *p* value threshold of 0.05. The analyses were performed using GraphPad Prism 8. Graphs and images were assembled by GraphPad Prism 8 and Biorender.com, respectively. All experiments were confirmed in 3 independent replicates (n=3) and all the procedures were carried out in accordance with the GLP guidelines adopted in our laboratories.

4.1.2 Results

4.1.2.1 SARS-CoV-2 infection of dopaminergic neurons

First, we proceeded with the characterization of the dopaminergic neurons in culture (Fig. 1). As depicted, we obtained 100% of dopaminergic (DA) neurons, according to the TH and MAP2 staining. In addition, as shown in Fig. 2 these neurons present a low expression of SOX 2 pluripotency marker and of the neuronal precursor markers PAX6 and Nestin. We found a scant staining of GLUK2 and GABA, suggesting a minor glutamatergic and GABAergic commitment.

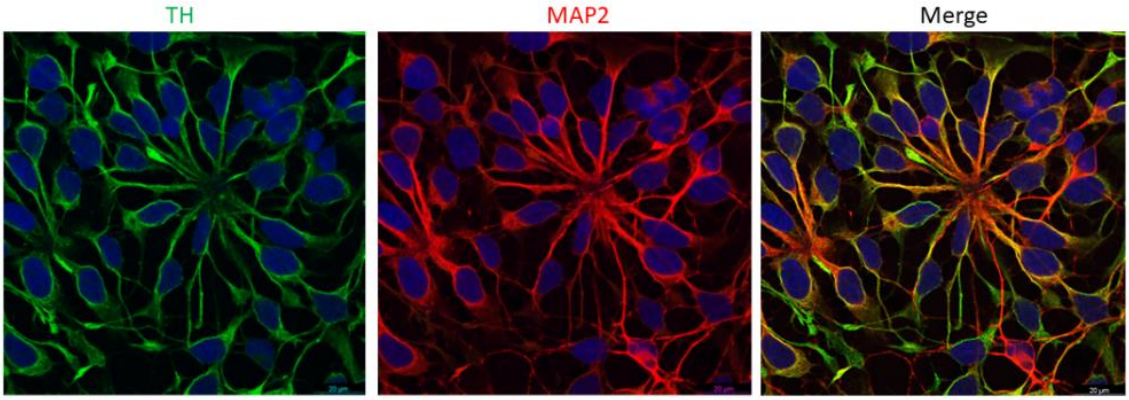


Figure 1. Human iPSC-derived dopaminergic neurons. ICC of DA neurons at day 30 of culture. Cells were stained for TH (green) and MAP2 (red). Nuclei were stained in blue. Images were acquired with a 40x magnification.

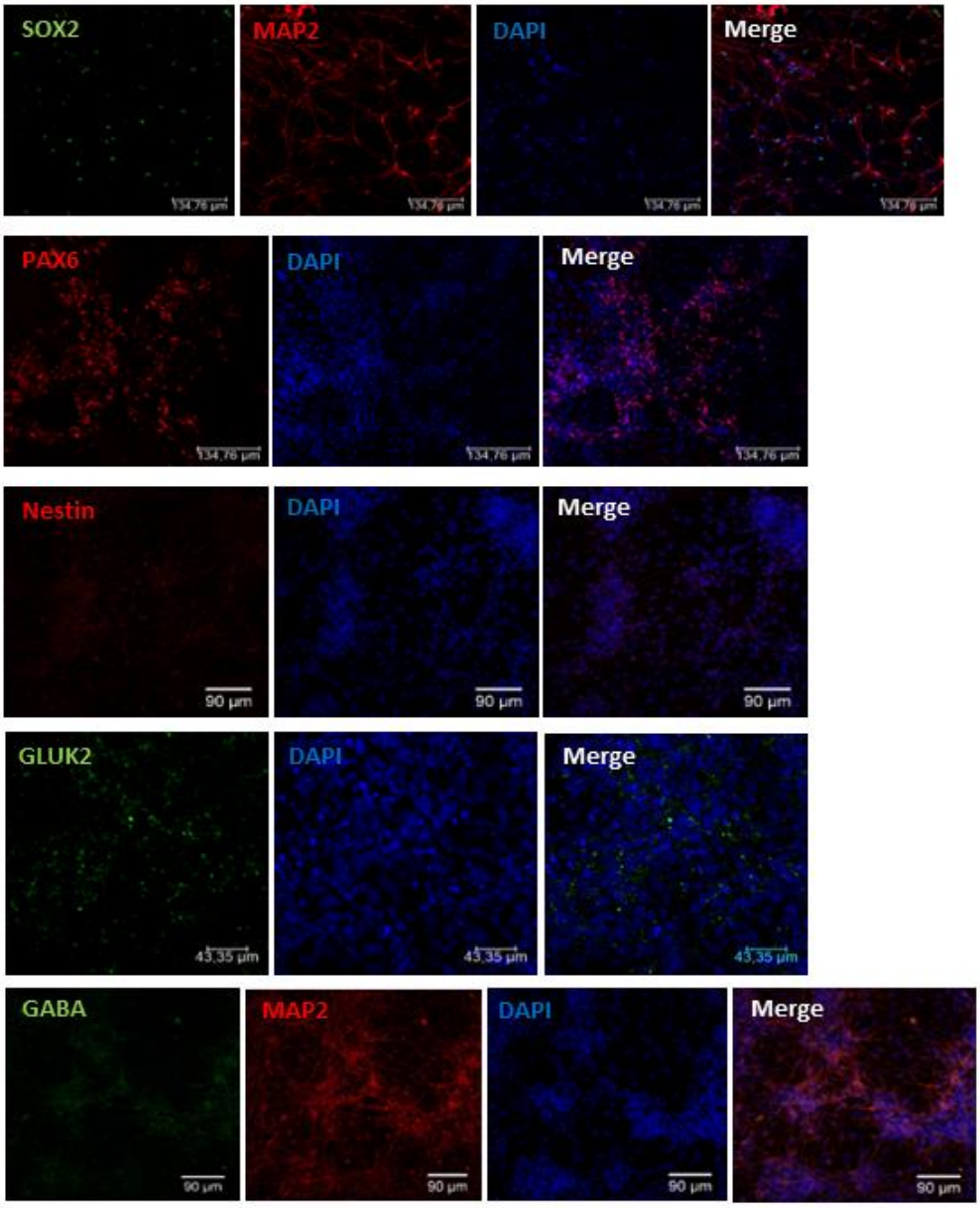
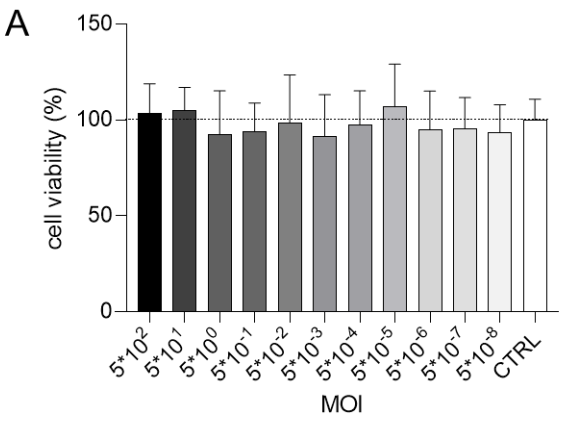


Figure 2. ICC of DA neurons at day 30 of differentiation. Cells were stained for Sox2, Gluk2, and GABA in green, and for MAP2, Pax6, and Nestin in red. Nuclei were stained with Hoechst and are visualized in blue. Images were obtained using Nikon Eclipse 90i Ni microscope at 200x magnification.

Then, we exposed the DA neurons to different concentrations of EU SARS-CoV-2, ranging from 5x102 to 5x10-8 MOI. At 96 hpi we detected no significant changes in cell viability compared to the uninfected control, by the means of an MTT assay (Fig. 3A). Similar results were obtained for Delta or Omicron SARS-CoV-2 variants (Fig. 4).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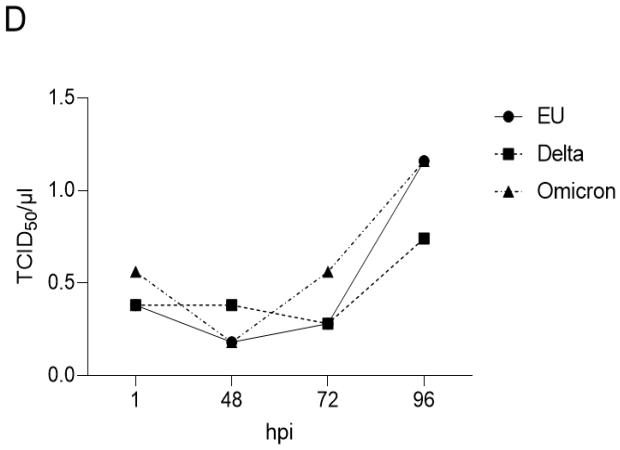
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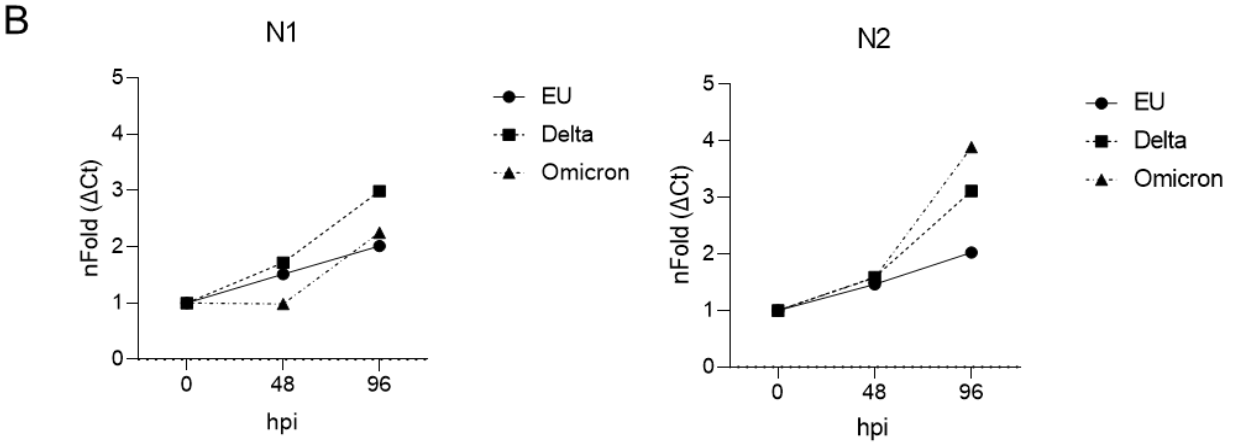
B
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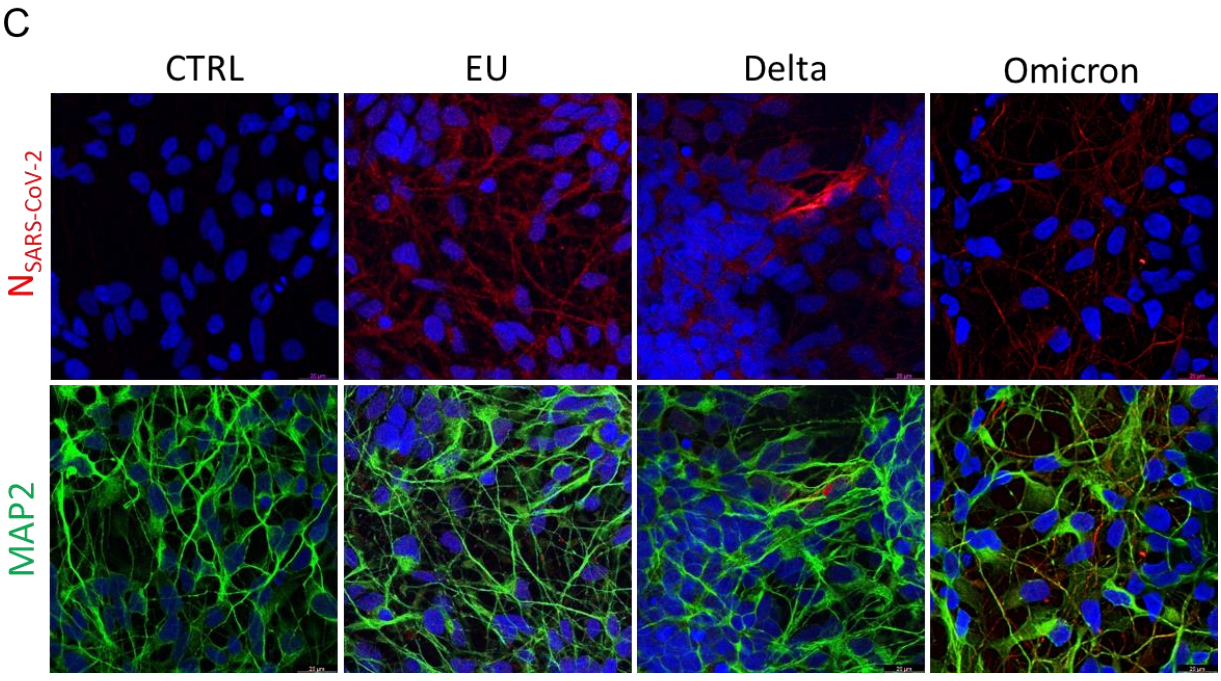

C
CTRL
EU
Delta
Omicron
NSARS-CoV-2
MAP2

Figure 3. ***In vitro*** **SARS-CoV-2 infection of human iPSC-derived dopaminergic neurons. Panel A)** MTT viability assay at 96 hpi with EU SARS-CoV-2. Viral concentrations range from 5*102 to 5*10-8 on a 1:10 dilution basis. Data are shown as percentage of the uninfected control. **Panel B)** Upon in vitro challenge of DA neurons with 5 MOI of SARS-CoV-2 (EU, Delta or Omicron), the infection was monitored at 0, 48 and 96 hpi. Real-time PCR for N1 (left, Anova time factor $p \leq 0.02$) and N2 (right, Anova time factor $p \leq 0.02$) viral genes was performed. Results are shown as nFold (ΔCt). **Panel C)** ICC of DA neurons at 96 hpi with 5 MOI of EU, Delta or Omicron SARS-CoV-2 variants, or mock infected (DAPI in blue, NSARS-CoV-2 in red, MAP2 in green). **Panel D)** Titration of progeny SARSCoV- 2 virus (EU, Delta or Omicron variants) in the DA neurons culture supernatant harvested at 0, 48, 72 and 96 hpi. Data are shown as TCID50/µl (Anova time factor $p \leq 0.005$).

Overtime, we could detect by real-time PCR a modest but steady increase of SARS-CoV-2 N1 and N2 RNA in DA neurons exposed to 5 MOI of SARS-CoV-2 (EU, Delta or Omicron) (Fig. 3B). The SARS-CoV-2 infection in DA neurons was confirmed by immunocytochemistry (ICC) at 96 hpi, by detecting the viral protein N at the intracellular level (Fig. 3C). We observed sporadic N2 positive DA neurons upon infection with 0.5 MOI as well (Fig. 5).

In order to test whether the progeny virus could be infectious, we collected the supernatants of the infected DA neurons throughout the 96-hour culture. Such supernatants were then tested by TCID50 assay on VeroE6 cells (Fig. 3D). Results show a modest infectious ability of the progeny virus for all the three SARS-CoV-2 variants considered, EU, Delta and Omicron, reaching at 96 hpi 1.16, 0.74 and 1.16 TCID50/µl, respectively.

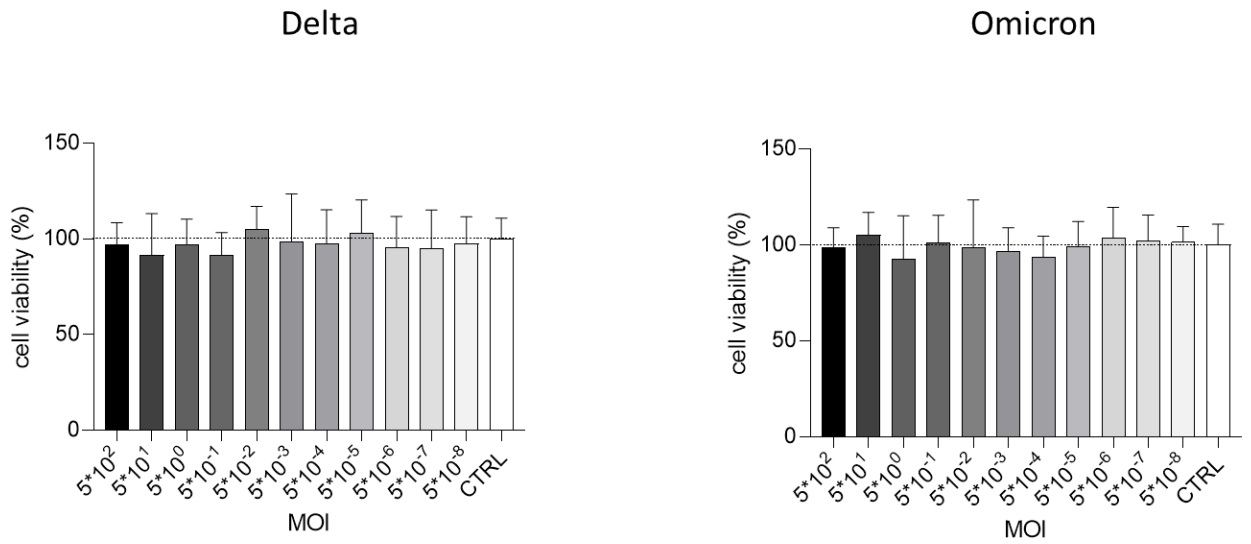


Figure 4. MTT viability assay at 96 hpi with Delta and Omicron SARS-CoV-2. Viral concentrations range from 5*102 to 5*10-8 on a 1:10 dilution basis. Data are shown as percentage of the uninfected control.

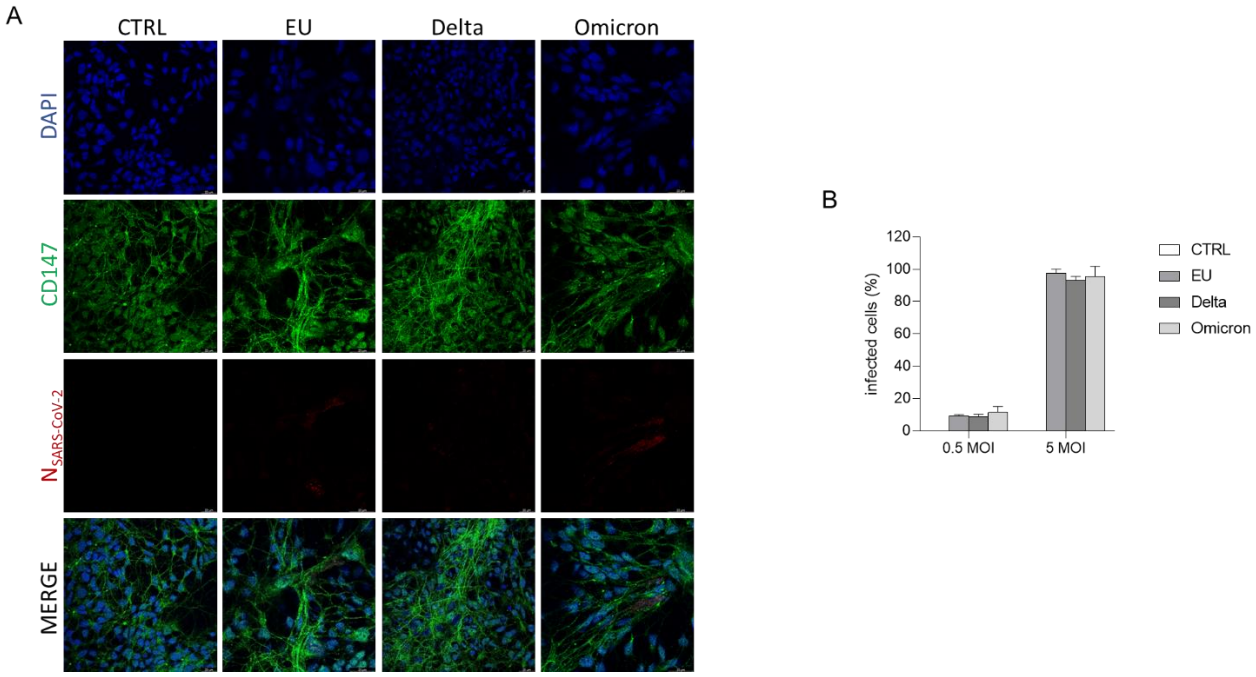


Figure 5. A) ICC of DA neurons at 96 hpi with 0.5 MOI of EU, Delta or Omicron SARS-CoV-2 variants, or mock infected (DAPI in blue, NSARS-CoV-2 in red, MAP2 in green). **B)** Percentages of SARS-CoV-2 positive cells are shown for *in vitro* infected DA neurons, challenged with 0.5 or 5 MOI (or mock - CTRL) EU, Delta or Omicron SARS-CoV-2 variants. Percentages were calculated on ICC images (n=3).

