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Astroglia-mediated neuroinflammation as a putative mechanism of neurological outcomes in COVID-19? Insights from a Brazilian cohort

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ABSTRACT

NeuroCOVID-19 has emerged as a significant global health concern, presenting a wide spectrum of neurological manifestations, including headaches, brain fog and anosmia. While mounting evidence indicates that SARS-CoV-2 infection compromises central nervous system (CNS) function, the precise processes underlying these effects remain incompletely understood. Although neurons have been extensively studied, astrocytes – critical regulators of brain homeostasis - have been largely overlooked in this context. In this study, we position astrocytes as central players in the neuropathological landscape of neuroCOVID-19, challenging their traditionally supportive role. We evaluated the frequent neurological symptoms in a Brazilian cohort of COVID-19 patients and investigated whether SARS-CoV-2 infection of cortical astrocytes induces neuroinflammation, glutamatergic imbalance, vasoregulatory disruption, and apoptosis as likely pathogenic processes. Among 162 COVID-19-positive patients, headache (53.09 %), brain fog (42.15 %), and anosmia (38.72 %) were the most commonly reported symptoms. Using human-induced pluripotent stem cell (hiPSC)-derived astrocytes, we found that SARS-CoV-2 infection promotes a pronounced pro-inflammatory response, evidenced by elevated levels of IL-6, IL-15, and IL-4 in the culture supernatant. Infected astrocytes also showed reduced mRNA expression of *KLK1* and *EAAT1*, key genes involved in vasodilation and glutamate clearance, respectively. Additionally, a significant increase in cleaved caspase-3-positive cells indicated enhanced apoptosis. Overall, these findings demonstrate that SARS-CoV-2 disrupts astrocyte homeostatic functions, leading to neuroinflammation, excitatory neurotransmission dysregulation, and cell death that may, hypothetically, underlie the neurological sequelae of COVID-19. By reframing astrocytes as active protagonists, this study highlights their essential role in CNS vulnerability. It also suggests potential targets for the future investigation in the development of therapies against the neurological complications of COVID-19.

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1. Introduction

Neurological symptoms such as hypo/ageusia, hypo/anosmia, headache, fatigue, seizures, brain fog, hallucinations, and stroke have been widely reported during the acute phase of COVID-19 (Jin et al., 2020). In addition, the long-term effects SARS-Cov-2 infection, commonly referred as long COVID – continue to emerge with many symptoms linked to the persistent neurological involvement aspects (Geng et al., 2025). Although the pandemic has officially ended, SARS-CoV-2 remains globally prevalent, and neurological complications persist across different viral variants (Huang et al., 2020; Grisanti et al., 2025). Initially, these neurological symptoms were primarily attributed to secondary effects of systemic infection, including hypoxia and cytokine-mediated neuroinflammation (Mangalmurti et al., 2020). However, accumulating evidence has established SARS-CoV-2 as a neurotropic virus, capable of directly infecting neural tissues, which may underlie a spectrum of neuropsychiatric and cognitive sequelae (Cheng et al., 2019; Conde et al., 2020). While much of these studies focused on the SARS-CoV-2 impact on neurons and microglia, astrocytes – despite their essential role in the CNS homeostasis – have received comparatively limited attention. Recent findings now indicate that SARS-CoV-2 may exhibit gliotropism, and the ability to infect and disrupt astrocytic function (Andrews et al., 2022). However, the downstream consequences of this SARS-Cov-2 infection on astrocyte function, and their broader implications for brain physiology and behavior remain insufficiently understood. Given that the neuron-to-astrocyte ratio in the central nervous system (CNS) is approximately 1:1, and considering astrocytes' critical roles in neurotransmitter synthesis and reuptake, regulation of cerebral blood flow, and maintenance of brain homeostasis, their involvement in Neuro-COVID-19 warrants deeper investigation (Izrael et al., 2021). It is worth noting that, astrocytes are often among the first CNS cells to be targeted by viruses, mounting an innate immune response characterized by cytokine production and release, contributing to neuroinflammation, cell death and long-term neurological dysfunctions (Jorgačevski and Potokar, 2023).

The impact of SARS-CoV-2 infection on human astrocytes can be effectively modeled using various *in vitro* approaches, including primary and immortalized cell cultures, as well as astrocytes derived from glioblastoma cell lines or differentiated from pluripotent stem cells (Jacob et al., 2020; Vanhulle et al., 2022). *In vivo* studies (Rockx et al., 2020; Chan et al., 2020), *postmortem* brain analyses (Younger, 2021; Menezes et al., 2022), three-dimensional *in vitro* models (Ramani et al., 2020; Zhang et al., 2020), and two-dimensional co-cultures of human neural cells have proven instrumental in elucidating the complex cellular interactions underlying Neuro-COVID-19 (Zhang et al., 2020). These models provide insights into how SARS-CoV-2 disrupts neural homeostasis and promotes neuropathology. In contrast, two-dimensional monoculture systems offer the examination of the specific response of a particular cell type to the virus, enabling precise characterization of astrocyte-specific independently of peripheral and adjacent neural cell types. Although all these *in vitro* and *in vivo* models have been useful in advancing our understanding of SARS-CoV-2 neuropathology, the astrogenesis from human pluripotent stem cells hiPSCs offers a particularly interesting platform with a relevant translational value (Luciani et al., 2024). Moreover, the genome stability of hiPSCs increases across passages, in contrast to immortalized or glioblastoma-derived cell lines, which can easily accumulate mutations (Beltrão-Braga et al., 2011). In this context, the present study aimed to investigate the prevalence of neurological symptoms in a COVID-19 Brazilian cohort and to explore, *in vitro,* the possible astrocytic disturbances caused by SARS-CoV-2 infection. Specifically, we examined the impact of SARS-CoV-2 infection on key astrocytic functions related to vasodilation, glutamatergic signaling, and inflammatory and apoptotic pathways. We hypothesized that SARS-CoV-2 gliotropism may trigger a pro-inflammatory response, disrupt gene expression of key proteins critical for proper glutamatergic neurotransmission and cerebral blood flow regulation, and promote apoptosis in cortical astrocytes. Taking into account the role that astrocyte-specific alterations play in headache and memory deficits *per se*, we shed light on these cells as potential contributors to the development of such symptoms in Neuro-COVID-19 context. Future studies should verify this hypothesis thoroughly.

