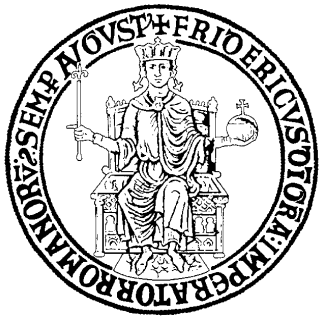
# “FEDERICO II” UNIVERSITY OF NAPLES

## SCHOOL OF MEDICINE




*PHD PROGRAM IN NEUROSCIENCE*

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PhD THESIS WORK:

# The Role of the SARS-CoV-2 Spike S1 Subunit in COVID-19-Associated Neurotoxicity: Molecular Mechanisms and Cellular Entry Pathways

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*Alla mia famiglia*

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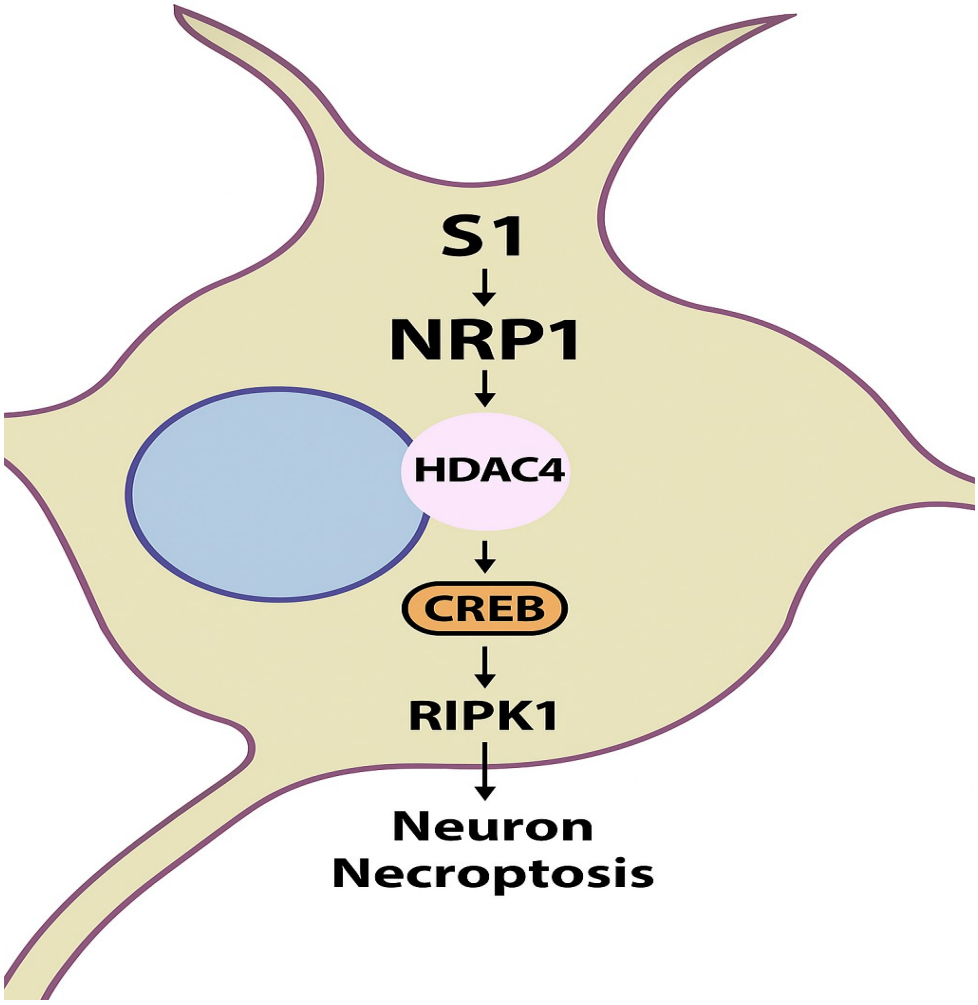
## 2. Abstract

Neurological manifestations of COVID-19, ranging from mild cognitive impairment to severe encephalopathies and stroke, have emerged as a significant clinical concern. Although SARS-CoV-2 is primarily a respiratory virus, growing evidence suggests that its structural proteins, particularly the Spike glycoprotein, may exert direct neurotoxic effects. The Spike protein is cleaved into two subunits: S1, which contains the receptor-binding domain, and S2, which facilitates membrane fusion. Notably, the S1 subunit has been detected in both plasma and cerebrospinal fluid of infected individuals and has demonstrated the capacity to cross the blood-brain barrier, raising critical questions about its role in central nervous system pathology. This study explores the hypothesis that the S1 subunit alone, independent of viral replication, can induce neuronal death through a defined molecular axis involving Neuropilin-1 (NRP1), histone deacetylase 4 (HDAC4), cAMP response element-binding protein (CREB), and receptor-interacting protein kinase 1 (RIPK1). Using differentiated SH-SY5Y neuroblastoma cells, the intracellular signaling pathways activated by S1 were dissected, revealing key molecular determinants of its neurotoxicity. Transfection with S1 significantly reduced cell viability and increased LDH release in a time-dependent manner, peaking at 72 hours, while the S2 subunit showed no such effects, confirming the specificity of S1-induced damage. The full-length Spike protein also induced toxicity, but the isolated S1 subunit was sufficient to replicate these effects, suggesting that S1 alone can act as a neurotoxic agent. Necrostatin-1, a selective RIPK1 inhibitor, partially rescued cell viability, implicating necroptosis as the dominant cell death pathway. RIPK1 expression was markedly upregulated in S1-transfected cells, consistent with clinical observations of elevated necroptotic markers in patients with moderate to severe COVID-19. Among HDAC isoforms, only HDAC4 knockdown reversed S1-induced toxicity. S1 overexpression increased HDAC4 levels, which correlated with RIPK1 upregulation, although HDAC4 did not directly bind the RIPK1 promoter, suggesting an indirect regulatory mechanism. Further analysis revealed that HDAC4-mediated deacetylation leads to CREB degradation, removing its transcriptional repression of RIPK1. CREB overexpression restored promoter activity and reduced RIPK1 expression, confirming its protective role. The study also demonstrated that NRP1 facilitates S1 internalization into neuronal cells, independent of ACE2, and that blocking endocytosis with Sodium Azide prevented cytosolic accumulation of S1 and NRP1. These findings establish NRP1 as the primary receptor mediating S1 entry and identify a mechanistic pathway, NRP1/HDAC4/CREB/RIPK1, through which S1 induces neuronal death. This paradigm shift from replication-dependent neurotoxicity to protein-mediated intracellular signaling offers new insights into the pathogenesis of neuro-COVID and may explain persistent

neurological symptoms in long COVID patients, even in the absence of detectable viral RNA. Therapeutically, the study highlights several promising targets: NRP1 antagonists to block S1 internalization, HDAC4 inhibitors to restore CREB function, RIPK1 inhibitors to prevent necroptosis, and proteasome stabilizers to preserve CREB levels. These strategies warrant further investigation in preclinical and clinical settings to assess their potential in mitigating COVID-19-associated neurotoxicity. Ultimately, this work delineates a novel molecular framework for understanding SARS-CoV-2-induced neuronal damage and underscores the importance of targeting viral protein interactions and epigenetic regulators in the treatment of neuro-COVID and its long-term sequelae.



# 3. Introduction

## 3.1 Overview of SARS-CoV-2

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is a novel, enveloped, positive-sense single-stranded RNA virus belonging to the *Betacoronavirus* genus within the *Coronaviridae* family, order *Nidovirales* (Lashgari et al., 2023; Mallick, et al., 2021; Mingaleeva, et al., 2023). It is the etiological agent of Coronavirus Disease 2019 (COVID-19), a pandemic that has reshaped global health, economics, and scientific paradigms since its emergence in late 2019. Phylogenetic analyses suggest a zoonotic origin, with bats serving as the primary reservoir and possible intermediate hosts such as pangolins facilitating cross-species transmission (Lashgari et al., 2023; Mallick, et al., 2021; Mingaleeva, et al., 2023). The virus shares significant genomic homology with SARS-CoV (2002) and MERS-CoV (2012), yet it exhibits distinct biological properties that have contributed to its rapid global dissemination and complex clinical profile (Yajima et al., 2024).

SARS-CoV-2 virions are spherical to pleomorphic particles, approximately 60–140 nm in diameter, with a lipid bilayer envelope derived from the host cell. Embedded within this envelope are three principal structural proteins, Spike (S), Membrane (M), and Envelope (E), while the Nucleocapsid (N) protein associates with the viral RNA genome internally (Sapkota & Aryal, 2025).

- **Spike (S) Glycoprotein:** The S protein is a trimeric class I fusion protein responsible for host cell recognition and entry. It comprises two functional subunits:
    - **S1:** Contains the receptor-binding domain (RBD), which binds to angiotensin-converting enzyme 2 (ACE2), the primary entry receptor.
    - **S2:** Mediates membrane fusion between the viral envelope and host cell membrane.
- **Membrane (M) Protein:** The most abundant structural protein, involved in virion assembly and envelope shaping.
- **Envelope (E) Protein:** A small transmembrane protein implicated in virion release and pathogenesis.
- **Nucleocapsid (N) Protein:** Binds to the RNA genome, stabilizing it and participating in replication and transcription.

The viral genome is approximately 29.9 kilobases in length, making it one of the largest among RNA viruses (Sapkota & Aryal, 2025). It is capped and polyadenylated, resembling host mRNA, and organized into several open reading frames (ORFs). ORF1a and ORF1b encode polyproteins

pp1a and pp1ab, which are cleaved into 16 non-structural proteins (nsps) forming the replication-transcription complex (RTC). Downstream ORFs encode structural proteins and accessory proteins such as ORF3a, ORF6, ORF7a, ORF8, and ORF10, which modulate host immune responses and enhance viral fitness (Sapkota & Aryal, 2025).

The replication cycle of SARS-CoV-2 is a multistep process involving precise coordination between viral and host cellular machinery:

1. **Attachment and Entry:** Viral entry is initiated by binding of the S1 subunit to ACE2 receptors on host cells. This interaction is stabilized by co-receptors such as neuropilin-1 (NRP1), CD147, and heparan sulfate proteoglycans. Host proteases, including TMPRSS2 and furin, cleave the S protein at the S1/S2 and S2′ sites, triggering conformational changes that facilitate membrane fusion. Alternatively, the virus may enter via clathrin-mediated endocytosis, with fusion occurring in acidified endosomes (Murgolo et al., 2021).
2. **Translation of Replication Machinery:** Upon entry, the viral RNA genome is released into the cytoplasm and translated by host ribosomes to produce the RTC. This complex includes RNA-dependent RNA polymerase (RdRp, nsp12), helicase (nsp13), exonuclease (nsp14), and other cofactors that ensure high-fidelity replication (Murgolo et al., 2021).
3. **Genome Replication and Subgenomic Transcription:** The RTC synthesizes a full-length negative-sense RNA template, which serves as a template for the production of new genomic RNA and a nested set of subgenomic RNAs. These subgenomic RNAs encode structural and accessory proteins and are generated via a unique discontinuous transcription mechanism involving transcription regulatory sequences (TRSs) (Murgolo et al., 2021).
4. **Assembly and Maturation:** Structural proteins are translated in the rough endoplasmic reticulum and trafficked to the ER-Golgi intermediate compartment (ERGIC), where they assemble with genomic RNA to form mature virions. The N protein plays a critical role in packaging the RNA genome and stabilizing the nucleocapsid (Murgolo et al., 2021).
5. **Release:** Mature virions are transported via vesicular pathways to the cell surface and released by exocytosis. This process can induce cytopathic effects, including syncytia formation and apoptosis, contributing to tissue damage (Murgolo et al., 2021).

SARS-CoV-2 exhibits broad tissue tropism, attributable to the widespread expression of ACE2 and other entry factors across various organ systems. The virus has been detected in the respiratory tract, gastrointestinal system, cardiovascular tissues, kidneys, and central nervous system (Singh & Yi, 2021). This systemic distribution underlies the diverse clinical manifestations of COVID-19, which

range from asymptomatic infection to severe pneumonia, thromboembolic events, multi-organ failure, and long-term sequelae.

The virus's ability to infect multiple cell types is further enhanced by its interaction with co-receptors and its capacity to evade innate immune responses. Viral proteins such as ORF6 and ORF8 interfere with interferon signaling and antigen presentation, contributing to immune dysregulation and hyperinflammatory states (Singh & Yi, 2021).

SARS-CoV-2 has demonstrated a remarkable capacity for genetic evolution, particularly within the Spike gene. Selective pressures, including host immunity, antiviral treatments, and vaccine deployment, have driven the emergence of multiple variants of concern (VOCs):

- **Alpha (B.1.1.7):** N501Y mutation enhances ACE2 binding affinity.
- **Beta (B.1.351) and Gamma (P.1):** E484K and K417N/T mutations reduce neutralization by convalescent and vaccine-induced antibodies.
- **Delta (B.1.617.2):** Exhibits increased transmissibility and partial resistance to neutralizing antibodies.
- **Omicron (B.1.1.529) and its subvariants (e.g., BA.2, XBB):** Harbor extensive mutations in the RBD and N-terminal domain (NTD), significantly impacting antigenicity and vaccine efficacy (Yajima et al., 2024; Singh & Yi, 2021).

These variants have reshaped the epidemiological landscape of COVID-19, necessitating continuous genomic surveillance and adaptation of public health strategies (Singh & Yi, 2021).

Since its emergence, SARS-CoV-2 has demonstrated sustained transmission across all continents. As of late 2025, over 770 million confirmed cases and more than 7 million deaths have been reported globally (Singh & Yi, 2021). The virus does not exhibit strict seasonality, unlike influenza viruses, and its transmission dynamics are influenced by population density, mobility, vaccination coverage, and behavioral factors.

The basic reproduction number ($R_0$) has varied across variants, with Delta and Omicron exhibiting significantly higher transmissibility compared to the ancestral strain. Superspreading events, asymptomatic transmission, and environmental stability have further complicated containment efforts (Singh & Yi, 2021).

The pandemic has exposed vulnerabilities in global health infrastructure, disrupted supply chains, and exacerbated socioeconomic inequalities. Lockdowns, travel restrictions, and overwhelmed healthcare systems have led to secondary crises in mental health, education, and chronic disease

management. The burden of post-acute sequelae of SARS-CoV-2 infection (PASC), commonly referred to as long COVID, has added complexity to recovery efforts (Singh & Yi, 2021).

Vaccination campaigns have played a pivotal role in reducing morbidity and mortality. However, disparities in vaccine access and hesitancy have hindered global containment. The emergence of immune-evasive variants has prompted the development of updated vaccines and booster strategies (Singh & Yi, 2021).

From a scientific perspective, SARS-CoV-2 has catalyzed advancements in virology, immunology, and public health policy. It has accelerated the adoption of mRNA vaccine platforms, genomic surveillance technologies, and international data-sharing frameworks. The pandemic has also underscored the importance of preparedness, interdisciplinary collaboration, and equitable healthcare delivery (Singh & Yi, 2021).

### 3.2 Structural Architecture of the SARS-CoV-2 Spike Protein and Its S1 Subunit

The Spike (S) glycoprotein of SARS-CoV-2 is a trimeric class I fusion protein that plays a central role in viral entry, tropism, and immune evasion. It is the most prominent surface protein of the virion and the primary target of neutralizing antibodies and vaccine design. Structurally, the Spike protein is composed of two functional subunits: S1, which mediates host receptor recognition, and S2, which facilitates membrane fusion. The S1 subunit, in particular, is responsible for binding to angiotensin-converting enzyme 2 (ACE2), the main entry receptor on host cells, and contains the receptor-binding domain (RBD) and other immunologically relevant regions (Nahalka, 2024).

The full-length Spike protein comprises approximately 1273 amino acids and is heavily glycosylated, with 22 N-linked glycosylation sites per monomer. It assembles into a homotrimer, forming a crown-like structure on the viral surface that gives coronaviruses their name (Sapkota & Aryal, 2025). Each monomer is divided into:

- **S1 Subunit (Residues 14–685)**: Responsible for receptor binding.
- **S2 Subunit (Residues 686–1273)**: Responsible for membrane fusion.

The Spike protein is synthesized as a single polypeptide and cleaved at the S1/S2 junction by host proteases such as furin. A second cleavage at the S2′ site, near the fusion peptide, is required for activation and fusion competence (Huang Y et al., 2020).

The trimeric Spike exists in a metastable prefusion conformation. Upon receptor engagement, it undergoes dramatic structural rearrangements that expose the fusion machinery and initiate viral

entry. These transitions are tightly regulated and influenced by pH, proteolytic processing, and host factors (Walls et al., 2020).

The S1 subunit is structurally subdivided into four domains:

1. **N-Terminal Domain (NTD)** Located at the apex of the Spike trimer, the NTD contributes to host cell attachment and may interact with sialic acids and other glycan moieties. It is also a target of neutralizing antibodies and exhibits significant antigenic variation among SARS-CoV-2 variants (McCallum et al., 2022).
2. **Receptor-Binding Domain (RBD)** The RBD spans residues 319–541 and adopts a twisted five-stranded β-sheet core flanked by α-helices. It exists in two conformational states:
    - **"Up" (open)**: Accessible for ACE2 binding.
    - **"Down" (closed)**: Shielded from immune recognition.

    This dynamic equilibrium regulates viral infectivity and immune evasion. Mutations in the RBD, such as N501Y, E484K, and L452R, enhance ACE2 affinity and reduce antibody neutralization, contributing to the emergence of variants of concern (Wolf et al., 2022).
3. **Subdomain 1 (SD1) and Subdomain 2 (SD2)** These domains flank the RBD and stabilize its orientation. SD2 contains the furin cleavage site (RRAR), which is unique to SARS-CoV-2 and absent in SARS-CoV, enhancing infectivity and tissue tropism (Liu et al., 2020).

The Spike protein is extensively glycosylated, forming a glycan shield that modulates immune recognition and protein folding. The S1 subunit contains multiple glycosylation sites, particularly in the NTD and RBD. These glycans serve dual roles:

- **Structural**: Stabilizing domain architecture and facilitating proper folding.
- **Immunological**: Masking epitopes from neutralizing antibodies and shaping antigenicity (Watanabe et al., 2020).

Glycan heterogeneity and site occupancy vary across viral strains and host cell types, influencing vaccine efficacy and immune escape. Certain glycan sites are conserved, while others are hotspots for mutation and adaptation (Harvey et al., 2021).

The Spike protein undergoes a series of conformational changes during viral entry:

1. **Prefusion State**: The RBDs toggle between "up" and "down" conformations. The S1 subunit stabilizes the trimer and prevents premature activation.

2. **ACE2 Binding**: Engagement of the RBD with ACE2 triggers destabilization of S1 and exposure of the S2 fusion machinery.
3. **Proteolytic Cleavage**: Furin and TMPRSS2 cleave the S1/S2 and S2′ sites, priming the Spike for fusion.
4. **Postfusion State**: The S2 subunit refolds into a six-helix bundle, driving membrane merger and viral entry (Huang Y et al., 2020; Wolf et al., 2022).

These transitions are tightly regulated and represent key targets for therapeutic intervention. Stabilizing the prefusion conformation or blocking RBD-ACE2 interaction can prevent infection (Schroeder & Bieneman, 2022).

The S1 subunit is the most variable region of the Spike protein. Mutations in the RBD and NTD alter receptor binding, antigenicity, and immune escape. For example:

- **Alpha (B.1.1.7)**: N501Y increases ACE2 affinity.
- **Beta (B.1.351)**: E484K and K417N reduce antibody binding.
- **Delta (B.1.617.2)**: L452R enhances infectivity.
- **Omicron (B.1.1.529)**: Over 30 mutations in S1, including multiple RBD and NTD changes, drastically reshape the Spike surface (Wolf et al., 2022; Harvey et al., 2021).

These structural alterations affect vaccine efficacy, monoclonal antibody performance, and diagnostic sensitivity. Mapping these changes is essential for updating immunogens and surveillance strategies.

### 3.3 COVID-19: Pathogenesis, Clinical Spectrum, Immunological Landscape, Epidemiology, and Global Consequences

Coronavirus Disease 2019 (COVID-19) is a complex, multisystemic disorder caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), a novel betacoronavirus that emerged in December 2019 in Wuhan, China. The virus rapidly disseminated across the globe, triggering a pandemic that has profoundly reshaped biomedical research, public health infrastructure, and global socio-economic dynamics. Its emergence has catalyzed a paradigm shift in infectious disease surveillance, vaccine development, and international health policy (Li et al.).

The pathogenesis of COVID-19 begins with the interaction between the viral Spike (S) glycoprotein and the host angiotensin-converting enzyme 2 (ACE2) receptor, which is expressed in epithelial

cells of the respiratory tract, gastrointestinal system, vascular endothelium, renal tissue, and central nervous system (Chung et al., 2024). Viral entry is facilitated by host proteases such as TMPRSS2 and furin, which cleave the S protein at the S1/S2 and S2′ sites, enabling membrane fusion or endosomal uptake (Parasher, 2021). Once inside the host cell, the viral RNA genome is translated into non-structural proteins that form the replication-transcription complex (RTC), responsible for synthesizing genomic and subgenomic RNAs (Lok et al., 2024).

The virus employs multiple strategies to evade host immune detection. Accessory proteins such as ORF3a, ORF6, and ORF8 interfere with interferon signaling, antigen presentation, and apoptosis regulation (Li et al.). These mechanisms contribute to viral persistence and immune dysregulation, particularly in severe cases. The host response is characterized by a biphasic pattern: an initial antiviral phase followed by a hyperinflammatory phase in susceptible individuals. The latter is marked by elevated levels of pro-inflammatory cytokines (e.g., IL-6, TNF-α), complement activation, and endothelial dysfunction, culminating in acute respiratory distress syndrome (ARDS), coagulopathy, and multi-organ failure (Blanco-Melo et al., 2020).

COVID-19 presents with a heterogeneous clinical spectrum. Mild cases often manifest with fever, dry cough, fatigue, and anosmia, while moderate cases may include dyspnea, chest pain, and gastrointestinal symptoms such as diarrhea and nausea (Guan et al., 2020). Severe cases progress to hypoxemia, bilateral pneumonia, and ARDS, requiring intensive care and mechanical ventilation. Laboratory findings typically include lymphopenia, elevated C-reactive protein (CRP), ferritin, D-dimer, and transaminases (Huang C et al., 2020).

Cardiovascular complications include myocarditis, arrhythmias, and thromboembolic events such as pulmonary embolism and stroke. Endothelial injury and platelet activation contribute to a prothrombotic state, exacerbated by cytokine-mediated vascular inflammation (Varga et al., 2020). Renal involvement ranges from transient proteinuria to acute kidney injury, particularly in patients with pre-existing comorbidities.

Neurological manifestations have gained increasing attention. These include headache, dizziness, encephalopathy, seizures, and cerebrovascular events. The neuroinvasive potential of SARS-CoV-2 is supported by the expression of ACE2 and neuropilin-1 in neuronal and glial cells, as well as evidence of viral RNA in cerebrospinal fluid and postmortem brain tissues (Ellul et al., 2020).

The immunological response to SARS-CoV-2 is multifaceted. Innate immunity involves pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and RIG-I-like receptors, which detect viral RNA and initiate interferon signaling. Adaptive immunity is characterized by the

activation of CD4+ and CD8+ T cells, B cell maturation, and antibody production (Sette & Crotty, 2021).

Neutralizing antibodies target the receptor-binding domain (RBD) of the Spike protein, preventing viral entry. However, antibody titers wane over time, and memory B cell responses vary among individuals. T cell responses are more durable and correlate with protection against severe disease. Vaccine-induced immunity mirrors natural infection but may be modulated by age, immunocompetence, and prior exposure (Goel et al., 2021).

Breakthrough infections have been reported, particularly with immune-evasive variants. Booster doses and bivalent vaccines have been introduced to enhance protection and broaden immune coverage. Studies have shown that hybrid immunity, resulting from vaccination and prior infection, confers superior protection against severe outcomes (Whitaker, et al., 2023).