4.1.2.2 Neuronal stress markers

DA neurons infected with 5 MOI of SARS-CoV-2 (EU) were assessed for innate immunity or stress markers 48, 72, 96 hpi. By real-time PCR, we measured the mRNA expression of different virus specific intracellular response genes (IFITM1, IFITM3 and MxA) and a neuronal stress-related marker (S100B) (Fig. 6A). While IFITM1 was not regulated by SARS-CoV-2 infection, we observed IFITM3, MxA and S100B to be upregulated over time (p=0.0139, p=0.0002, p<0.0001) reaching a significant increase at 96 hpi. Therefore, we tested also the effect of Delta and Omicron variants in these very same conditions. As shown in Fig. 6B the only observed effect was an upregulated expression for MxA upon infection of DA neurons with the Delta variant. By immunoblotting analyses, the expression of MAP2 and Tau protein was evaluated in SARS-CoV-2 infected cells at 96 hpi. Both of them were found to be decreased by EU- SARS-CoV-2 infection (p=0.0191 and p=0.0158, respectively) (Fig. 6C), but not by Delta or Omicron- SARS-CoV-2 infection (Fig. 7 A and B, respectively). The data was supported also by immunocytochemistry against MAP2, which showed a fragmented staining in neurons infected by EU- SARS-CoV-2 (Fig. 8). These data suggest that at 96 hpi with SARS-CoV-2- EU dopaminergic neurons undergo neuronal stress.

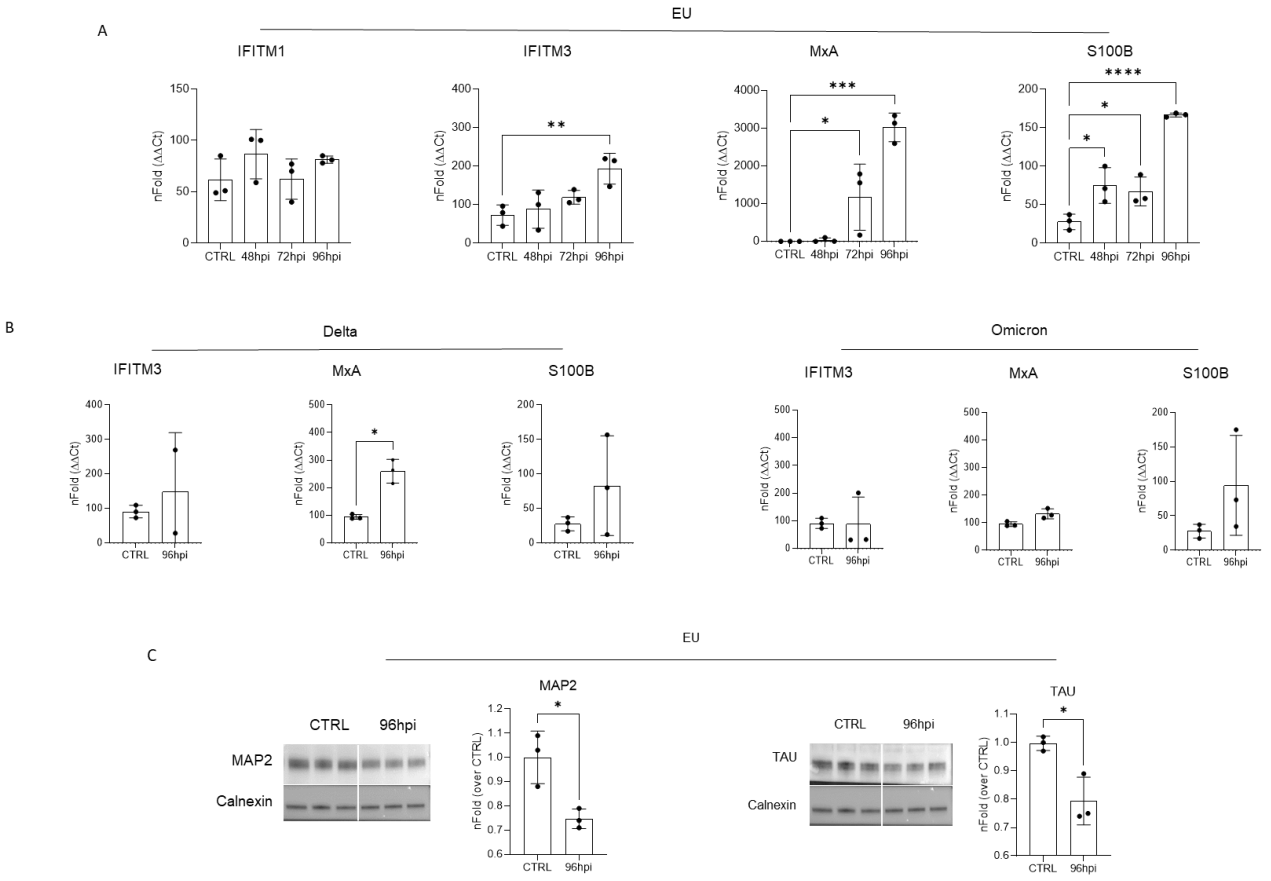


Figure 6. Effect of SARS-CoV-2 variants on antiviral and neuronal stress markers. Panel A) Real-time PCR expression analyses, expressed as nFold (ΔΔCt), of the antiviral innate neuronal immune response markers MxA, IFITM1 and IFITM3 and the neuronal stress marker S100B, triggered by the EU SARS-CoV-2 variant at 48, 72 and 96 hours post infection (hpi). **Panel B)** Realtime PCR expression analyses of MxA, IFITM3 and S100B triggered by the Delta (left) and Omicron (right) SARS-CoV-2 variant at 96 hpi. **Panel C)** Western blot analyses of the neuronal marker MAP2 and TAU in DA neurons infected by the EU SARS-CoV-2 variant at 96 hpi. Band intensity of each antigen was normalized over calnexin and expressed as fold change over the control. Statistical significance was calculated by multiple comparison test or by Student's T test, where appropriate. * ≤0.05; ** ≤0.01; *** ≤0.005; **** ≤0.0001 (n=3).

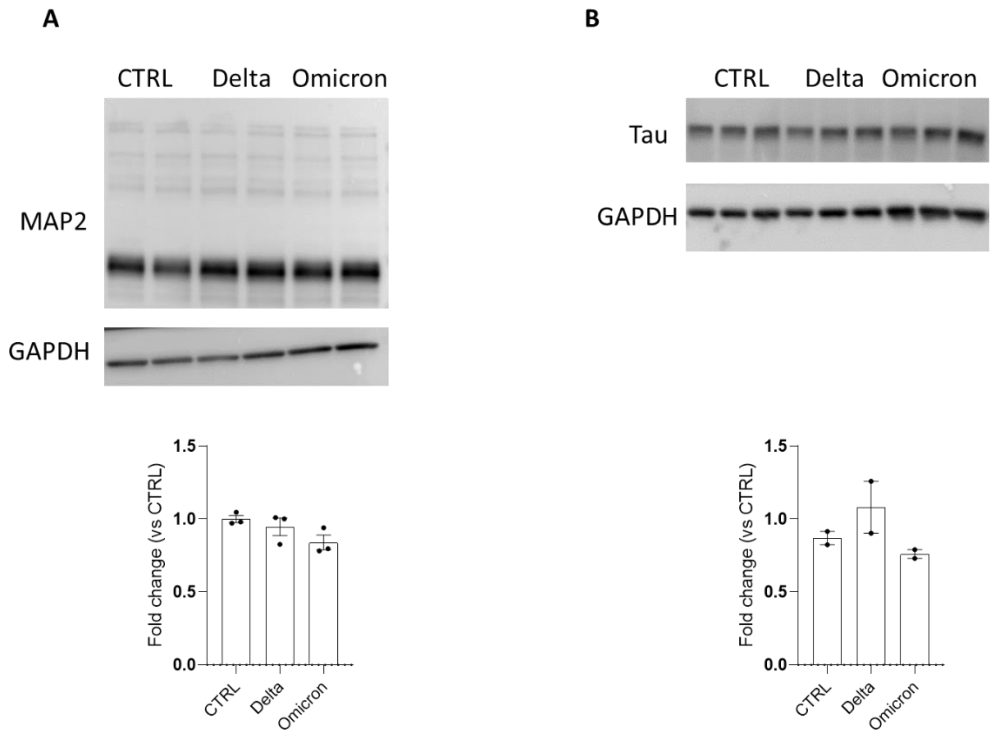


Figure 7. Western blot analyses of the neuronal marker MAP2 and TAU (**panel A** and **B**, respectively) in DA neurons infected by the Delta and the Omicron SARS-CoV-2 variants at 96 hpi. Band intensity of each antigen was normalized over calnexin and expressed as fold change over the control.

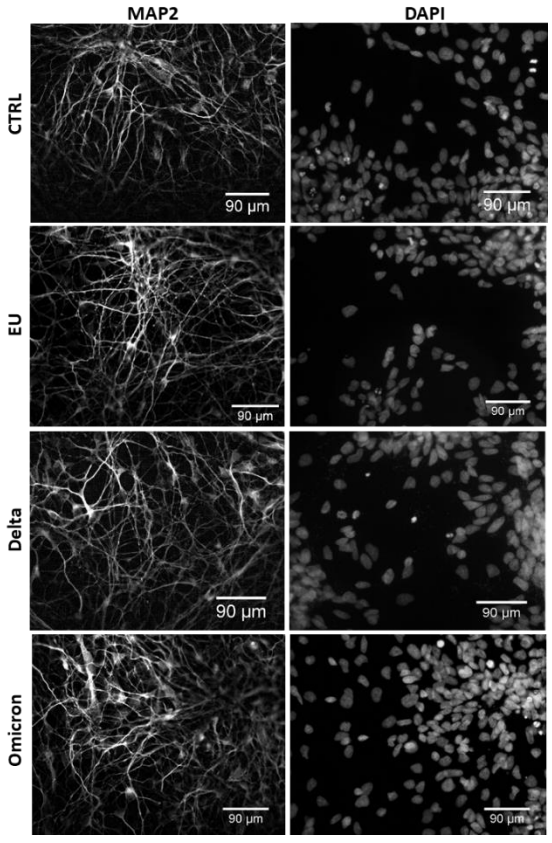


Figure 8. ICC of DA neurons infected by the EU, Delta or Omicron SARS-CoV-2 variant at 96 hpi. Neurons were stained for MAP2 and nuclei were stained with Hoechst. Images were obtained using Nikon Eclipse 90i Ni microscope at 200× magnification.

4.1.2.3 Dopamine metabolic pathway

Considering the symptomatology manifested by COVID-19 patients, which seems to involve the dopaminergic tone, we focused our attention on the dopamine metabolic pathway. First, we measured by ELISA the intracellular dopamine content (Fig. 9A) and the amount secreted in the extracellular environment (Fig. 9B). In addition, we evaluated if the SARS-CoV-2 effect on dopamine production might be virus-dose or variant dependent. In order to do so, we challenged DA neurons with 0.05, 0.5 or 5 MOI of SARS-CoV-2 EU, Delta, or Omicron variants. Results at 96 hpi show that both the EU and the Delta SARS-CoV-2 variants were able to hamper the dopamine production and secretion, while no significant effect was detected in Omicron-infected neurons. In particular, for the EU and Delta SARS-CoV-2 variant the dopamine content inversely correlates with the MOI of virus employed (Anova $p \leq 0.02$ and $p \leq 0.01$, respectively).

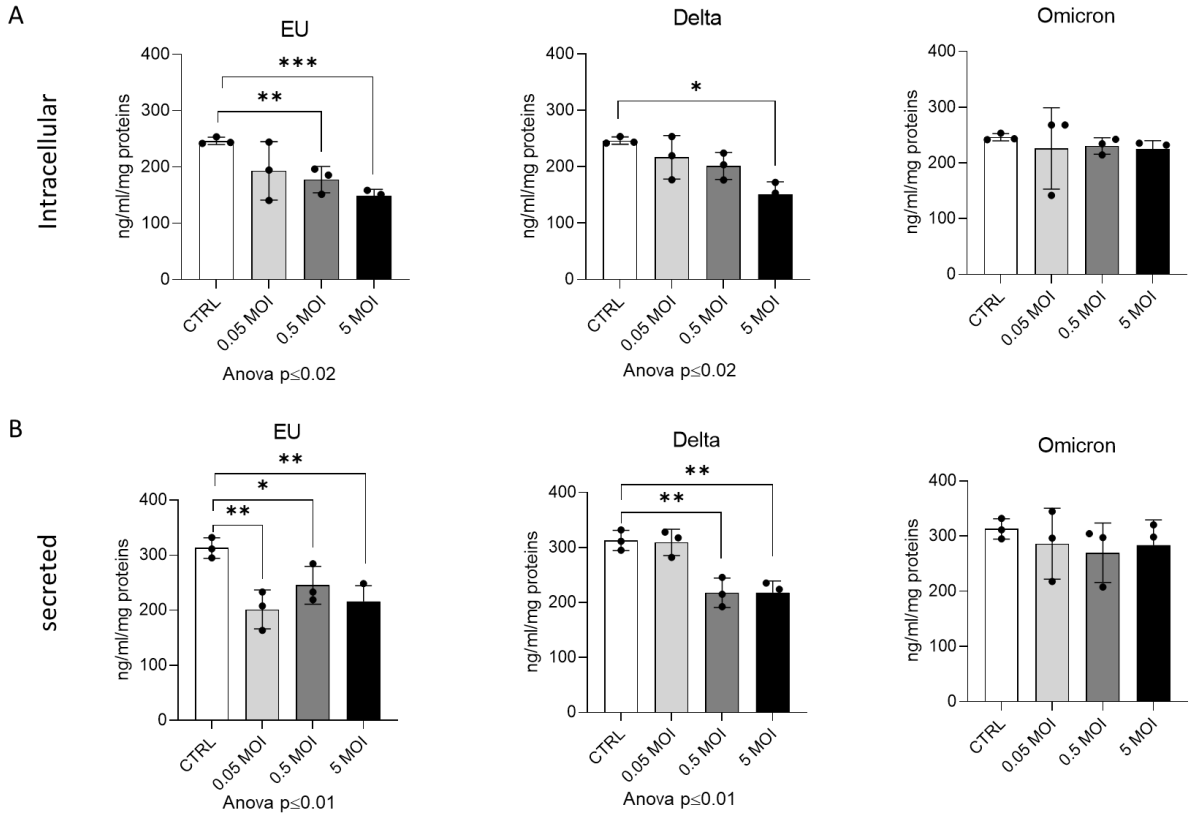


Figure 9. Effect of SARS-CoV-2 variants on dopamine production. Panel A) Intracellular dopamine quantification by ELISA upon challenge with 0.05 MOI, 0.5 MOI, and 5 MOI of EU (Anova $p \leq 0.02$) (left), Delta (middle) (Anova $p \leq 0.02$), and Omicron (right) SARS-CoV-2 variants, or the uninfected control, at 96 hpi. **Panel B)** Secreted dopamine quantification by ELISA upon challenge with 0.05 MOI, 0.5 MOI, and 5 MOI of EU (left), Delta (middle) (Anova $p \leq 0.001$), and Omicron (right) SARS-CoV-2 variants, or the uninfected control, at 96 hpi. The depicted statistical significance is relative to multiple comparison t-test only. * ≤0.05; ** ≤0.01; *** ≤0.005 (n = 3).

On the other hand, the reduction in dopamine secretion for the EU- SARS-CoV-2 variant was not virus-dose dependent, whereas for the Delta- SARS-CoV-2 variant (Anova $p \leq 0.001$), we observed a decrease of about 30% only upon the infection with 5 or 0.5 MOI. To better investigate this aspect, 96 hpi we evaluated the protein expression and the mRNA levels of the main players involved in dopamine metabolism (Fig 10). The protein levels of tyrosine hydroxylase (TH), the enzyme that converts tyrosine to DOPA, resulted strongly reduced upon infection with both EU and Delta variants ($p \leq 0.05$ and $p \leq 0.005$, respectively) (Fig. 10A). Conversely, in DA neurons, the mRNA expression of TH was significantly upregulated ($p<0.0001$) only upon infection with 5 MOI of SARS-CoV-2- EU and Delta (Fig. 10B).

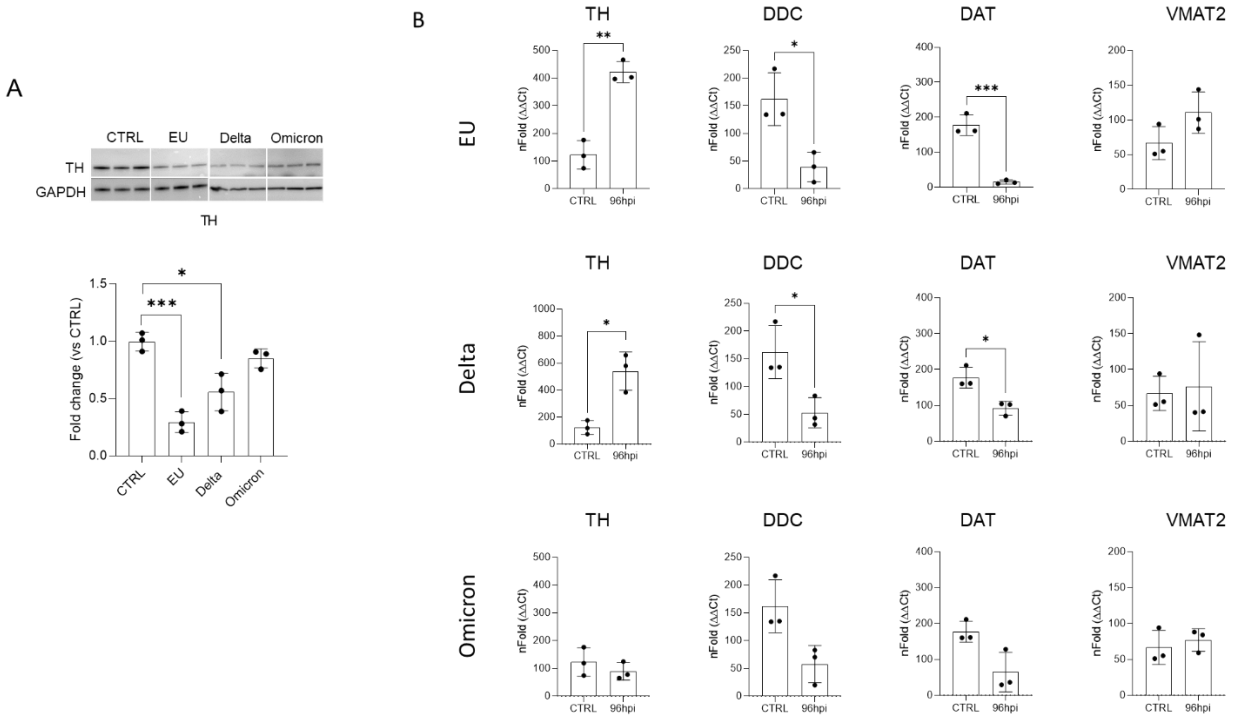


Figure 10. Effect of SARS-CoV-2 variants on the dopamine biosynthetic pathway. Panel A) Western blot analyses of TH upon challenge with EU, Delta and Omicron SARS-CoV-2 variants at 96 hpi. Band intensity of each antigen was normalized over GAPDH and expressed as fold change over the control (Anova $p \leq 0.0002$). **Panel B**) Real-time PCR expression analyses, expressed as nFold (ΔΔCt), of the tyrosine hydroxylase (TH), the DOPA decarboxylase (DDC), the dopamine transporter (DAT) and the vesicular monoamine transporter 2 (VMAT2) mRNA, upon challenge with the EU (top), Delta (middle) and Omicron (bottom) SARS-CoV-2 variant at 96 hpi. Statistical significance was calculated by Student's t-test. * ≤0.05; ** ≤0.01; *** ≤0.005 (n = 3).