2. Methods

2.1. Subjects anamnesis and molecular triage

The studies involving human participants were approved by the Human Research Ethics Committee of the Institute of Biomedical Sciences, of the University of São Paulo (#4.036.252). The human data presented here were obtained from the database of the project entitled "SARS-CoV-2 in the Metropolitan Region of São Paulo - Emergency Action" and the associated biorepository (#CAAE #3146620.6.0000.5467) maintained by the *Institut Pasteur de São Paulo* collected during the task force against COVID-19 (Cunha et al., 2021). Clinical and molecular data, including anamnesis and RT-qPCR diagnostic results were extracted from institutional databases. Data processing and analysis were conducted using Python. Raw datasets were merged based on anonymized patient identification codes to ensure correlation between clinical and molecular parameters.

2.2. SARS-CoV-2 isolation and amplification

The SARS-CoV-2 isolate used in this study belonged to the B lineage (GenBank: MT350282.1) and was isolated from the nasopharyngeal swab from the second brazilian patient who confirmed for COVID-19 in Brazil (Araujo et al., 2020). All experiments with SARS-CoV-2 were conducted in the biosafety level 3 (BSL3) laboratory at the Institut Pasteur of São Paulo following established biosafety guidelines. Viral amplification was carried out in Vero CCL81 cells using DMEM high glucose medium (Gibco, MA, USA), supplemented with 10 % fetal bovine serum (LGC, SP, Brazil). The medium was removed 72 h after inoculation, and aliquots were harvested and stored at −80 °C. Viral titers were determined according as previously described (Araujo et al., 2020).

2.3. Astrocyte generation

The Astrocytes used in this study were derived from human-induced pluripotent stem cells (hiPSCs) generated through the reprogramming of stem cells from human exfoliated deciduous teeth (SHED), obtained from children's teeth donations (CEP-ICB/USP: CAAE: 37309420.0.0000.5467). These individuals were recruited through the "The Tooth Fairy Project" initiative at the University of São Paulo. Ethical approval for the study was granted by the Ethical Committee of the Institute of Biomedical Sciences at the University of São Paulo, Brazil (CEP-ICB/USP #1001; Biorepository: CAAE #58219416.0.0000.5467). The protocols for SHED culture and subsequent reprogramming using the Sendai virus (CytoTune; Thermo Fisher Scientific, MA, USA) have been previously described (Beltrão-Braga et al., 2011; Russo et al., 2018).

Neural Progenitor Cell (NPC) generation, hiPSCs were seeded on Matrigel (Corning, NY, USA) and maintained in culture until achieving 80 % confluence. Subsequently, the culture medium was switched to N2: DMEM/F12 supplemented with 1x N2 supplement (Invitrogen, MA, USA) and dual SMAD inhibitors—1 μM dorsomorphin (Tocris, BRS, UK) and 10 μM SB431542 (Stemgent, MA, USA). Colonies were then manually detached and cultured in suspension to form embryoid bodies (EBs) for 5 day at 90 rpm in an N2 medium. Following the suspension period, EBs were plated onto Matrigel-coated plates and transitioned to neural basal factor (NBF) medium, consisting of DMEM/F12 supplemented with 0.5x N2, 0.5x B27 (Gemini Bioproducts, LIV, UK), 20 ng/

mL FGF2 (Gibco), and 1 % penicillin/streptomycin (Gibco). Neural emerging rosettes containing NPCs were manually selected, dissociated, and plated on double-coated plates with poly-L-ornithine (10 μg/mL; Sigma, MA, USA) and laminin (2.5 μg/mL, Thermo Fisher Scientific) double-coated plates. NPCs were subsequently expanded using the NBF medium.

Cortical astrocytes were generated using a previously validated protocol (Russo et al., 2018). Confluent NPC cultures in a 100 mm diameter plate were incubated with dPBS (Gibco) at 37 °C for 5 min and then detached to form neurospheres. These cells were gently dissociated by pipetting and transferred to a six-well plate under continuous agitation (90 rpm). The medium was refreshed 24 h post-suspension, upon visible neurosphere formation. A ROCK inhibitor was added at a final concentration of 5 μM for 48 h in the absence of FGF2 media. After removing the ROCK inhibitor, NB media, without FGF2, was used for one week. Subsequently, Astrocyte Growth Media (AGM; Lonza, Basel, CH) was added to the spheres for two weeks and maintained at constant shaking (90 rpm), with media changed every 4–5 days. Mature spheres were then plated on double-coated poly-L-ornithine/laminin coated dishes, where mitotically active astrocytes were expanded and maintained in AGM media. Astrocytes were enzymatically detached using Accutase (Cellgro, VI, USA) once cultures reached about 80 % confluence. For all the experiments described herein, astrocytes were characterized by immunostaining and utilized at passages three to four.

2.4. SARS-CoV-2 infection

Cortical astrocytes were infected with SARS-CoV-2 at 37 °C in a BSL3 laboratory, using a multiplicity of infection (MOI) of 0.5. For viral adsorption, the plate was maintained with a thin layer of medium, and after 1 h, the remaining medium was added without removing the virus to prevent cell detachment. The cells were incubated at 37 °C with 5 % CO2 and observed daily. Samples were collected 24, 48, 72, and 96 h post-infection (hpi).