Post-acute sequelae of SARS-CoV-2 infection (PASC), commonly referred to as long COVID, encompass a constellation of symptoms persisting beyond four weeks after acute infection. These include fatigue, cognitive dysfunction ("brain fog"), dyspnea, palpitations, and autonomic disturbances such as postural orthostatic tachycardia syndrome (POTS) (Davis et al., 2021).

Pathophysiological mechanisms remain under investigation but may involve persistent viral reservoirs, immune dysregulation, microvascular injury, and autonomic imbalance. Neuroinflammation and mitochondrial dysfunction have been implicated in cognitive and neurological symptoms. Long COVID affects individuals regardless of initial disease severity and poses a significant burden on healthcare systems and workforce productivity (Nalbandian et al., 2021).

Multidisciplinary management is recommended, including rehabilitation, psychological support, and symptom-targeted therapies. Biomarker discovery and longitudinal cohort studies are essential for elucidating mechanisms and developing targeted interventions.

SARS-CoV-2 has demonstrated sustained human-to-human transmission via respiratory droplets, aerosols, and contaminated surfaces. The basic reproduction number ($R_0$) of the ancestral strain was estimated at 2.5–3.0, but subsequent variants such as Delta and Omicron have exhibited $R_0$ values exceeding 5–8, reflecting enhanced transmissibility (Liu & Rocklöv, 2021).

Superspreading events, asymptomatic carriers, and environmental stability have contributed to rapid global dissemination. The virus does not exhibit strict seasonality, although transmission may be influenced by temperature, humidity, and population behavior. Urban density, mobility patterns, and public health interventions such as masking and lockdowns have played critical roles in modulating

transmission (Baker et al., 2020).

Global surveillance has revealed disparities in case detection, reporting, and genomic sequencing. Low- and middle-income countries face challenges in testing capacity, vaccine access, and healthcare infrastructure, exacerbating the pandemic's impact.

SARS-CoV-2 has undergone extensive genetic evolution, particularly within the Spike gene. Variants of concern (VOCs) have emerged with mutations that enhance receptor binding, increase transmissibility, and confer partial resistance to neutralizing antibodies.

- Alpha (B.1.1.7): N501Y mutation increases ACE2 affinity and transmissibility (Volz et al., 2021).
- Beta (B.1.351) and Gamma (P.1): E484K and K417N/T mutations reduce antibody neutralization (Tegally et al., 2021).
- Delta (B.1.617.2): L452R and T478K mutations enhance infectivity and immune escape (Mlcochova et al., 2021).
- Omicron (B.1.1.529) and subvariants (e.g., BA.2, XBB): Exhibit over 30 mutations in the Spike protein, significantly altering antigenicity and vaccine efficacy (Cao et al., 2022).

Recombinant lineages and convergent evolution have been observed, underscoring the virus's adaptability. Continuous genomic surveillance and real-time data sharing are essential for tracking viral evolution and informing public health responses.

Vaccination has been the cornerstone of pandemic mitigation. mRNA-based vaccines (e.g., BNT162b2, mRNA-1273), adenoviral vector vaccines (e.g., ChAdOx1, Ad26.COV2.S), and protein subunit vaccines have demonstrated efficacy in reducing severe disease and mortality (Polack et al., 2020). Booster doses and variant-specific formulations have been introduced to address waning immunity and immune escape.

Therapeutic approaches include antiviral agents such as remdesivir, molnupiravir, and nirmatrelvir/ritonavir, as well as immunomodulators like dexamethasone and IL-6 inhibitors (e.g., tocilizumab) (Beigel et al., 2020). Monoclonal antibodies targeting the Spike protein have shown promise, although their efficacy may be compromised by emerging variants (Weinreich et al., 2021).

Non-pharmaceutical interventions (NPIs), including masking, social distancing, ventilation, and contact tracing, remain essential components of public health strategy. The integration of digital tools, such as exposure notification apps and telemedicine platforms, has enhanced pandemic

response capabilities.

The pandemic has exposed systemic inequities in healthcare access, digital infrastructure, and global preparedness. It has accelerated the adoption of telemedicine, remote work, and digital epidemiology. Collaborative initiatives such as COVAX and GISAID have exemplified the potential of global cooperation in addressing public health crises.

From a scientific standpoint, COVID-19 has revolutionized vaccine development, genomic surveillance, and data sharing. The rapid deployment of mRNA platforms and real-time tracking of viral evolution represent milestones in translational medicine. Ethical debates on resource allocation, vaccine equity, and individual freedoms have emerged, highlighting the need for inclusive and resilient health systems.

### 3.4 Neurological Manifestations and Neurotoxicity of SARS-CoV-2

Since its emergence in late 2019, SARS-CoV-2 has been primarily characterized as a respiratory pathogen. However, accumulating evidence has revealed that the virus exerts profound effects on multiple organ systems, including the central and peripheral nervous systems. Neurological manifestations of COVID-19 have been reported across all stages of infection, from prodromal symptoms to long-term sequelae, and range from mild sensory disturbances to severe neuroinflammatory and neurodegenerative conditions. The neurotropic and neurotoxic potential of SARS-CoV-2 has prompted a reevaluation of its pathophysiological profile, positioning it as a virus with systemic and neuroinvasive capabilities (Pang et al., 2024).

The prevalence of neurological symptoms in COVID-19 patients is substantial. Studies estimate that more than 30% of hospitalized individuals exhibit neurological signs, including headache, dizziness, anosmia, ageusia, encephalopathy, and cerebrovascular events (Tyagi et al., 2023). These symptoms may arise from direct viral invasion, immune-mediated damage, or secondary effects of systemic inflammation and hypoxia. Importantly, neurological complications are not limited to severe cases; even individuals with mild respiratory symptoms have reported persistent cognitive and sensory deficits (World Health Organization, 2024).

SARS-CoV-2 may access the nervous system through multiple pathways. The most widely studied route involves trans-synaptic transfer via the olfactory nerve. The olfactory epithelium expresses high levels of ACE2 and TMPRSS2, facilitating viral entry and retrograde transport to the olfactory bulb and adjacent brain regions (Vashisht et al., 2024). This mechanism is supported by the frequent occurrence of anosmia in early COVID-19 and by postmortem studies demonstrating viral RNA and

proteins in the olfactory tract (Guadarrama-Ortiz et al., 2020).

Another proposed route is hematogenous dissemination. The virus may cross the blood-brain barrier (BBB) by infecting endothelial cells or via a “Trojan horse” mechanism involving infected leukocytes. SARS-CoV-2 has been shown to induce endothelial dysfunction, increase BBB permeability, and promote neuroinflammation through cytokine release and complement activation (Meinhardt et al., 2021). These processes facilitate the entry of viral particles and inflammatory mediators into the central nervous system (CNS), contributing to neuronal injury and glial activation.

Peripheral nerve involvement has also been documented. Guillain-Barré syndrome (GBS), an acute demyelinating polyneuropathy, has been reported in temporal association with SARS-CoV-2 infection. The pathogenesis of GBS in COVID-19 is thought to involve molecular mimicry and aberrant immune responses, rather than direct viral invasion (Toscano et al., 2020).

The initial neurological manifestations of COVID-19 are often nonspecific but clinically significant. Headache and dizziness are among the most common complaints and may reflect early CNS involvement or systemic effects such as hypoxia and dehydration. Anosmia and ageusia are hallmark symptoms, frequently preceding respiratory signs and serving as early indicators of infection (Lechien et al., 2020).

These sensory deficits are believed to result from viral damage to olfactory and gustatory neurons, as well as supporting cells in the nasal and oral mucosa. Histopathological studies have revealed degeneration of olfactory epithelium and inflammation of olfactory bulbs in affected individuals (de Melo et al., 2021). While most patients recover these senses within weeks, a subset experiences prolonged or permanent deficits, suggesting potential neurodegeneration or impaired neuronal regeneration.

Cognitive disturbances, including confusion, attention deficits, and memory impairment, have been reported in both acute and post-acute phases. These symptoms may be exacerbated by systemic inflammation, hypoxia, and metabolic derangements. In hospitalized patients, delirium is a frequent complication and is associated with increased morbidity and mortality (Helms et al., 2020).

SARS-CoV-2 infection triggers a cascade of neuroinflammatory responses that may contribute to both acute and chronic neurological dysfunction. The virus induces activation of microglia and astrocytes, leading to the release of pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α within the central nervous system (CNS) (Song et al., 2021). This cytokine milieu disrupts neuronal homeostasis, impairs synaptic function, and promotes excitotoxicity through glutamate

dysregulation.

Postmortem studies have revealed microglial nodules, astrogliosis, and perivascular lymphocytic infiltration in the brains of COVID-19 patients, even in the absence of direct viral detection in neural tissue (Matschke et al., 2020). These findings suggest that neuroinflammation may be driven by systemic immune activation and endothelial dysfunction rather than by viral replication within neurons.

Chronic neuroinflammation is a known contributor to neurodegenerative processes. SARS-CoV-2 has been implicated in accelerating pathological mechanisms associated with Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS). For instance, elevated levels of neurofilament light chain (NfL), a biomarker of axonal injury, have been detected in COVID-19 patients with neurological symptoms (Kanberg et al., 2020). Moreover, the virus may disrupt blood-brain barrier integrity, facilitating the entry of peripheral immune cells and neurotoxic molecules that exacerbate neurodegeneration.

COVID-19 is associated with a heightened risk of cerebrovascular events, including ischemic stroke, intracerebral hemorrhage, and cerebral venous sinus thrombosis. These complications are particularly prevalent in patients with severe disease, underlying cardiovascular risk factors, or hypercoagulable states (Merkler et al., 2020).

The pathophysiology of COVID-19-associated stroke involves endothelial injury, platelet activation, and thromboinflammation. SARS-CoV-2 infects endothelial cells via ACE2, leading to apoptosis, disruption of tight junctions, and exposure of subendothelial procoagulant surfaces (Varga et al., 2020). Concurrently, elevated levels of D-dimer, fibrinogen, and antiphospholipid antibodies contribute to a prothrombotic milieu.

Neuroimaging studies have documented large-vessel occlusions, multifocal infarcts, and hemorrhagic lesions in COVID-19 patients. These findings underscore the need for early recognition and aggressive management of cerebrovascular complications, including anticoagulation and neuroprotective strategies.

Post-acute sequelae of SARS-CoV-2 infection (PASC), commonly referred to as Long COVID, encompass a wide array of neurological symptoms that persist beyond the resolution of acute illness. These include fatigue, cognitive impairment, sleep disturbances, paresthesia, and dysautonomia (Davis et al., 2021).

Neurocognitive deficits, often described as "brain fog", are among the most disabling features of Long COVID. Patients report difficulties with attention, memory, executive function, and language

processing. Functional MRI studies have revealed altered connectivity in frontoparietal and limbic networks, suggesting persistent neuroinflammation and synaptic dysfunction (Douaud et al., 2022).

Autonomic dysfunction manifests as orthostatic intolerance, tachycardia, and gastrointestinal dysmotility. These symptoms may reflect damage to brainstem nuclei or peripheral autonomic fibers. Additionally, persistent headache, tinnitus, and mood disturbances have been reported, indicating widespread neural involvement.

The etiology of Long COVID remains multifactorial. Hypotheses include viral persistence in immune-privileged sites, autoimmunity, mitochondrial dysfunction, and dysbiosis of the gut-brain axis. Longitudinal studies are essential to delineate the natural history, risk factors, and therapeutic targets for these syndromes.

Emerging evidence suggests that the SARS-CoV-2 Spike protein, particularly the S1 subunit, may exert direct neurotoxic effects independent of viral replication. The S1 protein has been shown to cross the blood-brain barrier and bind to neuronal and glial receptors, triggering inflammatory cascades and oxidative stress (Rhea et al., 2021).

In vitro studies demonstrate that exposure to recombinant S1 protein induces apoptosis in neuronal cultures and activates microglia. Animal models reveal that intranasal administration of S1 leads to behavioral changes, neuroinflammation, and synaptic loss (Buzhdygan et al., 2020). These findings raise concerns about the long-term neurological impact of Spike protein exposure, particularly in the context of persistent antigenemia or vaccine-induced Spike expression.

Recent investigations have identified Spike protein accumulation in the meninges and skull bone marrow up to four years post-infection, suggesting a mechanism for chronic neuroinflammation and neurodegeneration in Long COVID patients. This persistent antigenic stimulation may contribute to cognitive decline, mood disorders, and increased risk of neurodegenerative diseases.

The neurological impact of COVID-19 is disproportionately severe in certain populations. Older adults, individuals with pre-existing neurological conditions, and those with metabolic or cardiovascular comorbidities are at heightened risk for neuroinvasive complications and poor outcomes (Cagnin et al., 2020).

Patients with Alzheimer’s disease or Parkinson’s disease may experience accelerated cognitive and motor decline following SARS-CoV-2 infection. The virus may exacerbate underlying pathologies through neuroinflammation, vascular injury, and disruption of neurotransmitter systems. Similarly, individuals with multiple sclerosis may face increased relapse rates and disease progression due to immune activation and treatment interruptions.

Children and adolescents, although less likely to develop severe respiratory illness, may exhibit neurological symptoms such as seizures, encephalitis, and multisystem inflammatory syndrome (MIS-C). The long-term neurodevelopmental consequences of pediatric COVID-19 remain under investigation.

Management of COVID-19-related neurological complications requires a multidisciplinary approach. Acute interventions include corticosteroids, anticoagulants, and immunomodulators to mitigate inflammation and thrombosis. Supportive care, rehabilitation, and neuropsychological assessment are essential for recovery and quality of life.

Experimental therapies targeting neuroinflammation, mitochondrial dysfunction, and autoimmunity are under development. These include monoclonal antibodies, neuroprotective agents, and stem cell-based approaches. Biomarker discovery, such as neurofilament light chain, glial fibrillary acidic protein, and cytokine profiles, may aid in diagnosis, prognosis, and treatment monitoring.

Future research should prioritize longitudinal cohort studies, mechanistic investigations, and randomized controlled trials. Understanding the interplay between viral factors, host immunity, and neural vulnerability will be critical for preventing and managing the neurological burden of COVID-19.

### 3.5 Mechanisms of Neuroinvasion and Neurotoxicity Induced by SARS-CoV-2, the Spike Protein, and Its S1 Subunit

SARS-CoV-2, the etiological agent of COVID-19, has demonstrated a remarkable capacity to affect the central and peripheral nervous systems. While initially characterized as a respiratory virus, mounting evidence has revealed its neuroinvasive and neurotoxic potential, mediated not only by the intact virion but also by its structural components, most notably the Spike glycoprotein and its S1 subunit. These viral elements have been implicated in a range of neuropathological processes, including blood-brain barrier disruption, neuroinflammation, synaptic dysfunction, and neuronal death. The neurological burden of COVID-19 spans acute manifestations, such as encephalopathy and stroke, to chronic sequelae including cognitive impairment and neurodegeneration, collectively referred to as neuro-PASC (post-acute sequelae of SARS-CoV-2 infection) (Menezes et al., 2024).

One of the most plausible and well-documented routes of SARS-CoV-2 neuroinvasion is via the olfactory epithelium. The nasal cavity provides a direct anatomical conduit to the brain through the cribriform plate. The olfactory neuroepithelium expresses high levels of ACE2 and TMPRSS2, facilitating viral entry and retrograde axonal transport to the olfactory bulb. Postmortem studies

have confirmed the presence of viral RNA and proteins in the olfactory tract and adjacent brain regions, supporting this mechanism (Meinhardt et al., 2021).

SARS-CoV-2 may also reach the brain via systemic circulation. The virus can infect endothelial cells of the blood-brain barrier (BBB), leading to increased permeability and allowing viral particles, inflammatory cytokines, and immune cells to infiltrate the CNS. The “Trojan horse” mechanism, whereby infected leukocytes cross the BBB, has also been proposed. Endothelial dysfunction and microvascular thrombosis further exacerbate barrier breakdown and neuroinflammation (Kim et al., 2021).

Peripheral nerves, including the vagus and trigeminal nerves, may serve as conduits for viral transport to the brainstem. This is particularly relevant in cases of dysautonomia and brainstem-related symptoms. Guillain-Barré syndrome, a post-infectious demyelinating neuropathy, has been reported in temporal association with SARS-CoV-2 infection, suggesting immune-mediated peripheral nerve involvement (Toscano et al., 2020).

The Spike protein, particularly its S1 subunit, has been shown to cross the BBB independently of viral replication. In murine models, radiolabeled S1 was detected in multiple brain regions following intravenous administration, indicating active transcytosis across endothelial cells. This process is facilitated by interactions with neuropilin-1 (NRP1), a co-receptor highly expressed in the CNS and vasculature (Rhea et al., 2021).

Once inside the brain, the S1 subunit binds to neuronal and glial receptors, including ACE2, NRP1, and Toll-like receptors. These interactions trigger intracellular signaling cascades that lead to oxidative stress, mitochondrial dysfunction, and apoptosis. In vitro studies using human neuronal cultures have demonstrated that S1 exposure induces caspase activation, synaptic loss, and neurite retraction (Wang F et al., 2024).

SARS-CoV-2 and its Spike protein elicit robust activation of microglia and astrocytes, the resident immune cells of the CNS. This leads to the release of pro-inflammatory cytokines (e.g., IL-6, TNF-α, IL-1β), chemokines, and reactive oxygen species. Chronic glial activation disrupts synaptic homeostasis, impairs neurogenesis, and promotes excitotoxicity through glutamate dysregulation (Menezes et al., 2024; Kim et al., 2021).

Postmortem analyses of COVID-19 patients have revealed microglial nodules, astrogliosis, and perivascular lymphocytic infiltration, even in cases without direct viral detection in brain tissue. These findings underscore the role of systemic inflammation and immune-mediated damage in COVID-19 neuropathology (Meinhardt et al., 2021).

The neurotoxic effects of SARS-CoV-2 and its Spike protein may accelerate neurodegenerative processes. In cellular models of synucleinopathy, the S1 subunit has been shown to enhance α-synuclein phosphorylation and aggregation, hallmarks of Parkinson's disease (Wang J et al., 2024). Similarly, the inflammatory milieu induced by viral components may promote tau hyperphosphorylation and amyloidogenesis, contributing to Alzheimer's disease pathology.

Elevated levels of neurofilament light chain (NfL), a biomarker of axonal injury, have been detected in COVID-19 patients with neurological symptoms. These changes correlate with cognitive decline and may persist for months post-infection (Menezes et al., 2024).

Recent studies have identified Spike protein persistence in the meninges and skull bone marrow up to four years post-infection. This chronic antigenic stimulation may sustain neuroinflammation, disrupt cerebrospinal fluid dynamics, and impair lymphatic clearance. The presence of Spike protein in immune-privileged sites raises concerns about long-term neurological sequelae and the potential for progressive neurodegeneration (Menezes et al., 2024).

The neurological burden of SARS-CoV-2 has prompted the development of targeted therapeutic strategies aimed at mitigating neuroinflammation, restoring neuronal function, and preventing long-term sequelae. Corticosteroids such as dexamethasone have demonstrated efficacy in reducing systemic inflammation and may indirectly benefit CNS outcomes by dampening cytokine storms and stabilizing the blood-brain barrier (BBB) (Pang et al., 2024). However, their direct neuroprotective effects remain under investigation.

Monoclonal antibodies targeting IL-6 (e.g., tocilizumab) and TNF-α have been proposed to counteract glial activation and neuroinflammatory cascades. These agents may reduce microglial reactivity and preserve synaptic integrity, particularly in patients with severe neuro-COVID presentations (Brown et al., 2023).

Neuroprotective compounds such as N-acetylcysteine, minocycline, and melatonin have shown promise in preclinical models by attenuating oxidative stress, modulating mitochondrial function, and inhibiting apoptotic pathways (Pang et al., 2024).

Rehabilitation strategies, including cognitive training, physical therapy, and neuromodulation, are essential for recovery in post-COVID neurological syndromes. Emerging interventions such as transcranial magnetic stimulation (TMS) and vagus nerve stimulation (VNS) are being explored for their potential to modulate neuroimmune interactions and enhance neuroplasticity.

Robust experimental models are critical for elucidating the mechanisms of SARS-CoV-2 neuroinvasion and neurotoxicity. In vitro systems using human-induced pluripotent stem cell

(iPSC)-derived neurons, astrocytes, and brain organoids have enabled detailed analysis of viral entry, replication, and cytopathic effects. These models recapitulate key features of human neurobiology and allow for high-throughput screening of antiviral and neuroprotective agents (Sanclemente-Alaman et al., 2020).

Animal models, including transgenic mice expressing human ACE2 (hACE2), Syrian hamsters, and non-human primates, have provided valuable insights into viral dissemination, BBB permeability, and neuroinflammatory responses. Intranasal and intravenous inoculation protocols have demonstrated CNS involvement, with viral RNA detected in the olfactory bulb, brainstem, and cortex (Paoletti et al., 2022).

Advanced imaging techniques such as two-photon microscopy and PET scans have been employed to monitor neurovascular changes and glial activation in vivo. These approaches facilitate longitudinal tracking of disease progression and therapeutic efficacy.

Despite their utility, current models have limitations in replicating the full spectrum of human neurological symptoms, particularly cognitive and psychiatric manifestations. Future efforts should focus on integrating multi-organ systems, refining humanized models, and incorporating genetic diversity to better reflect clinical heterogeneity.

SARS-CoV-2 shares several neuroinvasive features with other neurotropic viruses, including herpes simplex virus (HSV), human immunodeficiency virus (HIV), and Zika virus. Like HSV, SARS-CoV-2 can access the CNS via peripheral nerves and establish latent reservoirs in immune-privileged sites. Both viruses induce neuroinflammation and neuronal apoptosis through similar cytokine profiles and glial activation (Carneiro, et al., 2022; Haddad et al., 2023; Molaverdi et al., 2023; Wan et al., 2021; Ehsanipur et al., 2023).

HIV-associated neurocognitive disorders (HAND) provide a framework for understanding chronic neuroinflammation and viral persistence. SARS-CoV-2 may similarly disrupt synaptic networks and neurotransmitter systems, leading to cognitive decline and mood disturbances. However, unlike HIV, SARS-CoV-2 does not integrate into the host genome, suggesting different mechanisms of long-term neuropathology.

Zika virus, known for its teratogenic effects, shares with SARS-CoV-2 the ability to infect neural progenitor cells and impair neurodevelopment. While Zika primarily affects fetal brains, SARS-CoV-2 has been implicated in pediatric neurological syndromes such as multisystem inflammatory syndrome in children (MIS-C), raising concerns about long-term neurodevelopmental outcomes (Miner & Diamond, 2017).

Comparative virology underscores the importance of host factors, viral tropism, and immune responses in shaping neurological outcomes. Understanding these parallels may inform cross-cutting therapeutic strategies and enhance preparedness for future neurotropic pandemics.

### 3.6 Interaction of the SARS-CoV-2 Spike Protein and S1 Subunit with Neuronal Receptors

The neuroinvasive potential of SARS-CoV-2 is closely linked to the molecular interactions between its Spike protein, particularly the S1 subunit, and host cell receptors expressed in neural tissues. While angiotensin-converting enzyme 2 (ACE2) is the primary receptor mediating viral entry, additional co-receptors and facilitators such as neuropilin-1 (NRP1), TMPRSS2, and nicotinic acetylcholine receptors (nAChRs) have been implicated in modulating viral tropism, neurotoxicity, and immune responses. These interactions not only facilitate viral access to the central nervous system (CNS) but also contribute to the neurological symptoms observed in COVID-19, including anosmia, encephalopathy, and cognitive dysfunction (Barroso da Silva et al., 2025).

ACE2 is widely expressed in multiple regions of the brain, including the cerebral cortex, brainstem, and olfactory bulb. Single-cell transcriptomic analyses have identified ACE2 mRNA in neurons, astrocytes, oligodendrocytes, and endothelial cells, although expression levels vary across regions and developmental stages (Yao et al., 2023). The receptor-binding domain (RBD) of the S1 subunit binds to ACE2 with high affinity, initiating conformational changes that expose the fusion machinery of the Spike protein.