Moreover, the infection with EU and Delta variants determined the downregulation in the mRNA expression of two key molecules of dopamine metabolism, the DOPA decarboxylase (DDC; $p \leq 0.05$) and the dopamine transporter (DAT; $p \leq 0.05$) (Fig. 10B). In addition, the mRNA level of the vesicular monoamine transporter 2 (VMAT2) displayed a modest increase only upon SARS-CoV-2- EU

infection (Fig. 10B). Any change was observed upon infection with Omicron SARS-CoV-2 (Fig. 10B).

To further assess whether the effect of SARS-CoV-2 on DA neurons was virus-specific or generally stress-related, we challenged these cells with other stimuli, such as LPS, the respiratory syncytial virus (RSV), or the heat-inactivated EU SARS-CoV-2 (iSARS). Any of these stimuli is able to significantly modulate the antiviral response (Fig. 11A) or to affect the dopamine metabolism (Fig. 11BC).

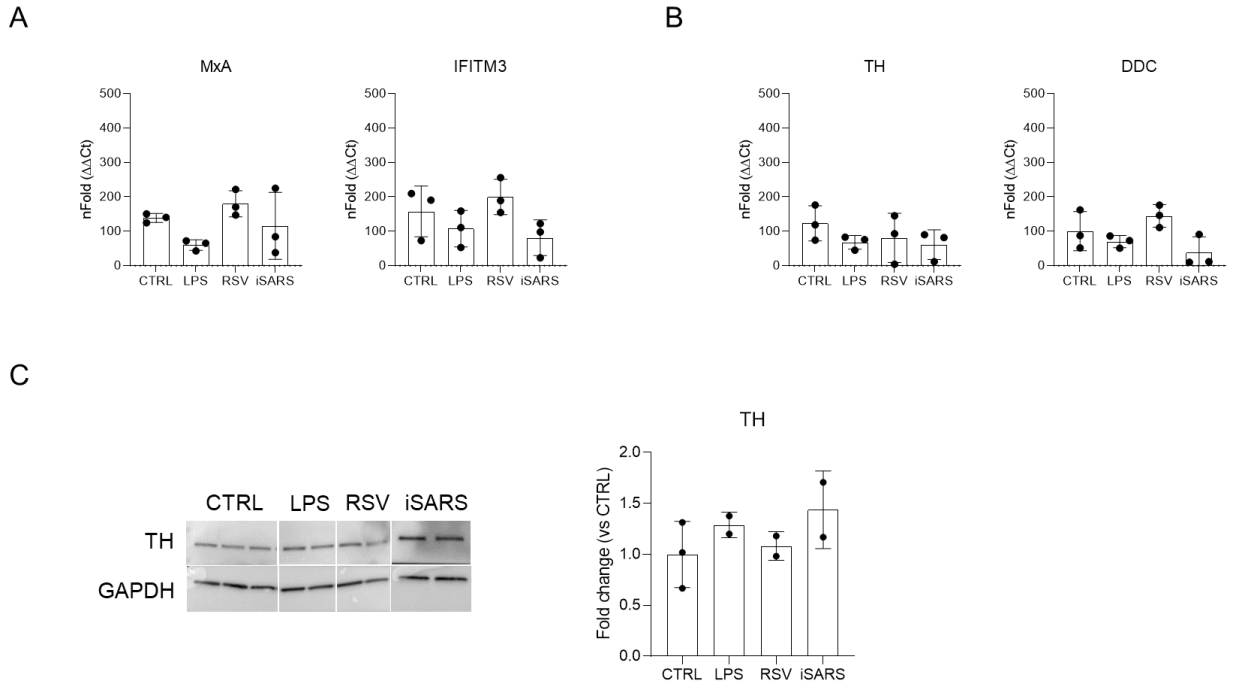


Figure 11. Effect of other stimuli on the antiviral response and the dopamine pathway. Panel A and B) Real-time PCR expression analyses, expressed as nFold (ΔΔCt), of MxA, IFITM3, TH and DDC upon challenge of DA neurons with LPS, and heat-inactivated SARS-CoV-2 (iSARS). **Panel C**) Western blot analyses of TH upon challenge of DA neurons with LPS, RSV and iSARS. Band intensity of each antigen was normalized over GAPDH and expressed as fold change over the control. Statistical significance was calculated by Student's t-test. (n = 3).

4.1.3 Discussion

The neurotropic effect of viruses represents an important item of virology, since the infection of an immune privileged site as the brain could have both short and long- term severe consequences, due to the post- mitotic stage of neurons. Although several viruses are reported to infect neurons, scant is the information related to the family Coronaviridae. Retrospective analyses on patients infected by H1N1 virus during the influenza pandemic of 1918, report an increased prevalence of lethargic encephalitis associated with Parkinsonism [336, 337]. Arboviruses are known to affect neurons and

to dampen catecholamine biosynthesis [338–340], while Herpes simplex virus specifically targets the TH [341, 342]. Emerging evidence supports also the effect of SARS-CoV-2 infection on both CNS and PNS. In particular, a large fraction of patients experiences symptoms such as post exertional malaise, brain fog, neurological sensations, headaches, memory issues, insomnia, muscles aches, dizziness, balance issues, speech issues, joint pain, sleep disturbance, anxiety and depression [195, 196, 343]. In addition, a broad spectrum of signs affecting the dopaminergic tone has been described among patients affected by COVID-19. Currently, the extent of SARS-CoV-2 infection in the brain is not well defined in humans. Despite this clinical evidence, poor is the information related to the effect of SARS-CoV-2 infection on neuronal homeostasis. In a recent paper published by Pedrosa et al. it has been demonstrated that the EU variant of SARS-CoV-2 presented a limited capability to infect neurons, whereas it showed an infectivity for astrocytes, which, by the induction of an inflammatory response, lead indirectly to neuronal damage [344]. The SARS-CoV-2 tropism for astrocytes, together with the consequent inflammatory response and cell dysfunction, was confirmed by multiple authors [345, 346]. Some of these observations were recapitulated in a mouse model, reporting an increased susceptibility to oxidative stress in DA neurons from infected mice [26]. However, the authors did not test for the actual infection of those neurons. Although Spike-bearing pseudoviruses corroborate the hypothesis of a direct infection of DA neurons [296], the SARS-CoV-2 infection of such neurons remains somehow elusive, so far. In our work we tried to address this issue exploiting the use of human DA neurons derived from iPSCs. Our differentiation protocol allows to obtain a neuronal population entirely expressing the dopaminergic marker TH. As usually occurs for iPSCs differentiation we observed also a scant expression of the neuronal precursor markers Nestin and PAX6. Nevertheless, compared to the neuronal marker MAP2 and TH, the expression level of these precursor markers is very low suggesting a late stage of maturation of DA neurons. Using this model, we found that the EU, Delta, and Omicron SARS-CoV-2 variants showed a low productive infection in DA neurons, even if administered at high MOI. Interestingly, we observed that the infection with EU and Delta SARS-CoV-2 promotes the neuronal innate immune response as demonstrated by the increased mRNA expression of MxA and IFITM3 [347–351]. Such genes are enclosed in the so-called interferon response and are known to be key players in the intracellular antiviral response, which can rely on different finely- tuned pathways. Indeed, in our model, IFITM3 and MxA were upregulated, while IFITM1 was not, underlying the specificity of the antiviral response triggered by SARS-CoV-2 in DA neurons. In addition, EU infection caused also the upregulation of the neuronal stress marker S100B, which was previously related to SARS-CoV-2 disease severity and neuronal damage [32, 270, 352]. These data clearly indicate that the infection with different variants of SARS-CoV-2 affects the homeostasis of DA neurons. Since DA neurons

are the main actors for the production of dopamine in brain, we investigated whether the SARS-CoV-2 infection affects this pathway. A proof that SARS-CoV-2 infection could affect the dopamine metabolism derives also from a recent paper by Mpekoulis G. et al. in which it has been described that the infection of non- neuronal cells determines a reduction in the mRNA expression of DDC [353, 354]. The order of magnitude that SARS-CoV-2 viral load could potentially reach in the human brain *in vivo* is currently unknown. Some hints might come from the observation that SARS-CoV-2 is endowed of a marked tropism for astrocytes [344, 346]. Andrews and colleagues detected a viral load between 104 and 105 PFU/ml as order of magnitude at 72 hpi in the supernatant of SARS-CoV-2-infected neurospheres, mainly ascribable to astrocytes [346]. Comparing this to our experimental setting, it would translate to an order of magnitude of 10-1 MOI, which is the intermediate dose among those tested. Overtime, the viral load could easily accumulate even higher. Although this does not prove the point, to our best knowledge, it is suggestive that the amount of SARS-CoV-2 employed in our experiments *in vitro* is likely to be potentially reached *in vivo*.

In our work, by titrating the progeny virus, we demonstrated that SARS-CoV-2 not only infects, but also propagates in DA neurons. Moreover, we demonstrated that SARS-CoV-2 infection of DA neurons is able to impair dopamine metabolism inducing a reduction in the production of dopamine and in its release in the extracellular environment, only upon the infection with the EU and Delta SARS-CoV-2 variants, but not with the Omicron one. We speculate that these data might relate to the clinical observation that EU or Delta -infected patients manifest the most severe neuronal symptomatology with respect to those infected by Omicron variant [355–358]. Indeed, it is currently unknown if the overall milder symptomatology observed in Omicron infected patients is due to the higher level of immunization of the population, or rather due to a reduced aggressiveness or, in this case, to a reduced neutropism of the Omicron variant. Our results suggest the latter one and are in line with those obtained on iPS-derived cortical neurons and astrocytes, or humanized mice/hamster models [359–361]. In addition, the opposite trend shown by TH mRNA and protein levels let to speculate about a compensatory mechanism activated by DA neurons in order to restore the dopamine production in a positive feedback manner. The observation of a functional deficit in DA neurons after SARS-CoV-2 infection stimulates some speculations on the potential translatability in the clinical setting. It is reasonable to hypothesize that the worsening that patients with Parkinson's disease experience during COVID-19 infection and in the subsequent period, short or long post COVID syndrome, may be associated with this functional deficit. This hypothesis could support a scenario in which Parkinson’s disease patients would have a transient worsening of symptoms, and then return to the pre-infection condition once fully recovered [362]. Further confirmations and insights of this mechanism are necessary in order to consolidate this data and guide clinicians towards an increasingly

targeted therapy for Parkinson’s disease patients suffering from COVID-19 and for patients suffering from post-COVID syndrome.

4.2 II. Human motor neurons derived from induced pluripotent stem cells are susceptible to SARS-CoV-2 infection.

Cappelletti G, Colombrita C, Limanaqi F, Invernizzi S, Garziano M, Vanetti C, Moscheni C, Santangelo S, Zecchini S, Trabattoni D, Silani V, Clerici M, Ratti A, Biasin M. Front Cell Neurosci. 2023 Dec 5;17:1285836. doi: 10.3389/fncel.2023.1285836. PMID: 38116398; PMCID:PMC10728732.

4.2.1 Materials & Methods

4.2.1.1 Cell Lines and Culture

VeroE6 cells (ATCC, VA, USA) and Human Lung Carcinoma Cells Expressing Human Angiotensin-Converting Enzyme 2 (A549-hACE2) (BEI Resources, Catalog No. NR-53821) were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Euroclone, Milan, Italy) containing 4 mM L-glutamine, supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C and 5% CO_2. Cells were regularly passaged and tested for the presence of mycoplasma contamination.

4.2.1.2 iPSC generation and MN differentiation

Induced Pluripotent Stem Cells (iPSC) were reprogrammed from 3 healthy donors (Table 1) after obtaining informed consent and approval from local ethics committee (approval number 2022_03_15_12). For fibroblast/blood cell reprogramming the CytoTune®-iPSC 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific) was used as previously described [363]. After picking and selecting about 6 clones per sample, one clone for each cell line was further grown in Essential 8 medium (Thermo Fisher Scientific) and fully characterized for the expression of stemness markers (TRA-1-60, OCT3/4, SOX2, NANOG) and for the capacity to spontaneously differentiate into the three germ layers lineages as assessed by the expression of alpha-feto protein (AFP) as endodermal, βIII tubulin as ectodermal and desmin as mesodermal markers and already shown in [364, 365]. Genome integrity of the iPSC clones was determined by Q-banding karyotype analysis [365].
iPSCs were differentiated into motoneurons (MNs) as previously described [363]. Briefly, iPSCs were grown in suspension for 21 days to obtain embryoid bodies (EBs) in HuES medium (DMEM/F12, 20% knock-out serum replacement, 2 mM L-glutamine, 10 U/ml penicillin, 10 µg/ml streptomycin, 0.1 mM MEM NEAA, 110 µM β-mercaptoethanol) for the first 3 days and then in neural induction medium (DMEM/F12, 2 mM L-glutamine, 10 U/ml penicillin, 10µg/ml streptomycin, 0.1 mM MEM NEAA, 2µg/ml heparin, 1% N2 supplement), supplemented with

specific factors. EBs were dissociated with 0.05% trypsin and cells were plated on poly-D-lysine/laminin-coated (Thermo Fisher Scientific) coverslips and cultured in neural differentiation medium (Neurobasal medium, 2 mM L-glutamine, 10 U/ml penicillin, 10 µg/ml streptomycin, 0.1 mM MEM NEAA, 1% N2 supplement, all from Thermo Fisher Scientific) with the addition of specific factors for 13 days to obtain iPSC-MNs.

4.2.1.3 In vitro SARS-CoV-2 infection assay

The European (EU - B.1) SARS-CoV-2 lineage was a kind gift of Dr. Davide Mileto, Clinical Microbiology, ASST Fatebenefratelli-Sacco, Milan, Italy. All the experiments with SARS-CoV-2 were performed in a BSL3 facility.

In order to generate a viral stock, SARS-CoV-2 was expanded in VeroE6 cells and infectious viral particles concentration was assessed by 50% tissue culture infectious dose (TCID50) assay, as elsewhere described [333].

iPSC-MNs were *in vitro* Mock- or SARS-CoV-2-infected with 1 multiplicity of infection (MOI). After an overnight incubation, cells were thoroughly washed three times with pre-warmed PBS and replenished with the complete neural differentiation medium. Supernatants were collected at 6 (T0), 24 (T1), 48 (T2) and 72 (T3) hours post infection (hpi) to monitor viral replication and to perform SARS-CoV-2 infection assays on VeroE6 cells for each iPSC-MN line (Table 1).

At 48 hpi, iPSC-MNs were fixed for immunofluorescence (IF) analyses, while cells harvested at 72 hpi were lysed for RNA extraction and appropriately stored at -80°C for further processing, as specified below.

At 72 hpi, cell viability was assessed by Trypan Blue exclusion assay. Briefly, iPSC-MNs were incubated in Accutase (Thermo Fisher Scientific) for 5 min at 37 °C. Then, an equal volume of fresh medium was added to the wells to stop the dissociation reaction and the cells were detached and centrifuged for 8 min at 1200 rpm. The supernatant was carefully discarded, and cells were resuspended in 1 mL of fresh medium. Ten µl of cell suspension were incubated with 10 µl of 0.4% Trypan Blue (Merck-Sigma, Milan, Italy) in 96-well plates. Ten µl of the mix were loaded on chamber slides and counted with the T20 Automated Cell Counter (Bio-Rad Laboratories, Hercules, CA, USA).

	Sex	Age	Real-time PCR for N1 and N2 viral genes	TCID50 on VERO E6 cells	Gene expression analysis by Real-Time PCR	Quantigene assay	IF	Trypan Blue exclusion assay
S1	M	37	n=5	n=1	n=5	n=2	n=5	n=5
S2	F	45	n=5	n=1	n=5	-	n=4	n=5
S3	F	49	n=4	n=1	n=4	n=1	n=4	n=4

S= subject

Table 1. Number of independent replicates performed for each assay with the iPSC-MN lines obtained from 3 healthy control subjects already described in *Gumina et al.*,[364] (S1 and S2) and in *Bossolasco et al.*, [365](S3). Features of the enrolled subjects (sex, age) are also reported.

4.2.1.4 Viral replication assessment

For SARS-CoV-2 replication assessment, RNA was extracted from iPSC-MN supernatants using the Maxwell® RSC Instrument with Maxwell® RSC Viral Total Nucleic Acid Purification Kit (Promega, Fitchburg, WI, USA). Viral RNA was reverse transcribed in a single-step RT-qPCR (GoTaq 1-Step RT-qPCR; Promega) on a CFX96 instrument (Bio-Rad, Hercules, CA, USA) using primers specifically designed to target two regions of the nucleocapsid (N1 and N2) gene [142] (2019-nCoV CDC qPCR Probe Assay emergency kit; IDT, Coralville, IA, USA), together with primers for the human RNase P gene. Viral copy number quantification (viral copy number/ml) was assessed by creating a standard curve from the quantified 2019-nCoV_N positive Plasmid Control (IDT).

VeroE6 cells were *in vitro* infected with supernatants collected from SARS-CoV-2-infected iPSC-MNs at different time points (24, 48, 72 hpi) and infectious viral particles concentration was assessed by TCID50, as previously described. Briefly, VeroE6 cells were seeded at 2×10^4 cells per well in a 96-well plate and cultured with serial dilutions (1:3) of the iPSC-MN supernatants collected at different time points. After 72 hpi, VeroE6 cell supernatants were removed, cells fixed by 4% paraformaldehyde (PFA - Sigma-Aldrich, MO, USA) for 1 h at RT and then stained with 0.2% crystal violet solution (Sigma-Aldrich) to assess cell death and to calculate TCID50.

4.2.1.5 QuantiGene Plex Gene Expression Assay

SARS-CoV-2 infection was further assessed on $5x10^4$ iPSC-MNs by QuantiGene Plex assay (Thermo Scientific), which uses signal amplification rather than target amplification for direct measurement of RNA transcripts directly from lysed cells. The following SARS-CoV-2 viral genes were analyzed: *ORF7A, ORF3A, ORF8, RDRP, E* and *N.* Signal was detected using a Luminex instrument and results were calculated relative to GAPDH, and PPIB as housekeeping genes, and expressed as ΔCt.