2.5. Immunofluorescence assay

For immunofluorescence, astrocytes were cultured in 24-well plates containing glass coverslips for cellular characterization until they reached approximately 70–80 % of confluence. Cells were washed with Dulbecco's phosphate-buffered saline (dPBS) and fixed in 4 % paraformaldehyde (Sigma-Aldrich) for 15 min at room temperature (RT). Permeabilization was performed using 0.1 % Triton X-100 (Sigma-Aldrich) for 15 min at RT, following by blocking in 2 % bovine serum albumin (BSA) (Sigma-Aldrich) for 4 h at RT. Cells were incubated overnight at 4 °C with the primary antibodies (GFAP 1:500; Sars-CoV-2 1:200; Casp3 1:200). Blood serum from an infected human patient was used as the primary antibody for SARS-CoV-2 detection. The following day, cells were washed thrice with dPBS and subjected to an additional blocking step with 2 % BSA for 1 h at RT. Secondary antibodies, conjugated with Alexa Fluor 488, Alexa Fluor 555, and Alexa Fluor 647 (Life Technologies, MA, USA, and Abcam, CA, UK) were applied for 1 h at RT in the dark. After triple-washes with dPBS, nuclei were counterstained using 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen, 1:10,000) diluted in a 1x dPBS solution for 5 min. Cells were washed once after the DAPI addition with dPBS and mounted using ProLong Gold Antifade Reagent (Invitrogen).

2.6. Immunofluorescence quantification

Images of total coverslips scanned by TissueFAXS imaging system (TissueGnostics, Vienna, Austria), were processed using Strataquest software Fluorescence image quantification was performed by generating masks that automatically detect individual nuclei and their respective cytosols. Mean fluorescence intensities for each fluorochrome channel were plotted as histograms to establish threshold values based on negative control. Density plots of the mean intensity of fluorochrome pairs were used to determine the percentage of populations. Graphs and statistical analyses were based on duplicates for each subject and performed using GraphPad Prism Software (GraphPad, CA, USA), applying unpaired *t*-test after confirming normality using the Shapiro-Wilk test.

2.7. Molecular analysis by RT-qPCR of cellular lysates

Total cellular RNA was extracted from cultured cells using Trizol Reagent (Life Technologies), according to the manufacturer's instructions. One microgram of total RNA was used for reverse transcription with the SuperScript™ III One-Step RT-PCR System (Invitrogen), according to the manufacturer's instructions. cDNA was amplified with the StepOne Real-Time PCR System (Thermo Fisher Scientific). The reaction was conducted in a 30 μL buffer containing cDNA, SYBR Green master mix (Life Technologies), and sequence-specific primer pairs (Supplementary Table 1). The initial denaturation was performed for 10 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and a 1-min annealing step at 60 °C. Gene expression was normalized to reference genes, peptidylprolyl isomerase A (PPIA) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH), whose expression levels remained stable across experimental conditions. Relative expression results were calculated using the ΔΔCt method and then converted to a GraphPad Prism software using an unpaired *t*-test after confirming the normal distribution of data with the Shapiro-Wilk test.

2.8. Virus replication by RT-qPCR in cellular supernatants

Cell culture supernatants were collected, and viral RNA was extracted using the MagMax Viral/Pathogen kit (Applied Biosystems, MA, USA) on the KingFisher™ Flex automatic extractor system (Thermo Fisher Scientific). One-step RT-qPCR was performed using the Allplex 2019-nCoV Assay kit (Seegene Inc., Seoul, KR) which targets multiple SARS-CoV-2 genomic regions, and analyzed on the QuantStudio 5 Real-Time PCR System (Applied Biosystems). PCR cycle conditions were identical to those used for cellular PCR, as described above. Given that RNA was extracted from cell-free supernatants, amplification of endogenous housekeeping genes was not feasible. Therefore, the analysis focused on the monitoring the cycle threshold (Ct) values across different time points to evaluate changes in viral RNA load over the course of infection. Statistical analysis and graph generation were performed in GraphPad Prism (GraphPad Software, CA, USA). Data normality was confirmed using the Shapiro-Wilk test, and group comparisons were conducted using ordinary one-way ANOVA followed by Bonferroni's multiple comparisons test.

2.9. Cytokine quantification

Cytokine concentration in cell culture supernatants were measured using the Luminex xMAP multiplexing platform (Thermo Fisher Scientific), employing a 27-plex magnetic bead-based panel designed to detect the following targets: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, eotaxin, bFGF, G-CSF, GM-CSF, IFN-γ, CXCL10 (IP-10), MCP-1 (MCAF), MIP-1α, MIP-1β, PDGF-BB, CCL5 (RANTES), TNF-α, and VEGF. The assay was designed according to the manufacturer's protocol to simultaneously quantify the different cytokines. Data analysis was performed in GraphPad Prism GraphPad Software, CA, USA), with normality assessed using the Shapiro-Wilk test, followed by unpaired *t*-tests for intergroup comparisons.

2.10. Glutamate measurement

Cell culture supernatants from SARS-CoV-2-infected astrocyte cultures were collected at defined time points without media replacement and stored at −80 °C until analysis. Glutamate concentrations were

measured using a commercial assay kit (Abcam), following the manufacturer's instructions. Absorbance was recorded at 450 nm using a microplate reader. Statistical analysis and data visualization were conducted using (GraphPad Software, CA, USA). Normality was assessed using the Shapiro-Wilk test, followed by two-way ANOVA with Geisser-Greenhouse correction followed by Bonferroni's multiple comparisons post hoc test.