In neurons, ACE2-mediated entry may lead to direct cytopathic effects, mitochondrial dysfunction, and synaptic disruption. Moreover, ACE2 downregulation following viral binding may impair neurovascular regulation and exacerbate inflammation, contributing to cerebrovascular complications and neurodegeneration (Saiz et al., 2021).

NRP1 is a transmembrane glycoprotein involved in axonal guidance, angiogenesis, and immune modulation. It is highly expressed in the olfactory epithelium, hippocampus, and neocortex, making it a plausible facilitator of SARS-CoV-2 neuroinvasion. The S1 subunit contains a polybasic furin cleavage site (RRAR) that, once cleaved, exposes a C-end rule (CendR) motif capable of binding NRP1 (Cantuti-Castelvetri et al., 2020).

Experimental studies have shown that NRP1 enhances Spike-mediated entry into cells with low ACE2 expression, suggesting a synergistic role in viral dissemination. In neural tissues, NRP1 may facilitate transcytosis across the blood-brain barrier and promote viral access to immune-privileged compartments (Sierra-Sánchez et al., 2025).

TMPRSS2 is a serine protease that cleaves the Spike protein at the S1/S2 and S2′ sites, priming it for membrane fusion. Although TMPRSS2 expression in the brain is lower than in the respiratory tract, it has been detected in choroid plexus epithelial cells and certain neuronal populations. Its activity may enhance viral infectivity and contribute to neuroinflammation by activating pro-inflammatory pathways (Hoffmann et al., 2020).

Recent computational and biochemical studies have proposed that the S1 subunit may interact with nAChRs, particularly the α7 subtype, which is abundant in the CNS and involved in cognitive function, neuroprotection, and inflammation regulation. This interaction may disrupt cholinergic signaling, contributing to neurocognitive symptoms and autonomic dysfunction observed in COVID-19 (Carlson et al., 2023).

Moreover, the cholinergic anti-inflammatory pathway, mediated by α7 nAChRs, plays a critical role in modulating systemic and neuroinflammation. Interference by the Spike protein may impair this regulatory axis, exacerbating cytokine release and glial activation (Tracey, 2002).

SARS-CoV-2 may also influence the expression of neuronal receptors through epigenetic mechanisms. Studies have shown that viral infection alters DNA methylation and histone acetylation patterns at ACE2 and NRP1 loci, potentially modifying receptor availability and susceptibility to reinfection. These changes may persist beyond acute infection and contribute to long-term neurological sequelae (Saiz et al., 2021).

The interaction of the SARS-CoV-2 Spike protein and its S1 subunit with neuronal receptors (including ACE2, NRP1, TMPRSS2, and nAChRs) represents a multifaceted mechanism of neuroinvasion and neurotoxicity. These molecular engagements facilitate viral entry, disrupt neural signaling, and promote inflammation, laying the foundation for the diverse neurological manifestations of COVID-19. Understanding these interactions is essential for developing targeted therapies and mitigating the long-term impact of SARS-CoV-2 on the nervous system.

### 3.7 Mechanisms Underlying the Toxicity of SARS-CoV-2, the Spike Protein, and the S1 Subunit

The pathophysiological impact of SARS-CoV-2 extends far beyond its role as a respiratory pathogen. Increasing evidence reveals that the virus, particularly its Spike glycoprotein and S1 subunit, exerts direct and indirect toxic effects on multiple organ systems through a complex interplay of receptor interactions, intracellular signaling disruptions, immune dysregulation, and epigenetic reprogramming. The S1 subunit, which contains the receptor-binding domain (RBD), is

not only essential for viral entry but also capable of triggering inflammatory and cytotoxic responses independent of viral replication, contributing to acute complications and long COVID symptoms. Understanding the molecular mechanisms of SARS-CoV-2 toxicity is critical for elucidating the etiology of acute COVID-19 complications and the persistent symptoms observed in post-acute sequelae (PASC), commonly referred to as long COVID (Nahalka, 2024).

The initial step in SARS-CoV-2 pathogenesis involves the binding of the Spike protein's S1 subunit to angiotensin-converting enzyme 2 (ACE2), a transmembrane receptor expressed in the lungs, heart, kidneys, gastrointestinal tract, and brain. This interaction facilitates viral entry via endocytosis or membrane fusion, but also leads to internalization and downregulation of ACE2 expression on host cells. ACE2 plays a protective role in the renin-angiotensin system (RAS) by converting angiotensin II into angiotensin-(1–7), a vasodilatory and anti-inflammatory peptide. Loss of ACE2 function results in elevated angiotensin II levels, promoting vasoconstriction, oxidative stress, and pro-inflammatory cytokine release (Barroso da Silva et al., 2025; Hazan et al., 2025). This imbalance contributes to endothelial dysfunction, thrombosis, and organ damage.

In the brain, reduced ACE2 activity may impair cerebral blood flow regulation and exacerbate neuroinflammation. The S1 subunit's ability to bind ACE2 in the absence of full viral particles suggests that circulating Spike fragments may perpetuate tissue injury even after viral clearance (Rhea et al., 2021).

The blood-brain barrier (BBB) is a highly selective interface that protects the central nervous system (CNS) from pathogens and toxins. SARS-CoV-2 has been shown to compromise BBB integrity through multiple mechanisms. The S1 subunit can cross the BBB via adsorptive transcytosis, facilitated by interactions with neuropilin-1 (NRP1) and heparan sulfate proteoglycans (Carlson et al., 2023). Once inside the CNS, S1 binds to neuronal and glial receptors, triggering inflammatory cascades and cellular stress responses.

Endothelial cells lining the BBB express ACE2 and are susceptible to Spike-mediated injury. Exposure to S1 induces tight junction disruption, increased permeability, and upregulation of adhesion molecules such as ICAM-1 and VCAM-1, promoting leukocyte infiltration and neuroinflammation (Lei et al., 2021; Perico et al., 2024; Robles et al., 2022; Montezano et al., 2023). These changes contribute to the neurological symptoms of COVID-19, including headache, confusion, and encephalopathy.

Microglia are the resident immune cells of the CNS and play a central role in maintaining neural homeostasis. Upon exposure to the S1 subunit, microglia undergo phenotypic transformation into a

pro-inflammatory state, characterized by the release of cytokines such as IL-6, TNF-α, and IL-1β (Mamedov et al., 2023). This neuroinflammatory milieu disrupts synaptic function, impairs neurogenesis, and promotes excitotoxicity through glutamate dysregulation.

Astrocytes also respond to S1 exposure by upregulating GFAP and releasing chemokines that recruit peripheral immune cells. Chronic glial activation has been implicated in the pathogenesis of neurodegenerative diseases and may underlie the cognitive deficits observed in long COVID patients. Notably, these effects occur even in the absence of detectable viral RNA in the brain, suggesting that Spike protein fragments alone are sufficient to induce neurotoxicity (Montezano et al., 2023).

Mitochondria are essential for cellular energy production and redox balance. SARS-CoV-2 infection and S1 exposure have been linked to mitochondrial fragmentation, depolarization, and impaired ATP synthesis. These changes are accompanied by increased production of reactive oxygen species (ROS), leading to oxidative damage of lipids, proteins, nucleic acids and disrupt calcium homeostasis and synaptic transmission (Singh et al., 2020). These phenomena contributing to fatigue, brain fog, and mood disturbances. The S1 subunit may also interfere with mitochondrial antiviral signaling (MAVS), dampening innate immune responses and facilitating viral persistence. Antioxidant therapies targeting mitochondrial pathways are being explored as potential interventions for neuro-COVID syndromes.

The endoplasmic reticulum (ER) is responsible for protein folding and trafficking. SARS-CoV-2 infection induces ER stress by overwhelming the protein synthesis machinery and triggering the unfolded protein response (UPR). The S1 subunit has been shown to activate UPR sensors such as PERK, IRE1, and ATF6, leading to translational arrest, apoptosis, and inflammatory signaling (Blanco-Melo et al., 2020). ER stress contributes to cytokine production, immune cell recruitment, and tissue damage. In the CNS, prolonged UPR activation may impair synaptic plasticity and promote neurodegeneration. Therapeutic strategies aimed at modulating ER stress responses are under investigation for mitigating COVID-19-related organ injury.

The Spike protein also interferes with innate immune signaling. The S2 subunit has been shown to disrupt the formation of the interferon-stimulated gene factor 3 (ISGF3) complex, thereby antagonizing type I interferon responses and facilitating immune evasion (Cai Z et al., 2024). This immunosuppressive effect may allow for prolonged viral persistence and chronic inflammation, further exacerbating tissue damage.

In addition, the S1 subunit may engage toll-like receptors (TLRs) and other pattern recognition

receptors (PRRs), amplifying inflammatory responses and contributing to cytokine storms. Dysregulated immune activation has been implicated in multisystem inflammatory syndrome (MIS) and autoimmune phenomena associated with COVID-19.

The toxicity of SARS-CoV-2, particularly its Spike protein and S1 subunit, is mediated through a constellation of molecular mechanisms that disrupt cellular homeostasis, immune regulation, and neural integrity. These include receptor-mediated signaling, mitochondrial and ER stress, epigenetic reprogramming, and immune antagonism. The ability of the S1 subunit to exert pathogenic effects independent of viral replication underscores its role as a key driver of COVID-19 complications and long-term sequelae. Continued research into these mechanisms will inform the development of targeted therapies and improve outcomes for individuals affected by neuro-COVID and systemic manifestations of SARS-CoV-2.

### 3.8 Mechanisms of Neurotoxicity Induced by SARS-CoV-2, the Spike Protein, and the S1 Subunit

SARS-CoV-2 has demonstrated a multifaceted capacity to affect the central nervous system (CNS), with neurological symptoms ranging from anosmia and encephalopathy to long-term cognitive impairment. These effects are not solely attributable to viral replication but are increasingly linked to the molecular actions of the Spike glycoprotein, particularly its S1 subunit. The S1 domain, which contains the receptor-binding domain (RBD), interacts with neuronal receptors, disrupts cellular homeostasis, and triggers inflammatory and degenerative cascades that may persist long after acute infection resolves (Wang F et al., 2024).

The S1 subunit facilitates neuroinvasion by binding to ACE2 and neuropilin-1 (NRP1), both expressed on brain endothelial cells. This interaction compromises the blood-brain barrier (BBB), allowing viral particles and inflammatory mediators to infiltrate the CNS. Experimental models have shown that S1 alone can cross the BBB and accumulate in regions such as the cortex and hippocampus (Cantuti-Castelvetri et al., 2020; Rhea et al., 2021).

Once inside the CNS, S1 activates microglia via pattern recognition receptors including TLR4 and NRP1. This leads to the release of pro-inflammatory cytokines (IL-6, TNF-α, IL-1β), chemokines, and reactive oxygen species (ROS), creating a neuroinflammatory environment that disrupts synaptic function and promotes neuronal injury (Carlson et al., 2023; Saucier et al., 2023).

Neurons are highly dependent on mitochondrial function. S1 exposure impairs mitochondrial dynamics, reduces ATP synthesis, and increases ROS generation. These changes lead to oxidative

damage of neuronal membranes and DNA, contributing to apoptosis and synaptic loss (Singh et al., 2020).

The S1 subunit accelerates phosphorylation and aggregation of α-synuclein in cellular models of synucleinopathy. This process is mediated by microglia-induced inflammation and mitochondrial ROS production, suggesting a mechanistic link between SARS-CoV-2 exposure and Parkinson's disease-like pathology (Chang et al., 2023; Sulzer et al., 2020).

The S1 subunits of different variants differentially activate IL-6 signaling in brain endothelial cells, leading to downstream microglial activation and vascular inflammation. IL-6 is a key mediator of the cytokine storm and has been associated with BBB disruption and neurovascular injury (Stangis et al., 2024).

Astrocytes respond to S1 exposure by undergoing reactive gliosis, characterized by GFAP upregulation and inflammatory mediator release. This disrupts glutamate clearance, impairs synaptic support, and contributes to excitotoxicity (Saucier et al., 2023; Sofroniew, 2020).

S1 impairs synaptic plasticity, reduces dendritic spine density, and alters neurotransmitter release. These effects are mediated by inflammatory signaling, mitochondrial dysfunction, and epigenetic repression of synaptic genes (Douaud et al., 2022).

In children, SARS-CoV-2 has been associated with multisystem inflammatory syndrome (MIS-C) and neurodevelopmental delays. S1 may interfere with neural progenitor cell differentiation and disrupt developmental signaling pathways (Zimmermann & Curtis, 2020).

Potential strategies include antioxidants to mitigate ROS and IL-6 antagonists to reduce inflammation. These approaches are under investigation for neuro-COVID syndromes (Stangis et al., 2024).

### 3.9 Management of COVID-19-Associated Neurotoxicity and Emerging Therapeutic Strategies

The neurological burden of COVID-19 has emerged as a significant public health concern, with neurotoxicity manifesting across a spectrum of acute and chronic presentations. These include encephalopathy, cerebrovascular events, seizures, neuroinflammatory syndromes, and post-acute sequelae such as cognitive impairment and fatigue. The pathogenesis of SARS-CoV-2-induced neurotoxicity involves direct viral invasion, immune-mediated injury, endothelial dysfunction, and the neurotoxic effects of viral proteins, particularly the Spike glycoprotein and its S1 subunit. Given

the multifactorial nature of these mechanisms, therapeutic strategies must be equally multidimensional, encompassing antiviral, anti-inflammatory, neuroprotective, and rehabilitative approaches (Vashisht et al., 2024; Pang et al., 2024).

In patients with acute neuroinflammatory presentations, such as encephalitis, acute disseminated encephalomyelitis (ADEM), or Guillain-Barré syndrome, high-dose corticosteroids (e.g., methylprednisolone) remain a cornerstone of therapy. These agents suppress cytokine production and reduce blood-brain barrier permeability. Intravenous immunoglobulin (IVIG) and plasma exchange (PLEX) are also employed in immune-mediated neuropathies and severe encephalopathies (Graham et al., 2022).

Tocilizumab, an IL-6 receptor antagonist, has shown promise in mitigating cytokine storm and neurovascular inflammation, particularly in patients with elevated inflammatory markers and CNS involvement (Brown et al., 2023).

While direct antiviral therapies such as remdesivir and nirmatrelvir/ritonavir (Paxlovid) are primarily used for systemic viral suppression, their role in reducing neurotoxicity is indirect but relevant. Early viral clearance may prevent CNS dissemination and reduce the burden of viral proteins that contribute to neuroinflammation (Pang et al., 2024).

Patients with long COVID frequently report “brain fog,” memory deficits, and executive dysfunction. Cognitive rehabilitation programs, including computerized cognitive training, occupational therapy, and neuropsychological counseling, are essential for functional recovery. These interventions aim to restore attention, processing speed, and working memory through structured exercises and compensatory strategies (Seo et al., 2025).

Low-dose corticosteroids and selective cytokine inhibitors may be considered in persistent neuroinflammatory states. Minocycline, a tetracycline antibiotic with anti-inflammatory and neuroprotective properties, has been investigated for its ability to inhibit microglial activation and reduce oxidative stress (Garrido-Mesa et al., 2013).

N-acetylcysteine (NAC), a glutathione precursor, has shown potential in mitigating mitochondrial dysfunction and ROS-mediated neuronal injury. Its antioxidant and anti-apoptotic effects make it a candidate for neuro-COVID management (Shi et al., 2020).

Neuropilin-1 facilitates Spike protein entry into neural and endothelial cells. Experimental NRP1 antagonists have demonstrated efficacy in reducing S1-mediated neuroinvasion and inflammation. Although not yet approved for clinical use, these agents represent a promising strategy for preventing CNS penetration of viral proteins (Vashisht et al., 2024).

The S1 subunit activates IL-6 signaling in brain endothelial cells, contributing to microglial activation and BBB disruption. IL-6 inhibitors such as tocilizumab and sarilumab may attenuate these effects and are being evaluated in clinical trials for neuro-COVID applications (Brown et al., 2023; Stangis et al., 2024).

COVID-19 is associated with a hypercoagulable state and increased risk of ischemic stroke. Management includes anticoagulation with low-molecular-weight heparin or direct oral anticoagulants (DOACs), alongside neuroprotective agents such as edaravone and citicoline. Early recognition and intervention are critical to prevent long-term disability (Graham et al., 2022).

Neuro-COVID often presents with mood disturbances, anxiety, and sleep disorders. Selective serotonin reuptake inhibitors (SSRIs), melatonin, and cognitive behavioral therapy (CBT) are employed to address these symptoms. Melatonin also exhibits antioxidant and anti-inflammatory properties, potentially contributing to neuroprotection (Seo et al., 2025).

Mesenchymal stem cells (MSCs) have immunomodulatory and regenerative properties. Preliminary studies suggest that MSCs may reduce neuroinflammation and promote neuronal repair in post-COVID syndromes. Ongoing trials are evaluating their safety and efficacy (Yao et al. 2022; Cai Y et al., 2023; Lu et al., 2024; Li et al., 2024).

Transcranial magnetic stimulation (TMS) and transcranial direct current stimulation (tDCS) are non-invasive neuromodulation techniques that may enhance neuroplasticity and cognitive recovery. These modalities are being explored in rehabilitation programs for long COVID patients with persistent cognitive deficits (Seo et al., 2025).

Effective management of COVID-19-associated neurotoxicity requires a multidisciplinary approach involving neurologists, immunologists, psychiatrists, rehabilitation specialists, and primary care providers. Integrated care pathways and long COVID clinics have been established in several countries to coordinate diagnostics, therapy, and follow-up (Pang et al., 2024; Seo et al., 2025).

## 4. Thesis Aim

The aim of this thesis is to investigate the molecular and cellular mechanisms by which the S1 subunit of the SARS-CoV-2 Spike protein contributes to neurotoxicity in the context of COVID-19. While the systemic effects of SARS-CoV-2 have been extensively studied, its impact on the central nervous system remains incompletely understood and clinically underestimated. This work focuses on the S1 subunit as a neuroactive viral component capable of crossing the blood-brain barrier, interacting with neuronal and glial receptors, particularly Neuropilin-1 (NRP1), and triggering intracellular cascades that culminate in neuronal dysfunction and death. By integrating evidence from virology, neurobiology, and epigenetics, the thesis aims to characterize the cellular entry pathways exploited by S1, delineate its role in activating the HDAC4/CREB/RIPK1 axis, and assess its contribution to necroptosis and neuroinflammation. Through in vitro modeling, the study seeks to provide a mechanistic framework for understanding COVID-19-associated neurotoxicity and to identify potential molecular targets for therapeutic intervention in neuro-COVID and post-acute sequelae.

## 5. State of the Art

The neurological manifestations of SARS-CoV-2 infection have garnered increasing scientific attention, as accumulating clinical reports and mechanistic studies reveal a complex interplay between viral components and central nervous system (CNS) integrity. Among these components, the Spike glycoprotein, particularly its S1 subunit, has emerged as a critical mediator of neurotoxicity, acting beyond its canonical role in viral entry. The S1 domain facilitates viral attachment to host cells via high-affinity interactions with angiotensin-converting enzyme 2 (ACE2), neuropilin-1 (NRP1), and other co-receptors, but recent evidence suggests that it can exert pathogenic effects independently of full viral replication. Notably, the S1 subunit has been shown to cross the blood-brain barrier (BBB) through mechanisms such as adsorptive transcytosis and endothelial disruption, gaining access to the CNS and initiating a cascade of deleterious cellular responses. Once within the brain parenchyma, S1 engages with neuronal and glial receptors, triggering intracellular signaling pathways that culminate in neuroinflammation, oxidative stress, mitochondrial dysfunction, and ultimately, neuronal death. Studies have demonstrated that S1 activates the IL-6 signaling axis in human brain endothelial cells, which promotes microglial activation and the release of pro-inflammatory cytokines, contributing to a sustained inflammatory milieu that may underlie many of the cognitive and neuropsychiatric symptoms observed in COVID-19 patients (Stangis et al., 2024). Furthermore, S1 has been implicated in the acceleration of α-synuclein phosphorylation and aggregation, a hallmark of Parkinsonian pathology, suggesting a mechanistic overlap between viral exposure and neurodegenerative processes (Qu et al., 2025). These effects are exacerbated by microglia-induced inflammation and mitochondrial reactive oxygen species (ROS) production, which compromise neuronal viability and synaptic architecture (Chang et al., 2023). In vitro and in vivo models have revealed that S1 exposure leads to reduced dendritic spine density, impaired synaptic plasticity, and altered neurotransmitter release, providing a cellular substrate for the cognitive impairments frequently reported in clinical settings. Additionally, S1 disrupts autophagic flux and lysosomal degradation, impairing proteostasis and promoting the accumulation of misfolded proteins, which may accelerate the onset of neurodegenerative conditions. These findings underscore the multifactorial nature of S1-mediated neurotoxicity, which involves not only direct neuronal injury but also intricate crosstalk between neurons, astrocytes, microglia, and the cerebrovascular endothelium. The convergence of receptor-mediated signaling, immune activation, metabolic disruption, and proteostatic imbalance positions the S1 subunit as a neuroactive agent capable of eliciting profound and persistent damage to the CNS. Its ability to hijack host cellular machinery and provoke systemic and localized inflammatory

responses makes it a compelling target for therapeutic intervention, particularly in the context of long COVID and post-viral neurodegeneration. As research continues to unravel the molecular underpinnings of S1-induced neuropathology, it becomes increasingly clear that this viral protein plays a central role in the neurological sequelae of SARS-CoV-2 infection, warranting further investigation into its mechanisms of action and potential for pharmacological modulation.

# 6. Results

## 6.1 Selective Neurotoxicity of Spike S1 Subunit in SH-SY5Y Cells

To investigate the neurotoxic potential of SARS-CoV-2 Spike protein subunits, SH-SY5Y cells were transiently transfected with constructs encoding either the full-length Spike protein (Spike Vector), the S1 subunit (S1 Vector), or the S2 subunit (S2 Vector), alongside an empty vector (EV) as control. Western blot analysis confirmed robust and specific expression of the respective proteins, validating transfection efficiency. Cell viability was assessed at 24, 48, and 72 hours post-transfection using MTT and LDH assays. A significant reduction in cell survival was observed in cells transfected with Spike and S1 vectors, with maximal toxicity at 72 hours, whereas S2-transfected cells showed no significant changes compared to EV controls. These findings suggest that the S1 subunit alone is sufficient to induce neuronal death, and that the S2 domain lacks intrinsic neurotoxic activity. This aligns with previous reports indicating that S1, but not S2, can cross the blood-brain barrier and accumulate in the CNS, potentially contributing to COVID-19-associated neurological symptoms. Importantly, the observed cytotoxicity was not attributable to transfection artifacts, as EV-transfected cells maintained stable viability across all time points. These results provide a cellular basis for the hypothesis that circulating S1 fragments may exert direct neurotoxic effects independent of viral replication, and reinforce the notion that S1 acts as a neuroactive agent capable of triggering intracellular death cascades (Figure 1).