4.2.1.6 Gene expression analyses

Total RNA was extracted from iPSC-MNs as previously described by Limanaqi *et* al., [354]. Gene expression analyses of the main SARS-CoV-2 receptors (ACE2, CD147, NRP1) and peptidases (FURIN, TMPRSS2) as well as N (nucleocapsid)1, N2, S (spike)1, S2 and E (envelope)1 SARS-CoV-2 sequences was performed by Real-time qPCR (CFX96 connect, Bio-Rad, Hercules, CA, USA) using SYBR Green PCR mix (Promega), according to the following thermal profile: initial denaturation (95 °C, 15 min), and 40 cycles with denaturation (15 s at 95 °C), annealing (1 min at 60 °C) and extension (20 s at 72 °C). A Ct value of 35 or higher was considered negative. Melting curves were also analyzed for amplicon characterization. Results for gene expression analyses were calculated by the $2^{-\Delta\Delta Ct}$ equation and presented as the average of the relative expression units to an internal reference sample and normalized to the expression of the GAPDH housekeeping gene. Samples with GAPDH Ct values above 20 were excluded from the analysis. Already optimized primers were purchased (PrimePCR, Bio-Rad, Segrate, Italy). Gene expression analyses of the main SARS-CoV-2 receptors was also assessed on RNA extracted from A549-hACE2 cells, as positive control.

The expression of 46 genes related to inflammatory, apoptotic, and antiviral pathways were analyzed by a PCR array including a set of optimized Real-time PCR primers (Bio-Rad) for the targets reported in Table 2. Gene expression analyses were performed in iPSC-MNs at 72 hpi in duplicates. Results were analyzed using the SABiosciences online software, expressed by the $2^{-\Delta\Delta Ct}$ equation and presented as the average of the relative expression units to an internal reference sample and normalized to the expression of the GAPDH and ACTB housekeeping genes.

Table 2. Scheme of target genes analysed by Real-time PCR array in SARS-CoV-2 infected iPSC-MNs. *ACTB* and *GAPDH* were used as housekeeping genes for data normalization.

ACE2	**CD147**	**ERK**	**HLA-A**	**MMP9**	**TGFB1**
ACTB	**C5AR1**	**EPHA4**	**IFITM1**	**MT2A**	**TIMP1**
ANG	**CLDN5**	**FOS**	**IFITM3**	**MX1**	**TLR1**
ANXA2	**CTSB**	**FURIN**	**IGF1**	**NANOS2**	**TLR2**
BAX	**CXCR4**	**GAPDH**	**IL1A**	**NFKB**	**TLR4**
BCL2	**CYFIP2**	**GRN**	**IL6**	**NRP1**	**TLR6**
BDNF	**ERAP1**	**GSK3B**	**IL8**	**S100B**	**TLR8**
CASP8	**ERAP2**	**HDAC1**	**JNK**	**S1PR1**	**VEGFA**

4.2.1.7 Immunofluorescence assays

iPSC-MNs were seeded on coverslips in a 24-well plate, cultured until differentiation, and infected as specified above. At 48 hpi, cells were fixed in PBS containing 4% PFA at RT for 10 min, followed by permeabilization with 0,1% TritonX-100 in PBS for 10 min. Cells were treated with 1% BSA in PBS for blocking at RT for 1h, and incubated at 4°C overnight with specific primary antibodies. The following primary antibodies were used: anti-beta III Tubulin (1:500, Abcam, Cambridge, UK), anti-SMI-312 (1:1000, Covance, Princetown NJ, USA) and anti-ChAT (1:200, Chemicon) to assess iPSC-MN differentiation; anti-ACE2 (1:200, Prodotti Gianni), anti-CD147 (1:100, Thermo Fisher Scientific) anti-NRP1 (1:100, Thermo Fisher Scientific) and anti-N Nucleocapsid SARS-CoV-2 (1:1000, BEI Resources) to assess SARS-CoV-2 receptors and infection. Coverslips were then stained with secondary antibodies (Alexa Fluor 488 or 647, 1:500, Abcam) for 45 min at RT and mounted using a medium containing DAPI (Enzo Life Sciences, Milan, Italy). Confocal images were acquired on a TCS SP8 System equipped with a DMi8 inverted microscope and a HC PL APO 40×/1.30 Oil CS2 (Leica Microsystems, Wetzlar, Germany) at a resolution of 1024 × 1024 pixels (single stack).

4.2.1.8 Statistical analyses

Overall, we performed 14 SARS-CoV-2 independent experiments by using iPSC-derived MNs from 3 healthy donors. The different analyses were assessed on such samples according to the scheme reported in Table 1.

Statistical analyses were performed using GraphPad Prism 8. Results are expressed as mean ± SEM of the indicated n values. The two-tailed *Student's t-test* was used with a *p* value threshold of 0.05.

4.2.2 Results

4.2.2.1 Expression of SARS-CoV-2 receptors in iPSC-derived MNs

To assess the potential susceptibility of motor neuronal cells to SARS-CoV-2 infection, we differentiated iPSC from 3 healthy donor individuals (1 male and 2 females, 37-49 years of age at biopsy collection; Table 1) into motor neurons (iPSC-MNs) expressing neuronal (bIII-tubulin and SMI-312) and motoneuronal (ChAT, HB9) markers (Fig.1 panel a). By Real-time qPCR we validated the gene expression of the main receptors used by the virus and observed that all SARS-CoV-2 receptors (ACE2, CD147, NRP1) and peptidases (TMPRSS2, FURIN) analyzed were expressed in iPSC-MNs, although with different degrees (Fig.1 panel b). In particular, by assessing the expression

of these receptors in A549-hACE2 cells, a cell line used as positive control, ACE2 and Furin gene expression was significantly lower in iPSC-MNs compared to A549-hACE2 cells (Fig.1 panel b). NRP1 gene expression was instead comparable in iPSC-MNs and A549-hACE2 cells, while CD147 and TMPRSS2 gene expression was significantly higher in iPSC-MNs than in A549-hACE2 cells (Fig.1 panel b). Gene expression data on SARS-CoV-2 human receptors were further confirmed by IF analysis for ACE2, CD147 and NRP1 markers on both iPSC-MNs (Fig.1 panel c) and A549-hAC2 cells (Fig.1 panel d). Indeed, as for the gene expression analysis, ACE2 fluorescence intensity was significantly lower in iPSC-MNs compared to A549-hACE2 cells (Fig. S1).

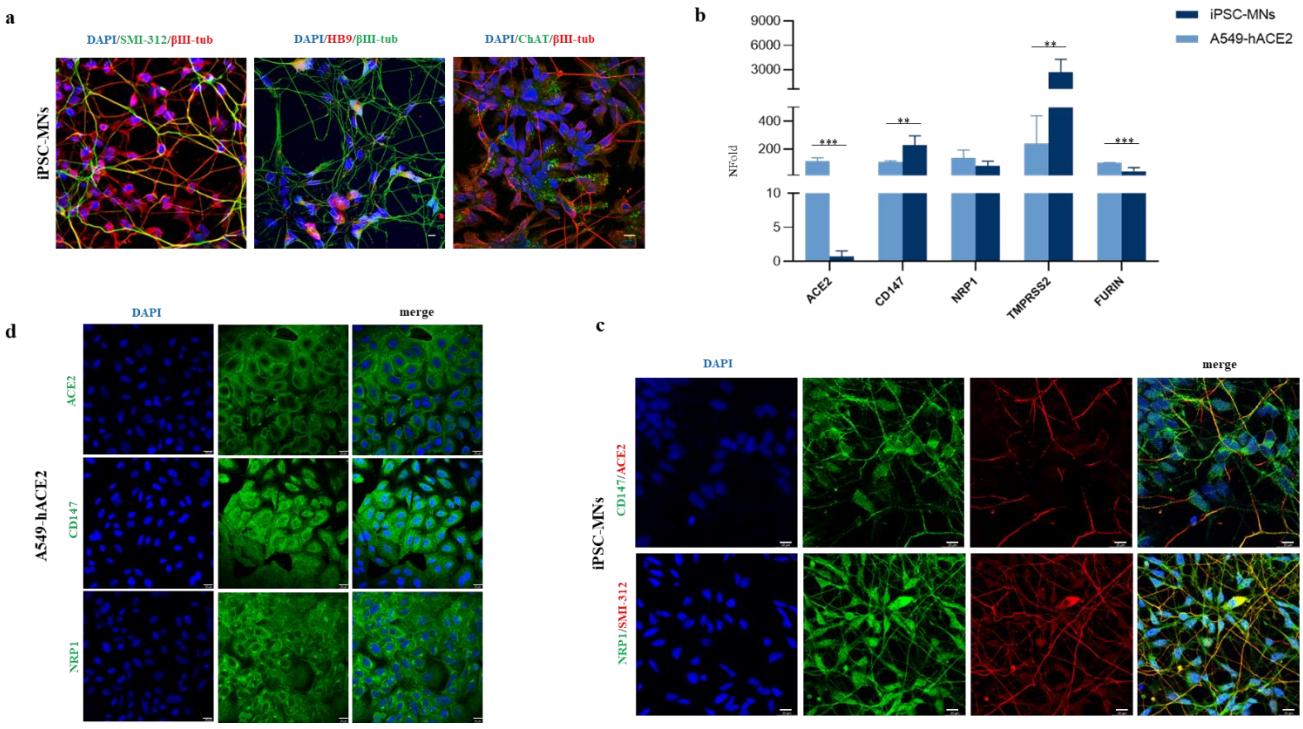


Figure 1. Expression of SARS-CoV-2 human receptors on iPSC-MNs and A549-hACE2 cells. (a) Representative images of differentiated iPSC-MNs obtained from 3 healthy control individuals. Expression of neuronal (bIII-Tubulin, red and green, and SMI-312, green) and motoneuronal (HB9, red and ChAT, green) markers is shown in merged images. Nuclei were stained with DAPI (blue). Bar, 10 mm. **(b)** Gene expression analyses of ACE2, CD147, NRP1, TMPRSS2 and FURIN in A549-hACE2 cells and iPSC- MNs by Real-time qPCR. Results are presented as mean ± SEM; n ≥ 4 for each cell line/iPSC-MN; the *Student's t-test* was used with the *p* value threshold of 0.05. Significance is indicated as follows: ** $p<0.01$; ***$p<0.001$. Representative immunofluorescence images for CD147 and NRP1 markers (green) in iPSC-MNs and in A549-hACE2 cells are shown in panel **(c)** and **(d),** respectively. The expression of ACE2 is shown in red (**c**) and in green (**d**). Nuclei were stained with DAPI (blue). The neuronal marker SMI-312 (red) is shown only in (**c**). Bars correspond to 20 mm in both **(c)** and **(d)**.

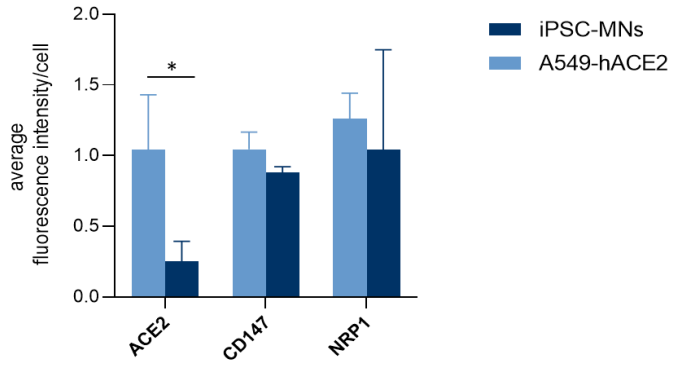


Fig. S1. Expression of SARS-CoV-2 human receptors on iPSC-MNs and A549-hACE2 cells. Quantification of Fluorescence intensity of ACE2, CD147, NRP1 in A549-hACE2 cells and iPSC- MNs obtained by Immunofluorescence assay. Results are presented as mean ± SEM; n ≥ 3 for each cell line/iPSC-MN; the *Student's t-test* was used with the *p* value threshold of 0.05. Significance is indicated as follows: *p<0.05.

4.2.2.2 SARS-CoV-2 viral replication in iPSC-MNs

To assess whether iPSC-MNs are productively infected by SARS-CoV-2, different experimental approaches were employed. We first assessed that cell viability, measured by Trypan blue assay, was not significantly modified in the 3 different iPSC-MN lines by comparing mock- and SARS-CoV-2-infected cells which showed more than 90% viability (Table 3), indicating a lack of cytopathic effect.

Table 3. Cell viability assessment in mock and SARS-CoV-2 infected iPSC-MNs

	Samples		
	S1	**S2**	**S3**
Mock x	93%	93%	96%
Mock y	93%	93%	95%
Mock z	93%	91%	96%
mean	**93%**	**92%**	**96%**
Infected x	93%	93%	92%
Infected y	94%	94%	96%
Infected z	95%	93%	92%
mean	**94%**	**93%**	**93%**

S= subject

By analyzing N1 and N2 viral nucleocapsid gene expression by Real-time qPCR in supernatants from iPSC-MN cultures over a timeframe of 72 hpi, we observed that human iPSC-MNs were productively infected by SARS-CoV-2 in a time-dependent manner (Fig.2 panel a), although viral replication was not accompanied by cytopathic effect as assessed by crystal violet assay (data not shown). Moreover, levels of viral replication were modest (at 72 hpi: mean viral copy number/ml±SEM, N1 = 4464.2±1281.7; N2 = 19139.4±5157.0) compared with SARS-CoV-2-susceptible VeroE6 cells (at 72

hpi: mean viral copy number/ml ±SEM, N1 = 18.18e+06± 3.2 e+06; N2 = 80.93e+06±17.29 e+06) (Fig 3).

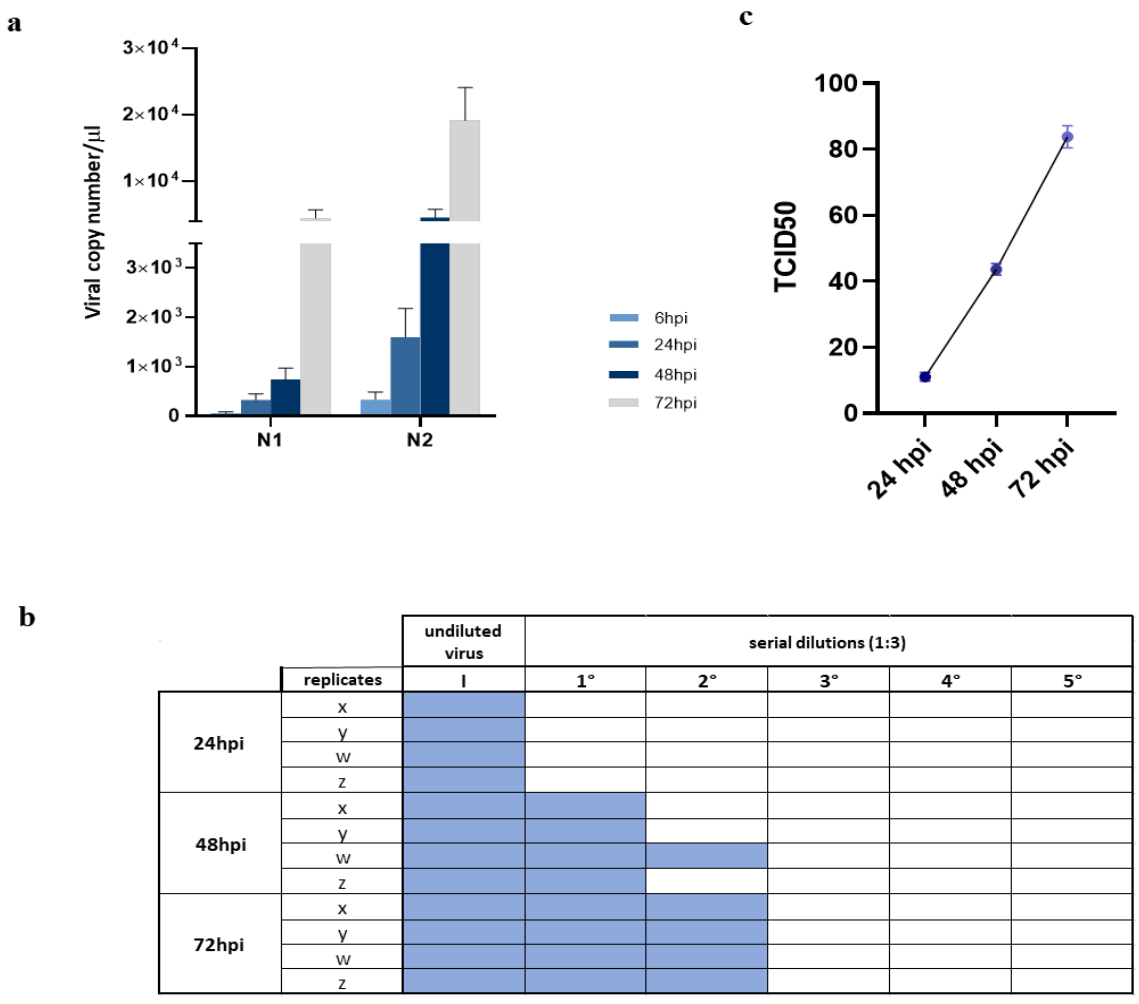


Figure 2. Assessment of viral replication in iPSC-MNs. (a) Upon *in vitro* challenge of iPSC-MNs with 1 MOI of SARS-CoV-2, the infection was monitored at 6, 24, 48 and 72 hpi. Results correspond to the absolute viral copy number/µl of the SARS-CoV-2 N1 and N2 target sequences from cell supernatants that were quantified through a single-step Real-time qPCR by referring to a standard curve for Ct values (IDT, Coralville, IA, USA). Results are presented as mean ± SEM from ≥4 independent replicates each on iPSC-MNs derived from the 3 enrolled healthy subjects (Table 1). **(b)** TCID50 analyses on VeroE6 cell infectability after exposure to iPSC-MN-infected supernatants, specifically the undiluted virus (I) and five serial dilutions (1:3), at 24, 48 and 72 hpi. Viral infection was assessed by cytopathic effect on VeroE6 cells as represented by colored well. The plate is representative of a single experiment which was performed once in quadruplicate (x, y, z, w) for each of the 3 different iPSC-MN lines. (**C**) Titration of SARS-CoV-2 virus in VeroE6 supernatants at 24, 48, and 72 hpi from data shown in (**B**). Data are shown as TCID50. Results are presented as mean ± SEM from 4 independent replicates each on iPSC-MNs derived from the 3 enrolled healthy subjects.

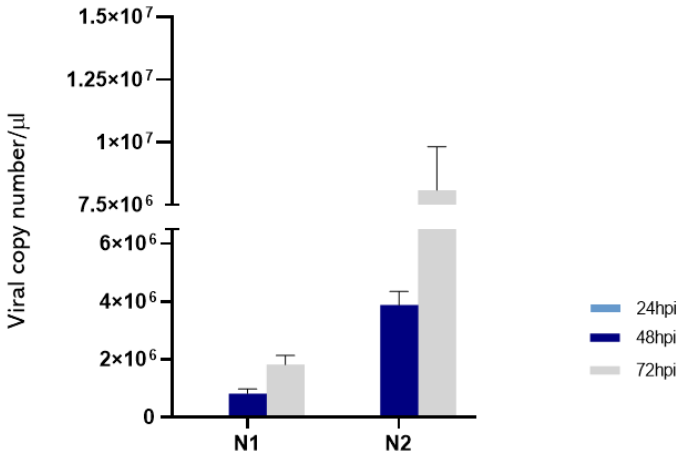


Figure 3. Assessment of viral replication in Vero E6 cells. Upon *in vitro* challenge of iPSC-MNs with 1 MOI of SARS-CoV-2 the infection was monitored at 24hpi, 48hpi and 72hpi. Results correspond to the absolute viral copy number/ml of the SARS-CoV-2 N1 and N2 target sequences from cell supernatants that were quantified through a single-step Real-time qPCR by referring to a standard curve for Ct values (IDT, Coralville, IA, USA). Results are presented as mean ± SEM from ≥3 independent replicates.

To further verify the productive infectability of iPSC-MNs, we then exposed VeroE6 cells to supernatants collected from infected iPSC-MNs at different time points (24, 48 and 72 hpi). VeroE6 cell infection resulted in an evident cytopathic effect which, as expected, increased according to the supernatant collection period over time (Fig.2 panel b), from 9.5 TCID50 at 24 hpi to 85.4 TCID50 at 72 hpi (Fig.2 panel c), mirroring the results obtained by Real-time qPCR.

