Numerous patients experience neurological and neuromuscular symptoms during and after infection with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Apart from the central nervous system (CNS), the peripheral nervous system (PNS) is also affected. In this study, the Italian authors investigated whether SARS-CoV-2 can infect and replicate in human motor neurons (MNs) differentiated *in vitro* from induced pluripotent stem cells (iPSC-MNs). They also examined whether iPSC-MNs express the main receptors for SARS-CoV-2 entry and whether SARS-CoV-2 infection of iPSC-MNs changes the expression of 46 genes involved in cell survival, metabolism, inflammatory response, apoptotic and antiviral pathways.

SARS-CoV-2 is an enveloped, positive-sense, single-stranded RNA virus. Its genome encodes four structural proteins, namely the spike (S), envelope (E), nucleocapsid (N), and membrane (M) proteins. It seems that SARS-CoV-2 uses various neuroinvasive strategies and entry pathways to invade the nervous system, such as infection of the nasal olfactory epithelium and axonal transport along the olfactory nerve, retrograde axonal transport, invasion by compromising the blood-brain barrier, retrograde virus spread from the lungs to the CNS *via* the vagus nerve, and the use of infected hematopoietic cells as "Trojan horses" (hematogenous route).

It remains unknown whether observed neuromuscular manifestations of SARS-CoV-2 infection are caused by a direct viral invasion of motor neurons, and/or are a collateral injury resulting from an uncontrolled innate immune response. Damage to motor neurons leads to the deterioration of muscle function, manifested as muscle weakness, atrophy, or paralysis.

Two host-cell factors are important for SARS-CoV-2 viral entry into many cell types: angiotensin-converting enzyme 2 (ACE2), which is bound by the S protein, and transmembrane serine protease 2 (TMPRSS2), which cleaves the S protein, allowing this binding to take place. In addition to ACE2 and TMPRSS2, the S protein has been reported to engage other cell-surface factors proposed to serve as attachment factors promoting SARS-CoV-2 entry.

The expression level of ACE2 is low in the human brain. In contrast, neuropilin 1 (NRP1), CD147, TMPRSS2, and furin are higher and broader expressed than ACE2, indicating that they may be putative mediators of SARS-CoV-2 entry into human nervous system cells. According to the authors of this study, the infection of human motor neurons with SARS-CoV-2 mainly relies on CD147 and/or NRP1 binding. Previous studies have shown that NRP1, known to bind furin-cleaved substrates, potentiates SARS-CoV-2 infectivity and that

the furin-cleaved S1 subunit of the S protein binds directly to cell surface NRP1.
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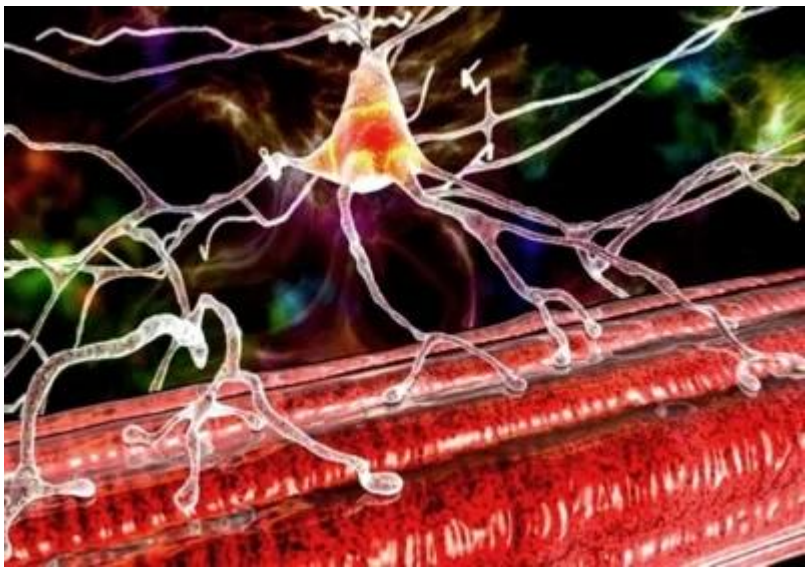

About the study

The authors used an *in vitro* model of human motoneurons (MNs) differentiated from induced pluripotent stem cells (iPSC-MNs) to investigate the infectability of these cells by SARS-CoV-2 and their expression of the main SARS-CoV-2 receptors. They also examined whether SARS-CoV-2 changes the expression of 46 genes involved in cell survival, metabolism, inflammatory response, and apoptotic and antiviral pathways.