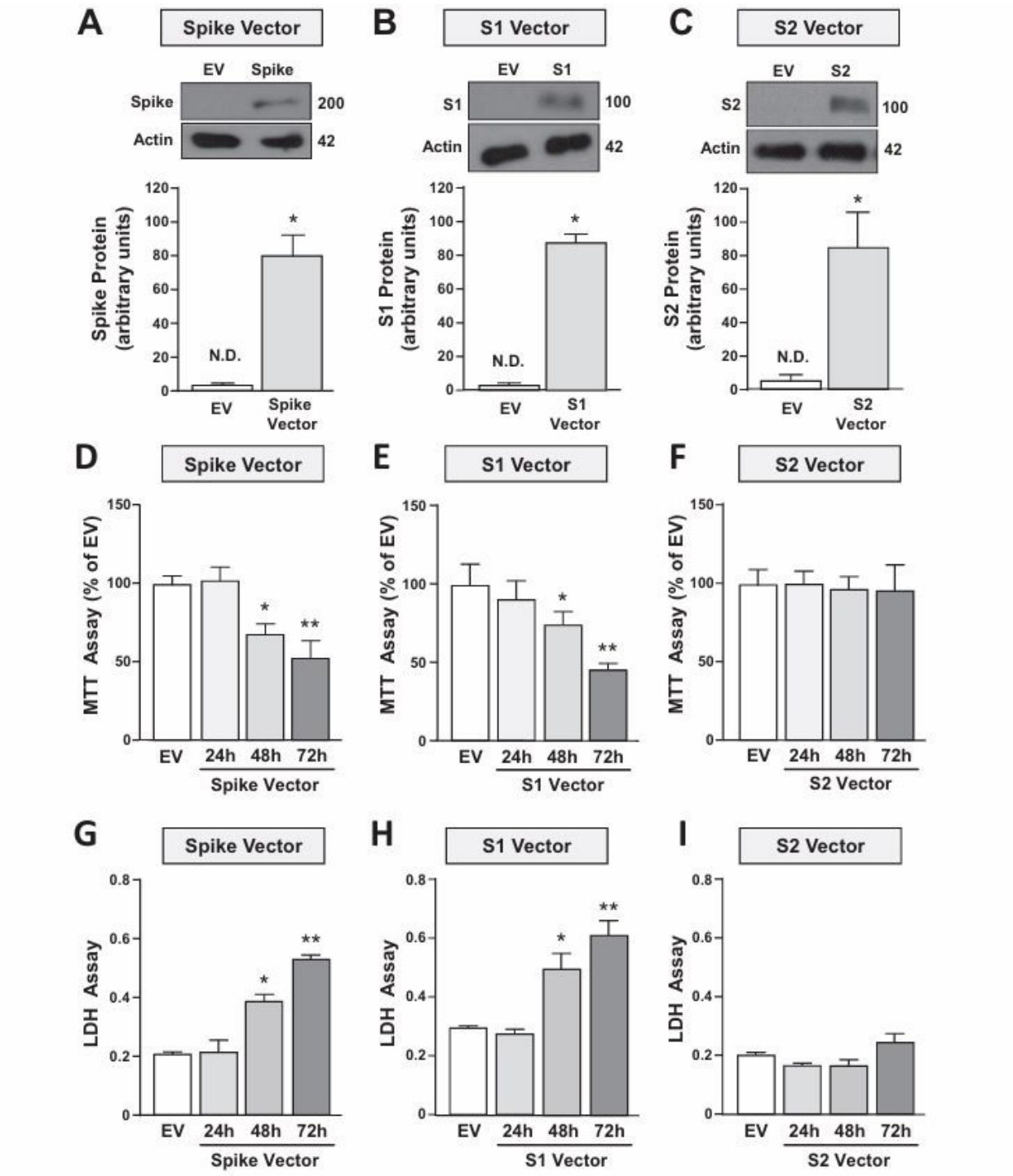



**Figure 1. Effect of transfection of the following constructs: (a) Empty Vector, (b) Spike S1+S2 Vector, (c) Spike S1 Vector and (d) Spike S2 Vector on Spike protein expression and cell viability in SH-SY5Y cells.** (A-C) Western blotting representative image and quantification of Spike protein in cells transfected for 48 hours with the following vectors: Spike-S1+S2 (Spike

Vector), Spike-S1 (S1 Vector) and Spike-S2 (S2 Vector) or with the Empty Vector (EV). (A) Spike was recognized by using an antibody against its C-terminal C9 tag, whereas (B) Spike S1 and (C) Spike S2 subunits were detected by using two different specific antibodies (See Material and Methods). *p≤0.05 vs EV by Student's t test (n=4). (D-F) Cell viability measured by MTT assay and (G-I) cell death measured by LDH assay in SH-SY5Y cells transfected with Spike Vector, S1 Vector and S2 Vector for 24, 48 or 72 hours. *p≤0,05 vs EV; **p≤0,05 vs all by 1-way ANOVA analysis followed by Tukey's post hoc test (n=4/5).

### 6.2 HDAC4-Mediated RIPK1 Upregulation Drives S1-Induced Cell Death

Given the established role of histone deacetylases (HDACs) in neurodegeneration and viral pathogenesis, we explored their involvement in S1-induced cytotoxicity. SH-SY5Y cells were co-transfected with S1 Vector and siRNAs targeting class I (HDAC1–3) and class II (HDAC4, 5, 7, 9) HDAC isoforms. Among all tested isoforms, only HDAC4 silencing significantly restored cell viability, implicating it as a key mediator of S1-induced neuronal death. Pharmacological inhibition experiments further demonstrated that Necrostatin-1, a necroptosis inhibitor, effectively counteracted S1-induced cell death, while inhibitors of apoptosis (ZVAD) and calpain-mediated necrosis (Calpeptin) were ineffective. Western blot and qRT-PCR analyses and confocal microscopic images revealed that S1 overexpression upregulated HDAC4 and RIPK1 at both protein and transcript levels (Figure 2, 5). Importantly, HDAC4 silencing reversed RIPK1 induction, confirming a functional link between HDAC4 activity and RIPK1-mediated necroptosis (Figure 2). These results suggest that HDAC4 acts upstream of RIPK1 and may serve as a molecular switch controlling necroptotic signaling in response to viral protein exposure. The specificity of this pathway was further validated by the absence of compensatory changes in other HDAC isoforms, reinforcing the unique role of HDAC4 in this context (Figure 2). Notably, the temporal correlation between HDAC4 and RIPK1 upregulation suggests a sequential activation cascade, with HDAC4 functioning as an early sensor of S1-induced stress. These findings are consistent with prior studies implicating HDAC4 in neurotoxic responses to environmental pollutants and ischemic insults, suggesting a broader role for this epigenetic regulator in CNS vulnerability.

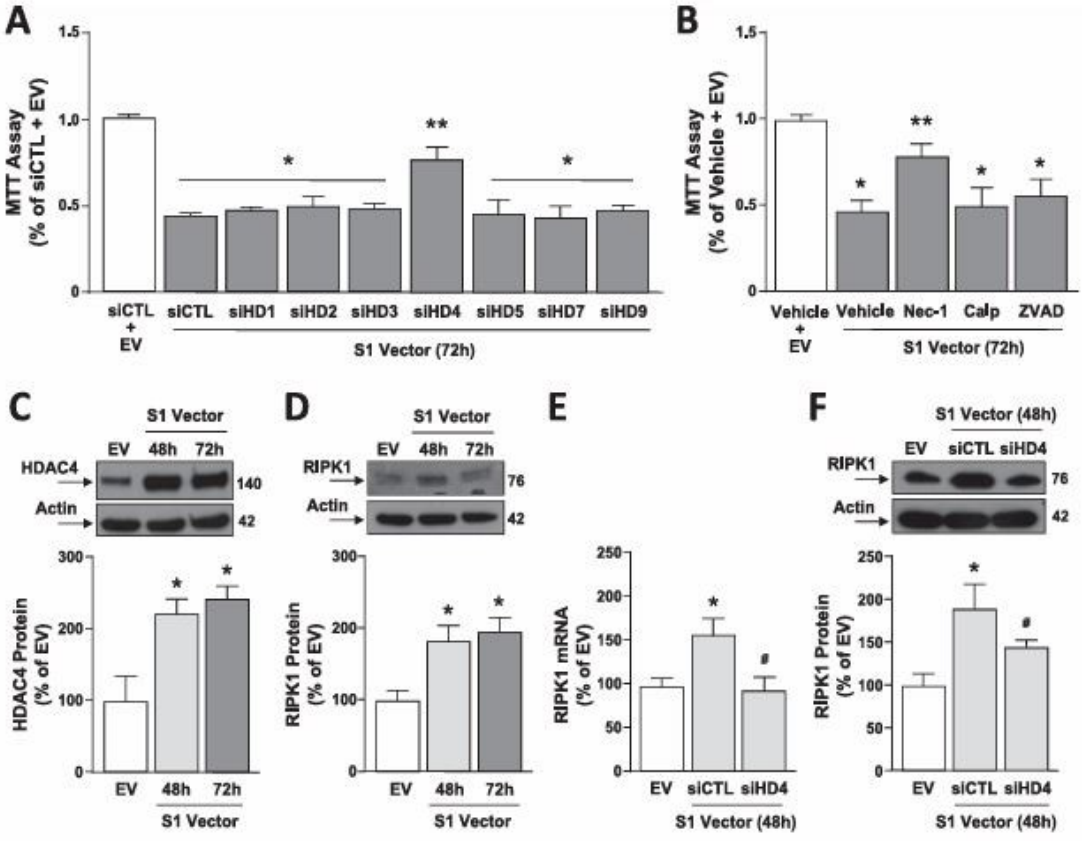



**Figure 2. Effect of HDAC4 knockdown and Necrostatin-1 to reduce S1 Vector-induced (1) cell death and (2) RIPK1 mRNA and protein increase in SH-SY5Y cells.** (A) Cell vitality measured by MTT assay in SH-SY5Y cells transfected with S1 Vector and treated with siRNA against HDAC1 (siHD1), HDAC2 (siHD2), HDAC3 (siHD3), HDAC4 (siHD4), HDAC5 (siHD5), HDAC7 (siHD7), HDAC9 (siHD9) or siRNA control (siCTL) for 72 hours. (B) Cell vitality measured by MTT assay in SH-SY5Y cells transfected with S1 Vector and treated with Vehicle, Nec-1 (10 μM), Calpeptin (30 μM) or ZVAD (50 μM) for 72 hours. *p≤0,05 respect to control (siCTL+EV or Vehicle+EV); **p≤0,05 vs all by one-way ANOVA analysis followed by Tukey's post hoc test (n=4/5). (C-D) Western blotting representative images and quantification of HDAC4 and RIPK1 proteins in SH-SY5Y cells after 48 and 72 hours transfection with S1 Vector. *p≤0,05 vs control (EV) by one-way ANOVA analysis followed by Tukey's post hoc test (n=4). Effect of siHDAC4 on RIPK1 at (E) transcriptional and (F) protein level after 48 hours of S1 Vector transfection. *p≤0,05 vs control (EV); #p≤0,05 vs siCTL+S1 Vector (48h) by one-way ANOVA analysis followed by Tukey's post hoc test (n=3/4).

### 6.3 HDAC4-Induced CREB Degradation Facilitates RIPK1 Transcription

To elucidate the transcriptional regulation of RIPK1, we investigated whether HDAC4 directly binds to the RIPK1 promoter. Chromatin immunoprecipitation (ChIP) assays using HDAC4 antibodies failed to detect promoter binding, suggesting an indirect regulatory mechanism (Figure 3). We hypothesized that HDAC4 may modulate RIPK1 expression via interaction with the transcription factor CREB, a known repressor of RIPK1 transcription. Immunoprecipitation experiments confirmed increased HDAC4–CREB interaction following S1 transfection. This was accompanied by a marked reduction in CREB protein levels, without changes in mRNA expression, indicating post-translational degradation. Treatment with the proteasome inhibitor MG132 or HDAC4 knockdown restored CREB protein levels and its binding to the RIPK1 promoter. Luciferase reporter assays demonstrated that CREB overexpression or HDAC4 inhibition suppressed RIPK1 promoter activity, while qRT-PCR confirmed reduced RIPK1 mRNA levels under these conditions. These findings support a model in which HDAC4 promotes CREB degradation, thereby relieving transcriptional repression of RIPK1 and facilitating necroptotic signaling. Notably, CREB overexpression not only restored transcriptional control over RIPK1 but also conferred partial neuroprotection, suggesting a dual role in transcriptional regulation and cell survival (Figure 4). Confocal microscopy further revealed a reduction in nuclear CREB localization in S1-transfected cells, reinforcing the hypothesis of HDAC4-mediated CREB destabilization (Figure 5). These results highlight a novel epigenetic mechanism by which viral proteins can hijack host transcriptional machinery to promote cell death, and position CREB as a critical node in the neuroprotective response to viral stress.

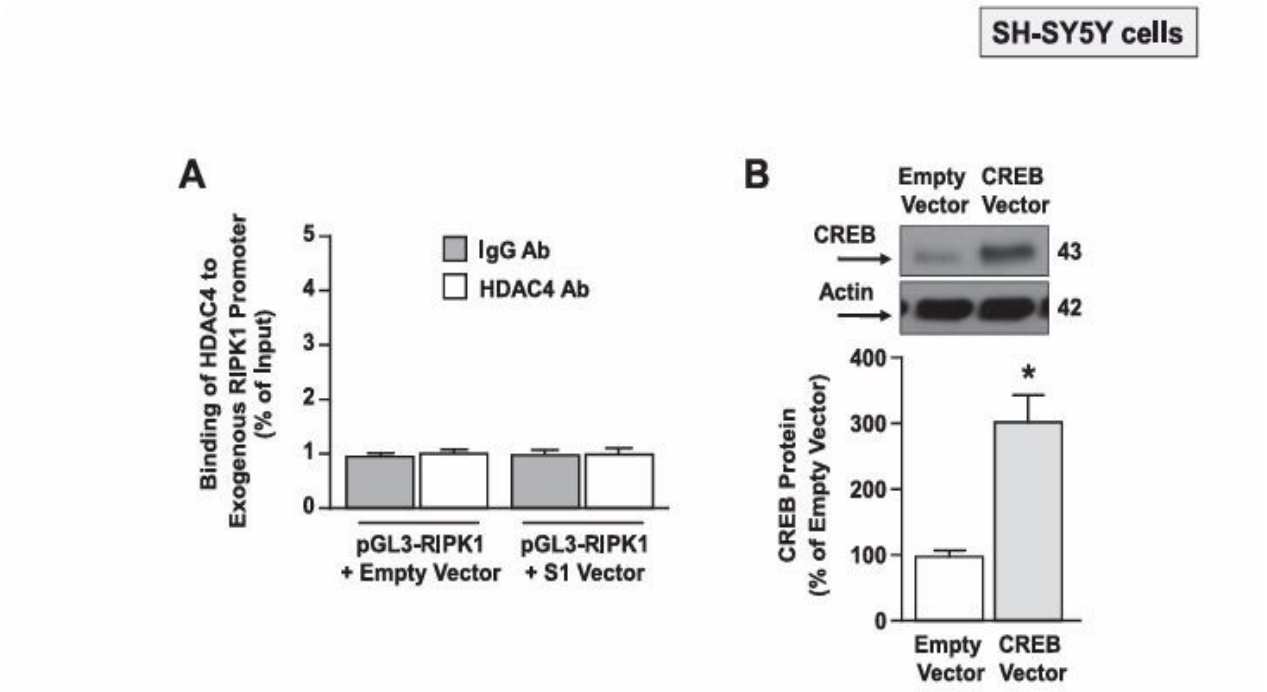



**Figure 3. HDAC4 does not bind the RIPK1 human promoter in S1 Vector-transfected SH-SY5Y cells.** (A) ChIP with HDAC4 and IgG antibodies followed by qPCR of the RIPK1 human promoter in SH-SY5Y cells transfected for 72 h with: (1) pGL3- RIPK1 + EV, (2) pGL3- RIPK1 + S1 Vector (n = 3). The pGL3- RIPK1 + EV group immunoprecipitated with IgG was defined as 1.0 and was used to compare the other experimental groups. (B) Western Blotting representative images with quantification of CREB protein levels in SH- SY5Y cells transfected 48 h with a vector overexpressing CREB. *$p \leq 0.05$ versus empty vector by unpaired Student's t test (n = 4).

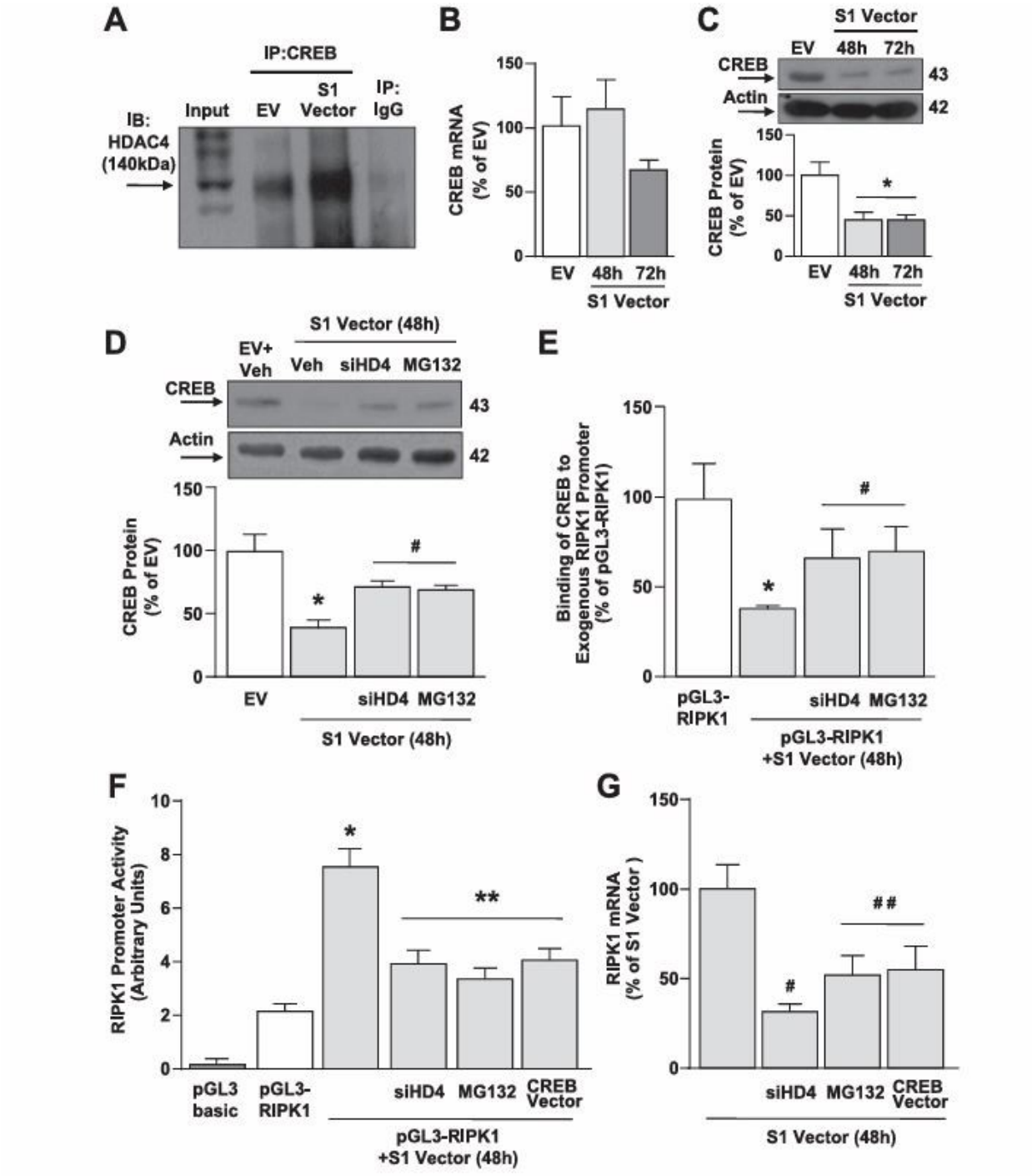



**Figure 4. Effect of siHDAC4 and proteasomal inhibitor MG132 on S1 Vector-induced down-regulation of CREB in SH-SY5Y cells.** (A) Representative Western Blotting of immunoprecipitation (IP) showing the interaction between CREB and HDAC4 in SH-SY5Y

transiently transfected with EV or S1 Vector. IP with IgG was used as negative control. The input represents the pre-immunoprecipitated total lysates (n=3). (B, C) Gene and protein expression of CREB in SH-SY5Y cells transiently transfected with S1 Vector for 48 and 72 hours. *p≤0,05 vs control (EV) by one-way ANOVA analysis followed by Tukey's post hoc test (n=3/4). (D) Western blotting images with quantification of CREB protein in SH-SY5Y cells transiently transfected and treated with: (1) EV; (2) S1 Vector, (3) S1 Vector + siHDAC4, and (4) S1 Vector + MG132. *p ≤ 0,05 vs EV; #p≤0,05 vs S1 Vector (48h) by one-way ANOVA analysis followed by Tukey's post hoc test (n=3). (E) ChIP with CREB antibody followed by qPCR of the RIPK1 human promoter in SH-SY5Y cells in the following conditions: (1) pGL3-RIPK1, (2) pGL3-RIPK1 + S1 Vector, (3) pGL3-RIPK1 + S1 Vector + siHDAC4, and (4) pGL3-RIPK1 + S1 Vector + MG132. *p ≤ 0,05 vs pGL3-RIPK1; #p≤0,05 vs pGL3-RIPK1 + S1 Vector by one-way ANOVA analysis followed by Tukey's post hoc test (n=3). (F) Luciferase assay in SH-SY5Y cells in the following experimental conditions: (1) pGL3basic, (2) pGL3-RIPK1, (3) pGL3-RIPK1+S1 Vector, (4) pGL3-RIPK1+S1 Vector + siHDAC4, (5) pGL3-RIPK1+S1 Vector + MG132, (6) pGL3-RIPK1 + S1 Vector + CREB Vector; *p≤0,05 vs pGL3-RIPK1; **p≤0,05 vs all by one-way ANOVA analysis followed by Tukey's post hoc test (n=6). (G) Effect of siHDAC4 and CREB construct transfection or MG132 treatment on RIPK1 mRNA levels evaluated by qRT-PCR in SH-SY5Y cells after 48h transfection of S1 Vector. #p≤0,05 vs S1 Vector, ##p≤0,05 vs all by one-way ANOVA analysis followed by Tukey's post hoc test (n=3).

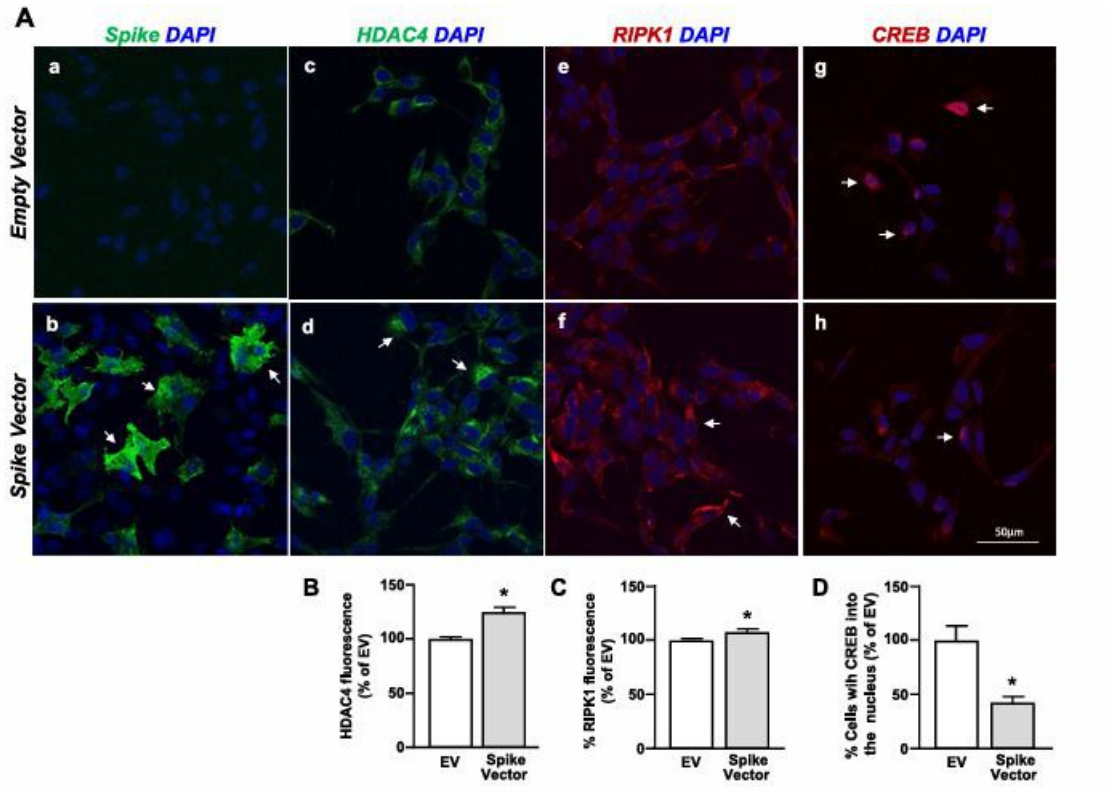



**Figure 5. Effect of Spike S1 overexpression on HDAC4, RIPK2, and CREB expression in SH-SY5Y cells.** (A) (a, b) Representative confocal microscopic images displaying the distribution of S1 immunoreactivity in SH-SY5Y transfected with empty vector (EV) (a) or S1 vector (b) for 72 h. (c–h) Representative confocal microscopic images displaying the distribution of HDAC4 (c, d), RIPK1 (e, f) and CREB (g, h) immunoreactivities in SH-SY5Y transfected with EV (a) or S1 Vector (b) for 72 h. Arrows in (d, f) point to intense HDAC4 or RIPK1 immunoreactive cells; arrows in (g, h) point to cells with CREB+- nucleus. Scale bars in (a–h): 50 µm. (B, C) Densitometric analysis of HDAC4+ (B) and RIPK1+ (C) fluorescence intensity in EV- or S1 Vector-transfected SH-SY5Y cells. (D) Quantitative analysis of cells with CREB+- nucleus in EV- or S1 Vector-transfected SH-SY5Y cells. Data in (B–D) were expressed as percentage of EV. *$p < 0.05$ versus EV by unpaired Student's t test ($n = 3$).