To further validate these results, the expression of some viral RNA targets was investigated in iPSC-MNs at intracellular level by three different methods: QuantiGene assay, Real-time qPCR and immunofluorescence (IF). As reported in the heatmap, the RNA of all the viral targets analyzed by QuantiGene (N, E, SPIKE, RDRP, ORF3A, ORF8 and ORF7A) were exclusively detected in SARS-CoV-2-infected cells, although at different levels (Fig.4 panel a). The mRNA expression of N1, S1, S2 and E2 in infected iPSC-MNs was further confirmed by Real-time qPCR (Fig.4 panel b).

Finally, by IF assay, the nucleocapsid (N) protein was detected exclusively in SARS-CoV-2-infected iPSC-MNs (Fig.4 panel c), mainly at perinuclear level in the soma and along the neurite extensions (Fig. 4 panel d), though the percentage of infected cells seems to be very low.

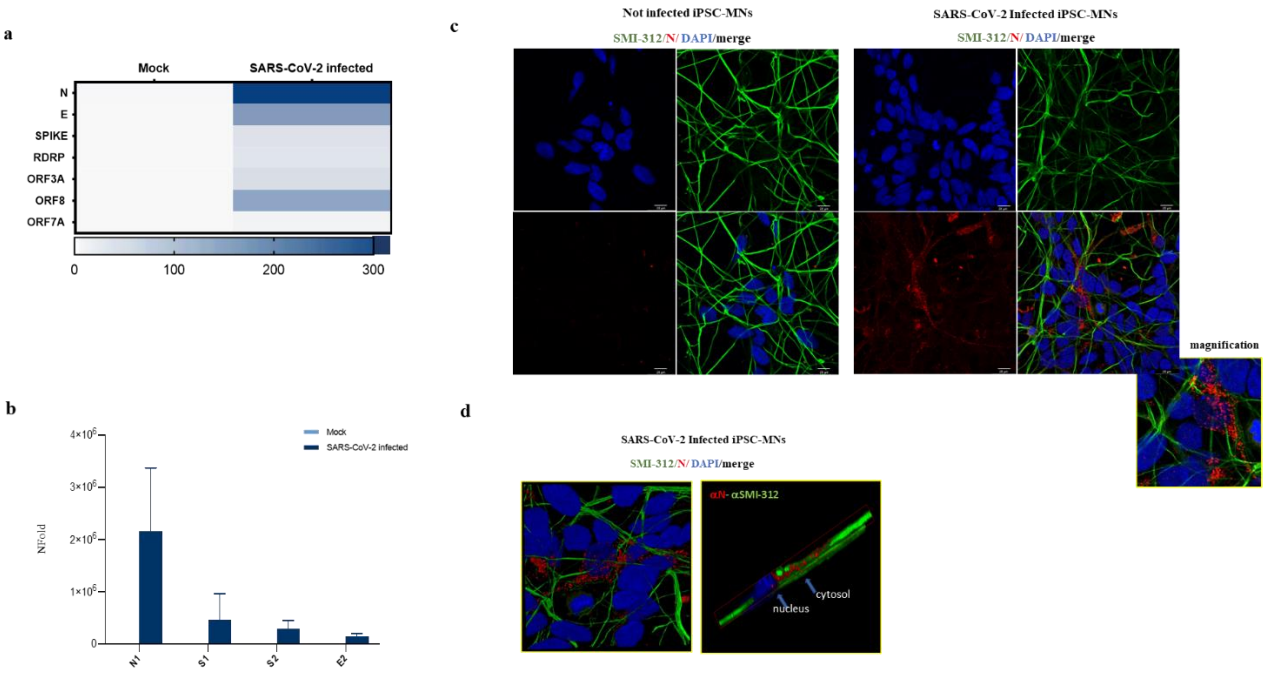


Figure 4. Assessment of viral replication in iPSC-MNs. (a) Expression of SARS-CoV-2 viral genes ORF7A, ORF3A, ORF8, RDRP, S, E and N by QuantiGene Plex Gene expression technology in uninfected (Mock) and SARS-CoV-2-infected iPSC-MNs. Results shown on the heatmap correspond to the mean ± SEM from ≥1 independent replicates on each iPSC-MNs derived from 2 healthy control subjects (Table 1). **(b)** Real-time qPCR expression analyses of N1, S1, S2 and E2 viral genes in uninfected (Mock) and SARS-CoV-2-infected iPSC-MNs. Results are presented as mean ± SEM from ≥4 independent replicates on iPSC-MNs derived from the 3 healthy control subjects (Table 1). **(c)** Representative immunofluorescence images of N protein (red) and neuronal SMI-312 marker (green) in Mock- and SARS-CoV-2-infected iPSC-MNs at 48 hpi. Nuclei were stained with DAPI (blue). Bars correspond to 20 mm. **(d)** Representative magnified immunofluorescence images and 3D reconstruction for N protein and SMI-312 marker in SARS-CoV-2-infected iPSC-MNs at 48 hpi.

4.2.2.3 Effect of SARS-CoV-2 infection on iPSC-MN gene expression

In order to assess whether SARS-CoV-2 infection fosters changes in iPSC-MNs gene expression, we evaluated the expression profile of a set of genes involved in the antiviral and immune-related response. Overall, among the 46 analyzed targets by a custom array (Table 2), we observed a widespread alteration of gene expression following SARS-CoV-2 exposure at 72 hpi, suggesting a deep alteration of cell homeostasis (Fig.5 panel a).

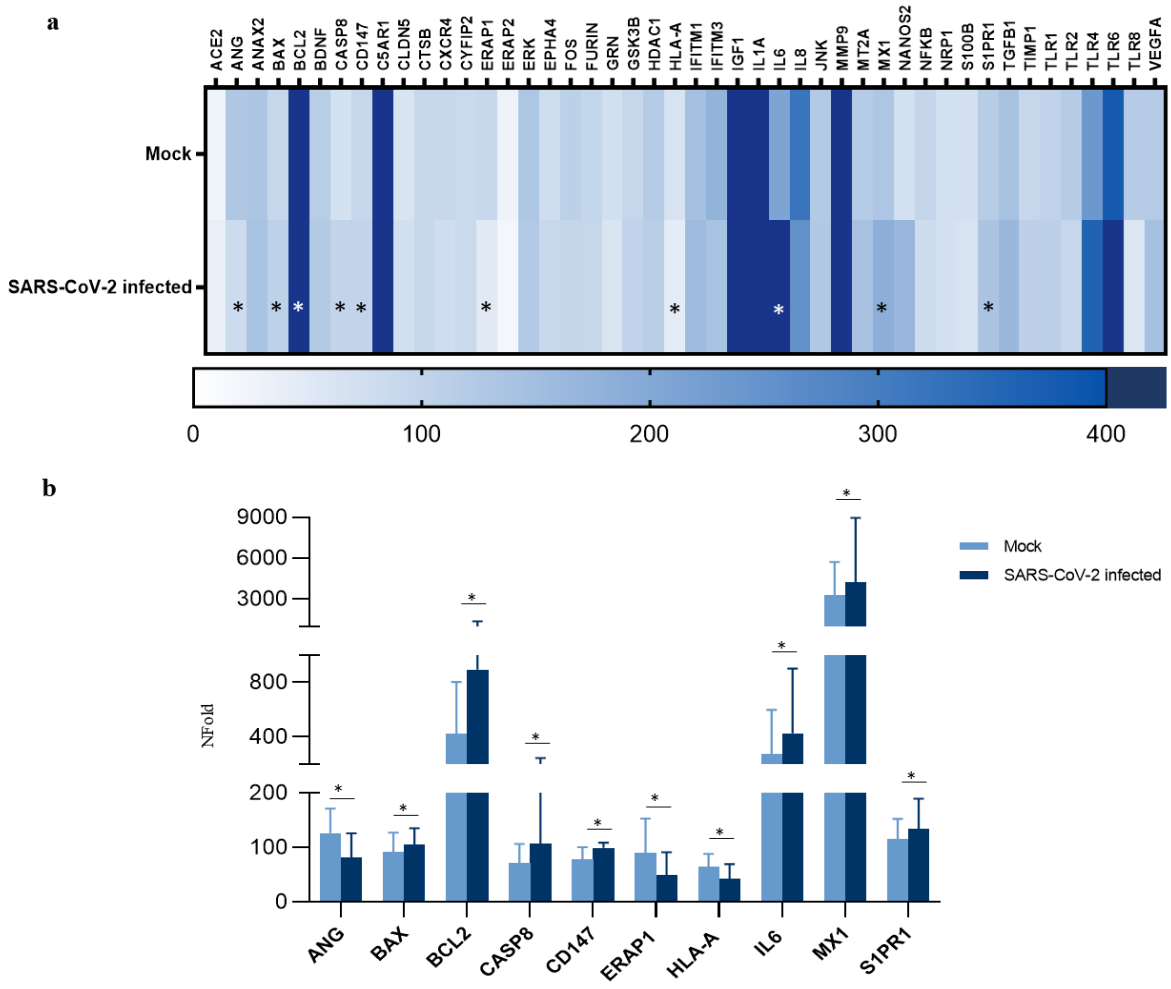


Figure 5. Gene expression analyses in SARS-CoV-2-infected iPSC-MNs. **(a)** Heatmap representation of Real-time qPCR expression data of 46 genes in uninfected (Mock) and SARS-CoV-2-infected iPSC-MNs at 72 hpi. **(b)**. Expression analyses of the statistically significant genes in SARS-CoV-2-infected vs uninfected (Mock) iPSC-MNs at 72hpi are shown in **(a)**. mRNA quantification was performed by Real-time qPCR and calculated by the $2^{-\Delta\Delta Ct}$ equation. Results in **(a)** and **(b)** correspond to the mean ± SEM from ≥4 independent replicates on each iPSC-MN derived from the 3 enrolled healthy subjects (Table 1); the *Student's t-test* was used with the *p* value threshold of 0.05. Significance is indicated as follows: follows: * $p<0.05$; ** $p<0.01$; ***$p<0.001$.

Notably, SARS-CoV-2-induced deregulation was significant for 10 genes involved in different intracellular pathways with both up-regulated (BAX, BCL2, CASP8, CD147, IL-6, MX1, S1PR1) and down-regulated (ANG, HLA-A, ERAP1) expression, as shown in Fig. 5 panel b. Notably, despite a slight rise in CASP8 expression, the BCL2/BAX ratio was significantly increased in SARS-CoV-2 infected iPSC-MNs insinuating different plausible speculations about the effect triggered by viral entry on the apoptotic pathway (Fig. 6).

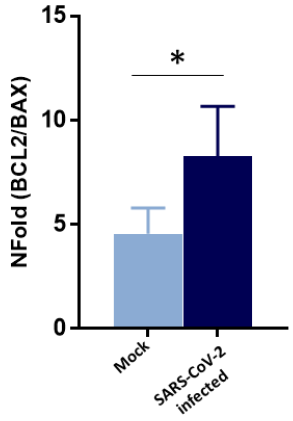


Figure 6. BCL2/BAX mRNA expression ratio. The ratio between the anti-apoptotic BCL2 and the pro-apoptotic BAX mRNA expression significantly increased in SARS-CoV-2 infected compared to uninfected (Mock) iPSC-MNs at 72hpi. Results are presented as mean ± SEM from ≥4 independent replicates on iPSC-MNs derived from the 3 enrolled subjects. Significance is indicated as follows: * = $p < 0.05$.

4.2.3 Discussion

Clinical observations from COVID-19 patients support evidence for the damages caused by SARS-CoV-2 infection on both central and peripheral components of the NS. Documented neurological abnormalities include postural tremor (13.8%), motor/sensory deficits (7.6%) [366], as well as persistent muscle pain (myalgia), muscle weakness (mild to severe), fatigue, exercise intolerance and arthralgia [367–369]. These are more frequent in severe manifestations of the disease and can differ among individuals [370, 371]. Rarely, clinically defined cases of acute disseminated encephalomyelitis, Guillain-Barré syndrome, and acute necrotizing encephalopathy have also been reported in COVID-19 patients [372]. Furthermore, COVID-19 has been shown to significantly affect amyotrophic lateral sclerosis (ALS) patients, causing a rapid neurological deterioration, accompanied by a marked decline in fine motor skills of hand and leg strength [373]. The damage of motor neurons, in turn, leads to the deterioration of muscle function, resulting in physical weakness, muscle atrophy, and paralysis. In addition, as reported by Li *et al.* [374], SARS-CoV-2 infection may stimulate the ALS-associated amyloid aggregation of host proteins, providing molecular evidence for the role of SARS-CoV-2 in triggering neurodegeneration and MNDs.

Despite a number of studies documenting SARS-CoV-2 infection of various neuronal cell populations [32–35, 289], to date, neither MN susceptibility to SARS-CoV-2 infection nor the molecular consequences of viral exposure on their homeostasis have been investigated. Therefore, it is still debated if the documented neurological/neuromuscular manifestations are secondary to a direct MN viral invasion and/or a collateral injury driven by an uncontrolled innate immune response fostering a pro-inflammatory milieu (immune mediated cytokines release), which is a hallmark of SARS-CoV-2 infection and COVID-19 severity.

In this study, we demonstrated for the first time that human iPSC-MNs are permissive to SARS-CoV-2 entry and production of infectious viral particles which are released in the cell culture supernatant. In fact, by using different experimental approaches, we verified that the virus is able to infect and replicate within this neuronal cell model. However, the levels of viral replication and the percentage of infected cells are significantly lower compared to those of susceptible cells, such as VeroE6 ones, possibly justifying the absence of cytopathic effect in iPSC-MNs. However, one should consider that this cellular model lacks the immunological component which, following SARS-CoV-2 infection, may favor the onset of a pro-inflammatory environment that is advantageous for viral infection/replication, and subsequent neuronal damage. It is therefore possible that in a pro-inflammatory setting, (i.e. in patients with pre-existing neuromuscular conditions) the rate of viral entry and replication would be substantially higher.

By profiling the expression of the main host cell receptors exploited by SARS-CoV-2, we confirmed previous data that ACE2 is scarcely expressed in neurons [375], here extending the observation to motor neuronal cells. We therefore speculate that iPSC-MN infection mainly relies on CD147 and/or NRP1 binding, as their expression on iPSC-MNs is high. Further confirming this assumption, it has already been demonstrated that these proteins have higher and broader patterns of expression in the human brain [100], advocating for their role as putative mediators of SARS-CoV-2 entry into human nerve cells. However, further analyses will be necessary to validate this hypothesis.

Interestingly, we also observed that SARS-CoV-2 infection promotes an alteration of iPSC-MNs transcriptome, involving different intracellular pathways. First, SARS-CoV-2 infection was associated with upregulation of MX1, a protective factor whose expression was reported to be switched-on in several viral infections including SARS-CoV-2 [376–379]. This suggests that SARS-CoV-2 prompts the activation of the antiviral response in iPSC-MNs.

Second, in SARS-CoV-2-infected iPSC-MNs we observed a conspicuous transcriptional upregulation of the pro-inflammatory cytokine IL-6. Remarkably, significantly elevated IL-6 levels have been reported in COVID-19 patients, which is associated with adverse clinical outcome [380]. In the NS, IL-6 levels increase in case of injury and/or inflammation, which may have both beneficial and detrimental effects on nerve cell survival and healing, depending on the cell type and context [381]. In the frame of neurodegenerative or neuropathic disorders, IL-6 exacerbates neurodegeneration and cell death, while blockade of IL-6 signaling improves the locomotor function in mice with spinal cord injury [382]. It is therefore plausible to assume that the neuromuscular complications of SARS-CoV-2 infection are due, at least in part, to an increased production of this inflammatory mediator, which is to some extent self-powered by infected MNs.

The expression of genes involved in the apoptotic pathway, such as BCL2, BAX and CASP8, was upregulated as well. Similar results were recently documented by Li *et al.* in SARS-CoV-2 infected lung epithelial cells where an increase of Caspase 8 responsible for cell death and inflammation was observed [383]. Nonetheless, we also observed that the ratio between the anti-apoptotic BCL2 and pro-apoptotic BAX genes was significantly increased in SARS-CoV-2 infected iPSC-MNs, suggesting that programmed cell death is somehow prevented following infection in these neuronal cells. Further analyses will be necessary to verify if this result reflects the virus' ability to manipulate, delay, or inhibit the host defense response, that usually exploits the apoptotic process to disrupt virus multiplication and propagation, thereby preserving other cells and surrounding tissue.

We also reported a modulation in the expression of factors involved in the antigen processing and presentation pathway. In particular, a statistically significant reduction in the expression of both HLA-A (MHC-I) and ERAP1 was observed. This is not surprising, as modifications in HLA-I and ERAPs expressions are found in many viral diseases. The downregulation of these targets represents one of the immune evasion strategies most widely used by viruses to block antigen presentation as well as CD8+ and NK cell response [384–386]. SARS-CoV-2 is no exception, as it was documented to downregulate cell surface MHC-I as a strategy to escape immune responses in various peripheral cell models [34, 387]. The role of MHC-I expression/activation in diseased MNs remains contradictory, as both detrimental and protective effects have been documented [388]. Besides immunological mechanisms, MHC-I has been firmly implicated in neuronal plasticity, regulation of synaptic density and axonal regeneration in the CNS and PNS, both during development and in brain diseases. Therefore, the downregulation of MHC-I along with ERAP1 detected in our model of SARS-CoV-2-infected iPSC-MNs might represent: i) an efficient antagonism of adaptive immune responses favoring successful viral replication, and/or also ii) a consequence of virus-induced alterations of neuronal homeostasis. This calls for additional focused investigations on the role and functional significance of neuronal MHC-I expression in the frame of SARS-CoV-2 infection.

In line with the possible alterations of neuronal homeostasis associated with SARS-CoV-2 infection in iPSC-MNs, we also detected a consistent downregulation of ANG (Angiogenin) gene expression. ANG is expressed in neurons during neuro-ectodermal differentiation and it exerts both neurotrophic and neuroprotective functions in the light of its role in multiple steps of RNA metabolism or processing [389]. Again, ANG acts as a circulating protein induced during inflammation and exhibits microbicidal activity against bacterial and fungal systemic pathogens, thus contributing to systemic responses to infection [390, 391]. Our data suggest that SARS-CoV-2 infection in iPSC-MNs goes

along with impaired ANG transcription, which might contribute to sustaining both virus-induced immune antagonism and dysregulation of neurotrophic responses.

Finally, in our model of SARS-CoV-2-infected iPSC-MNs, we detected an upregulation of CD147 and S1PR1 (Sphingosine 1-phosphate Receptor 1) genes. CD147 silencing was shown to reduce SARS-CoV-2 replication in epithelial lung cells [74]. Remarkably, CD147 upregulation has been documented in neurons, axons, and capillaries of Alzheimer's disease brain tissues [392]. These data configure CD147 as a potential target at the crossroad of SARS-CoV-2 infection and related neurological abnormalities, which remains to be confirmed in other neuronal models.

S1P is a bioactive sphingolipid with pleiotropic functions in many tissues, including the NS, where it regulates neurogenesis and inflammation through the S1P/S1PR axis [393]. For instance, accumulation of S1P in the brain promotes glial cell activation, TRL4 upregulation, and IL-6 secretion [394, 395]. In SARS-CoV-2 infection the study of the role of S1P at peripheral level has led to contradictory results [396, 397]. Remarkably, Fingolimod (FTY720), an approved drug for the treatment of multiple sclerosis (MS), which promotes the irreversible internalization and degradation of bound S1PR, has also been tested for COVID-19 treatment, in the light of its potential ability to inhibit the cytokine storm [393]. Thus, the observed S1PR1 upregulation might play an important role in SARS-CoV-2 infection in MNs, possibly by fostering pro-inflammatory cytokine release.