The induced pluripotent stem cells (iPSCs) from three healthy donors (1 male and 2 females, aged between 37 and 49 years) differentiated into motor neurons that expressed both, neuronal (bIII-tubulin and SMI-312) and motoneuronal (ChAT, HB9) markers.

To verify the infectability of iPSC-MNs by SARS-CoV-2, VeroE6 cells were exposed to supernatants collected from iPSC-MNs infected with SARS-CoV-2. Reverse transcription polymerase chain reaction (rt-PCR) was used to analyze the expression of SARS-CoV-2-specific ORF7A, ORF3A, ORF8, RDRP, S, E, and N genes in uninfected (mock) and infected iPSC-MNs.

The expression of main SARS-CoV-2 receptors (ACE2, CD147, NRP1) and peptidases (TMPRSS2, furin) was assessed in iPSC-MNs infected with SARS-CoV-2 and the A549-

hACE2 cells, as a positive control.

Results

To validate the infectability of iPSC-MNs, VeroE6 cells were exposed to supernatants collected from infected iPSC-MNs at different time points. The results showed that supernatants collected from infected iPSC-MNs could re-infect VeroE6 cells. Furthermore, the SARS-CoV-2-specific genes (N, E, S, ORF3A, ORF8, and ORF7A) were detected exclusively in infected iPSC-MNs. The expression of SARS-CoV-2 N1, S1, S2, and E2 genes in infected iPSC-MNs was subsequently confirmed by rt-PCR.

The expression of SARS-CoV-2 specific N1 and N2 genes was identified in supernatants from infected iPSC-MN cultures by rt-PCR, confirming that SARS-CoV-2 can infect and replicate in human motor neurons. However, the viral replication level was lower in iPSC-MNs than in VeroE6 cells. In addition, SARS-CoV-2 replication in infected iPSC-MNs was not accompanied by a cytopathic effect as assessed by the crystal violet assay. In addition, the immunofluorescence assay detected N protein in infected iPSC-MNs, mainly at the perinuclear level, in the soma, and along the neurite extensions. However, the percentage of infected iPSC-MNs was very low.

Further analysis demonstrated that iPSC-MNs expressed the main entry receptors of SARS-CoV-2, including ACE2, CD147, NRP1, and TMPRSS2, but at different levels. ACE2 and furin were expressed at lower levels, whereas CD147 and TMPRSS2 were expressed at higher levels in infected iPSC-MNs compared to the control A549-hACE2 cell line. The NRP1 expression was comparable between iPSC-MNs and A549-hACE2 cells. The immunofluorescence assay for ACE2, CD147, and NRP1 confirmed these results.

In iPSC-MNs, SARS-CoV-2 infection changed the expression of 10 genes involved in cell survival, metabolism, antiviral, and inflammatory response. The virus up-regulated the expression of B-cell lymphoma-2 family protein (BCL2), BCL2-associated X protein (BAX), caspase 8, CD147, proinflammatory interleukin-6, and sphingosine-1-phosphate receptor 1, involved in the regulation of lymphocyte trafficking, brain and cardiac function, vascular permeability, and vascular and bronchial tone. The virus down-regulated the expression of human leukocyte antigen-A and endoplasmic reticulum aminopeptidase 1, involved in antigen processing and presentation, and angiogenin, which exerts neuroprotective functions and contributes to the systemic response to infection.

Interestingly, an increased ratio between the expression of anti-apoptotic BCL2 and pro-apoptotic BAX gene suggests that programmed cell death was somehow prevented in iPSC-

MNs after the infection.

Conclusion

The authors concluded that this study has shown, for the first time, that SARS-CoV-2 can infect and replicate in iPSC-derived human motor neurons. However, viral replication and the percentage of infected cells were lower than in VeroE6 cells, susceptible to SARS-CoV-2.

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