### 6.4 NRP1-Dependent Internalization of S1 Protein Triggers Neuronal Death

To assess whether S1 protein can induce neurotoxicity independently of viral replication, differentiated SH-SY5Y cells were treated with recombinant S1 protein (S1rp). Dose-response and time-course experiments revealed that S1rp induces significant LDH release at concentrations ≥0.05 μg/mL, with maximal effect at 0.1 μg/mL after 72 hours. ACE2 expression was low and only modestly increased upon retinoic acid-induced differentiation, whereas NRP1 was constitutively expressed and progressively upregulated (Figure 6). Knockdown of NRP1, but not ACE2, significantly reduced S1rp-induced cell death, implicating NRP1 as the primary receptor mediating S1 internalization. Subcellular fractionation and confocal microscopy demonstrated that S1rp treatment led to NRP1 internalization and cytosolic accumulation of S1 protein. This process was abrogated by sodium azide, an endocytosis inhibitor, confirming that S1 enters the cytoplasm via NRP1-mediated endocytosis (Figure 7). These results establish a mechanistic link between NRP1-mediated S1 uptake and neuronal death, highlighting NRP1 as a potential therapeutic target to mitigate Spike-induced neurotoxicity. Importantly, the redistribution of NRP1 from the plasma membrane to the cytosol was accompanied by a loss of membrane integrity and increased perinuclear accumulation of S1, suggesting that endosomal trafficking may play a role in downstream signaling events. The absence of ACE2 involvement in this process reinforces the notion that NRP1 is the dominant entry route for S1 in neuronal cells, consistent with its high expression in the CNS and its known role in axonal guidance and vascular permeability. These findings provide compelling evidence that S1 acts as a neuroactive ligand capable of triggering intracellular death cascades through receptor-mediated internalization, independent of viral replication or systemic inflammation. Moreover, the ability of S1 to exploit NRP1 for cytosolic entry and initiate HDAC4/CREB/RIPK1 signaling underscores the convergence of viral and host pathways in mediating neurodegeneration.

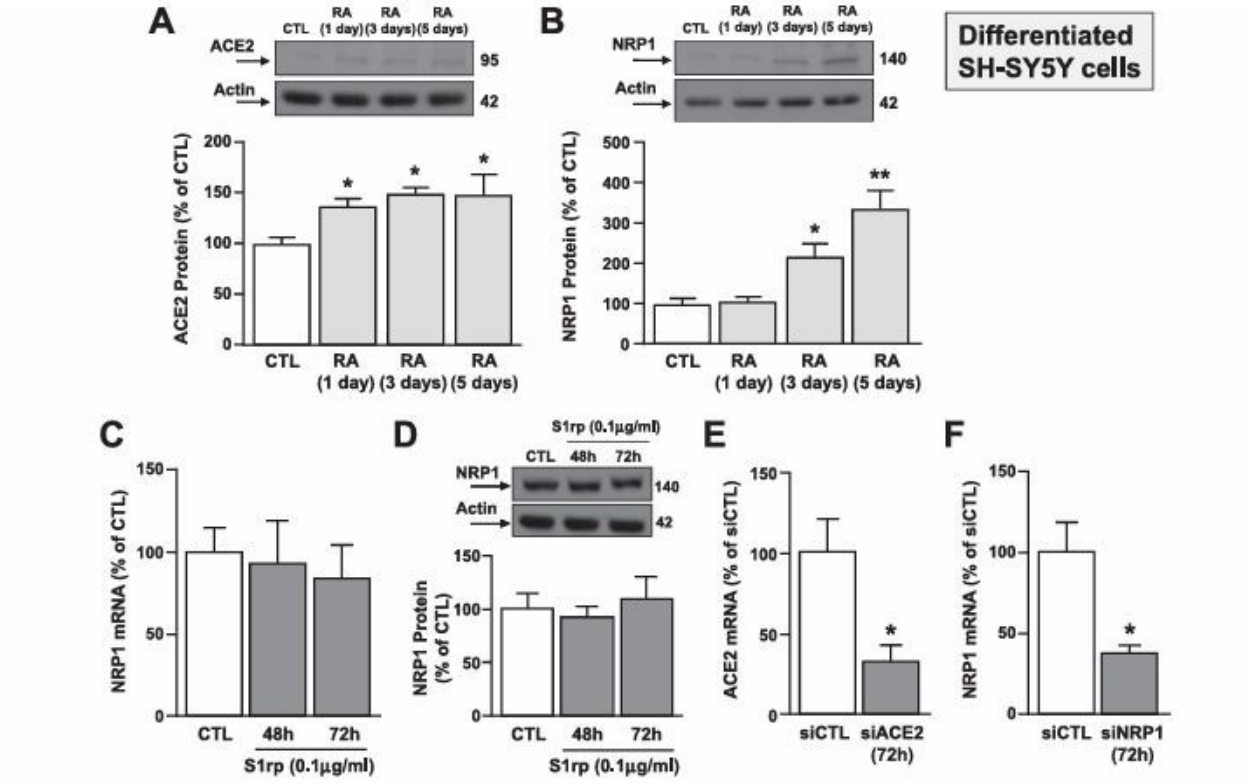



**Figure 6. Effect of Retinoic Acid on ACE2 and NRP1 protein expression and of S1rp on NRP1 gene and protein expression in SH-SY5Y cells.** (A, B) Western blotting images with relative quantification of ACE2 and NRP1 protein levels in SH-SY5Y cells differentiated with retinoic acid (RA) 10 μM for 1, 3 or 5 days. *p ≤ 0.05 versus CTL, **p ≤ 0.05 vs all by one-way ANOVA analysis followed by Tukey's post hoc test (n = 3). (C, D) Effect of 48 and 72 h of S1rp treatment (0.1 μg/mL) on NRP1 gene and protein expression. *p ≤ 0.05 versus CTL by one-way ANOVA analysis followed by Tukey's post hoc test (n = 3). (E, F) Effect of siRNAs against ACE2 (siACE2) and NRP1 (siNRP1) on ACE2 and NRP1 mRNA levels by qRT-PCR in SH-SY5Y cells differentiated with retinoic acid 10 μM for 5 days. *p ≤ 0.05 versus siCTL by Student's t test (n = 3).

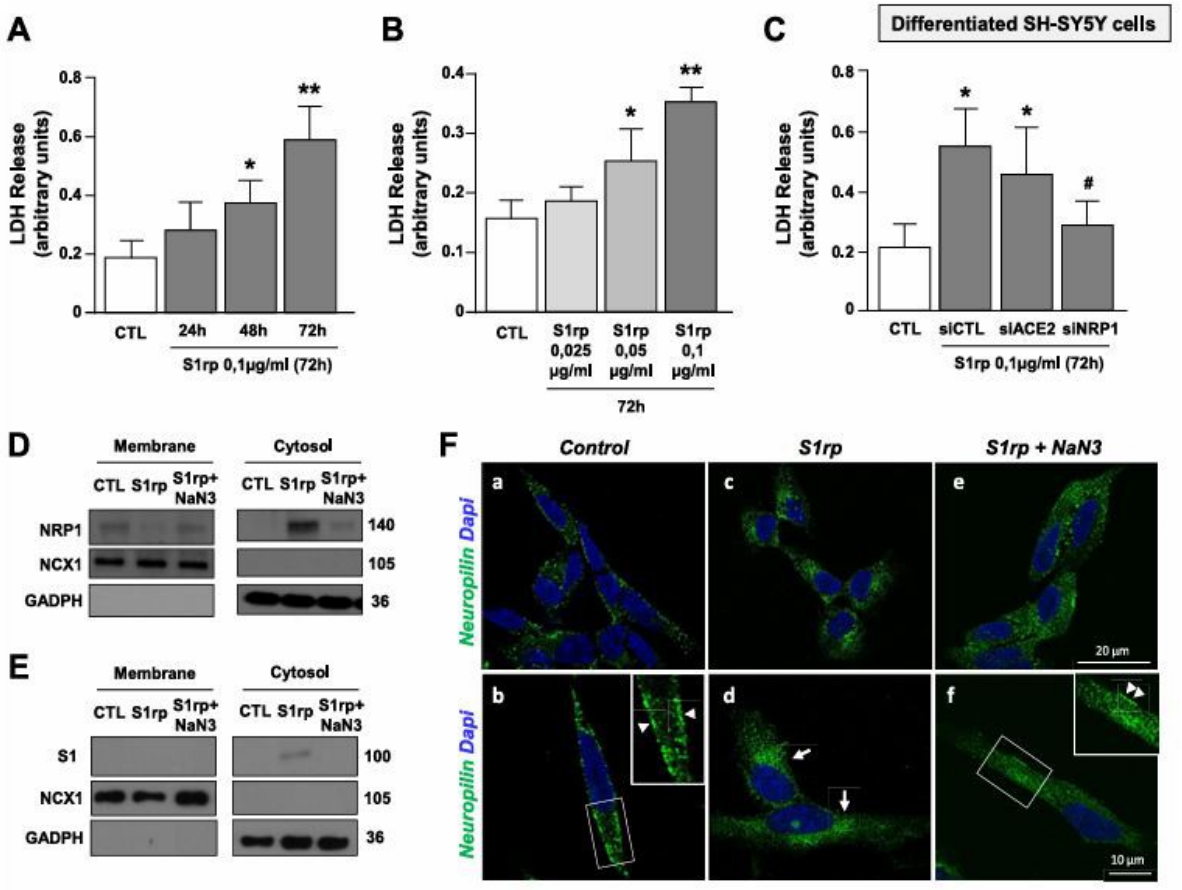



**Figure 7. Effect of S1 recombinant protein (S1rp) to induce cell death via internalization of the NRP1 receptor in differentiated SH-SY5Y cells.** (A, B) LDH release assay in differentiated SH-SY5Y cells treated with Spike-S1rp: (A) at different times points (24, 48, and 72 h) at 100 ng/mL con centration and (B) at different concentrations (25–100 ng/mL) for 72 h. *p ≤ 0.05 versus Vehicle; **p ≤ 0.05 versus all by one-way ANOVA analysis fol lowed by Tukey's post hoc test (n = 4/5). (C) Effect of siACE2 and of siNRP1 on LDH release in cells exposed to 72 h of S1 protein (0.1 µg/mL). *p ≤ 0.05 versus Vehicle, #p ≤ 0.05 versus siCTL + Spike- S1 protein (n = 5). (D, E) Western blot of NRP1 and Spike S1 subunit (S1) protein levels in membrane and cytosolic fraction of differentiated SH-SY5Y exposed for 72 h to Spike-S1 recombinat protein (100 ng/mL), alone or in combination with NaN3. GAPDH and NCX1 were used to verify the integrity of the cytosolic and membrane fraction of the samples (n = 3). (F) Representative confocal microscopic images

displaying the distribution of neuropilin immunoreactivity in SH-SY5Y cells under control conditions (a, b) and after exposure to Spike S1 recombinant protein, in the absence (c, d) or in the presence of NaN3 (e, f). Arrowheads in panels (b, f) point to neuropilin plasma membrane immunoreactivity. Arrows in (d) point to perinuclear neuropilin staining. Scale bars in (a, c, e): 20 μm; in (b, d, f): 10 μm.

# 7. Materials and Methods

## 7.1 Reagents, Drugs and Antibodies

All reagents were supplied by Sigma (Milan, IT). Oligonucleotides were synthesized by Eurofins Genomics (Ebersberg, Germany). The following siRNAs were used for human proteins: HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, HDAC7, and HDAC9 (Formisano et al., 2015; Sanguigno et al., 2023), NRP1 (sc- 36038) and ACE2 (sc- 41400). The following siRNAs were used for rat proteins: NRP1 (orb1830264), ACE2 (orb1829102), HDAC4 (Formisano et al., 2019), RIPK1 (Guida et al., 2017), and CREB purchased from Dharmacon. For all experiments, the siRNA CONTROL (siCTL) was from QIAGEN (All Stars Negative Control siRNA, cod: 1027280). The SARS-CoV2 expression plasmid constructs were: (1) pcDNA3.1 + SARS- Spike- C9, (Addgene Cod: 145031) for Spike Vector, (2) pCMV3- S1, (Sino Biological, Cod: VG40591- UT) for S1 subunit vector, and (3) pCMV3- S2, (Sino Biological, Cod: VG40590- UT) for S2 subunit vector. The plasmid used for CREB overexpression was gen tly gifted by Marc Montminy (Plasmid #22394). For all experiments with constructs transfection, pcDNA3.1 has been used as the empty vector (EV). Necrostatin-1 (Nec-1;10 μM), z-VAD-fmk (ZVAD; 50 μM), Calpeptin (Calp; 30 μM), MG132 (10 μM) (Guida et al., 2014; Guida et al., 2017; Sanguigno et al., 2023) and Retinoic Acid (RA; 10 μM) (Kovalevich et al., 2013) were prepared and used as previously reported. Sodium Azide (SIGMA, Cod: S2002, 30 mM). Culture media and sera were purchased from Invitrogen. All chemicals were diluted in cell culture medium. 0.1% dimethyl sulfoxide (DMSO) was the final concentration used as the vehicle that did not cause cellular toxicity. SARS- CoV2 Spike S1 recombinant protein (S1rp) was purchased from GeneScript (Cod. Z03501-100; stock solution 2.01 mg/ mL). The antibodies used were the following: Anti-C9 tag (MyBioSource, MBS430088); Anti-SARS-CoV-2 Spike Protein (Invitrogen, PA5-114451); Anti-Spike S2 (GeneTex, 135386); Anti-Spike S1 (Invitrogen, PA5- 114451); Anti-NRP1 (GeneTex, GXGTX127947); Anti-ACE2 (SantaCruz, sc-390851); Anti-CREB (Cell Signaling, 9194S). Anti-HDAC4 (Formisano et al., 2019); Anti-RIPK1; Anti-CREB; and Anti-Actin (Guida et al., 2017), which have been used in previous studies.

## 7.2 Cell Cultures

Human neuroblastoma SH- SY5Y cells were obtained and maintained as already reported (Guida et al., 2017; Sanguigno et al., 2023). To induce differentiation, retinoic acid (RA) was added 24 h after plating at a final con centration of 10 μM in culture medium for 5 days. The cellular density for: (1)

MTT, LDH, and Luciferase assays was 1 × 106 cells/well in 24 well plates; (2) qRT- PCR was 5 × 106 cells/plate in 60 mm plates; (3) Western blot and Immunoprecipitation was 15 × 106 cells/plate in 100 mm plates.

### 7.3 Transfection of SH- SY5Y Cells

SH-SY5Y cells were transfected with Lipofectamine 2000 (Thermo Fisher, cod: 11668027), following the manufacturer's instructions. Twenty-four hours after plating, SH- SY5Y cells (50% confluence) were singly transfected with the following constructs: (1) pcDNA3.1, (2) Spike Vector, (3) S1 Vector, and (4) S2 Vector. The transfection was blocked after 24, 48, and 72 h for time course experiments. Protein expression generated from: (1) Spike Vector transfection was detected with Anti-C9 tag; (2) S1 Vector transfection was detected with Anti-Spike S1; and (3) S1 Vector transfection was detected with Anti-Spike S2. The siRNAs against specific HDACs (50 nM) and the plasmid overexpressing CREB were co-transfected with S1 Vector for 48 or 72 h. The day after S1 Vector transfection, cells were treated with vehicle, Nec- 1, ZVAD, Calpeptin, and MG132 for 3 h, diluted in fresh medium (1% FBS); the experiments were blocked 24 h after drug treatment. Transfection efficiency was almost 50%.

### 7.4 Spike Protein Treatment in Differentiated SH- SY5Y Cells

S1rp (Stock solution 2 mg/mL PBS) was dissolved in the culture medium of differentiated SH-SY5Y cells to reach different concentrations (0.025–0.1 μg/mL) for dose–response experiments. The culture medium was changed and fresh S1rp was added daily. CTLs were pretreated with vehicle (PBS) siRNA trans fection (50 nM) was performed with Lipofectamine 2000 after 5 days RA differentiation in SH-SY5Y cells (Sanguigno et al., 2023). Twenty-four hours after siRNA transfection, cells were treated with S1rp 100 ng/mL for 72 h.

### 7.5 Western Blot Analysis, Immunoprecipitation, and Cell Fractionation

Cells were washed and collected in cold Phosphate Buffered Saline (PBS; Sigma, Milan IT). The cell pellet was resuspended in a RIPA lysis buffer (sc-24948, Santa Cruz Biotechnology) with 1× protease inhibitor and incubated on ice for 2 h. The lysate was centrifuged at 14,000 rpm for 20 min at 4°C to obtain total proteins. Immunoprecipitation was performed as previously reported (Guida et al., 2014) Specifically, 1.5 mg of protein was immunoprecipitated overnight at 4°C using 2 μg of

the following antibodies: Anti- CREB mouse monoclonal (cod. sc-271, Santa Cruz Biotechnology) and Normal Mouse IgG- AC (cod: sc-2343, Santa Cruz Biotechnology) as a negative control. The immune complexes were then precipitated through the use of 50 μL of Protein A/G PLUS-Agarose beads (sc-2003, Santa Cruz Biotechnology). The immunoprecipitates were then subjected to Western blot analysis for HDAC4 antibody.

Isolation of cytosolic and membrane fraction was performed as previously described (Nishiumi et al., 2007). Specifically, cells were collected in a 15-mL falcon tube and centrifuged at 1000 g for 5 min. The pellet was resuspended in 300 μL of buffer solution (Buffer A), which is composed of: 50 mM TRIS pH 8.0; 0.5 mM DDT, 0.1% NP- 40; protease and phosphatase inhibitors, and homogenized with a hand microtube homogenizer. Next, the lysate was transferred to a 1 mL syringe and passed through a 26-gauge needle (10 times) and disrupted. The suspension was centrifuged at 1000 g for 10 min. After this centrifugation, the pellet that contains membrane fraction (from endosomes, Golgi, plasma membrane, endoplasmic reticulum and secretory vesicles) was suspended in NP-40 free Buffer A, stood on ice for 10 min, and re-centrifuged at 1000 g for 10 min. The precipitate obtained was suspended again in Buffer A containing 1% (v/v) NP- 40, stood on ice for 60 min, and further centrifuged at 16,000 g for 20 min. The supernatant obtained was the cytosol fraction. All the procedure was performed in a cold room to limit the action of proteases and phospholipase. The purity of the preparation was assessed by evaluating the expression of Tubulin and NCX1 as cytosolic and membrane markers (Ren et al., 2010), respectively. All protein extracts were quantified by Bradford Protein Assay (Bradford Reagent, Biorad, #5000006) and separated on polyacrylamide gels (Precast Protein Gels 4%–20%, Biorad, #4561096). Proteins were transferred onto nitrocellulose membranes (Nitrocellulose Transfer Packs, Biorad, #1704158) using the Semi-Dry TransBlot System. Primary and secondary antibodies used are reported in the Reagents, Drugs and Antibodies paragraph. Analysis and quantification have been performed as already reported (Sanguigno et al., 2023).

### 7.6 qRT- PCR

Total RNA extraction from cells was performed using TRI Reagent (Sigma, cod: T9424), according to the vendor's di rections. Retrotranscription was performed on 2 μg of RNA, using the reagents contained in the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, cod: 4368814) following the manufacturer's directions and protocol (10 min at 25°C, 2 h at 42°C, 5 min at 85°C, ∞ 4°C). The obtained cDNA samples were amplified by RT-qPCR with SYBR Green Real-Time PCR Master Mix (Thermo Fisher, cod: 4309155) in triplicate

with a protocol previously described (Guida et al., 2017). PCR data were collected and analyzed by using ABI Prism 7000 SDS software (Applied Biosystems). The expression of the genes of our interest was normalized to the expression of the house-keeping gene encoding for Ribosomal Protein L19 for human genes and HPRT for rat genes (Guida et al., 2017). Changes in mRNA levels were shown as the average of relative quantification (RQ) values, obtained as the threshold cycle difference (ΔCt) between the target gene and the reference gene ($2^{-\Delta Ct} = RQ$) (Sanguigno et al., 2023). The primers used in this paper were the following: ACE2 human FW: 5′- AGA AAG CAG TCT GCC ATC CC- 3′; ACE2 human RV: 5′- GCT GTC AGG AAG TCG TCC AT- 3′; NRP1 human FW: 5′- TAG CTC CAA CGG GGA AGA CT- 3′; NRP1 human RV: 5′- TAG CTC CAA CGG GGA AGA CT- 3′; RIPK1 human FW: 5′- GGAGACTAGGTGGCAGGGTA- 3′; RIPK1 human RV: 5′- TCTGCGATCTCGGCTTTCAG- 3′.

### 7.7 Transient Chromatin Immunoprecipitation Assay

For the transient chromatin immunoprecipitation (ChIP) assay, cells at 60% confluence were co-transfected for 24 h with: (1) pGL3-RIPK1- promoter and (2) S1 Vector or EV. Antibodies used in the procedure include anti-HDAC4, anti-CREB, and polyclonal IgG as a negative control. Transfection conditions, ChIP assay procedures, and primer sequences have already been described by our group (Guida et al., 2017). Amplification and quantification were performed as previously reported (Guida et al., 2017). Results obtained from three different PCR experiments were normalized for the DNA input.

### 7.8 Luciferase Assay

The RIPK1 promoter (indicated as pGL3-RIPK1) was cloned in the pGL3basic vector as previously published (Guida et al., 2017). Specifically, to study the RIPK1 promoter activity, SH- SY5Y cells were co-transfected with 1.5 mg of total DNA vectors, including: (1) 800 ng of reporter constructs, that are pGL3-basic or pGL3-RIPK1; (2) 200 ng of the pRL-TK vector; (3) 500 ng of S1 Vector. For siRNAs transfection, 50 nM of siCTL or siHDAC4 were co-transfected with other plasmids. To overexpress CREB, 500 ng of CREB construct or empty vector were co-transfected. For the MG132 experimental group, cells were treated 24 h after transfection with MG132 for 3 h. All experimental groups were analyzed 48 h after transfection with the Dual-Luciferase Reporter Assay System Kit (Promega Italy, E1910). Luciferase activity was expressed as firefly-to-renilla ratio in arbitrary units.