Overall, these data are in line with those profiled in other SARS-CoV-2 infected neuronal cell models [398–401], suggesting an embedded mess-up of the transcriptional machinery affecting the cellular equilibrium. Notwithstanding, it appears rather difficult to pinpoint a shared and precise expression profile fostered by SARS-CoV-2 infection in this motor neuronal cell model.

The aforementioned lack of the immune system, a certain level of heterogeneity in cell differentiation, and the restricted number of enrolled subjects represent limiting factors in the interpretation of the results. Last but not least, the lack of an *in vivo* blood-CNS barrier represents a major inherent limitation of the present model. However, we believe that the present results contribute to adding to previous evidence that supports the ability of SARS-CoV-2 to affect different neuronal populations after entering the CNS parenchyma via direct or indirect BBB impairment (32-36). Indeed, our data are the first to document the susceptibility of iPSC-MNs to productive SARS-CoV-2 infection and the ensuing alteration of their homeostasis. Yet, the neuroinvasive routes of SARS-CoV-2 entry within the gray matter of the spinal cord specifically, remain to be investigated by employing in vivo models. Remarkably, during the revision of the present paper, a preprint was published on bioRxiv, documenting that SARS-CoV-2 can productively infect spinal cord neurons in mice models [402].

The study suggest that SARS-CoV-2 infection of spinal cord motor neurons might occur following virus spread from brainstem neurons. It also detected productive infection in sensory ganglionic neurons of the PNS [402], suggesting the existence of an alternative, retrograde axonal pathway that fosters trans-synaptic viral spread from periphery to sensory ganglionic neurons, interneurons and motor neurons. Coupled with our results, these data suggest that SARS-CoV-2 is able to establish productive infection in unassessed nervous system sites, potentially causing sensory and neuromuscular symptoms associated with COVID-19 [403]. Further research is needed to understand how changes in motor neurons might contribute to the acute and long term neurologic sequelae. These findings could help uncover the biological basis of neuromuscular disorders and identify new therapeutic targets to counteract the neurological symptomatology.

4.3 III. iPSC- derived human cortical organoids display profound alterations of cellular homeostasis following SARS- CoV- 2 infection and Spike protein exposure

Cappelletti G, Brambilla L, Strizzi S, Limanaqi F, Melzi V, Rizzuti M, Nizzardo M, Saulle I, Trabattoni D, Corti S, Clerici M, Biasin M. iPSC-derived human cortical organoids display profound alterations of cellular homeostasis following SARS-CoV-2 infection and Spike protein exposure. FASEB J. 2025 Feb 28;39(4):e70396. doi: 10.1096/fj.202401604RRR. PMID: 39950320; PMCID: PMC11826378.

4.3.1 Materials & Methods

4.3.1.1 Cell Lines, virus and Reagents

Vero E6 cells (ATCC, VA, USA) were grown in DMEM (Dulbecco's Modified Eagle's Medium) (Euroclone, Milan, Italy) with 10% FBS, 4 mM L-glutamine, 100 µg/mL streptomycin, and 100 U/mL penicillin at 37 °C and 5% CO_2. Regular mycoplasma contamination checks were performed using PCR, and experiments were carried out with cells from passages 15 to 25. The Delta (B.1.617.2) SARS-CoV-2 lineage (kindly provided by the Clinical Microbiology, Virology and Bioemergency diagnosis Unit – ASST Fatebenefratelli Milan, Italy) was grown into VeroE6 cells to create a viral stock. The concentration of viral particles was calculated as 50 percentage tissue culture infectious dose ($TCID_{50}$), as previously described [333]. SARS-CoV-2 recombinant S protein was acquired from BEI Resources NIAID, NIH (Cat. N. NR52397; www.beiresources.org).

4.3.1.2 iPSCs generation and Human Cortical Organoids (HCO) differentiation

Induced Pluripotent Stem Cells (iPSCs) were generated from Peripheral Blood Mononuclear Cells (PBMCs) obtained from a total of four healthy subjects (Table 1), through a non-integrating reprogramming protocol via a modified protocol of Sendai Virus to deliver the four reprogramming factors: Oct4, Sox2, Klf4, and c-Myc (CytoTune®-iPSC 2.0 Sendai Reprogramming kit, Thermo Fisher Scientific). iPSCs were cultured in Cultrex-coated (1% for 1h at 37°C) 6-well plates and grown in complete Essential 8™ Medium (E8, Thermo Fisher Scientific). Before proceeding with the HCO generation, iPSCs were checked for chromosomal abnormalities by karyotyping. Furthermore, the vector-free status of iPSCs clones was assessed by performing a RT-PCR for the detection of SeV genome and transgenes.

iPSCs were differentiated into HCOs based on the approach that Miura et al. [404] detailed. Upon reaching 80–90% confluence, the cells were seeded at a density of 10^4 cells per well in a 96-well

plate, using complete E8TM Medium supplemented with 10 µM Rock inhibitor. Accutase was used to detach the cells (5 min, 37°C). iPSCs were grown in 96 well plates to form embryoid bodies (EBs) until DIV12. From DIV0 to DIV 6, E8^{TM} medium was enriched with 2,5 µM dorsomorphin (DM) and 10 µM SB-431542 with changes made every other day. At DIV6, the neural induction medium was replaced with neural differentiation one, consisting of Neurobasal medium, 2% B27 supplement, 1% Glutamax, and 1% penicillin-streptomycin, supplemented with 20 ng/mL epidermal growth factor (EGF) and 20 ng/mL basic fibroblast growth factor (bFGF). Up until DIV22, the medium was switched every two days. At DIV12, EBs were transferred in 6 well plates. From DIV22 to DIV40, EGF and bFGF were replaced with 20 ng/mL brain-derived neurotrophic factor (BDNF), 20 ng/mL neurotrophin-3 (NT3), 200 µM ascorbic acid (AA), 50 µM dibutyryl-cAMP sodium salt and 10 µM docosahexaenoic acid (DHA). At DIV40, cortical organoids were used for the infection assay.

	Sex	Age	Real-time PCR for N1 and N2 viral genes	TCID50 on VERO E6 cells	Gene expression analysis by Real-Time PCR	Gene expression by Digital	IF
S1	M	69	n=3	n=3	n=3	n=3	n=3
S2	F	64	n=3	n=3	n=3	n=3	n=3
S3	M	65	n=3	n=3	n=3	n=3	n=3
S4	F	33	n=3	n=3	n=3	-	n=3

Abbreviation: S, subject.

Table 1. Sex, age and number of independent replicates performed for each assay with iPS-HCOs lines obtained from four healthy donors are reported.

4.3.1.3 HCO In Vitro SARS-CoV-2 infection assay

In vitro HCOs infection was performed with SARS-CoV-2 at 0.001 MOI (multiplicity of infection). The MOI was calculated considering a value of 3x10 ^{5} cells per single HCO. Following overnight incubation, HCOs were rinsed with PBS and filled with complete neural differentiation medium. For the assessment of SARS-CoV-2 replication and the execution of a re-infection test on VeroE6 cells in quadruplicate, supernatants were harvested at four different time points. The first point was set at an early stage of infection, whereas the last one was set in a late stage to appreciate a viral infection-kinetics over time. Thus, time point-0 (T0), -1 (T1), -2 (T2) and -3 (T3) have been scheduled at 6-, 24-, 48-, and 72 hours post-infection (hpi), respectively. At 72 hpi, HCOs were either fixed for

immunofluorescence (IF) analyses or lysed for RNA extraction and subsequently, as mentioned below, kept at -80°C for further processing.

4.3.1.4 In vitro SARS-CoV-2 replication assessment in HCO

RNA genome from SARS-CoV-2 infected HCO supernatants was isolated, retrotranscribed, and amplified as previously reported [405]. Concurrently, Vero E6 cells were plated in a 96-well plate at a density of 2×10^4 cells per well. Subsequently, these cells were cultured with serial dilutions (1:3) of supernatants obtained from SARS-CoV-2 infected HCOs at various time points, specifically at 24-, 48-, and 72 hours post-infection (hpi). After 72 hours, supernatants from Vero E6 cells were collected and fixed using 4% paraformaldehyde (PFA - Sigma-Aldrich, MO, USA) for 1 hour at room temperature. Cell death was then evaluated and the $TCID_{50}$ was determined with a crystal violet solution at 0.2% (Sigma-Aldrich), as previously reported [333].

4.3.1.5 iPSC-HCO exposure to SARS-CoV-2 S-protein

iPSC-HCOs were challenged with viral S Protein (0,08µg/ml) (BEI resources). At 72 h post-stimulation, iPSC-Human cortical organoids were fixed for immunofluorescence (IF) analyses or were lysed for RNA extraction.

4.3.1.6 Gene expression analyses

As reported earlier by Limanaqi *et* al. [354], total RNA was extracted and reverse transcribed from HCOs. The gene expression of 33 targets related to SARS-CoV-2 inflammatory, host receptors and co-receptors, apoptotic, and antiviral pathways was assessed using Real-time qPCR (CFX96 connect, Bio-Rad). SYBR Green PCR mix (Promega) and specific Real-time PCR primers (PrimePCR, Bio-Rad, Segrate, Italy) listed in Table 2 were employed for the analysis. N1 and S1 SARS-CoV-2 sequences were also analyzed as well. A negative Ct value was defined as 35 or greater, and all the samples displayed GAPDH Ct values below 20; thus, none were excluded from the analysis. Gene expression analyses were assessed as previously described [406].

Gene expression analyses of ACE2, CD147, NRP1, and Furin were conducted using droplet digital PCR (ddPCR QX200, Bio-Rad). This analysis can increase the detection of rare transcripts and provide absolute quantification of RNA molecules. For CD147 and NRP1 gene analyses, 3 µL of 1:100 diluted cDNA and 3uL of ACE2 cDNA were mixed with specific primers (Qiagen, Hilden, Germany) and EvaGreen ddPCR SuperMix (Bio-Rad). The mixture was blended with droplet

generator oil (Bio-Rad) utilizing a QX200 droplet maker, in compliance with the producer's guidelines.

The droplets were transferred to a 96-well reaction plate, and a pierceable foil sheet (PX1, PCR plate sealer, Bio-Rad) was utilized to heat-seal the plate. The PCR amplification was carried out using a T100 thermal cycler (BioRad). Further steps were performed by a QX200 droplet reader (Bio-Rad), and QuantaSoft software (version 1.7.4.0917) was used to analyze ddPCR data (Bio-Rad). Results are shown as median and Interquartile Range (IQR) (25th and 75th percentile).

ACE2	b2-micro	CD147	ERAP2	HLA-A	IL10	IL37	N2	NRP1	S100B	TAP
BAX	CASP1	cGAS	FURIN	IFITM1	IL18	MAVS	NFKB	RIG1	STAT1	TOMM70
BCL2	CASP8	ERAP1	GSK3B	IFITM3	IL33	MCP1	NLRP3	S1	STAT3	VEGFA

Table 2. Target genes analysed by Real-time PCR in mock, SARS-CoV-2 infected and S-exposed HCOs. Data were normalized on *GAPDH* housekeeping gene.

4.3.1.7 Immunofluorescence assays

72h post-infection and S-stimulation, HCOs were fixed in PBS plus 4% PFA at 4°C overnight. One millilitre of 30% sucrose/PBS for cryopreservation was then added at 4°C overnight. The cells were then prepared for cryosection. Sections were gathered on Superfrost Plus slides. For immunostaining, the cells were washed three times in PBS and blocked for 1 hour at RT with 10% normal goat serum (NGS), 0.2% Triton-X, and 0.1% BSA in PBS. Cells were incubated overnight at 4°C with the following primary antibodies to characterize the cell model, as well as to assess SARS-CoV-2 host entry factors, SARS-CoV-2 replication, and neuronal stress conditions: TUBB3 (1:500, BioLegend), SOX2 (1:200, Abcam), PAX6 (1:100, Invitrogen), MAP2 (1:100, Merck), DCX (1:200, Abcam), Nestin (1:200, Abcam), SYNAPSIN-1 (1:200, Merck), ACE2 (1:200, Prodotti Gianni), NRP1 (1:100, Thermo Fisher Scientific), CD147 (1:100, Thermo Fisher Scientific), CASP3 (1:200, Abcam) and SARS-CoV-2 Nucleocapsid protein (1:1000, BEI Resources). Following, sections were stained at room temperature for 45 minutes with secondary antibodies (Alexa Fluor 488 or 647, 1:500, Abcam) and mounted using a medium containing DAPI (Enzo Life Sciences, Milan, Italy). Analyses by confocal microscopy were conducted using a Leica TCS SP5 AOBS microscope (Leica Microsystems, Wetzlar, Germany).

4.3.1.8 Statistical analyses

GraphPad Prism 8 was used to conduct the statistical analysis. The mean ± SEM of the specified n values was employed to express the results, and a threshold of 0.05 was applied when two tailed Student's t-test was performed. In total, we utilized HCOs from 3 healthy subjects to perform 3 individual testing.

4.3.2 Results

4.3.2.1 iPSC-derived HCO characterization

Brain organoids represent a valuable and innovative model for studying the complexity of the human brain, closely resembling the *in vivo* cellular microenvironment. As a matter of fact, brain organoids can develop various discrete, although interdependent, brain regions, including the cerebral cortex.

We generated iPSC-derived HCOs from healthy controls (n=3), following an established protocol[404]. At day 30 of differentiation, iPSC-derived HCOs were comprehensively characterized through immunocytochemistry to assess the expression of key markers related to pluripotency, neural precursor identity, and neuronal differentiation (Fig.1). Despite the process of neuronal commitment, HCOs continued to exhibit persistent expression of the pluripotency marker SOX2, specifically within progenitor cell populations. This observation highlights the heterogeneous cellular composition of the organoids, where differentiated neurons coexist with progenitor cells that retain stem cell-like properties. Such heterogeneity is a hallmark of developing brain-like structures, where a dynamic balance between progenitor maintenance and neuronal differentiation is crucial for proper development. The presence of SOX2 in these progenitor populations after one month of differentiation underlines the ongoing neurogenic potential within the organoid, reflecting the complexity and the developmental trajectory of these in vitro model which reflects the human CNS development [318, 407, 408]. Markers associated with neuronal progenitors, such as PAX6 (paired box 6) and NESTIN, were analyzed to assess the transition to neural precursor stages, confirming the presence of a robust population of neural progenitor cells, which are crucial for the early stages of brain development. These markers indicate that the organoids successfully recapitulate the neurogenic niches typically observed during the embryonic development of the human brain.

Additionally, the detection of neuronal differentiation markers, including TUBB3 (beta-III tubulin), MAP2 (microtubule-associated protein 2), DCX (doublecortin), and Synapsin-1, provide evidence of advanced neuronal development. TUBB3 and MAP2 expression revealed the formation of differentiated neurons with stabilized microtubule networks, essential for axonal and dendritic

growth. The presence of DCX, a marker of migrating neurons, suggests that neuronal migration is occurring within the organoids. Additionally, Synapsin-1 expression is indicative of synaptogenesis, pointing to the establishment of functional connections between neurons and the emergence of nascent neuronal networks.

These findings collectively demonstrate that the organoids not only exhibit a heterogeneous cellular composition, encompassing both progenitor and mature neuronal populations, but also mirror key developmental processes such as neurogenesis, neuronal migration, and synaptogenesis.

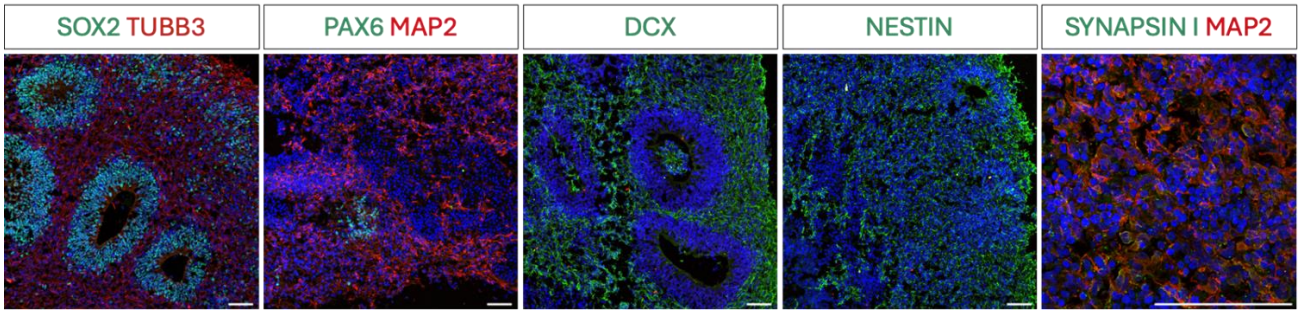


Figure 1. Immunofluorescence on HCOs. Immunostaining for stem cell, precursor and neuronal markers in HCOs at day 30 (SOX2, green; TUBB3, red; PAX6, green; MAP2, red; DCX, green; NESTIN, green; Synapsin-1, green). Nuclei were stained with DAPI, blue. Scale bar: 100 μm.

4.3.2.2 SARS-CoV-2-specific human receptor and co-receptor expression in HCOs

The assessment of the expression of key human receptors, co-receptors (ACE2, CD147, and NRP1), and the peptidase (Furin) employed by SARS-CoV-2 [409, 410] was performed on HCOs through digital PCR. HCOs expressed each of these entry factors, but with different gene expression levels (Fig.2A). Specifically, ACE2 and Furin were substantially less expressed than CD147 and NRP1, according to the gene expression study. However, protein expression of the SARS-CoV-2 entry factors assessed by IF assay (Fig.2B) did not confirm this finding. Indeed, ACE2 expression was slightly higher compared to CD147 and NRP1 (Fig.2C), suggesting the occurrence of compensatory cellular mechanisms balancing mRNA transcription and protein translation turnover.

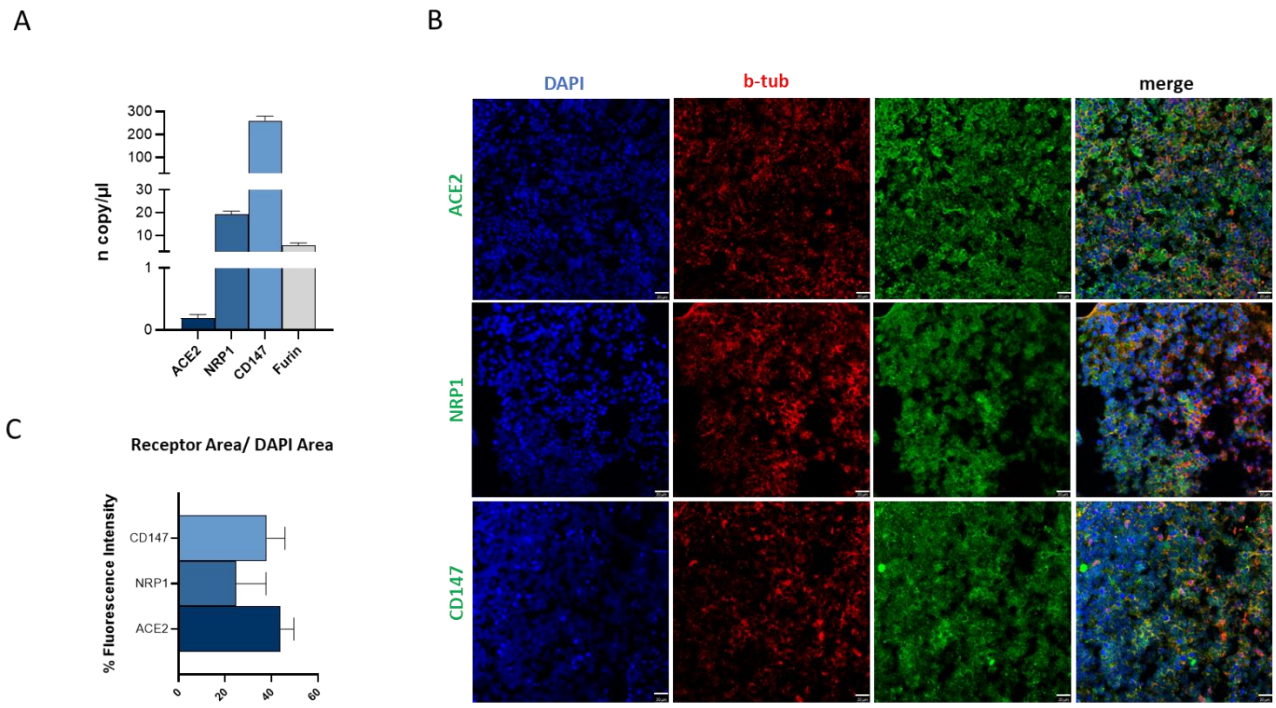


Figure 2. Expression of human SARS-CoV-2 primary entry factors on HCOs. A. Digital gene expression analyses were conducted on iPSC-HCOs to investigate the mRNA expression of ACE2, NRP1, CD147, and Furin entry factors targeted by SARS-CoV-2. Results are shown as copy/ul. and derived from a minimum of three separate experimental runs each one on HCOs obtained from 3 healthy individuals. **B.** Representative IF images of ACE2, CD147 and NRP1 markers (green) and nuclei in DAPI (blue) in HCOs. Bars are equivalent to 20 µm. **C.** Quantification of ACE2, CD147 and NRP1 receptor fluorescence intensity, expressed in percentage, obtained by analysing the ratio of receptor Area/DAPI Area from three different images acquired from the same HCO section.