3. Results

3.1. Neurological symptom prevalence in SARS-CoV-2-positive patients from the São Paulo Metropolitan Region

As part of the COVID-19 task force initiative at the *Institut Pasteur de São Paulo*, data were collected from individuals in São Paulo Metropolitan Region to assess the frequency of neurological symptoms in SARS-CoV-2-infected patients. A structured anamnesis, aligned with the Brazilian Ministry of Health guidelines, was administered to categorize participants as symptomatic, asymptomatic or negative for SARS-Cov-2. Diagnosis was confirmed through RT-qPCR detection of the viral E gene from naso-oropharyngeal swabs. Individuals with confirmed infections were monitored longitudinally to account for potential reinfections. Between March 2020 and October 2022, a total of 2166 samples were analyzed. The mean age of the participants was 35.08 years (Male = 36.6 years; Female = 33.56 years) with the cohort comprising 75,4 % male and 24,5 % female participants. SARS-CoV-2 infection was confirmed in 7.48 % of the investigated cases, with a comparable infection rates between sexes (male positives = 7.22 %; female positives = 8.29 %) (Fig. 1A). Neurological symptoms assessed included asthenia, brain fog, olfactory and gustatory agnosia, and headache (Fig. 1C). Among SARS-CoV-2-positive individuals, 66.67 % reported at least one of the listed neurological symptoms. Headache emerged as the most prevalent, reported by 79.63 % symptomatic individuals, and present in 53.09 % of all positive cases. The distribution of neurological symptom occurrence was similar across sexes (Fig. 1B), suggesting no sex-based disparity in neurological manifestations during SARS-CoV-2 infectIon.

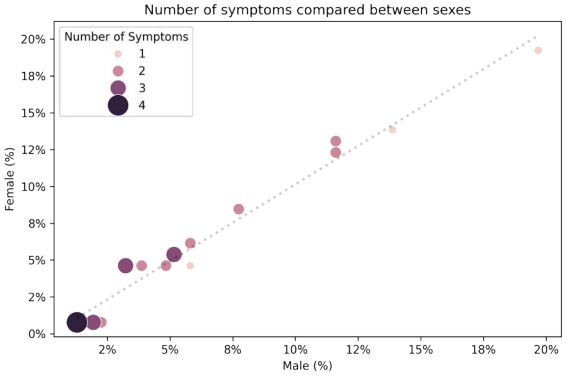
3.2. hiPSC-derived astrocytes are susceptible but not permissive to SARS-CoV-2 infection

Astrocytes differentiated from hiPSC demonstrated susceptibility to SARS-CoV-2 infection, as evidenced by the colocalization of viral and astrocytic markers (Fig. 2A, B, and C) via immunofluorescence analysis and by intracellular viral RNA quantification (Fig. 2D). These findings suggest that the virus is capable of entering astrocytes, likely using the same cellular entry receptors described for neural progenitor cells (NPC) and neurons. To investigate whether the infection modulates the expression of candidate viral entry receptors, mRNA levels of ACE1, ACE2, and CD147 were assessed. Although a slight increase was observed, no statistically significant changes were detected following infection (Fig. 2E; G), indicating that astrocyte susceptibility is not associated with dynamic regulation of these receptors post-entry. Despite evidence of infection, no increase in viral RNA levels was detected in cell culture supernatant upon 96 h post-infection (hpi) for the three viral genomic targets assessed (Fig. 2H). These data indicate that while astrocytes are susceptible to SARS-CoV-2 entry, they are not permissive to productive viral replication. Furthermore, no prominent cytopathic effects or significant cell death were observed under bright-field microscopy after infection up to 72 hpi (Fig. 2I), supporting the notion that astrocytes maintain structural integrity despite viral

A.

	Sex					
SARSCOV2_Gene_E RTQPCR subjects	**Male**		**Female**		**Sum**	*percentage upon all exams*
positives	118	*7.22%*	44	*8.29%*	162	7.48%
negatives	1438	*87.95%*	477	*89.83%*	1915	88.45%
inconclusive	1	*0.06%*	0	*0.00%*	1	0.05%
missing	78	*4.77%*	10	*1.88%*	88	4.06%
Average age (mean ± sd)	36.60 ± 12.53		33.56 ± 13.29			
Average age from positive samples (mean ± sd)	36.00 ± 12.44		38.85 ± 17.31			
Total of subjects by sex	1635	75.48%	531	24.52%		
Total of exams	2166					

B.



C.

	Sex						
SARS-CoV-2 neurological symptoms	**Male**	*total percentage in positive male group*	**Female**	*total percentage in positive female group*	**Sum**	*percentage upon all positive samples presenting neurological symptoms*	*percentage upon all positive samples in the screening*
Asthenia	43	*36.44%*	18	*40.91%*	61	*56.48%*	37.65%
Brain fog	18	*15.25%*	6	*13.64%*	24	*22.22%*	14.81%
Olfactory and gustatory agnosia	36	*30.51%*	16	*36.36%*	52	*48.15%*	32.10%
Headache	61	*51.69%*	25	*56.82%*	86	*79.63%*	53.09%
Total of samples presenting any of the above symptoms	78		30				
Total	108						
Percentage of neurological symptoms over all positive samples	66.67%						

Fig. 1. Results of anamnesis from suspected cases of SARS-CoV-2 in Brazilian patients. A) SARS-CoV-2 test data were divided by sex into categories of positives (total positives = 7.48 %), negatives (total negatives = 88.45 %), inconclusive (total inconclusive = 0.05 %), and missing (total missing = 4.06 %) outcomes. The missing outcomes account for any exam that lacks one or more values from the total information collected in anamnesis. Inconclusive results account for the inconclusive RT-PCR Gene E results. **B)** Positive SARS-CoV-2 tests were divided by sex into categories of neurological symptoms (asthenia, brain fog, loss of smell and taste, and headache). **C)** Comparison of the percentage of positive COVID-19 cases between sexes, considering the number of neurological symptoms.