### 7.9 MTT and LDH Assays

The 3- [4,5- dimethylthiazol- 2- yl]- 2,5 diphenyl tetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays were performed as previously described in SH-SY5Y (Sanguigno et al., 2023). For the MTT assay, culture medium was removed and cells were incubated in 500 μL of a 0.5 mg/mL MTT solution for 2 h at 37°C. Incubation was blocked by adding 500 μL of deacidified isopropanol to solubilize the formazan salt, and viability was read by measuring absorbance at 540 nm. The amount of LDH released into the extracellular medium was measured by using the kit from Cayman (cod: E-BC-K771-M) DBA (Milan, Italy) following the manufacturer's instructions. For LDH experiments, 1% Triton X100- treated cells were used as a positive control and its value was considered to be 100% cell death.

### 7.10 Confocal Immunofluorescence Analysis

Confocal immunofluorescence procedures in SH-SY5Y cultures were performed as previously described (Boscia et al., 2017, Cammarota et al., 2023). Cell cultures were fixed in 4% wt/vol paraformaldehyde in phosphate buffer for 30 min. After blocking with 3% BSA, cells were incubated with the primary antibodies for 24 h. The primary antibodies used were the following: rabbit polyclonal anti-C9 Tag (1:1000, MyBioSource, MBS430088); SARS-CoV2 Spike (1:500; #PA5-114451, Invitrogen); rabbit polyclonal anti-HDAC4 (1:200, sc-11418; Santa Cruz Biotechnology); goat polyclonal anti-RIPK1 (1:200, sc-41169, Santa Cruz Biotechnology); mouse monoclonal anti-CREB (1:400, 9194S, Cell Signaling); rabbit polyclonal anti-Neuropilin 1 (1:400, GXGTX12794, GeneTex). Then, cells were incubated with corresponding fluorescence-labeled secondary antibodies (Alexa488- or Alexa594- conjugated anti-mouse or anti-rabbit IgG). Hoechst-33342 (Merck, Millipore) was used to stain nuclei. Images were observed using a Zeiss LSM 700 laser (Carl Zeiss) scanning confocal microscope. Single images were taken with an optical thickness of 0.7 μm and a resolution of 1024 × 1024. All staining and morphological analyses were blindly conducted. Images were processed and analyzed with the public domain Java-based image processing software ImageJ (National Institutes of Health, Bethesda, Maryland, USA). Quantification of HDAC4 and RIPK1 fluorescence was quantified in terms of pixel intensity by using the NIH image software, as described previously (Cammarota et al., 2023). Briefly, digital images were taken with a 63× objective, and identical laser power settings and exposure times were applied to all the photographs from each experimental set. The number of cells displaying CREB immunoreactivity in the nucleus was determined by manual counting at ×40 magnification.

### 7.11 Statistical Analysis

Data were analyzed by Graph Pad Prism 5 software (Graph Pad Software Inc.). All bars in the figures represent the mean ± SD. Statistical differences between two experimental groups were analyzed with the Student's t-test. Statistically significant differences between more than two experimental groups were evaluated by one-way ANOVA, followed by Tukey's multiple comparison test.

## 8. Discussion and Conclusions

This study presents a comprehensive and mechanistically rich investigation into the neurotoxic effects of the SARS-CoV-2 Spike S1 subunit, revealing a novel intracellular signaling axis, NRP1/HDAC4/CREB/RIPK1, that plays a central role in mediating neuronal cell death. Through a multidisciplinary approach integrating molecular biology, pharmacological inhibition, gene silencing, and protein interaction assays, we delineate a pathway that not only advances our understanding of viral neurotoxicity but also reframes the broader discourse on virus-host interactions in the central nervous system.

The selective neurotoxicity of the S1 subunit, as opposed to S2, reinforces the notion that S1 possesses unique biological properties beyond its role in viral entry. Its ability to cross the blood-brain barrier (Rhea et al., 2021), accumulate in brain parenchyma, and interact with neuronal receptors positions it as a neuroactive fragment capable of initiating intracellular stress responses. This is supported by both human and animal studies: Nuovo et al. (2021a) demonstrated the presence of Spike protein in cortical neurons and endothelial cells of COVID-19 patients, and showed that injection of recombinant S1 protein in mice was sufficient to induce endothelial injury and neuroinflammation, even in the absence of viral replication (Nuovo et al., 2021b).

The observation that S1, but not S2, induces a time-dependent reduction in SH-SY5Y cell viability underscores its pathogenic potential and supports previous reports of S1-induced neuritic dystrophy and synaptic dysfunction (Datta et al., 2021; Fontes-Dantas et al., 2023). A central contribution of this study is the delineation of the NRP1/HDAC4/CREB/RIPK1 axis as a key mediator of S1-induced neuronal death. The identification of HDAC4 as a pivotal upstream regulator of RIPK1 transcription adds a new layer to our understanding of epigenetic control in neurodegeneration (Demyanenko, et al., 2021; Zhou, et al., 2024; Mielcarek, et al., 2015; Shukla, et al., 2020; Guida et al., 2014; Formisano et al., 2019; Wu et al., 2017). Our data show that HDAC4 does not directly bind the RIPK1 promoter but instead interacts with CREB, leading to its proteasomal degradation and subsequent derepression of RIPK1 transcription. This mechanism is supported by the rescue effect of MG132 and CREB overexpression, which restore CREB levels and suppress RIPK1 activation.

CREB is a master transcription factor involved in neuronal survival, synaptic plasticity, and memory formation (Lonze & Ginty, 2002; Feldmann et al., 2019; Kitagawa et al., 2007; Park et al., 2004). Its degradation may contribute to the cognitive deficits observed in COVID-19 patients, particularly those with long-term neurological symptoms (Ding & Zhao, 2023). Strategies aimed at

stabilizing CREB or enhancing its activity may offer neuroprotection in the context of viral protein exposure.

The role of NRP1 as the primary receptor mediating S1 internalization in neuronal cells is another key finding. While ACE2 has been widely studied as the canonical entry receptor for SARS-CoV-2 (Yan et al., 2020), its expression in neuronal tissues is limited, and our data confirm its negligible involvement in S1-induced neurotoxicity. In contrast, NRP1 is abundantly expressed in the CNS and has been shown to facilitate viral entry and neuroinvasion (Cantuti-Castelvetri et al., 2020). Our experiments demonstrate that NRP1 knockdown prevents S1-induced cell death, and that S1 internalization is blocked by sodium azide, confirming a NRP1-dependent endocytic mechanism. These findings are consistent with recent studies showing that NRP1 inhibitors protect against Spike protein-induced neurite shortening and synaptic loss (Mignolet et al., 2023).

The redistribution of NRP1 from the plasma membrane to the cytosol following S1 exposure, as observed in our fractionation and confocal microscopy experiments, suggests that endosomal trafficking may be a prerequisite for downstream signaling (Castellani et al., 2004). Cytosolic accumulation of S1 may facilitate its interaction with intracellular targets, including mitochondrial and nuclear components. S1-induced mitochondrial ROS production and α-synuclein aggregation (Chang et al., 2023), endolysosomal dysfunction (Datta et al., 2021), and TBK1-mediated inflammatory cascades (Ngo et al., 2025) all point to a disruption of cellular homeostasis and activation of neurodegenerative pathways. Similar mitochondrial vulnerabilities have been observed in other studies of viral protein trafficking (Wang F et al., 2024).

The specificity and robustness of the NRP1/HDAC4/CREB/RIPK1 axis are validated by pharmacological and genetic interventions. Silencing HDAC4 or inhibiting RIPK1 with Necrostatin-1 significantly attenuates S1-induced neurotoxicity (Degterev et al., 2005; Degterev et al., 2008), confirming the functional relevance of each component and suggesting actionable points for therapeutic intervention. HDAC4 inhibitors such as MC1568 have shown efficacy in models of environmental neurotoxicity (Guida et al., 2014; Chuang et al., 2009), while RIPK1 blockers are being explored for neuroinflammatory and neurodegenerative conditions (Yuan et al., 2019). NRP1 antagonists and CREB stabilizers represent promising candidates for neuroprotective strategies.

While systemic inflammation, hypoxia, and vascular complications have been widely implicated in COVID-19-related neurological symptoms (Ellul et al., 2020), our findings suggest that direct interactions between viral proteins and neuronal signaling pathways may play a more central role. The presence of S1 in cerebrospinal fluid and its ability to cross the blood-brain barrier support the notion that viral components, even without active replication, exert substantial neurobiological

effects.

This paradigm shift, from viewing neurotoxicity as a consequence of systemic factors to recognizing the direct impact of viral proteins, has profound implications. It invites further investigation into viral modulation of neuronal gene expression, epigenetic landscapes, and cell death pathways. Clinically, it suggests that blocking specific viral-host interactions may offer neuroprotective benefits even in the absence of antiviral activity.

The methodological rigor of this study, including the use of differentiated neuronal cell lines, targeted gene silencing, and quantitative assays, provides a robust framework for future investigations. Validation in primary human neurons, brain organoids, and in vivo models will be essential for assessing translational relevance. Exploring the temporal dynamics of S1-induced signaling and its contribution to cognitive deficits will be critical for understanding the full spectrum of SARS-CoV-2 neuropathology.

In a broader biomedical context, these insights may be applicable to other neurotropic viruses that exploit similar entry pathways or trigger comparable epigenetic disruptions. Understanding how viral proteins interface with host transcriptional machinery could inform pan-viral neuroprotective strategies and enhance preparedness for future neuroinvasive outbreaks.

Finally, the societal relevance of this research is significant. As the global population continues to grapple with long COVID-related neurological and cognitive impairments (Taquet et al., 2021), the need for mechanistic clarity and targeted interventions becomes increasingly urgent. By elucidating the molecular basis of S1-induced neuronal damage, this study contributes to a growing body of knowledge that may inform public health strategies, clinical diagnostics, and therapeutic development in the post-pandemic era.

In conclusion, this work delineates a novel and clinically relevant molecular framework through which the SARS-CoV-2 S1 subunit compromises neuronal integrity. By identifying and characterizing the NRP1/HDAC4/CREB/RIPK1 axis, we not only deepen our understanding of COVID-19-associated neurological complications but also contribute to the broader field of neurovirology. These insights lay the groundwork for future therapeutic strategies aimed at preserving neuronal health and offer a new lens through which to view the complex interplay between viral proteins and host cellular responses. Ultimately, this study affirms the importance of dissecting virus-host interactions at the molecular level and highlights the potential of targeted interventions to mitigate the long-term neurological consequences of viral exposure. As the scientific community continues to confront the multifaceted impacts of SARS-CoV-2, studies such

as this one underscore the necessity of integrating molecular insights with clinical observations to develop comprehensive strategies for diagnosis, prevention, and treatment. The implications of this work extend beyond the immediate context of COVID-19, offering a conceptual and methodological blueprint for addressing the neurological effects of future viral threats and advancing the frontier of neuroinfectious disease research.

## 9. List of Abbreviation

- ACE2 – Angiotensin-Converting Enzyme 2
- AD – Alzheimer's Disease
- ADEM – Acute Disseminated Encephalomyelitis
- ARDS – Acute Respiratory Distress Syndrome
- ATF6 – Activating Transcription Factor 6
- BBB – Blood-Brain Barrier
- Calp – Calpeptin
- CBT – Cognitive Behavioral Therapy
- ChIP – Chromatin Immunoprecipitation
- CNS – Central Nervous System
- COVID-19 – Coronavirus Disease 2019
- CREB – cAMP Response Element-Binding Protein
- CRP – C-Reactive Protein
- DAMPs – Damage-Associated Molecular Patterns
- DEHP – Di(2-Ethylhexyl)phthalate
- DMSO – Dimethyl Sulfoxide
- DOACs – Direct Oral Anticoagulants
- ER – Endoplasmic Reticulum
- ERGIC – Endoplasmic Reticulum-Golgi Intermediate Compartment
- EV – Empty Vector
- GFAP – Glial Fibrillary Acidic Protein
- GBS – Guillain-Barré Syndrome
- HDAC – Histone Deacetylase
- HDAC4 – Histone Deacetylase 4

- HDAC9 – Histone Deacetylase 9
- HIF-1α – Hypoxia-Inducible Factor 1-alpha
- ICAM-1 – Intercellular Adhesion Molecule 1
- IL-1B – Interleukin 1 Beta
- IL-6 – Interleukin 6
- IL-16 – Interleukin 16
- iPSC – Induced Pluripotent Stem Cells
- ISGF3 – Interferon-Stimulated Gene Factor 3
- IVIG – Intravenous Immunoglobulin
- LDH – Lactate Dehydrogenase
- MAVS – Mitochondrial Antiviral Signaling
- MIS-C – Multisystem Inflammatory Syndrome in Children
- MLKL – Mixed Lineage Kinase Domain-Like Protein
- MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
- NAC – N-Acetylcysteine
- NfL – Neurofilament Light Chain
- NPIs – Non-Pharmaceutical Interventions
- NRP1 – Neuropilin-1
- NTD – N-Terminal Domain
- ORF – Open Reading Frame
- PASC – Post-Acute Sequelae of SARS-CoV-2 Infection
- PBS – Phosphate-Buffered Saline
- PLEX – Plasma Exchange
- PRRs – Pattern Recognition Receptors
- qRT-PCR – Quantitative Reverse Transcription Polymerase Chain Reaction

- RA – Retinoic Acid
- RAS – Renin-Angiotensin System
- RBD – Receptor-Binding Domain
- REST – RE1-Silencing Transcription Factor
- RIPK1 – Receptor-Interacting Protein Kinase 1
- RIPK3 – Receptor-Interacting Protein Kinase 3
- RNA – Ribonucleic Acid
- RdRp – RNA-Dependent RNA Polymerase
- Ro – Basic Reproduction Number
- ROS – Reactive Oxygen Species
- RTC – Replication-Transcription Complex
- S1/S2 – Spike Subunit 1 / Subunit 2
- S1rp – Recombinant Spike S1 Protein
- SH-SY5Y – Human Neuroblastoma Cell Line SH-SY5Y
- siCTL – Control Small Interfering RNA
- siRNA – Small Interfering RNA
- Sp1/Sp3 – Specificity Protein 1 / 3
- SSRIs – Selective Serotonin Reuptake Inhibitors
- TBK1 – TANK-Binding Kinase 1
- TfR1 – Transferrin Receptor 1
- TLRs – Toll-Like Receptors
- TMPRSS2 – Transmembrane Protease Serine 2
- TNF-α – Tumor Necrosis Factor Alpha
- TRSs – Transcription Regulatory Sequences
- TMS – Transcranial Magnetic Stimulation

- tDCS – Transcranial Direct Current Stimulation
- VCAM-1 – Vascular Cell Adhesion Molecule 1
- VOCs – Variants of Concern
- ZVAD – z-VAD-fmk (Pan-Caspase Inhibitor)

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## 11. References


- Baker, R. E., Park, S. W., Yang, W., Vecchi, G. A., Metcalf, C. J. E., & Grenfell, B. T. (2020). *The impact of COVID-19 nonpharmaceutical interventions on the future dynamics of endemic infections.* Proceedings of the National Academy of Sciences of the United States of America, 117(48), 30547–30553. DOI: 10.1073/pnas.2013182117
- Barroso da Silva, F. L., Paco, K., Laaksonen, A., & Ray, A. (2025). *Biophysics of SARS-CoV-2 spike protein's receptor-binding domain interaction with ACE2 and neutralizing antibodies.* Biophysical Reviews, 17, 309–333. DOI: 10.1007/s12551-025-01276-z
- Beigel, J. H., Tomashek, K. M., Dodd, L. E., Mehta, A. K., Zingman, B. S., Kalil, A. C., Hohmann, E., Chu, H. Y., Luetkemeyer, A., Kline, S., Lopez de Castilla, D., Finberg, R. W., Dierberg, K., Tapson, V., Hsieh, L., Patterson, T. F., Paredes, R., Sweeney, D. A., Short, W. R., Touloumi, G., Lye, D. C., Ohmagari, N., Oh, M. D., Ruiz-Palacios, G. M., Benfield, T., Fätkenheuer, G., Kortepeter, M. G., Atmar, R. L., Creech, C. B., Lundgren, J., Babiker, A. G., Pett, S., Neaton, J. D., Burgess, T. H., Bonnett, T., Green, M., Makowski, M., Osinusi, A., Nayak, S., & Lane, H. C. (2020). *Remdesivir for the treatment of COVID-19 — Final report.* New England Journal of Medicine, 383, 1813–1826. DOI: 10.1056/NEJMoa2007764
- Blanco-Melo, D., Nilsson-Payant, B. E., Liu, W. C., Uhl, S., Hoagland, D., Møller, R., Jordan, T. X., Oishi, K., Panis, M., Sachs, D., Wang, T. T., Schwartz, R. E., Lim, J. K., Albrecht, R. A., & tenOever, B. R. (2020). *Imbalanced host response to SARS-CoV-2 drives development of COVID-19.* Cell, 181(5), 1036–1045.e9. DOI: 10.1016/j.cell.2020.04.026
- Boscia, F., Pannaccione, A., Ciccone, R., Casamassa, A., Franco, C., Piccialli, I., Sannino, S., Secondo, A., Scorziello, A., Adornetto, A., Taglialatela, M., Di Renzo, G., & Annunziato, L. (2017). *The expression and activity of K(V)3.4 channel subunits are precociously upregulated in astrocytes exposed to Aβ oligomers and in astrocytes of Alzheimer's disease Tg2576 mice*. Neurobiology of Aging, *54*, 187–198. https://doi.org/10.1016/j.neurobiolaging.2017.03.008
- Brown, R. L., Benjamin, L., Lunn, M. P., Bharucha, T., Zandi, M. S., Hoskote, C., McNamara, P., & Manji, H. (2023). *Pathophysiology, diagnosis, and management of neuroinflammation in COVID-19.* BMJ, 382, e073923. DOI: 10.1136/bmj-2022-073923
- Buzhdygan, T. P., DeOre, B. J., Baldwin-Leclair, A., Bullock, T. A., McGary, H. M., Khan, J. A., Razmpour, R., Hale, J. F., Galie, P. A., Potula, R., Andrews, A. M., & Ramirez, S. H.

(2020). *The SARS-CoV-2 spike protein alters barrier function in 2D static and 3D microfluidic in-vitro models of the human blood–brain barrier.* Neurobiology of Disease, 146, 105131. DOI: 10.1016/j.nbd.2020.105131

- Cagnin, A., Di Lorenzo, R., Marra, C., Bonanni, L., Cupidi, C., Laganà, V., Rubino, E., Vacca, A., Provero, P., Isella, V., Vanacore, N., Agosta, F., Appollonio, I., Caffarra, P., Pettenuzzo, I., Sambati, R., Quaranta, D., Guglielmi, V., Logroscino, G., Filippi, M., Tedeschi, G., Ferrarese, C., Rainero, I., & Bruni, A. C. (2020). *Behavioral and psychological effects of coronavirus disease-19 quarantine in patients with dementia.* Frontiers in Psychiatry, 11, 578015. DOI: 10.3389/fpsyt.2020.578015
- Cai, Y., Hu, M., & Dai, R. (2023). *Safety and efficacy of mesenchymal stem cells in COVID-19 patients: A systematic review and meta-analysis.* Immunity, Inflammation and Disease, 11(9), e1000. DOI: 10.1002/iid3.1000
- Cai, Z., Ni, W., Li, W., Wu, Z., Yao, X., Zheng, Y., Zhao, Y., Yuan, W., Liang, S., Wang, Q., Tang, M., Chen, Y., Lan, K., Zhou, L., & Xu, K. (2024). *SARS-CoV-2 S protein disrupts the formation of ISGF3 complex through conserved S2 subunit to antagonize type I interferon response.* Journal of Virology, 99(1), e01516-24. DOI: 10.1128/jvi.01516-24
- Cammarota, M., Ferlenghi, F., Vacondio, F., Giacovazzo, G., De Filippis, B., Giacovazzo, G., Bertini, S., Bertini, R., Mor, M., & Pittaluga, A. (2023). *Combined targeting of fatty acid amide hydrolase and melatonin receptors promotes neuroprotection and stimulates inflammation resolution in rats*. British Journal of Pharmacology, *180*, 1316–1338. https://doi.org/10.1111/bph.16014
- Cantuti-Castelvetri, L., Ojha, R., Pedro, L. D., Djannatian, M., Franz, J., Kuivanen, S., van der Meer, F., Kallio, K., Kaya, T., Anastasina, M., Smura, T., Levanov, L., Szirovicza, L., Tobi, A., Kallio-Kokko, H., Österlund, P., Joensuu, M., Meunier, F. A., Butcher, S. J., Winkler, M. S., Mollenhauer, B., Helenius, A., Gokce, O., Teesalu, T., Hepojoki, J., Vapalahti, O., Stadelmann, C., Balistreri, G., & Simons, M. (2020). *Neuropilin-1 facilitates SARS-CoV-2 cell entry and infectivity.* Science, 370(6518), 856–860. https://doi.org/10.1126/science.abd2985
- Cao, Y., Wang, J., Jian, F., Xiao, T., Song, W., Yisimayi, A., Huang, W., Li, Q., Wang, P., An, R., Wang, J., Wang, Y., Niu, X., Yang, S., Liang, H., Sun, H., Li, T., Yu, Y., Cui, Q., Liu, S., Yang, X., Du, S., Zhang, Z., Hao, X., Shao, F., Jin, R., Wang, X., Xiao, J., Wang, Y., & Xie, X. S. (2022). *Omicron escapes the majority of existing SARS-CoV-2 neutralizing antibodies.*

Nature, 602(7898), 657–663. DOI: 10.1038/s41586-021-04385-3

- Carlson, E. C., Macsai, M., Bertrand, S., Bertrand, D., & Nau, J. (2023). *The SARS-CoV-2 virus and the cholinergic system: Spike protein interaction with human nicotinic acetylcholine receptors and the nicotinic agonist varenicline.* International Journal of Molecular Sciences, 24(6), 5597. DOI: 10.3390/ijms24065597

- Carneiro, V. C. S., Alves-Leon, S. V., Sarmento, D. J. S., Coelho, W. L. C. N. P., Moreira, O. C., Salvio, A. L., Ramos, C. H. F., Ramos Filho, C. H. F., Marques, C. A. B., Gonçalves, J. P. C., Leon, L. A. A., & de Paula, V. S. (2022). *Herpesvirus and neurological manifestations in patients with severe coronavirus disease*. Virology Journal, *19*, Article 101. https://doi.org/10.1186/s12985-022-01828-9

- Castellani, V., De Angelis, E., Kenwrick, S., & Rougon, G. (2004). *Clathrin-mediated endocytosis of neuropilin-1 modulates axonal guidance.* Nature Cell Biology, 6(5), 381–388. DOI: 10.1038/ncb1129

- Chang, M. H., Park, J. H., Lee, H. K., Choi, J. Y., & Koh, Y. H. (2023). *SARS-CoV-2 Spike Protein 1 Causes Aggregation of α-Synuclein via Microglia-Induced Inflammation and Production of Mitochondrial ROS: Potential Therapeutic Applications of Metformin.* Biomedicines, 12(6), 1223. DOI: 10.3390/biomedicines12061223

- Chuang, D.-M., Leng, Y., Marinova, Z., Kim, H.-J., & Chiu, C.-T. (2009). *Multiple roles of HDAC inhibition in neurodegenerative conditions.* Trends in Neurosciences, 32(11), 591–601. DOI: 10.1016/j.tins.2009.06.002

- Chung, Y. S., Lam, C. Y., Tan, P. H., Tsang, H. F., & Wong, S. C. C. (2024). *Comprehensive Review of COVID-19: Epidemiology, Pathogenesis, Advancement in Diagnostic and Detection Techniques, and Post-Pandemic Treatment Strategies.* International Journal of Molecular Sciences, 25(15), 8155. DOI: 10.3390/ijms25158155