4.3.2.3 SARS-CoV-2-infecion and Replication in HCOs

Different experimental approaches were employed to verify if HCOs could be successfully infected by SARS-CoV-2. First, the presence of specific viral products within HCOs was evaluated at the intracellular level by using Real-time qPCR and IF approaches. The first one confirmed mRNA expression of SARS-CoV-2 nucleocapsid (N1) and Spike (S1) target sequences in infected HCOs (Fig.3A). Likewise, the viral nucleocapsid (N) protein was identified solely in SARS-CoV-2 infected HCOs, predominantly localized in the external region (Fig.3B and Supplementary Fig.1), though the percentage of infected cells was apparently scant.

In parallel, gene expression analyses of both N1 and N2 viral nucleocapsid sequences in supernatants from HCO cultures, over a 72-hpi period, proved an effective and progressive SARS-CoV-2 infection and replication in HCOs (Fig.3C).

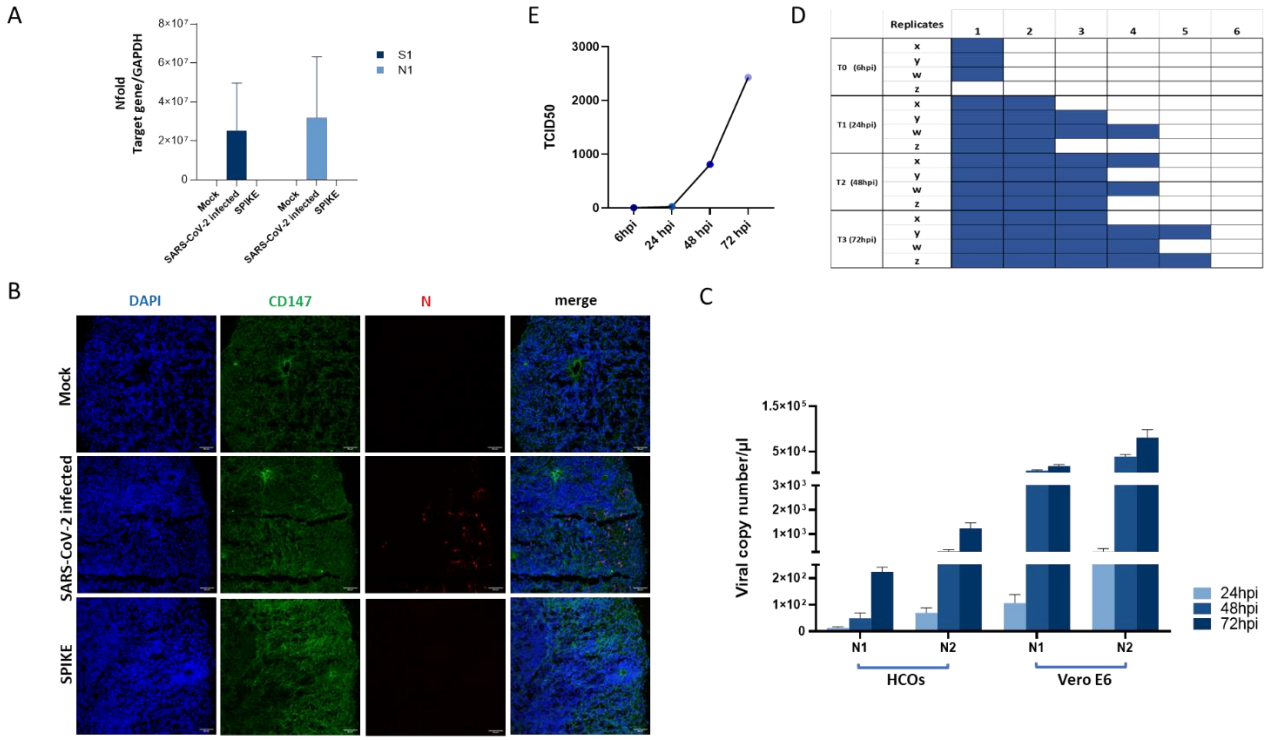
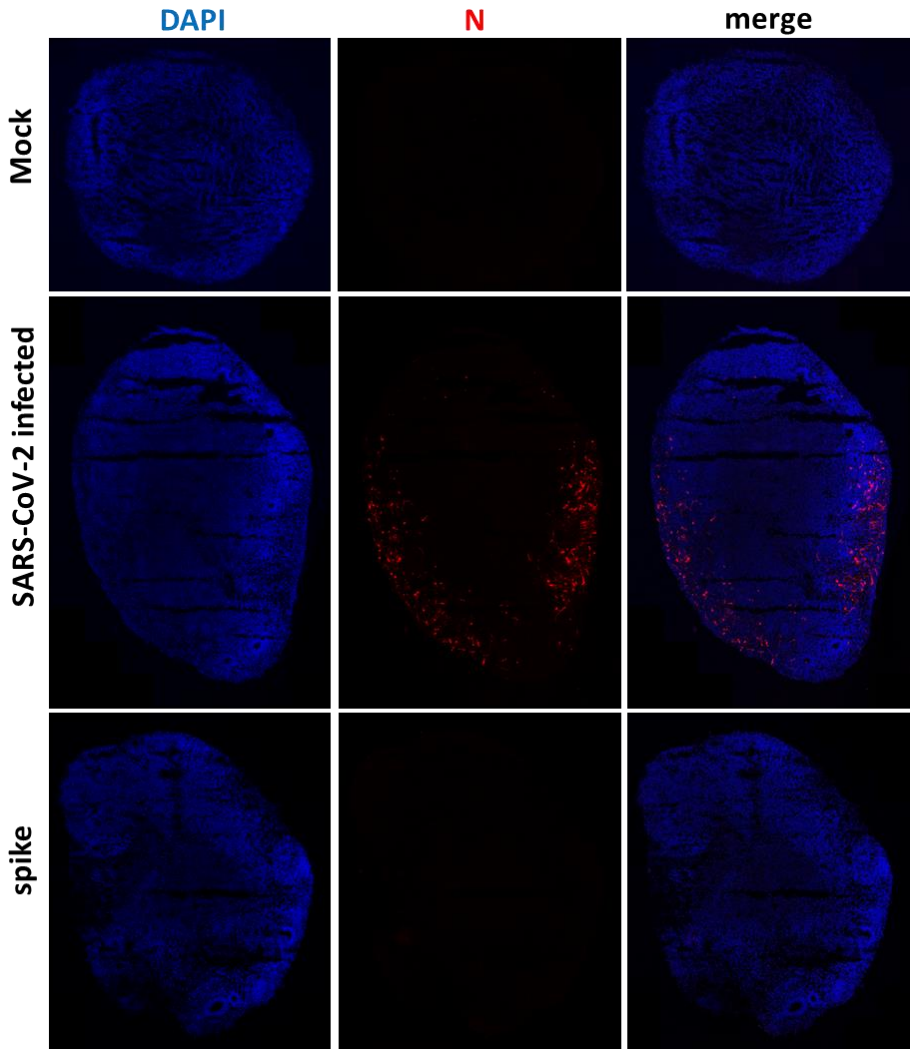


Figure 3. Assessment of SARS-CoV-2 *in vitro* infection and replication in HCOs. A. mRNA expression of S1 (Spike) and nucleocapsid (N1) viral sequences were investigated in SARS-CoV-2-infected, spike (S)-exposed, and uninfected (Mock) HCOs by Real-time qPCR. Results are displayed as the average value plus standard error of the mean (SEM) derived from a minimum of three separate experimental runs each one on HCOs obtained from 4 healthy individuals. **B.** Comparative immunofluorescence imaging of nucleocapsid viral protein (N) (red), CD147 receptor (green) and nuclei (blue) in uninfected (Mock), infected with SARS-CoV-2 and S-exposed HCOs 72-hpi. Bars correspond to 60 µm.**C.** Viral replication was evaluated at 24-, 48- and 72-hours post-infection (hpi) in SARS-CoV-2-infected HCO and Vero E6 supernatants (MOI 0.001). Viral copies per microliter was assessed by Real-Time PCR quantifying two regions of SARS-CoV-2 Nucleocapsid gene (N1 and N2). Data are displayed as the average value plus standard error of the mean (SEM) and were derived from a minimum of four separate experimental runs. **D.** $TCID_{50}$ analyses were conducted to assess the infectivity of VeroE6 cells following exposure to SARS-CoV-2-infected HCO- supernatants. This included the undiluted supernatant (1) and 5 serial 1:3 dilutions, at 6, 24, 48, and 72 hpi. The presented plate serves as a representation of a singular experiment performed in quadruplicate (X,Y,W,Z). Analyses of cytopathic effect, represented by coloured well in blue, was used to verify Vero E6 infectability by SARS-CoV-2. **E.** The quantification of SARS-CoV-2 in the Vero E6 supernatants was performed at 6, 24, 48, and 72 hpi, using the data reported in **(D).** $TCID_{50}$ values are displayed, with results reported as the average value with the standard error of the mean (SEM) and were derived from four separate experimental runs each one on HCOs obtained from four healthy individuals.

Nevertheless at 72 hpi, SARS-CoV-2 replication in HCOs was significantly lower (average SARS-CoV-2 copy number/ ul ±SEM: N1 = 222.99±17.64; N2 = 1229.38±228.17) than that detected in susceptible Vero E6 cells (average SARS-CoV-2 copy number/ ul ±SEM: N1 = 15.17e+06± 2.2 e+06; N2 = 77.94e+06±19.27 e+06) (Fig.3C).

To further confirm HCO potential to sustain productive infection, supernatants gathered from infected HCOs at 6, 24, 48 and 72 hpi were used to re-infect Vero E6 cells. As a result, Vero E6 cells showed a clearly visible cytopathic effect, which increased throughout the supernatant collecting period (Fig.3D). Indeed, the virus titers displayed a rise from 27 $TCID_{50}$ at 24 hpi to 2430 $TCID_{50}$ at 72 hpi (Fig.3E), replicating the outcomes of Real-time qPCR.



Supplementary Figure 1. *In vitro* SARS-CoV-2 infection in iPSC-Human cortical organoids. Intracellular analysis. Representative immunofluorescence images of N protein (red) in Mock, SARS-CoV-2-infected and S-exposed iPSC-HCOs at 72 hpi. Nuclei were stained with DAPI (blue).

4.3.2.4 Transcriptome analyses in HCO following in vitro SARS-CoV-2-infecion or S-exposure.

To assess whether SARS-CoV-2 infection or S-exposure alter cellular homeostasis and metabolism, including the antiviral and immune response in HCOs, we evaluated the expression of 31 different genes of interest at 72 h post infection or stimulation. Results summarized in Fig.4A demonstrate that both SARS-CoV-2 infection and S-stimulation foster transcriptional dysregulation in several of the analyzed targets. Notably, the trend of the various targets exhibited a similar pattern following both SARS-CoV-2 infection and S-exposure. (Fig.4A).

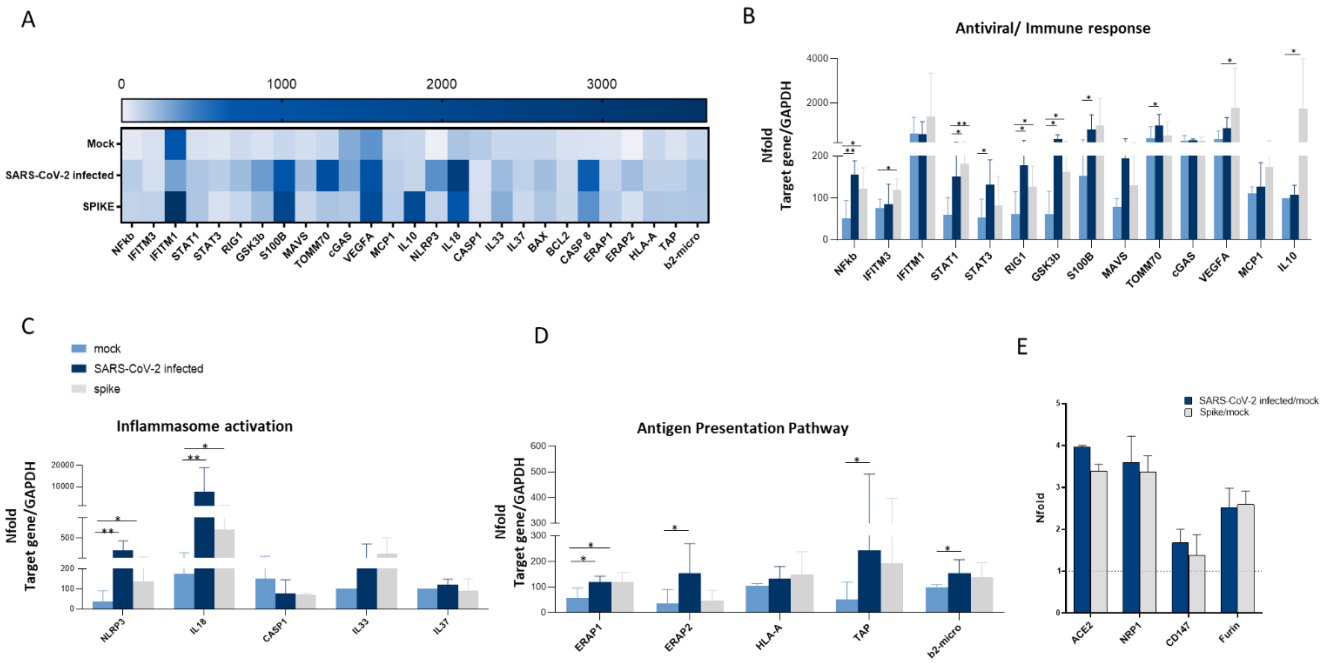


Figure 4. Transcriptional pathways altered in HCOs post SARS-CoV-2-infection or S-exposure. Gene expression analyses. A. At 72 hours post-infection, mRNA levels of 27 different genes were evaluated in HCOs uninfected (Mock), infected with SARS-CoV-2 and stimulated with the Spike protein. Results are displayed in a Heatmap. Gene expression (nfold) is shown as a colour scale from white to dark blue (0 to +3500). Real-Time qPCR was employed to quantify mRNA levels of genes related to the immune and antiviral response **(B)**, inflammasome activation **(C)**, antigen processing and presentation pathway **(D)**. As for entry factors NFold of ACE2, NRP1, CD147 and Furin peptidase, measured by Real Time-PCR, was reported as increase of the HCOs SARS-CoV-2 infected, and S-exposed conditions in comparison to the uninfected one **(E)**. The $2^{-\Delta\Delta Ct}$ equation was used to quantify expression level. Data are displayed as the average value with the standard error of the mean (SEM) and were derived from a minimum of three separate experimental runs each one on HCOs obtained from 4 healthy individuals. *=p<0.05; **=p<0.01.

In detail, results show that both SARS-CoV-2 infection and S-exposure augmented the mRNA expression of many intracellular target peculiar to virus contact, including interferon-induced transmembrane proteins (IFITM3, IFITM1), interferon-stimulated genes (NFKB, STAT1, STAT3, RIG1), as well as a neuronal stress-related marker (S100B), and GSK3b, a multifunctional kinase involved in neuronal homeostasis, differentiation, and metabolism (Fig.4B). We also observed a

consistent rise in NLRP3 and IL-18 mRNA expression, which are key components of the inflammasome pathway (Fig.4C).

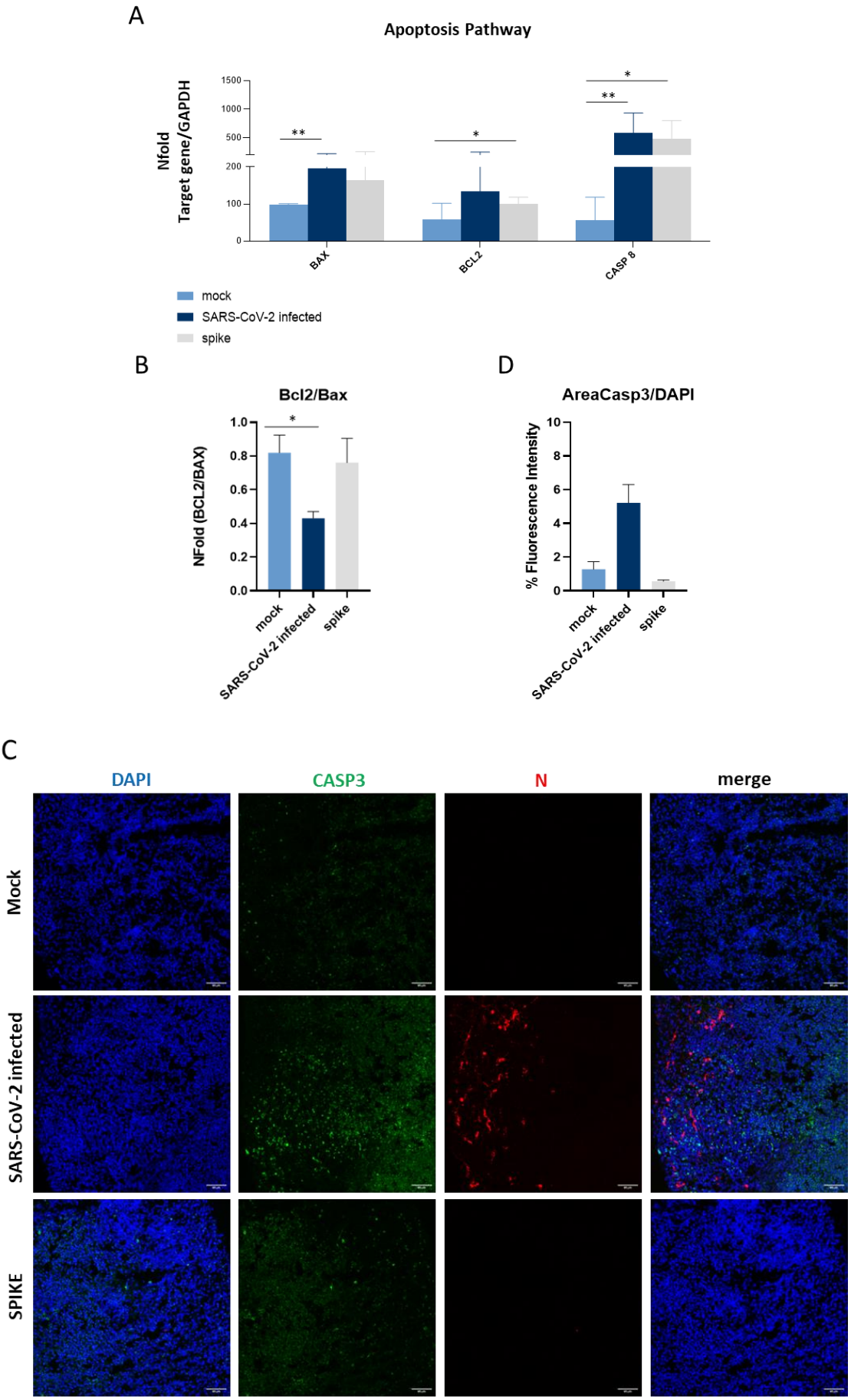
Of note, significant modulation of genes implicated in the antigen processing and presentation pathway (ERAP1, ERAP2, TAP, b2-microglobulin) was observed as well (Fig.4D). Interestingly, the induction of this pathway was evident following both viral infection and S exposure, but statistically significant differences were evident post-SARS-CoV-2 infection alone. This implies an active viral replication is needed to fully boost antigen processing and presentation.