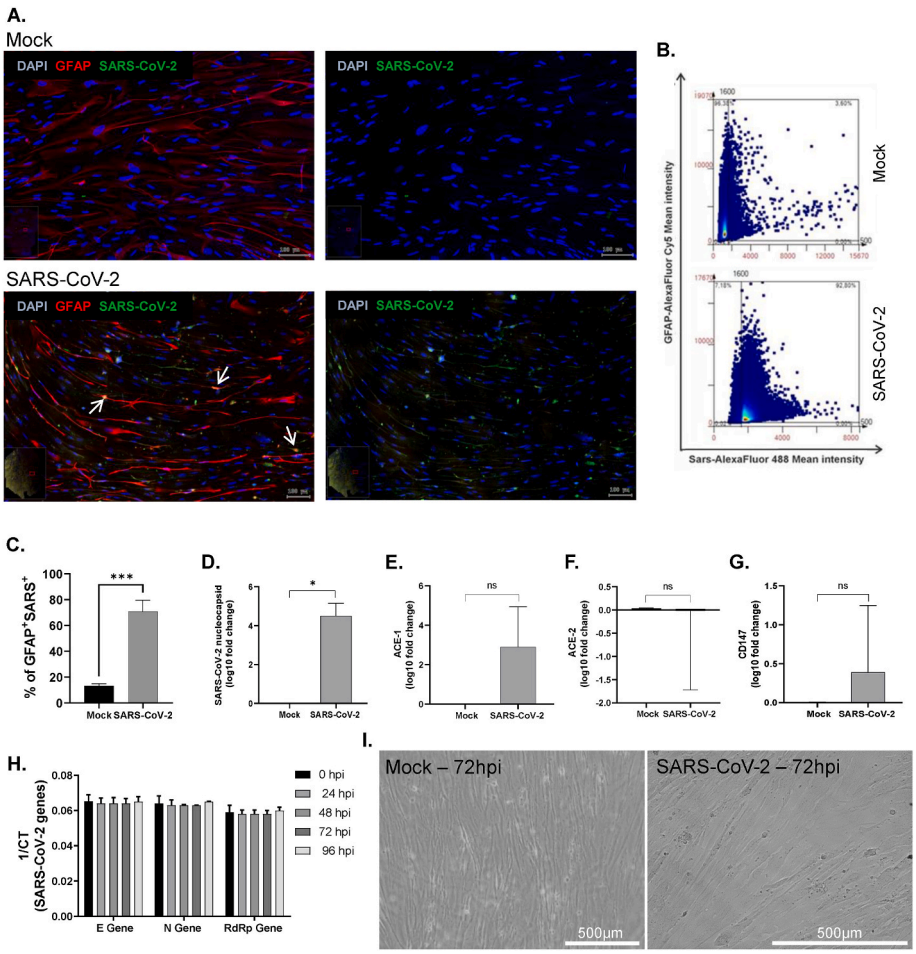


Fig. 2. SARS-CoV-2 gliotropism on cortical astrocytes derived from hiPSCs. Photomicrographs of immunofluorescence staining of uninfected (MOCK) and SARS-CoV-2-infected cortical astrocytes at 96 h post-infection **(A)**. The cellular nucleus was labeled with DAPI. Astrocytes were identified as GFAP + cells (red). SARS-CoV-2 was labeled in green, and GFAP + SARS-CoV-2+ cells are indicated by arrows. Scale bar: 100 µm. **B)** Density plot of Alexa Fluor 647 (GFAP) mean intensity × Alexa Fluor 488 (SARS-CoV-2) mean intensity. **C)** The percentage of GFAP + SARS-CoV-2+ cells was determined by immunofluorescence analysis. The relative expression of **D)** SARS-CoV-2 nucleocapsid, **E)** ACE1, **F)** ACE2, **G)** CD147 mRNA (log10) in cortical astrocytes at 72 h post-infection (hpi) was measured by RT-qPCR. Quantification of **H)** SARS-CoV-2 genes in the cortical astrocyte supernatant was performed by RT-qPCR over the days of infection to evaluate viral replication, using the envelope (E gene), nucleocapsid (N gene), and RNA-dependent RNA polymerase (RdRp gene) as targets. **I)** Bright field microscopy images of uninfected (Mock) or SARS-CoV-2 infected cortical astrocytes at 72 hpi. The data are expressed as mean ± SD. ***p < 0.005, unpaired *t*-test.

presence.

3.3. *SARS-CoV-2 triggers apoptosis in infected astrocytes, induces neuroinflammation, and disrupts the glutamate cycle*

Although SARS-CoV-2 did not result in clear cytopathic effects in hiPSC astrocytes, a significant increase in cleaved caspase-3 expression was detected (Fig. 3A–C), indicating activation of the apoptotic pathway. In parallel, SARS-CoV-2 infection elicited a pro-inflammatory response in astrocytes, as evidenced by enhanced secretion of interleukin-6 (IL-6), interleukin-4 (IL-4), and interleukin-15 (IL-15) (Fig. 3D–F, and Supplementary Fig. 1). Notably, this cytokine response was preserved even at lower viral loads, suggesting a robust innate immune activation upon infection. No significant changes were observed for other cytokines in the panel (Supplemental Fig. 1).

Tissue kallikrein (KLK1), a key modulator of neurovascular function known for its anti-apoptotic and anti-inflammatory properties via the production of vasoactive kinins (Chao et al., 2010), was found to be downregulated in infected astrocytes (Fig. 3G). The reduction of *KLK1* expression may contribute to increase cellular vulnerability and impaired neuroprotective signaling in the infected microenvironment.