- Datta, G., Miller, N. M., Halcrow, P. W., Khan, N., Colwell, T., Geiger, J. D., & Chen, X. (2021). *SARS-CoV-2 S1 Protein Induces Endolysosome Dysfunction and Neuritic Dystrophy.* Frontiers in Cellular Neuroscience, 15, 777738. DOI: 10.3389/fncel.2021.777738

- Davis, H. E., Assaf, G. S., McCorkell, L., Wei, H., Low, R. J., Re'em, Y., Redfield, S., Austin, J. P., & Akrami, A. (2021). *Characterizing Long COVID in an International Cohort: 7 Months of Symptoms and Their Impact.* EClinicalMedicine, 38, 101019. DOI: 10.1016/j.eclinm.2021.101019

- de Melo, G. D., Lazarini, F., Levallois, S., Hautefort, C., Michel, V., Larrous, F., Verillaud, B., Aparicio, C., Wagner, S., Gheusi, G., Kergoat, L., Kornobis, E., Donati, F., Cokelaer, T., Hervochon, R., Madec, Y., Roze, E., Salmon, D., Bourhy, H., Lecuit, M., & Lledo, P. M. (2021). *COVID-19-related anosmia is associated with viral persistence and inflammation in human olfactory epithelium and brain infection in hamsters.* Science Translational Medicine, 13(596), eabf8396. DOI: 10.1126/scitranslmed.abf8396
- Degterev, A., Huang, Z., Boyce, M., Li, Y., Jagtap, P., Mizushima, N., Cuny, G. D., Mitchison, T. J., Moskowitz, M. A., & Yuan, J. (2005). *Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury.* Nature Chemical Biology, 1(2), 112–119. DOI: 10.1038/nchembio711
- Degterev, A., Hitomi, J., Germscheid, M., Ch'en, I. L., Korkina, O., Teng, X., Abbott, D., Cuny, G. D., Yuan, C., Wagner, G., Hedrick, S. M., Gerber, S. A., Lugovskoy, A., & Yuan, J. (2008). Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nature Chemical Biology*, 4(5), 313–321. DOI: 10.1038/nchembio.83
- Demyanenko, S., Dzreyan, V., & Sharifulina, S. (2021). *Histone deacetylases and their isoform-specific inhibitors in ischemic stroke*. Biomedicines, *9*(10), 1445. https://doi.org/10.3390/biomedicines9101445
- Ding, H., & Zhao, Y. (2023). *Long COVID and neurological symptoms: emerging mechanisms and clinical implications.* Nature Reviews Neurology, 19(2), 85–97. DOI: 10.1038/s41582-022-00791-2
- Douaud, G., Lee, S., Alfaro-Almagro, F., Arthofer, C., Wang, C., McCarthy, P., Lange, F., Andersson, J. L. R., Griffanti, L., Duff, E., Jbabdi, S., Taschler, B., Keating, P., Winkler, A. M., Collins, R., Matthews, P. M., Allen, N., Miller, K. L., Nichols, T. E., & Smith, S. M. (2022). *SARS-CoV-2 is associated with changes in brain structure in UK Biobank.* Nature, 604(7907), 697–707. DOI: 10.1038/s41586-022-04569-5
- Ehsanipur, F., Vafaee-Shahi, M., Tahernia, L., & Riahi, A. (2023). *HSV-2 induced cerebellitis: A case report of HSV-2 reactivation by SARS-CoV-2 infection*. The Open Neurology Journal, *17*, e1874205X259383. https://doi.org/10.2174/011874205X259383231031051625
- Ellul, M. A., Benjamin, L., Singh, B., Lant, S., Michael, B. D., Easton, A., Kneen, R., Defres, S., Sejvar, J., & Solomon, T. (2020). *Neurological associations of COVID-19.* The Lancet Neurology, 19(9), 767–783. DOI: 10.1016/S1474-4422(20)30221-0

- Feldmann, K. G., Chowdhury, A., Becker, J. L., McAlpin, N., Ahmed, T., Haider, S., Xia, J. X. R., Diaz, K., Mehta, M. G., & Mano, I. (2019). *Non-canonical activation of CREB mediates neuroprotection in a Caenorhabditis elegans model of excitotoxic necrosis*. Journal of Neurochemistry, *148*(4), 531–549. https://doi.org/10.1111/jnc.14629
- Fontes-Dantas, F. L., Fernandes, G. G., Gutman, E. G., De Lima, E. V., Antonio, L. S., Hammerle, M. B., Mota-Araujo, H. P., Colodeti, L. C., Araújo, S. M. B., Froz, G. M., da Silva, T. N., Duarte, L. A., Salvio, A. L., Pires, K. L., Leon, L. A. A., Vasconcelos, C. C. F., Romão, L., Savio, L. E. B., Silva, J. L., da Costa, R., Clarke, J. R., Da Poian, A. T., Alves-Leon, S. V., Passos, G. F., & Figueiredo, C. P. (2023). *SARS-CoV-2 Spike protein induces TLR4-mediated long-term cognitive dysfunction recapitulating post-COVID-19 syndrome in mice.* Cell Reports, 43(4), 112100. DOI: 10.1016/j.celrep.2023.112100
- Formisano, L., Guida, N., Laudati, G., Mascolo, L., Di Renzo, G., & Canzoniero, L. M. (2015). *MS-275 inhibits Aroclor 1254-induced SH-SY5Y neuronal cell toxicity by preventing the formation of the HDAC3/REST complex on the Synapsin-1 promoter.* Journal of Pharmacology and Experimental Therapeutics, *352*, 236–243. https://doi.org/10.1124/jpet.114.219345
- Formisano, L., Laudati, G., Guida, N., Mascolo, L., Serani, A., Cuomo, O., Cantile, M., Boscia, F., Molinaro, P., Anzilotti, S., Pizzorusso, V., Di Renzo, G., Pignataro, G., & Annunziato, L. (2019). *HDAC4 and HDAC5 form a complex with DREAM that epigenetically down-regulates NCX3 gene and its pharmacological inhibition reduces neuronal stroke damage*. Journal of Cerebral Blood Flow & Metabolism, *40*(10), 2081–2097. https://doi.org/10.1177/0271678X19884742
- Garrido-Mesa, N., Zarzuelo, A., & Gálvez, J. (2013). *Minocycline: far beyond an antibiotic*. British Journal of Pharmacology, *169*(2), 337–352. https://doi.org/10.1111/bph.12139
- Goel, R. R., Painter, M. M., Apostolidis, S. A., Mathew, D., Meng, W., Rosenfeld, A. M., Lundgreen, K. A., Reynaldi, A., Khoury, D. S., Pattekar, A., Gouma, S., Kuri-Cervantes, L., Hicks, P., Dysinger, S., Hicks, A., Sharma, H., Herring, S., Korte, S., Baxter, A. E., Oldridge, D. A., Giles, J. R., Weirick, M. E., McAllister, C. M., Awofolaju, M., Tanenbaum, N., Drapeau, E. M., Dougherty, J., Long, S., D'Andrea, K., Hamilton, J. T., McLaughlin, M., Williams, J. C., Adamski, S., Kuthuru, O., Frank, I., Betts, M. R., Vella, L. A., Grifoni, A., Weiskopf, D., Sette, A., Hensley, S. E., Davenport, M. P., Bates, P., Luning Prak, E. T., Greenplate, A. R., & Wherry, E. J. (2021). *mRNA vaccines induce durable immune memory to SARS-CoV-2 and variants of concern*. Science, *374*(6572), abm0829.

https://doi.org/10.1126/science.abm0829

- Graham, E. L., Koralnik, I. J., & Liotta, E. M. (2022). *Therapeutic approaches to the neurologic manifestations of COVID-19*. Neurotherapeutics, *19*(5), 1435–1466. https://doi.org/10.1007/s13311-022-01267-y
- Guadarrama-Ortiz, P., Choreño-Parra, J. A., Sánchez-Martínez, C. M., Pacheco-Sánchez, F. J., Rodríguez-Nava, A. I., & García-Quintero, G. (2020). *Neurological aspects of SARS-CoV-2 infection: mechanisms and manifestations*. Frontiers in Neurology, *11*, Article 1039. https://doi.org/10.3389/fneur.2020.01039
- Guan, W., Ni, Z., Hu, Y., Liang, W., Ou, C., He, J., Liu, L., et al. (2020). *Clinical characteristics of coronavirus disease 2019 in China*. New England Journal of Medicine, *382*(18), 1708–1720. https://doi.org/10.1056/NEJMoa2002032
- Guida, N., Laudati, G., Galgani, M., Santopaolo, M., Montuori, P., Triassi, M., Di Renzo, G., Canzoniero, L. M. T., & Formisano, L. (2014). *Histone deacetylase 4 promotes ubiquitin-dependent proteasomal degradation of Sp3 in SH-SY5Y cells treated with di(2-ethylhexyl)phthalate (DEHP), determining neuronal death*. Toxicology and Applied Pharmacology, *280*(1), 190–198. https://doi.org/10.1016/j.taap.2014.07.014
- Guida, N., Laudati, G., Serani, A., Mascolo, L., Molinaro, P., Montuori, P., Di Renzo, G., Canzoniero, L. M. T., & Formisano, L. (2017). *The neurotoxicant PCB-95 by increasing the neuronal transcriptional repressor REST down-regulates caspase-8 and increases RIPK1, RIPK3 and MLKL expression determining necroptotic neuronal death*. Biochemical Pharmacology, *142*, 229–241. https://doi.org/10.1016/j.bcp.2017.06.135
- Haddad, M., Sheybani, F., Olfati, N., Nahayati, M. A., Boostani, R., Layegh, P., & Rashid-Nejad, A. (2023). *Central nervous system reactivation of herpesviridae family in patients with COVID-19*. Journal of NeuroVirology, *29*, 211–217. https://doi.org/10.1007/s13365-023-01132-4
- Harvey, W. T., Carabelli, A. M., Jackson, B., Gupta, R. K., Thomson, E. C., Harrison, E. M., Ludden, C., Reeve, R., Rambaut, A., Peacock, S. J., & Robertson, D. L. (2021). *SARS-CoV-2 variants, spike mutations and immune escape*. Nature Reviews Microbiology, *19*, 409–424. https://doi.org/10.1038/s41579-021-00573-0
- Hazan, A., Saperi, A. A., Zulkifli, N., Mat Rahim, N.-A., Tiong, V., Lee, H. Y., & AbuBakar, S. (2025). *Toxicity study of SARS-CoV-2 spike protein expressed on Bacillus subtilis spores*. BMC Microbiology, *25*, Article 633. https://doi.org/10.1186/s12866-025-04186-y

- Helms, J., Kremer, S., Merdji, H., Clere-Jehl, R., Schenck, M., Kummerlen, C., Collange, O., Boulay, C., Fafi-Kremer, S., Ohana, M., Anheim, M., & Meziani, F. (2020). *Neurologic features in severe SARS-CoV-2 infection*. New England Journal of Medicine, *382*(23), 2268–2270. https://doi.org/10.1056/NEJMc2008597

- Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., Schiergens, T. S., Herrler, G., Wu, N.-H., Nitsche, A., Müller, M. A., Drosten, C., & Pöhlmann, S. (2020). *SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor.* Cell, *181*(2), 271–280.e8. https://doi.org/10.1016/j.cell.2020.02.052

- Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., Zhang, L., Fan, G., Xu, J., Gu, X., Cheng, Z., Yu, T., Xia, J., Wei, Y., Wu, W., Xie, X., Yin, W., Li, H., Liu, M., Xiao, Y., Gao, H., Guo, L., Xie, J., Wang, G., Jiang, R., Gao, Z., Jin, Q., Wang, J., & Cao, B. (2020). *Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China*. Lancet, *395*(10223), 497–506. https://doi.org/10.1016/S0140-6736(20)30183-5

- Huang, Y., Yang, C., Xu, X.-F., Xu, W., & Liu, S.-W. (2020). *Structural and functional properties of SARS-CoV-2 spike protein: potential antivirus drug development for COVID-19*. Acta Pharmacologica Sinica, *41*(9), 1141–1149. https://doi.org/10.1038/s41401-020-0485-4

- Kanberg, N., Ashton, N. J., Andersson, L.-M., Yilmaz, A., Lindh, M., Nilsson, S., Price, R. W., Blennow, K., Zetterberg, H., & Gisslén, M. (2020). *Neurochemical evidence of astrocytic and neuronal injury commonly found in COVID-19*. Neurology, *95*(12), e1754–e1759. https://doi.org/10.1212/WNL.0000000000010111

- Kim, E. S., Jeon, M.-T., Kim, K.-S., Lee, S., Kim, S., & Kim, D.-G. (2021). *Spike proteins of SARS-CoV-2 induce pathological changes in molecular delivery and metabolic function in the brain endothelial cells*. Viruses, *13*(10), 2021. https://doi.org/10.3390/v13102021

- Kitagawa, K. (2007). *CREB and cAMP response element-mediated gene expression in the ischemic brain*. FEBS Journal, *274*(13), 3210–3217. https://doi.org/10.1111/j.1742-4658.2007.05890.x

- Kovalevich, J., & Langford, D. (2013). *Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology*. In Methods in Molecular Biology, *1078*, 9–21. https://doi.org/10.1007/978-1-62703-640-5_2

- Lashgari, N.-A., Shayan, M., Momeni Roudsari, N., Khayatan, D., Momtaz, S., Abdolghaffari, A. H., Sathyapalan, T., & Sahebkar, A. (2023). *Biology of SARS-CoV-2 coronavirus; origin, structure, and variants*. In M. Banach (Ed.), Cardiovascular Complications of COVID-19 (pp. 3–18). Contemporary Cardiology (SpringerNature). Humana, Cham. https://doi.org/10.1007/978-3-031-15478-2_1
- Lechien, J. R., Chiesa-Estomba, C. M., De Siati, D. R., Horoi, M., Le Bon, S. D., Rodriguez, A., Dequanter, D., Blecic, S., El Afia, F., Distinguin, L., Chekkoury-Idrissi, Y., Hans, S., Lopez Delgado, I., Calvo-Henriquez, C., Lavigne, P., Falanga, C., Barillari, M. R., Cammaroto, G., Khalife, M., Leich, P., Souchay, C., Rossi, C., Journe, F., Hsieh, J., Edjlali, M., Carlier, R., Ris, L., Lovato, A., De Filippis, C., Coppee, F., Fakhry, N., Ayad, T., & Saussez, S. (2020). *Olfactory and gustatory dysfunctions as a clinical presentation of mild-to-moderate forms of the coronavirus disease (COVID-19): a multicenter European study*. European Archives of Oto-Rhino-Laryngology (SpringerNature), *277*(8), 2251–2261. https://doi.org/10.1007/s00405-020-05965-1
- Lei, Y., Zhang, J., Schiavon, C. R., He, M., Chen, L., Shen, H., Zhang, Y., Yin, Q., Cho, Y., Andrade, L., Shadel, G. S., Hepokoski, M., Lei, T., Wang, H., Zhang, J., Yuan, J. X., Malhotra, A., Manor, U., Wang, S., Yuan, Z. Y., & Shyy, J. Y. (2021). *SARS-CoV-2 Spike Protein Impairs Endothelial Function via Downregulation of ACE2*. Circulation Research, *128*(9), 1323–1326. https://doi.org/10.1161/CIRCRESAHA.121.318902
- Li, J., He, S., Yang, H., Zhang, L., Xiao, J., Liang, C., & Liu, S. (2024). *The main mechanisms of mesenchymal stem cell-based treatments against COVID-19*. Tissue Engineering and Regenerative Medicine, *21*(4), 545–556. https://doi.org/10.1007/s13770-024-00633-5
- Li, J., Peng, W., Zhang, Y., Liu, S., Han, M., Song, R., Zhang, Y., Jin, R., & Wang, X. (2024). *A comparative study of clinical characteristics and COVID-19 vaccine effectiveness against SARS-CoV-2 variants: Wild-Type, Alpha, Delta, and Omicron in Beijing, China*. Infection and Drug Resistance, *17*, 5147–5161. https://doi.org/10.2147/IDR.S483098
- Li, X., Mi, Z., Liu, Z., & Rong, P. (2024). *SARS-CoV-2: Pathogenesis, therapeutics, variants, and vaccines*. Frontiers in Microbiology, *15*, Article 1334152. https://doi.org/10.3389/fmicb.2024.1334152
- Liu, Y., & Rocklöv, J. (2021). *The reproductive number of the Delta variant of SARS-CoV-2 is far higher compared to the ancestral SARS-CoV-2 virus*. Journal of Travel Medicine,

*28*(7), taab124. https://doi.org/10.1093/jtm/taab124

- Liu, Z., Xiao, X., Wei, X., Li, J., Yang, J., Tan, H., Zhu, J., Zhang, Q., Wu, J., & Liu, L. (2020). *Composition and divergence of coronavirus spike proteins and host ACE2 receptors predict potential intermediate hosts of SARS-CoV-2*. Journal of Medical Virology, *92*(6), 595–601. https://doi.org/10.1002/jmv.25726
- Lok, L. S. C., Sarkar, S., Lam, C. C. I., Law, C. F., Chau, S. T., Leung, C. Y. T., Cheang, W. H., Li, T., Monteiro, O., & Baptista-Hon, D. T. (2024). *Long COVID across SARS-CoV-2 variants: Clinical features, pathogenesis, and future directions*. MedComm – Future Medicine, *3*(4), Article e70004. https://doi.org/10.1002/mef2.70004
- Lonze, B. E., & Ginty, D. D. (2002). *Function and regulation of CREB family transcription factors in the nervous system*. Neuron, *35*(4), 605–623. https://doi.org/10.1016/S0896-6273(02)00828-0
- Lu, W., Yan, L., Tang, X., Wang, X., Du, J., Zou, Z., Li, L., Ye, J., & Zhou, L. (2024). *Efficacy and safety of mesenchymal stem cells therapy in COVID-19 patients: A systematic review and meta-analysis of randomized controlled trials*. Journal of Translational Medicine, *22*, 550. https://doi.org/10.1186/s12967-024-05358-6
- Mallick, R., & Duttaroy, A. K. (2021). *Origin and structural biology of novel coronavirus (SARS-CoV-2)*. Advances in Experimental Medicine and Biology, *1352*, 1–13. https://doi.org/10.1007/978-3-030-85109-5_1
- Mamedov, T., Yuksel, D., Gurbuzaslan, I., Gulec, B., Mammadova, G., Ozdarendeli, A., Pavel, S. T. I., Yetiskin, H., Kaplan, B., Uygut, M. A., & Hasanova, G. (2023). *SARS-CoV-2 spike protein S1 subunit induces potent neutralizing responses in mice and is effective against Delta and Omicron variants*. Frontiers in Plant Science, *14*, Article 1290042. https://doi.org/10.3389/fpls.2023.1290042
- Matschke, J., Lütgehetmann, M., Hagel, C., Sperhake, J. P., Schröder, A. S., Edler, C., Mushumba, H., Fitzek, A., Allweiss, L., Dandri, M., Dottermusch, M., Heinemann, A., Pfefferle, S., Schwabenland, M., Magruder, D. S., Bonn, S., Prinz, M., Gerloff, C., Püschel, K., Krasemann, S., Aepfelbacher, M., & Glatzel, M. (2020). *Neuropathology of patients with COVID-19 in Germany: a post-mortem case series*. The Lancet Neurology, *19*(11), 919–929. https://doi.org/10.1016/S1474-4422(20)30308-2
- McCallum, M., Czudnochowski, N., Rosen, L. E., Zepeda, S. K., Bowen, J. E., Walls, A. C.,

Hauser, K., Joshi, A., Stewart, C., Dillen, J. R., Powell, A. E., Croll, T. I., Nix, J., Virgin, H. W., Corti, D., Snell, G., & Veesler, D. (2022). *Structural basis of SARS-CoV-2 Omicron immune evasion and receptor engagement*. Science, *375*(6583), 864–868. https://doi.org/10.1126/science.abn8652

- Meinhardt, J., Radke, J., Dittmayer, C., Franz, J., Thomas, C., Mothes, R., Laue, M., Schneider, J., Brünink, S., Greuel, S., Lehmann, M., Hassan, O., Aschman, T., Schumann, E., Chua, R. L., Conrad, C., Eils, R., Stenzel, W., Windgassen, M., Rößler, L., Goebel, H.-H., Gelderblom, H. R., Martin, H., Nitsche, A., & Heppner, F. L. (2021). *Olfactory transmucosal SARS-CoV-2 invasion as a port of central nervous system entry in individuals with COVID-19*. Nature Neuroscience, *24*(2), 168–175. https://doi.org/10.1038/s41593-020-00758-5
- Menezes, F., Palmeira, J. F., Oliveira, J. S., Argañaraz, G. A., Soares, C. R. J., Nóbrega, O. T., Ribeiro, B. M., & Argañaraz, E. R. (2024). *Unraveling the SARS-CoV-2 spike protein long-term effect on neuro-PASC*. Frontiers in Cellular Neuroscience, *18*, Article 1481963. https://doi.org/10.3389/fncel.2024.1481963
- Merkler, A. E., Parikh, N. S., Mir, S., Gupta, A., Kamel, H., Lin, E., Lantos, J., Schenck, E. J., Goyal, P., Bruce, S. S., Kahan, J., Lansdale, K. N., LeMoss, N. M., Murthy, S. B., Stieg, P. E., Fink, M. E., Iadecola, C., Segal, A. Z., Cusick, M., Campion, T. R. Jr., Diaz, I., Zhang, C., & Navi, B. B. (2020). *Risk of ischemic stroke in patients with coronavirus disease 2019 (COVID-19) vs patients with influenza*. JAMA Neurology, *77*(11), 1366–1372. https://doi.org/10.1001/jamaneurol.2020.2730
- Mielcarek, M., Zielonka, D., Carnemolla, A., Marcinkowski, J. T., & Guidez, F. (2015). *HDAC4 as a potential therapeutic target in neurodegenerative diseases: a summary of recent achievements*. Frontiers in Cellular Neuroscience, *9*, Article 42. https://doi.org/10.3389/fncel.2015.00042
- Mignolet, M., Gilloteaux, J., Halloin, N., Gueibe, M., Willemart, K., De Swert, K., Bielarz, V., Suain, V., Pastushenko, I., Gillet, N. A., & Nicaise, C. (2023). *Viral entry inhibitors protect against SARS-CoV-2-induced neurite shortening in differentiated SH-SY5Y cells*. Viruses, *15*(10), 2020. https://doi.org/10.3390/v15102020
- Mingaleeva, R. N., Nigmatulina, N. A., Sharafetdinova, L. M., Romozanova, A. M., Gabdoulkhakova, A. G., Filina, Y. V., Shavaliyev, R. F., Rizvanov, A. A., & Miftakhova, R. R. (2023). *Biology of the SARS-CoV-2 coronavirus*. Biochemistry (Moscow), *87*(12–13),

1662–1678. https://doi.org/10.1134/S0006297922120215

- Miner, J. J., & Diamond, M. S. (2017). *Zika virus pathogenesis and tissue tropism*. Cell Host & Microbe, *21*(2), 134–142. https://doi.org/10.1016/j.chom.2017.01.004

- Mlcochova, P., Kemp, S. A., Dhar, M. S., Papa, G., Meng, B., Ferreira, I. A. T. M., Datir, R., Collier, D. A., Albecka, A., Singh, S., Pandey, R., Brown, J., Zhou, J., Goonawardane, N., Mishra, S., Whittaker, C., Mellan, T., Marwal, R., Datta, M., Sengupta, S., Ponnusamy, K., Radhakrishnan, V. S., Abdullahi, A., Charles, O., Chattopadhyay, P., Devi, P., Caputo, D., Peacock, T. P., Wattal, C., Goel, N., Satwik, A., Vaishya, R., Agarwal, M., Indian SARS-CoV-2 Genomics Consortium (INSACOG), ... Gupta, R. K. (2021). *SARS-CoV-2 B.1.617.2 Delta variant replication and immune evasion*. Nature, *599*(7883), 114–119. https://doi.org/10.1038/s41586-021-03944-y

- Molaverdi, G., Kamal, Z., Safavi, M., Shafiee, A., Mozhgani, S.-H., Zarei Ghobadi, M., & Goudarzvand, M. (2023). *Neurological complications after COVID-19: A narrative review*. eNeurologicalSci, *33*, 100485. https://doi.org/10.1016/j.ensci.2023.100485

- Montezano, A. C., Camargo, L. L., Mary, S., Neves, K. B., Rios, F. J., Stein, R., Lopes, R. A., Beattie, W., Thomson, J., Herder, V., Szemiel, A. M., McFarlane, S., Palmarini, M., & Touyz, R. M. (2023). *SARS-CoV-2 spike protein induces endothelial inflammation via ACE2 independently of viral replication*. Scientific Reports, *13*, Article 14086. https://doi.org/10.1038/s41598-023-41115-3

- Murgolo, N., Therien, A. G., Howell, B., Klein, D., Koeplinger, K., Lieberman, L. A., Adam, G. C., Flynn, J., McKenna, P., Swaminathan, G., Hazuda, D. J., & Olsen, D. B. (2021). *SARS-CoV-2 tropism, entry, replication, and propagation: Considerations for drug discovery and development*. PLOS Pathogens, *17*(2), e1009225. https://doi.org/10.1371/journal.ppat.1009225

- Nahalka, J. (2024). *1-L transcription of SARS-CoV-2 spike protein S1 subunit*. International Journal of Molecular Sciences, *25*(8), 4440. https://doi.org/10.3390/ijms25084440

- Nalbandian, A., Sehgal, K., Gupta, A., Madhavan, M. V., McGroder, C., Stevens, J. S., Cook, J. R., Nordvig, A. S., Shalev, D., Sehrawat, T. S., Ahluwalia, N., Bikdeli, B., Dietz, D., Der-Nigoghossian, C., Liyanage-Don, N., Rosner, G. F., Bernstein, E. J., Mohan, S., Beckley, A. A., Seres, D. S., Choueiri, T. K., Uriel, N., Ausiello, J. C., Accili, D., Freedberg, D. E., Baldwin, M., Schwartz, A., Brodie, D., Garcia, C. K., Elkind, M. S. V., Connors, J.