Moreover, as shown in Fig. 4E, following 72 h of either SARS-CoV-2 infection or S-stimulation, we observed, by Real Time PCR, a generalized increase in in the expression of all human SARS-CoV-2 entry factors compared to the mock HCOs.

4.3.2.5 Apoptotic pathway alteration in SARS-CoV-2-infected or S-exposed HCOs

To further determine the effects of viral infection and S-exposure on HCO viability, we investigated the apoptotic pathway 72 h post infection or stimulation. Thus, the mRNA expression of some pro-apoptotic genes, including BAX, BCL2, and Caspase 8, was established. Results showed that all these genes were upregulated upon both SARS-CoV-2 infection and S-protein exposure (Fig.5A). A significant decrease in the BCL2/BAX ratio was nevertheless observed only after HCO SARS-CoV-2 infection, suggesting various plausible speculations about how viral entry affects the apoptotic pathway (Fig.5B).

Through immunofluorescence analyses, we assessed the expression of Caspase 3, a key modulator of apoptosis in neuronal cells and organoids. As demonstrated by the representative immunofluorescence panel (Fig.5C) and corroborated by fluorescence quantification analysis (Fig.5D), Caspase 3 expression was significantly increased in HCOs following SARS-CoV-2 infection compared to the uninfected (mock) condition.


A
Apoptosis Pathway
Nfold
Target gene/GAPDH
1500
1000
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0
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*
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BAX
BCL2
CASP 8
mock
SARS-CoV-2 infected
spike
B
Bcl2/Bax
NFold (BCL2/BAX)
1.0
0.8
0.6
0.4
0.2
0.0
*
mock
SARS-CoV-2 infected
spike
D
AreaCasp3/DAPI
% Fluorescence Intensity
10
8
6
4
2
0
mock
SARS-CoV-2 infected
spike
C
DAPI
CASP3
N
merge
Mock
SARS-CoV-2 infected
SPIKE

Figure 5. Apoptotic pathway alteration in SARS-CoV-2-infected or S-exposed HCOs. A. Real-Time qPCR was utilized to quantify mRNA levels of genes related to the apoptotic pathway (BAX, BCL2 CASP8), in uninfected (Mock), SARS-CoV-2-infected, and S-exposed HCOs. The $2^{-\Delta\Delta Ct}$ equation was used to quantify expression level. **B.** The ratio between mRNA from the anti-apoptotic (BCL2) and pro-apoptotic (BAX) genes was analyzed at 72 hpi or spike stimulation. Data are displayed as the average value with the standard error of the mean (SEM) and were derived from a minimum of three separate experimental runs. *=p<0.05; **=p<0.01. **C.** Caspase 3 protein (green), viral nucleocapsid protein (red) and nuclei (blue) analysed by immunofluorescence assay in Mock, SARS-CoV-2-infected and S-exposed HCOs at 72 hpi. Bars are equivalent to 60 µm. **D.** Quantification of Caspase 3 fluorescence intensity, expressed in percentage, obtained by analyzing the ratio of Casp3 area/DAPI area from three different images acquired from the same HCO section.

4.3.3 Discussion

While the Blood-Brain-Barrier (BBB) serves as a highly effective safeguard, preventing peripheral immune cells and external molecules from entering the brain parenchyma, some viruses, including the highly pathogenic coronaviruses MERS and SARS, have developed neuroinvasive abilities to bypass this protective barrier and infect the CNS. This, in turn, causes both immediate and long-term adverse outcomes [248, 411–413]. Likewise, neurological symptoms, like dementia or encephalopathy, reported during the recent SARS-CoV-2 pandemic, suggest that this coronavirus could injure the central as well as the peripheral nervous systems. It is hypothesized that an excessive inflammatory response plays a major role in this context; however, direct infection of neural cells may also contribute to the onset of neurological symptoms. Notwithstanding, there is limited and conflicting information regarding the impact of SARS-CoV-2 infection on neuronal homeostasis. For example, Pedrosa *et al.,* reported that SARS-CoV-2 has a constrained capacity of neuronal infection, while astrocyte infectability was evident and triggered inflammatory responses and neuronal damage [344]. Conversely, in several *in vitro* cell culture models, SARS-CoV-2 was proved capable of efficiently infecting neurons derived from both human embryonic stem cells and iPSCs [34, 293, 294, 414]. To further dissect this controversial issue, we take advantage of human iPSC-derived brain organoids, which are *in vitro* 3D models mimicking the molecular, cellular, and functional aspects of the human brain.

We first assessed the presence of different entry factors (ACE2, NRP1, CD147, Furin) exploited by SARS-CoV-2 to infect cells on HCOs. Gene expression profiling and IF techniques showed that all these molecules are expressed on HCOs. Moreover, their expression was consistently induced following both SARS-CoV-2 infection and S-exposure, indicating that HCOs recognize the virus/viral components and react to such encounters. Though preliminary, these findings suggest that SARS-CoV-2 may directly infect HCOs, potentially exploiting the interactions with each of the entry factors

analyzed. This result is particularly relevant considering that CD147 and NRP1 proteins are significantly expressed in the human brain [415], and their utilization by SARS-CoV-2 has been extensively documented [74, 77–79, 111]. Newly emerging evidence, indeed, indicates that CD147 and NRP1 proteins display a more comprehensive and widespread expression pattern in the human brain compared to ACE2 [77, 78, 100, 150, 297, 299, 345, 415, 416].

In line with this speculation, herein we demonstrated through different experimental approaches that SARS-CoV-2 productively infects HCOs and replicates in this 3D cellular model. These findings corroborate the previously established evidence of productive SARS-CoV-2 infection and replication reported in the 2D neuronal cortical HCN-2 cell line [399], as well as in HCO conducted by other research groups [32, 34, 288, 417–420]. The viral replication rate in HCOs was notably constrained compared to that observed in cell lines known for their susceptibility to SARS-CoV-2 infection, such as Vero E6 cells. However, in this 3D model, the immunological components, mainly represented by astrocytes and microglia, are missing. Microglia, the primary immune cells in the brain, swiftly activate in response to viral infections, creating a pro-inflammatory milieu where cytokines, chemokines, and reactive oxygen species potentially enhance viral infection and replication, leading to neuronal damage [17, 229, 257, 260–263, 266–268, 421]. Therefore, it is conceivable that under pro-inflammatory conditions, particularly in individuals with pre-existing neuronal pathology, there might be a substantial escalation in viral entry and replication mechanisms.

At the same time, it should be underlined that in this HCO model, the protective effect physiologically exerted by the BBB is completely absent. Previous findings have consistently demonstrated SARS-CoV-2's capacity to pass through the BBB using a transcellular pathway, leading to an amplification of the inflammatory response [17, 35, 295, 414, 422–424]. Analyses performed in Cerebrospinal Fluid (CSF) of COVID-19 patients, nevertheless, revealed the presence of viral particles exclusively in individuals with concurrent pathologies linked to BBB impairment, emphasizing the crucial role of the BBB in preventing SARS-CoV-2 infection [425]. To mimic the few viral particles, which are expected to cross the BBB, we performed the *in vitro* HCO-SARS-CoV-2 infection assay with a very low viral input (0.001 MOI). However, further experiments in more complex human brain organoid models, designed to recapitulate the vasculature and microenvironment of the BBB, are currently underway and are expected to provide further insights into the ability of SARS-CoV-2 to breach the BBB and infect neurons.

In our model, we observed that iPSC-HCO homeostasis is altered even with a very low viral input. In fact, transcriptomic analyses showed a relevant modification in the expression of genes partaking in different intracellular pathways. Among the upregulated genes some are involved in signalling transduction (NFKB, STAT1, STAT3) and interferon pathway (IFITM1, IFITM3) and are recognized

as essential components of protective antiviral responses [348, 351]. As these pathways are triggered even just following S-exposure, these results suggest that HCOs can recognize viral particles and initiate defensive responses even in the lack of a productive infection.

Notably, both infection and S-exposure also upregulated the expression of the neuronal stress marker S100B. This calcium-binding protein is released in biological fluids and serves as a consistent indicator of stressful conditions. Indeed, increased S100B serum concentrations have been associated with COVID-19 severity and neuronal damage [32, 426, 427]. The initiation of an inflammatory reaction in SARS-CoV-2-infected HCOs was further substantiated by the increased expression of genes associated with the inflammasome pathway, particularly NLRP3 and IL-18. Notably, NLRP3 inflammasome activation has been observed in the brains of SARS-CoV-2-infected patients [263, 428, 429], signifying its pivotal involvement in CNS lesions associated with SARS-CoV-2. Of note, as the expression of these markers was increased even following S-exposure, it is worthwhile to wonder if, even in the absence of an overt infection, Spike alone could induce neurotoxic effects in the CNS, as already observed in other cellular models [265, 430–435]. Indeed, it has already been demonstrated that the S protein could alter the barrier function [279] as well as cross the BBB and enter the brain in mice [436].

The antigen presentation pathway also exhibited significant activation following SARS-CoV-2 infection, as evidenced by the increased ERAP1/ ERAP2, TAP and b2-microglobulin expression. This result is not unexpected, as the modulation of HLA-I and ERAP expression has already been documented in several cell lines following viral infections, including SARS-CoV-2 [406, 437], and is generally considered as a protective mechanism activating viral-specific lymphocyte responses. However, in the CNS, this phenomenon could display negative repercussions. Neuronal HLA-I expression plays central roles in the formation, function, and alteration of synapses; yet, an abnormal increase is known to occur upon immune activation, including viral infections, and also during neurodegeneration [438]. HLA-I presents viral antigens to CD8+ T lymphocytes and activate cytotoxicity to prevent lethal viral spread [439]. Excessive or misdirected cytotoxicity can nevertheless be responsible for autoimmune responses, potentially supporting a role for virus-induced HLA-I upregulation in the development of neurodegenerative diseases [438, 440]. Therefore, in the next future it will be interesting to verify whether HLA-I upregulation does contribute to SARS-CoV-2-associated neuropathology.

Finally, the disruptions of neuronal homeostasis caused by either HCO SARS-CoV-2 infection or S-protein exposure also promoted the upregulation of Caspase 8, BAX, and BCL2 expression, mediators of the apoptotic pathway. It is noteworthy, though, that the anti-apoptotic (BCL2)/ pro-

apoptotic (BAX) ratio significantly decreased only in SARS-CoV-2-infected HCOs, indicating that after infection, apoptosis is somewhat enhanced in this neural model. These results seem to confirm previous findings showing that SARS-CoV-2-infected neurons develop cellular stress, promoting cell death [32, 288, 414, 420, 441]. Likewise, Li *et al.* observed an increase in Caspase 8 levels, responsible for apoptosis and inflammation, in SARS-CoV-2-infected lung epithelial cells, indicating a cellular response to viral infection [383]. Remarkably, in our 3D model, we also observed a significant rise in the expression of Caspase 3, a crucial mediator of apoptosis in neuronal cells and organoids [32, 295, 420]. Considering its substantial role in the break of amyloid-beta A precursor, which is linked to Alzheimer's disease-related neuronal degeneration [442, 443], the augmented expression of this protein after HCO SARS-CoV-2 infection could potentially be correlated with the initiation of subsequent neurological symptoms.

Overall, these findings confirm that HCOs may be infected by SARS-CoV-2, further supporting other studies in the fields that also showed a significant infection of cells such as cortical neurons, astrocytes and certain neuronal progenitor population [32, 34, 288, 288, 295, 296, 345, 346, 420, 441, 444–446], mainly localized in the periphery [447] of brain organoids. Such infection potentially occurs exploiting the interaction with ACE2, CD147, and NRP1 proteins. Furthermore, exposure to the S protein alone exhibited the capacity to disrupt their equilibrium and induce neurotoxic effects, potentially playing a role in the onset of long-COVID symptoms.

However, SARS-CoV-2 has a wide range of viral proteins, beyond the spike, that can induce host immune responses even without direct infection. For instance, current researches indicate that nucleocapsid protein plays a significant role in modulating immune responses [448–451]. It would, therefore, be interesting to test the effects of other viral proteins, primarily the N protein on HCOs, to further explore the 'armamentarium' of SARS-CoV-2 and its effects on the host immune system. Moreover, it’s worthwhile to consider that the disrupted phenotype caused by SARS-CoV-2 infection/replication could be massively diluted by most of the cells which, according to IF data, are not infected. In the next future, we, therefore, intend to verify how viral infection/replication affects mRNA transcription, by adopting a single cell approach.

Despite the acknowledged limitations, this study offers relevant insights into the molecular mechanisms that might be behind the neurological symptoms of COVID-19 and long-COVID, providing an opportunity to identify novel therapeutic targets to prevent or control the onset of neurological symptoms.

5. CONCLUSIONS

Since the emergence of Coronavirus Disease-19 (COVID-19) and related neurological manifestations, the ability of SARS-CoV-2 to enter and productively infect CNS cells has been extensively studied. To date, is not well established whether and how SARS-CoV-2 is able to infect neurons but what is becoming day by day more evident is that SARS-CoV-2 infection can affect the brain parenchima, probably by triggering an inflammatory response which in turn can cause neurological disorders. Indeed, the percentage of patients with SARS-CoV-2-associated CNS impairments during the post-acute phase, which are part of the wide spectrum of complications associated with long-COVID, can be up to 30–60% [229, 452–454]. Neurological and cognitive symptoms are a major feature of long-COVID, including sensorimotor symptoms, memory loss, cognitive impairment, paraesthesia, dizziness and balance issues, sensitivity to light and noise, loss of smell or taste, and autonomic dysfunction, often impacting activities of daily living. Currently available data indicates that the effects of SARS-CoV-2-associated CNS complications seem relatively confined in the acute phase but become more conspicuous in the post-acute phase. Anyway, both of these phases of SARS-CoV-2 infection are associated with a wide range of complications, which might be related, at least in part, to host factors include age, sex, metabolic status, or pre-existing neurological conditions, comorbidities, immune status of the host, virus variants, or other variables [229, 455–457] that may affect the risk of developing severe disease from COVID-19.

Furthermore, there is a need for additional research to explore potential distinctions in neuroinvasiveness, neurotropism, or neurovirulence among various SARS-CoV-2 variants. It has been demonstrated that SARS-CoV-2 variants diverge in their approach to respiratory disease pathogenesis [229]. There is still much to uncover regarding the fundamental mechanisms responsible for SARS-CoV-2-induced neuropathology.

Albeit no conclusive evidence on whether the observed neurological sequelae are attributable to direct CNS invasion by the virus, or Spike exposure, a plethora of studies converge in documenting heterogeneous manifestations which support its neuropathological potential.

In accordance with this information, taken together, our preliminary data suggest that: 1) the exposure to different SARS-CoV-2 variants affects Dopaminergic neuron homeostasis; 2) SARS-CoV-2 could infect human iPSC-derived MN probably by binding CD147 and NRP1 receptors and that MN-SARS-CoV-2 infection is accompanied by the activation of the antiviral response; 3) SARS-CoV-2 could infect iPSC-HCO, probably by binding CD147 receptor and iPSC-HCO infection as well as exposure to the Spike protein alters HCO homeostasis and results in neurotoxic effects. Overall, these data are in line with those profiled in other SARS-CoV-2 infected neuronal cell models [398–401],

suggesting an embedded mess-up of the transcriptional machinery affecting the cellular equilibrium.

Despite the gathering of so many results, my PhD project displays some limitations and further analyses in each of the three sub-projects are necessary to validate and expand the data obtained. For example, in our HCO model the protective effect physiologically exerted by the BBB is completely absent, and this could significantly affect the results obtained. The BBB effectively prevents foreign molecules and immune cells from reaching the brain, but some viruses have developed neuroinvasive capabilities to infect CNS tissue [413]. However, previous studies provide evidence supporting that SARS-CoV-2 can cross the BBB in a transcellular pathway leading to an exacerbation of the inflammatory response [422, 423], which highlights the importance of this structure in preventing SARS-CoV-2 infection to the CNS [425]. However, further experiments in more complex models encompassing HCO plus BBB are ongoing in our laboratory to confirm SARS-CoV-2 capability to pass through the BBB and effectively infect neurons.

Likewise, in the Motor neuron model the lack of the immune system, of an in vivo blood-CNS barrier, a certain level of heterogeneity in cell differentiation, and the restricted number of enrolled subjects represent limiting factors in the interpretation of the results. Indeed, it is possible that the low rate of viral replication in MNs depends on the absence of the immunological component which, following SARS-CoV-2 infection, may favour the onset of a pro-inflammatory environment that is advantageous for viral infection/replication, and subsequent neuronal damage. It is therefore possible that in a pro-inflammatory setting, (i.e. in patients with pre-existing neuromuscular conditions) the rate of viral entry and replication would be substantially higher.

Despite these experimental limits in the adopted models these results might partially explain some of the neurological symptoms characterizing the so-called long COVID symptomatology and suggest the possibility to exploit the reported pathways as new therapeutic targets or biomarkers of disease progression to be searched in human biological fluids or tissues.

For instance, it might be interesting to quantify the dopamine (DA) metabolites in infected patients, such as homovanillic acid (HVA), which can be measured in urine. These analyses would confirm whether a reduction in total DA levels is coupled with the neuronal deficit, both in the acute phase and/or in the long-COVID phase, several months later. Based on these results, analyses could be performed on different cohorts distinguishing patients who experienced neuro-difficulties exclusively during the infection or even afterward, as well as between those who had a brief infection and those with a prolonged one. Furthermore, if the focal point of the issue is represented by the absence of the TH enzyme and this would be confirmed in vivo, DA could be considered as a new predictive marker

of neuronal damage or long-COVID. In a therapeutic context, new or existing strategies could then be adopted, such as the administration of Levodopa, to mitigate or eliminate the neurological problems associated with COVID-19.

Overall, the data collected through my PhD project are solid and consistent and being based on three independent experimental models they prove that SARS-CoV-2 may significantly affect both the central and peripheral nervous system. The direct infection/replication of these cells activates different molecular pathways responsible of the neurological problems observed in SARS-CoV-2 infection and long COVID symptoms. As previously mentioned further analyses are necessary to validate the results obtained and more sophisticated models should be adopted to properly mimic the physiological conditions. Notwithstanding, such information are key to correctly address still unanswered inquiries concerning the neurotoxic effect triggered by SARS-CoV-2 on neuronal cells and to test possible remedies to counteract such complications. Moreover, comparing the results among these different experimental models is expected to provide a novel, comprehensive scenario on the molecular virus-host interactions occurring in the CNS/PNS, meanwhile elucidating potential neuropathological mechanisms related with long-term health consequences of SARS-CoV-2 and identifying biomarkers and targets of disease.

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