Regarding the glutamate metabolism, SARS-CoV-2 infection significantly downrecycle in cultured astrocytes, the infection regulated the expression of key glutamate transporters at 72 hpi (Fig. 3HandI). However, glutamate concentrations in the culture supernatants remained unchanged (Fig. 3J), possibly reflecting early-stage disturbances or compensatory mechanisms that transiently maintain glutamate homeostasis.

4. Discussion

Beyond its well documented respiratory manifestations, COVID-19 has emerged as a systemic disease with wide-spred effects, including significant neurological involvement. A diverse array of neurological symptoms ranging from headaches and sensory disturbances to cognitive impairments such as brain-fog and asthenia – have been reported both during the acute phase of infection and persisting for months thereafter. This constellation of symptoms is now recognized as Neuro-COVID-19 and constitutes a major component of post-COVID syndrome or Long COVID. In our Brazilian cohort, we observed a high prevalence of neurological symptoms in SARS-CoV-2-positive individuals, particularly headaches, along with cognitive and sensory disturbances, in alignment with global reports. While extensive efforts have been made to elucidate the effects of SARS-CoV-2 infection on neurons as a

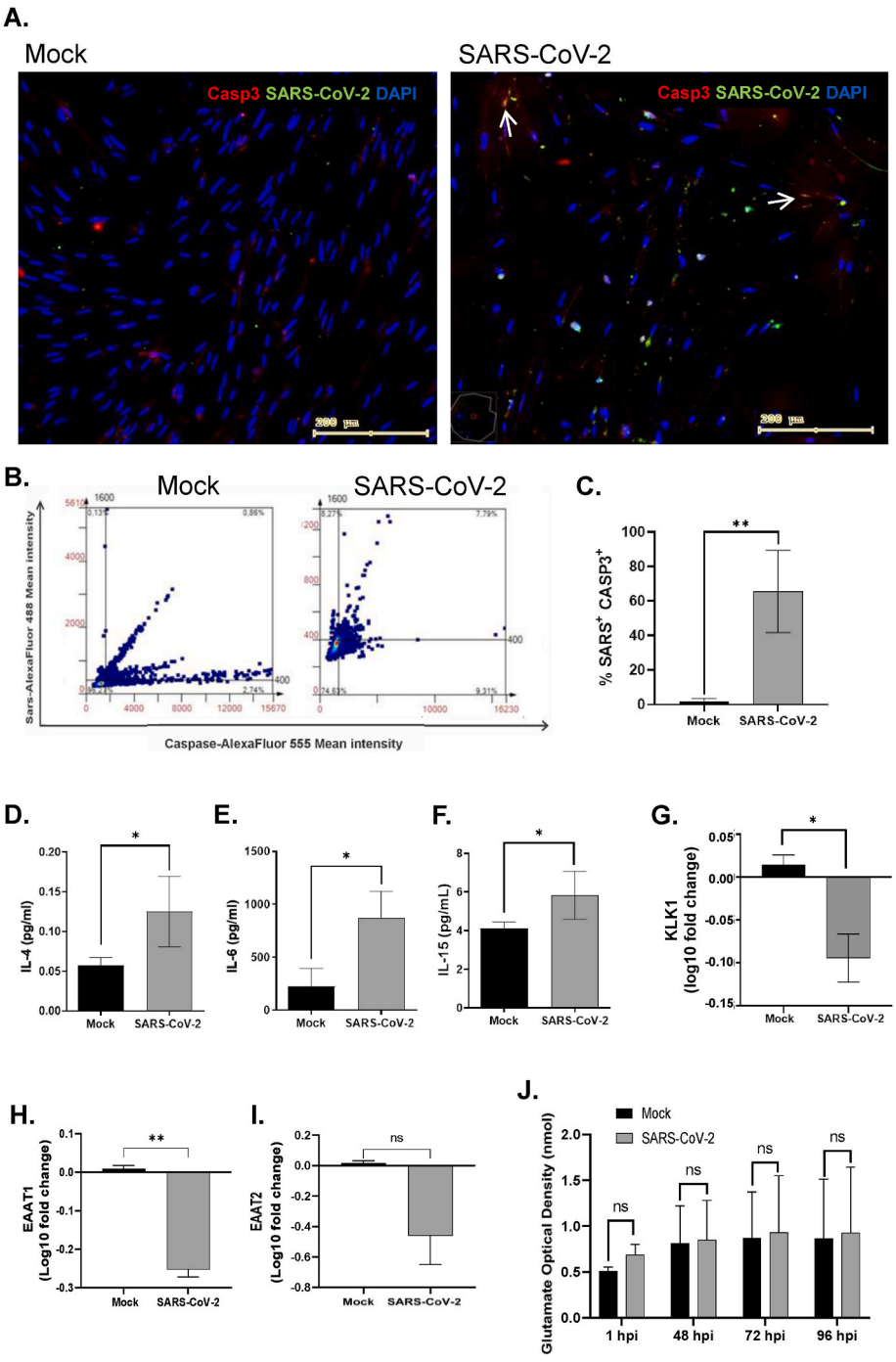


Fig. 3. Analysis of apoptosis related to neuroinflammation, KLK1 expression downregulation, and glutamatergic disturbance in cortical astrocytes post-SARS-CoV-2 infection. Immunofluorescence photomicrographs of cortical astrocytes cultured under standard conditions, Mock or infected **(A)** with SARS-CoV-2 at 96 hpi, with nuclei stained with DAPI, activated caspase-3 in red, and SARS-CoV-2 in green. **B)** Density plot of Alexa Fluor 488 (SARS-CoV-2) mean intensity × Alexa Fluor 555 (caspase-3) mean intensity. SARS + CASP3+ cells are indicated by arrows. Scale bar: 200 μm. The percentage of SARS + CASP3+ cells was determined by immunofluorescence analysis **(C)**. Quantification of **D)** IL-4, **E)** IL-6, and **F)** IL-15 in the cortical astrocyte supernatant at 72 hpi by Luminex xMAP technology. **G)** Cortical astrocyte intracellular relative expression of KLK1, **H)** EAAT1, and **I)** EAAT2 mRNA (log10) at 72 hpi by RT-qPCR. **J)** Glutamate optical density (nmol) at different times post-SARS-CoV-2 infection of the cortical astrocyte. The data are expressed as mean ± SD. *p < 0.05; **p < 0.01, unpaired *t*-test.