M., Bilezikian, J. P., Landry, D. W., & Wan, E. Y. (2021). *Post-acute COVID-19 syndrome*. Nature Medicine, *27*(4), 601–615. https://doi.org/10.1038/s41591-021-01283-z

- Ngo, H., Kang, Y. J., Diep, Y. N., Tran, V. T. A., Kim, S., Park, S., Lee, L. P., & Cho, H. (2025). *SARS-CoV-2 S1-inducing reactive astrocyte and proteinopathies via TANK-binding kinase 1 in human neuroglia model*. BioChip Journal, *15*(3), Article 226. https://doi.org/10.1007/s13206-025-00226-x
- Nishiumi, S., & Ashida, H. (2007). *Rapid preparation of a plasma membrane fraction from adipocytes and muscle cells: Application to detection of translocated glucose transporter 4 on the plasma membrane*. Bioscience, Biotechnology, and Biochemistry, *71*, 2343–2346. https://doi.org/10.1271/bbb.70342
- Nuovo, G. J., Magro, C., Shaffer, T., Awad, H., Suster, D., Mikhail, S., He, B., Michaille, J.-J., Liechty, B., & Tili, E. (2021) (a). *Endothelial cell damage is the central part of COVID-19 pathology: evidence from tissue studies*. Journal of Medical Virology, 93(5), 2507–2515. https://doi.org/10.1002/jmv.26658
- Nuovo, G. J., Magro, C., Shaffer, T., Awad, H., Suster, D., Mikhail, S., He, B., Michaille, J.-J., Liechty, B., & Tili, E. (2021) (b). *Endothelial cell damage is the central part of COVID-19 and a mouse model induced by injection of the S1 subunit of the spike protein*. Annals of Diagnostic Pathology, 51, 151682. https://doi.org/10.1016/j.anndiagpath.2020.151682
- Pang, Z., Tang, A., He, Y., Fan, J., Yang, Q., Tong, Y., & Fan, H. (2024). *Neurological complications caused by SARS-CoV-2*. Clinical Microbiology Reviews, *37*(4), e00131-24. https://doi.org/10.1128/cmr.00131-24
- Paoletti, A. M., Melilli, M. G., & Vecchio, I. (2022). *Experimental models of SARS-CoV-2 infection in the central nervous system*. Journal of Central Nervous System Disease, *14*, 11795735221102231. https://doi.org/10.1177/11795735221102231
- Parasher, A. (2021). *COVID-19: Current understanding of its pathophysiology, clinical presentation and treatment*. Postgraduate Medical Journal, *97*(1147), 312–320. https://doi.org/10.1136/postgradmedj-2020-138577
- Park, E. M., Joh, T. H., Volpe, B. T., Chu, C. K., Song, G., & Cho, S. (2004). *A neuroprotective role of extracellular signal-regulated kinase in n-acetyl-o-methyldopamine-treated hippocampal neurons after exposure to in vitro and in vivo ischemia*. Neuroscience, *123*(1), 147–154. https://doi.org/10.1016/j.neuroscience.2003.08.023

- Perico, L., Benigni, A., & Remuzzi, G. (2024). *SARS-CoV-2 and the spike protein in endotheliopathy*. Trends in Microbiology, *32*(1), 53–67. https://doi.org/10.1016/j.tim.2023.06.004
- Polack, F. P., Thomas, S. J., Kitchin, N., Absalon, J., Gurtman, A., Lockhart, S., Perez, J. L., Pérez Marc, G., Moreira, E. D., Zerbini, C., Bailey, R., Swanson, K. A., Roychoudhury, S., Koury, K., Li, P., Kalina, W. V., Cooper, D., Frenck, R. W. Jr., Hammitt, L. L., Türeci, Ö., Nell, H., Schaefer, A., Ünal, S., Tresnan, D. B., Mather, S., Dormitzer, P. R., Şahin, U., Jansen, K. U., & Gruber, W. C. (2020). *Safety and efficacy of the BNT162b2 mRNA COVID-19 vaccine*. New England Journal of Medicine, *383*(27), 2603–2615. https://doi.org/10.1056/NEJMoa2034577
- Qu, Y., An, K., Wang, D., Yu, H., Li, J., Min, Z., Xiong, Y., Xue, Z., & Mao, Z. (2025). *Short-chain fatty acid aggregates alpha-synuclein accumulation and neuroinflammation via GPR43-NLRP3 signaling pathway in a model of Parkinson's disease*. Molecular Neurobiology, *62*, 6612–6625. https://doi.org/10.1007/s12035-025-04726-7
- Ren, X., Nicoll, D. A., Xu, L., Qu, Z., & Philipson, K. D. (2010). *Transmembrane segment packing of the $Na^+/Ca^{2+}$ exchanger investigated with chemical cross-linkers*. Biochemistry, *49*, 8585–8591. https://doi.org/10.1021/bi101173c
- Rhea, E. M., Logsdon, A. F., Hansen, K. M., Williams, L. M., Reed, M. J., Baumann, K. K., Holden, S. J., Raber, J., Banks, W. A., & Erickson, M. A. (2021). *The S1 protein of SARS-CoV-2 crosses the blood–brain barrier in mice*. Nature Neuroscience, *24*(3), 368–378. https://doi.org/10.1038/s41593-020-00771-8
- Robles, J. P., Zamora, M., Adan-Castro, E., Siqueiros-Marquez, L., Martinez de la Escalera, G., & Clapp, C. (2022). *The spike protein of SARS-CoV-2 induces endothelial inflammation through integrin α5β1 and NF-κB signaling*. Journal of Biological Chemistry, *298*(3), 101695. https://doi.org/10.1016/j.jbc.2022.101695
- Saiz, M. L., DeDiego, M. L., López-García, D., Corte-Iglesias, V., Baragaño Raneros, A., Astola, I., Asensi, V., López-Larrea, C., & Suárez-Álvarez, B. (2021). *Epigenetic targeting of the ACE2 and NRP1 viral receptors limits SARS-CoV-2 infectivity*. Clinical Epigenetics, *13*, Article 187. https://doi.org/10.1186/s13148-021-01168-5
- Sanclemente-Alaman, I., Moreno-Jiménez, L., Benito-Martín, M. S., Canales-Aguirre, A., Matías-Guiu, J. A., Matías-Guiu, J., & Gómez-Pinedo, U. (2020). *Experimental models for the study of central nervous system infection by SARS-CoV-2*. Frontiers in Immunology, *11*,

2163. https://doi.org/10.3389/fimmu.2020.02163

- Sanguigno, L., Guida, N., Anzilotti, S., Cuomo, O., Mascolo, L., Serani, A., Brancaccio, P., Pennacchio, G., Licastro, E., Pignataro, G., Molinaro, P., Annunziato, L., & Formisano, L. (2023). *Stroke by inducing HDAC9-dependent deacetylation of HIF-1 and Sp1, promotes TfR1 transcription and GPX4 reduction, thus determining ferroptotic neuronal death*. International Journal of Biological Sciences, *19*(9), 2695–2710. https://doi.org/10.7150/ijbs.80735
- Sapkota, A., & Aryal, S. (2025). *SARS-CoV-2 Structure & Genome: Key Proteins & RNA Features*. MicrobeNotes. Retrieved from https://microbenotes.com/structure-and-genome-of-sars-cov-2
- Saucier, J., Comeau, D., Robichaud, G. A., & Chamard-Witkowski, L. (2023). *Reactive gliosis and neuroinflammation: prime suspects in the pathophysiology of post-acute neuroCOVID-19 syndrome*. Frontiers in Neurology, *14*, Article 1221266. https://doi.org/10.3389/fneur.2023.1221266
- Schroeder, J. T., & Bieneman, A. P. (2022). *The S1 subunit of the SARS-CoV-2 spike protein activates human monocytes to produce cytokines linked to COVID-19: Relevance to Galectin-3*. Frontiers in Immunology, *13*, Article 831763. https://doi.org/10.3389/fimmu.2022.831763
- Seo, Y. B., Choi, Y. J., Seo, J.-W., Kim, E. J., Lee, J., & Song, J. Y. (2025). *Therapeutic options for the treatment of post-acute sequelae of COVID-19: a scoping review.* BMC Infectious Diseases, *25*(1), Article 731. https://doi.org/10.1186/s12879-025-11131-x
- Sette, A., & Crotty, S. (2021). *Adaptive immunity to SARS-CoV-2 and COVID-19*. Cell, *184*(4), 861–880. https://doi.org/10.1016/j.cell.2021.01.007
- Shi, Z., & Puyo, C. A. (2020). *N-Acetylcysteine to combat COVID-19: An evidence review.* Therapeutics and Clinical Risk Management, *16*, 1047–1055. https://doi.org/10.2147/TCRM.S273700
- Shukla, S., & Tekwani, B. L. (2020). *Histone Deacetylases Inhibitors in Neurodegenerative Diseases, Neuroprotection and Neuronal Differentiation*. Frontiers in Pharmacology, *11*, Article 537. https://doi.org/10.3389/fphar.2020.00537
- Sierra-Sánchez, V. M., Blancas-Napoles, C. M., Sánchez-Maldonado, A. D., Medina, I., Romero-Nava, R., Huang, F., Hong, E., Aguilera-Méndez, A., Ocampo-Ortega, S. A., & Villafaña, S. (2025). *Neuropilin-1: A conserved entry receptor for SARS-CoV-2 and a*

*potential therapeutic target*. Biomedicines, *13*(7), Article 1730. https://doi.org/10.3390/biomedicines13071730

- Singh, D., & Yi, S. V. (2021). *On the origin and evolution of SARS-CoV-2*. Experimental & Molecular Medicine, *53*(4), 537–547. https://doi.org/10.1038/s12276-021-00604-z
- Singh, K. K., Chaubey, G., Chen, J. Y., & Suravajhala, P. (2020). *Decoding SARS-CoV-2 hijacking of host mitochondria in COVID-19 pathogenesis*. American Journal of Physiology - Cell Physiology, *319*(2), C258–C267. https://doi.org/10.1152/ajpcell.00224.2020
- Sofroniew, M. V. (2020). *Astrocyte reactivity: subtypes, states, and functions in CNS innate immunity.* Trends in Immunology, *41*(9), 758–770. https://doi.org/10.1016/j.it.2020.07.004
- Song, E., Zhang, C., Israelow, B., Lu-Culligan, A., Prado, A. V., Skriabine, S., Lu, P., Weizman, O.-E., Liu, F., Dai, Y., Szigeti-Buck, K., Yasumoto, Y., Wang, G., Castaldi, C., Heltke, J., Ng, E., Wheeler, J., Alfajaro, M. M., Levavasseur, E., Fontes, B., Ravindra, N. G., Van Dijk, D., Mane, S., Gunel, M., Ring, A., Kazmi, S. A. J., Zhang, K., Wilen, C. B., Horvath, T. L., & Iwasaki, A. (2021). *Neuroinvasion of SARS-CoV-2 in human and mouse brain*. Journal of Experimental Medicine, *218*(3), e20202135. https://doi.org/10.1084/jem.20202135
- Stangis, M., Adesse, D., Sharma, B., Castro, E., Kumar, K., Kumar, N., Minevich, M., & Toborek, M. (2024). *The S1 subunits of SARS-CoV-2 variants differentially trigger the IL-6 signaling pathway in human brain endothelial cells and downstream impact on microglia activation*. NeuroImmune Pharmacology and Therapeutics, *3*(1), Article 100015. https://doi.org/10.1515/nipt-2023-0024
- Sulzer, D., Antonini, A., Leta, V., Nordvig, A., Smeyne, R. J., Goldman, J. E., Al-Dalahmah, O., Zecca, L., Sette, A., Bubacco, L., Meucci, O., Moro, E., Harms, A. S., Xu, Y., Fahn, S., & Chaudhuri, K. R. (2020). *COVID-19 and possible links with Parkinson's disease and parkinsonism: from bench to bedside*. NPJ Parkinson's Disease, *6*, Article 18. https://doi.org/10.1038/s41531-020-00123-0
- Taquet, M., Geddes, J. R., Husain, M., Luciano, S., & Harrison, P. J. (2021). *6-month neurological and psychiatric outcomes in 236,379 survivors of COVID-19: a retrospective cohort study.* The Lancet Psychiatry, *8*(5), 416–427. https://doi.org/10.1016/S2215-0366(21)00084-5
- Tegally, H., Wilkinson, E., Giovanetti, M., Iranzadeh, A., Fonseca, V., Giandhari, J.,

Doolabh, D., Pillay, S., San, E. J., Msomi, N., Mlisana, K., von Gottberg, A., Walaza, S., Allam, M., Ismail, A., Mohale, T., Glass, A. J., Engelbrecht, S., Van Zyl, G., Preiser, W., Petruccione, F., Sigal, A., Hardie, D., Marais, G., Hsiao, N.-Y., Korsman, S., Davies, M.-A., Tyers, L., Mudau, I., York, D., Maslo, C., Goedhals, D., Abrahams, S., Laguda-Akingba, O., Alisoltani-Dehkordi, A., Godzik, A., Wibmer, C. K., Sewell, B. T., Lourenço, J., Alcantara, L. C. J., Kosakovsky Pond, S. L., Weaver, S., Martin, D., Lessells, R. J., Bhiman, J. N., Williamson, C., & de Oliveira, T. (2021). *Detection of a SARS-CoV-2 variant of concern in South Africa*. Nature, *592*(7854), 438–443. https://doi.org/10.1038/s41586-021-03402-9

- Tyagi, K., Rai, P., Gautam, A., Kaur, H., Kapoor, S., Suttee, A., Jaiswal, P. K., Sharma, A., Singh, G., & Barnwal, R. P. (2023). *Neurological manifestations of SARS-CoV-2: complexity, mechanism and associated disorders*. European Journal of Medical Research, *28*, Article 307. https://doi.org/10.1186/s40001-023-01293-2

- Toscano, G., Palmerini, F., Ravaglia, S., Ruiz, L., Invernizzi, P., Cuzzoni, M. G., Franciotta, D., Baldanti, F., Daturi, R., Postorino, P., Cavanna, L., & Micieli, G. (2020). *Guillain–Barré Syndrome associated with SARS-CoV-2*. New England Journal of Medicine, *382*(26), 2574–2576. https://doi.org/10.1056/NEJMc2009191

- Tracey, K. J. (2002). *The inflammatory reflex*. Nature, *420*(6917), 853–859. https://doi.org/10.1038/nature01321

- Varga, Z., Flammer, A. J., Steiger, P., Haberecker, M., Andermatt, R., Zinkernagel, A. S., Mehra, M. R., Schuepbach, R. A., Ruschitzka, F., & Moch, H. (2020). *Endothelial cell infection and endotheliitis in COVID-19*. The Lancet, *395*(10234), 1417–1418. https://doi.org/10.1016/S0140-6736(20)30937-5

- Vashisht, A., Vashisht, V., Singh, H., Ahluwalia, P., Mondal, A. K., Williams, C., Farmaha, J., Woodall, J., & Kolhe, R. (2024). *Neurological Complications of COVID-19: Pathophysiological Underpinnings and Therapeutic Implications*. Viruses, *16*(8), Article 1183. https://doi.org/10.3390/v16081183

- Volz, E., Mishra, S., Chand, M., Barrett, J. C., Johnson, R., Geidelberg, L., Hinsley, W. R., Laydon, D. J., Dabrera, G., O'Toole, Á., Amato, R., Ragonnet-Cronin, M., Harrison, I., Jackson, B., Ariani, C. V., Boyd, O., Loman, N. J., McCrone, J. T., Gonçalves, S., Sillitoe, J., Kwiatkowski, D. P., Ferguson, N. M., Rambaut, A., & Connor, T. R. (2021). *Assessing transmissibility of SARS-CoV-2 lineage B.1.1.7 in England*. Nature, *593*(7858), 266–269. https://doi.org/10.1038/s41586-021-03470-x

- Walls, A. C., Park, Y. J., Tortorici, M. A., Wall, A., McGuire, A. T., & Veesler, D. (2020). *Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein*. Cell, *181*(2), 281–292.e6. https://doi.org/10.1016/j.cell.2020.02.058
- Wan, D., Du, T., Hong, W., Chen, L., Que, H., Lu, S., & Peng, X. (2021). *Neurological complications and infection mechanism of SARS-CoV-2*. Signal Transduction and Targeted Therapy, *6*, Article 406. https://doi.org/10.1038/s41392-021-00818-7
- Wang, F., Han, H., Wang, C., Wang, J., Peng, Y., Chen, Y., He, Y., Deng, Z., Li, F., Rong, Y., Wang, D., Liu, W., Chen, H., & Zhang, Z. (2024). *SARS-CoV-2 membrane protein induces neurodegeneration via affecting Golgi-mitochondria interaction*. Translational Neurodegeneration, *13*, Article 68. https://doi.org/10.1186/s40035-024-00458-1
- Wang, J., Dai, L., Deng, M., Xiao, T., Zhang, Z., & Zhang, Z. (2024). *SARS-CoV-2 Spike Protein S1 Domain Accelerates α-Synuclein Phosphorylation and Aggregation in Cellular Models of Synucleinopathy.* Molecular Neurobiology, *61*, 2446–2458. https://doi.org/10.1007/s12035-023-03726-9
- Watanabe, Y., Allen, J. D., Wrapp, D., McLellan, J. S., & Crispin, M. (2020). *Site-specific glycan analysis of the SARS-CoV-2 Spike*. Science, *369*(6501), 330–333. https://doi.org/10.1126/science.abb9983
- Weinreich, D. M., Sivapalasingam, S., Norton, T., Ali, S., Gao, H., Bhore, R., Musser, B. J., Soo, Y., Rofail, D., Im, J., Perry, C., Pan, C., Hosain, R., Mahmood, A., Davis, J. D., Turner, K. C., Hooper, A. T., Hamilton, J. D., Baum, A., Kyratsous, C. A., Kim, Y., Cook, A., Kampman, W., Kohli, A., Sachdeva, N., Graber, X., Kowal, B., DiCioccio, T., Stahl, N., Lipsich, L., Yancopoulos, G. D., & Trial Investigators. (2021). *REGN-COV2, a neutralizing antibody cocktail, in outpatients with COVID-19*. New England Journal of Medicine, *384*, 238–251. https://doi.org/10.1056/NEJMoa2035002
- Whitaker, H. J., Tsang, R. S. M., Byford, R., Aspden, C., Button, E., Pillai, P. S., Jamie, G., Kar, D., Williams, J., Sinnathamby, M., Marsden, G., Elson, W. H., Leston, M., Anand, S., Okusi, C., Fan, X., Linley, E., Rowe, C., DArcangelo, S., & Otter, A. D. (2023). *COVID-19 vaccine effectiveness against hospitalisation and death of people in clinical risk groups during the Delta variant period: English primary care network cohort study*. Journal of Infection, *87*(4), 315–327. https://doi.org/10.1016/j.jinf.2023.08.005
- Wolf, K. A., Kwan, J. C., & Kamil, J. P. (2022). *Structural Dynamics and Molecular Evolution of the SARS-CoV-2 Spike Protein*. mBio, *13*(2), e02030-21.

https://doi.org/10.1128/mbio.02030-21

- World Health Organization. *Neurology and COVID-19: scientific brief.* WHO Publications. August 2024. WHO Reference Number: B09134 URL: Neurology and COVID-19: scientific brief

- Wu, Q., Yang, X., Zhang, L., Zhang, Y., & Feng, L. (2017). *Nuclear accumulation of histone deacetylase 4 (HDAC4) exerts neurotoxicity in models of Parkinson's disease*. Molecular Neurobiology, *54*(9), 6970–6983. https://doi.org/10.1007/s12035-016-0199-2

- Yajima, H., Nomai, T., Okumura, K., Maenaka, K., The Genotype to Phenotype Japan (G2P-Japan) Consortium, Ito, J., Hashiguchi, T., & Sato, K. (2024). *Molecular and structural insights into SARS-CoV-2 evolution: from BA.2 to XBB subvariants*. mBio, *15*(10), e03220-23. https://doi.org/10.1128/mbio.03220-23

- Yao, W., Dong, H., Qi, J., Zhang, Y., & Shi, L. (2022). *Safety and efficacy of mesenchymal stem cells in severe/critical patients with COVID-19: A systematic review and meta-analysis*. eClinicalMedicine, *51*, 101545. https://doi.org/10.1016/j.eclinm.2022.101545

- Yao, W., Li, Y., Ma, D., Hou, X., Wang, H., Tang, X., Cheng, D., Zhang, H., Du, C., Pan, H., Li, C., Lin, H., Sun, M., Ding, Q., Wang, Y., Gao, J., & Zhong, G. (2023). *Evolution of SARS-CoV-2 Spikes shapes their binding affinities to animal ACE2 orthologs*. Microbiology Spectrum, *11*(6), e02676-23. https://doi.org/10.1128/spectrum.02676-23
- Yan, R., Zhang, Y., Li, Y., Xia, L., Guo, Y., & Zhou, Q. (2020). *Structural basis for the recognition of SARS-CoV-2 by full-length human ACE2*. Science, *367*(6485), 1444–1448. https://doi.org/10.1126/science.abb2762

- Yuan, J., Amin, P., & Ofengeim, D. (2019). *Necroptosis and RIPK1-mediated neuroinflammation in CNS diseases*. Nature Reviews Neuroscience, *20*(1), 19–33. https://doi.org/10.1038/s41583-018-0093-1

- Zhou, C., Zhao, D., Wu, C., Wu, Z., Zhang, W., Chen, S., Zhao, X., & Wu, S. (2024). *Role of histone deacetylase inhibitors in non-neoplastic diseases*. Heliyon, *10*(13), e33997. https://doi.org/10.1016/j.heliyon.2024.e33997

- Zimmermann, P., & Curtis, N. (2020). *COVID-19 in children, pregnancy and neonates: a review of epidemiologic and clinical features*. Pediatric Infectious Disease Journal, *39*(6), 469–477. https://doi.org/10.1097/INF.0000000000002700