potential mechanism underlying such dysfunctions, astrocytes-despite their critical role in maintaining CNS homeostasis – have received comparatively limited attention. Here, we sought to address this gap by investigating the impact of SARS-CoV-2 on hiPSC-derived cortical astrocytes; our findings suggest it as a possible piece in the intricate puzzle of Neuro-COVID-19.

In this study, we demonstrate that SARS-CoV-2 infection induces a pro-inflammatory phenotype in hiPSC-derived cortical astrocytes, accompanied by transcriptional dysregulation of genes associated with the kinin-kallikrein system, glutamatergic signaling, and apoptotic pathways. Notably, even a low viral inoculum (MOI 0,05) was sufficient to infect astrocytes, as confirmed by immunofluorescence colocalization of GFAP and SARS-CoV-2, as well as molecular detection of viral RNA in cell lysates. The increased frequency of GFAP/SARS-CoV-2 double-positive cells in the infected group supports the susceptibility of astrocytes to viral entry. The low-level background signal observed in mock

controls is likely attributable to the use of a polyclonal primary antibody derived from convalescent patient serum, which may exhibit nonspecific binding. Nonetheless, RT-qPCR analysis of cellular RNA confirmed infection in the exposed group and the absence of viral RNA in mock samples, reinforcing the specificity of the findings.

Although no statistically significant changes were observed, infected astrocytes showed an increase in the expression of non-canonical SARS-CoV-2 receptors, such as CD147 and ACE-1, while ACE2 levels remained unchanged. This receptor profile corroborate with emerging data suggesting that astrocytes predominantly use alternative entry pathways – including CD147 and Neuropilin-1 proteins rather than ACE2 for SARS-CoV-2 internalization (Malik et al., 2023; Kong et al., 2022). These data support the hypothesis that SARS-CoV-2 may upregulate specific receptors to enhance its tropism or persistence in glial populations.

Of importance, the stable Ct values of viral genes in the cell culture supernatants across 96 hpi suggest astrocytes, while susceptible, are not permissive to productive viral replication. This non-productive infection profile is consistent with previous reports pointing out astrocytes as reservoirs for other neurotropic viruses, possibly due to their early exposure via hematological routes (Ojeda et al., 2018). In this study, we show that even a nonproductive infection is sufficient to induce astrocyte apoptosis, as evidenced by increased expression of cleaved caspase-3. This pro-apoptotic outcome appears to be driven, at least in part, by the pro-inflammatory cytokine profile elicited upon infection, characterized by elevated secretion of IL-4, IL-6, and IL-15. Such an innate antiviral response is consistent with the canonical role of astrocytes in preserving brain parenchyma homeostasis. However, when dysregulated or long-lasting, this response may become maladaptive, amplifying local neuroinflammation and promoting cell death not only in astrocytes themselves but also in neighboring neurons that are exposed to pro-inflammatory signaling and viral components (Jorgačevski and Potokar, 2023). It is possible that the upregulation of the anti-inflammatory IL-4 secretion by infected astrocytes may represent a compensatory mechanism counterbalancing the deleterious effects IL-15 and IL-6. This dualistic behavior reflects the complex functional plasticity of astrocytes during neuroinflammation, wherein they can simultaneously mediate protective antiviral actions and contribute to neurotoxicity. Such a dynamic balance may play a pivotal role in the neurological manifestations of COVID-19.

Concerning the detrimental outcomes possibly associated with neuroinflammation, its relation with headaches was pointed out as plausible. Pro-inflammatory cytokines, such as IL-6, are known to contribute to cortical depression spread and activation of calcitonin gene-related peptide release (CGRP) by the hypothalamus, ultimately promoting vasodilation of meningeal vessels and, consequently, the perception of pain (Biscetti et al., 2023). The clinical efficacy of anti-inflammatory drugs in alleviating COVID-19-related headache further supports this link. In parallel, the reduction of *KLK1* gene expression in infected astrocytes may also reflect a compensatory response, as kallikrein-1 is positively involved with inflammatory signaling and vascular regulation (Raidoo et al., 1999). Given KLK1's role in producing vasoactive kinins that typically promote vasodilation and protect against oxidative stress (Chao et al., 2010), its suppression may result in reduced cerebral perfusion. This vasoconstrictive effect could plausibly contribute to symptoms such as headache and brain fog through diminished oxygen and glucose delivery to neural tissues. Moreover, the cognitive disturbances associated with brain fog may be further exacerbated by the inflammatory environment. Pro-inflammatory cytokines have been shown to reduce neurogenesis and synaptic plasticity, which are critical components of cognitive function. Infected astrocytes, directly stimulated by viral presence or secondarily activated by glutamatergic signaling, may thus initiate a vicious cycle of inflammation and glutamate-induced toxicity, further worsening neural dysfunction.

In the current study, glutamate levels in astrocyte supernatant remained stable over time, despite SARS-CoV-2 infection. This may be explained by the fact that only a subset of astrocytes are specialized to gliotransmitter release, while the majority supply glutamine to neurons, which then synthesize glutamate (de Ceglia et al., 2023; Andersen, 2025). We observed a significant reduction in *EAAT1* expression and a non-significant but consistent decrease in *EAAT2*. Since this model consists only of astrocytic monoculture, these changes can be attributed specifically to viral infection, independent of interactions with other cell types.

While no extracellular glutamate accumulation was observed in cultures, the implications of transporter downregulation are more apparent in models like co-culture with neurons or brain organoids. In these complex systems, SARS-CoV-2 infection leads to both increased glutamate levels and a decreased transporter expression, indicating impaired glutamate uptake by astrocytes from the synaptic cleft. It is well documented that an excess of glutamate in the synaptic cleft can cause glutamatergic excitotoxicity, and as such trigger a mechanism of neuronal injury driven by extracellular excess of glutamate (Vaarmann et al., 2013). This observation is particularly relevant in hippocampal and cortical circuits, which are predominantly glutamatergic and essential for memory processing (Nelson et al., 2022). Thus, the brain fog reported by participants in our cohort may, possibly, be linked to glutamatergic dysregulation originating from astrocytic dysfunction. Supporting this hypothesis, an elegant study revealed glucose hypometabolism in the brains of long COVID patients with brain fog, possibly driven by impaired glutamatergic astrocyte activity (Horowitz et al., 2023). In addition, our findings about the reduced expression of glutamate transporters in astrocytes reinforce such hypotheses.

Furthermore, impaired glutamate uptake could compromise the glutamine supply to neurons, subsequently affecting both glutamate synthesis and energy production via glutaminolysis (de Oliveira et al., 2022), further disrupting glutamatergic signaling pathways involved in cognition. Beyond cognitive symptoms, glutamate-mediated excitotoxicity may also, hypothetically, contribute to the high prevalence of headaches observed in our cohort. Excess of glutamate is known to promote downstream mechanisms such as neuroinflammation and vasodilation—two major contributors to headache pathogenesis (Martami and Holton, 2023). The role of the glutamatergic system in headache has become increasingly evident, particularly in light of pharmacological treatments that act by enhancing GABAergic activity while inhibiting glutamatergic signaling (Weatherall, 2015).

Taken together, the findings presented in this study reinforce the direct impact of SARS-CoV-2 infection on astrocytes, corroborating the gliotropism of the virus, and its possible contribution to the onset of Neuro-COVID. Here, we highlight an underexplored cell type and demonstrate that even a non-productive infection should not be underestimated. SARS-CoV-2 exposure alone was sufficient to induce a pro-inflammatory state, alter the expression of genes involved in glutamatergic signaling and cerebrovascular regulation, and ultimately activate apoptotic pathways. From a translational perspective, the astrocytic dysfunction observed in our model offers mechanistic insights that may help explain the neurological symptoms of COVID-19. However, we were not able to confirm this putative association since the current *in vitro* model did not originate from the cohort patients. Indeed, the exact relation between astroglia damage and behavioral deficits of Neuro-COVID-19 would be more properly determined by correlating symptoms with alterations found in astrocytes exposed to sera or derived from cohort participants, which should be performed in future studies. Data from *postmortem* studies correlating alterations in SARS-CoV-2-infected astrocytes with retrospective reports about neurological impairments would also be ideal to establish a mechanistic relation. In spite of it, our data align with growing evidence that glial cells play a central role in the neuropathophysiology of COVID-19.

Understanding the cellular and molecular mechanisms underlying SARS-CoV-2' s impact on the CNS is essential for identifying potential therapeutic targets and informing the development of preventive strategies. We encourage further studies into specific neurotropic effects of circulating SARS-CoV-2 variants, particularly their interactions with

astrocytes and other glial populations. Such research is critical to mitigating the long-term neurological consequences of COVID-19 and reducing burden of post-COVID neurological disabilities.

CRediT authorship contribution statement

Ethiane Segabinazi: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. **Fernando R. Tocantins:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Talita Glaser:** Conceptualization, Data curation, Formal analysis, Methodology, Writing – review & editing. **Tamires Maglio:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Nathalia C. Oliveira:** Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Andrelissa Gorete Castanha:** Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Fabiele Baldino Russo:** Conceptualization, Formal analysis, Methodology, Writing – review & editing. **Paulo Emílio Corrêa Leite:** Methodology, Writing – review & editing. **Anita Brito:** Writing – review & editing. **Camila Vieira Molina:** Data curation, Writing – review & editing. **Gabriela Prado Paludo:** Methodology. **Raquel de Oliveira Souza:** Methodology. **Simone Ravena Maia Alves:** Methodology. **Marielton dos Passos Cunha:** Writing – review & editing. **Henning Ulrich:** Formal analysis, Methodology, Resources, Writing – review & editing. **Edison Luiz Durigon:** Methodology, Writing – review & editing. **Paola Minoprio:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Patricia C.B. Beltrão-Braga:** Conceptualization, Writing – review & editing, Funding acquisition, Project administration, Resources, Supervision.

Ethical statements

The studies involving human participants were approved by the Human Research Ethics Committee of the Institute of Biomedical Sciences, of the University of São Paulo (#4.036.252). The human data presented here were obtained from the database of the project entitled "SARS-CoV-2 in the Metropolitan Region of São Paulo - Emergency Action" and the associated biorepository (#CAAE #3146620.6.0000.5467) maintained by the *Institut Pasteur de São Paulo* collected during the task force against COVID-19 (Cunha et al., 2021).

Declaration of competing interest

We would like to declare that Henning Ulrich has an ongoing scientific advisory role with TissueGnostics (Vienna, Austria). The other authors of "**Astroglia-Mediated Neuroinflammation as a Mechanism of Neurological Outcomes in COVID-19? Insights from a Brazilian Cohort**" declare no conflict interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2025.101115.

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