

Central Nervous System Targets and Routes for SARS-CoV-2: Current Views and New Hypotheses

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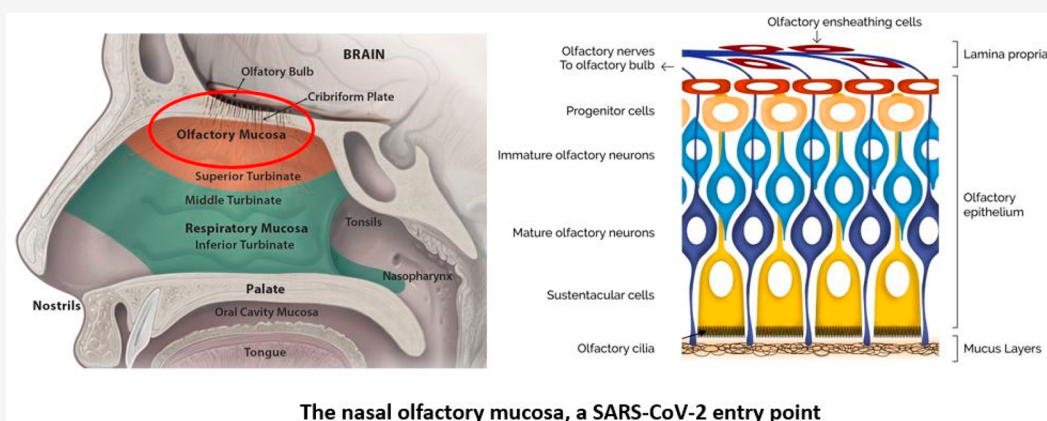
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The nasal olfactory mucosa, a SARS-CoV-2 entry point

ABSTRACT: As the coronavirus disease 2019 (COVID-19) pandemic unfolds, neurological signs and symptoms reflect the involvement of targets beyond the primary lung effects. The etiological agent of COVID-19, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), exhibits neurotropism for central and peripheral nervous systems. Various infective mechanisms and paths can be exploited by the virus to reach the central nervous system, some of which bypass the blood–brain barrier; others alter its integrity. Numerous studies have established beyond doubt that the membrane-bound metalloprotease angiotensin-converting enzyme 2 (ACE2) performs the role of SARS-CoV-2 host-cell receptor. Histochemical studies and more recently transcriptomics of mRNA have dissected the cellular localization of the ACE2 enzyme in various tissues, including the central nervous system. Epithelial cells lining the nasal mucosae, the upper respiratory tract, and the oral cavity, bronchoalveolar cells type II in the pulmonary parenchyma, and intestinal enterocytes display ACE2 binding sites at their cell surfaces, making these epithelial mucosae the most likely viral entry points. Neuronal and glial cells and endothelial cells in the central nervous system also express ACE2. This short review analyzes the known entry points and routes followed by the SARS-CoV-2 to reach the central nervous system and postulates new hypothetical pathways stemming from the enterocytes lining the intestinal lumen.

KEYWORDS: COVID-19, SARS-CoV-2, neurotropic virus, angiotensin-converting enzyme 2, ACE2, receptor, brain, viral infection, TMPRSS2

INTRODUCTION

The *Coronaviridae* are a family of enveloped viruses carrying between 26 and 32 kilobases of single-stranded, positive-sense RNA, the largest so far detected for an RNA virus.^{1,2} Coronaviruses (CoVs) infect a wide range of avian and mammalian species. A first set of human CoVs (HCoV-OC43, HCoV-229E, HCoV-NL63, and HKU1-CoV) generally causes mild and self-limiting respiratory diseases. A second set of HCoVs are more pathogenic and include the etiological agents of the two epidemics occurring earlier this century, namely, the severe acute respiratory syndrome (SARS-CoV) and the Middle East respiratory syndrome (MERS-CoV) viruses. The ongoing outbreak of coronavirus disease 2019 (COVID-19) is caused by

the recently discovered seventh human CoV, the severe acute respiratory syndrome coronavirus 2 virus (SARS-CoV-2).

Various neurotropic viruses exert pathogenic effects on the peripheral and central (CNS) nervous systems. To reach these targets, viruses use different strategies adapted in the course of evolution to exploit cell-surface molecules normally fulfilling

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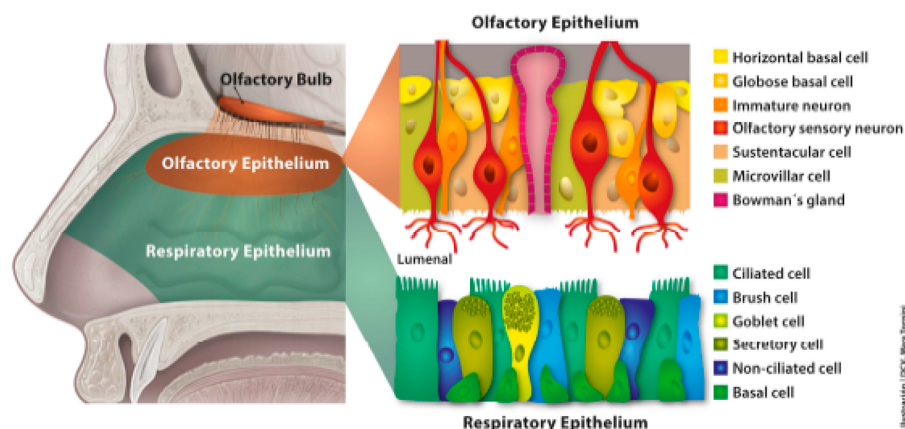


Figure 1. Nasal mucosae (left panel) and cellular composition of the olfactory and respiratory regions of the nasal mucosae (right panel). Color code depicts the various cellular components, some of which may be used by SARS-CoV-2 to produce the primary infection and serve as a starting point to reach the brain by neural or non-neural paths.

completely different functions in the cell, to serve as binding partners. Binding of viruses to these adapted molecules, usually transmembrane enzymes, is the first step in viral infection. CoVs have perfected these strategies using metalloproteases as recognition molecules and other membrane-bound enzymes such as the transmembrane serine protease 2 (TMPRSS2) for activation of a key viral protein during the subsequent step of the infection process. SARS-CoV-2 engages its spike S glycoprotein, interacting in sequential order with these two proteins before fusing with the host membrane.³

Other viruses such as the mouse hepatitis virus (MHV) beta-CoV rely on the S protein N-term domain to bind to the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1).⁴ A zinc metalloprotease, aminopeptidase N (APN, CD13), serves as a host-cell receptor molecule for human H229E-CoV, transmissible gastroenteritis virus, porcine epidemic diarrhea, and feline infectious peritonitis virus. The enzyme dipeptidyl-peptidase 4 (DPP4), also known as a cluster of differentiation 26 (CD26), present in the apical surface of unciliated bronchial epithelial cells, acts as the receptor for MERS-CoV.⁵ MERS-CoV can also infect human pulmonary epithelial cells through highly specific but low affinity interactions with sialic acid residues present in host cell-surface glycoproteins⁶ using a different region of its spike protein S.⁷

During the first weeks of the pandemic, individuals admitted to hospital presented the typical initial symptomatology of lower respiratory tract infection, associated with fever, fatigue with or without myalgia, sore throat, shortness of breath, dry cough, and in moderate-to-severe cases, dyspnea; rhinorrhea was seldom observed.^{8,9} Fever was by far the most frequent sign.^{9,10} Computer tomography revealed ground-glass opacity in roughly 50% of hospitalized patients with lower respiratory tract symptoms.^{10–12} As the number of cases rose worldwide, the spectrum of symptoms reported for COVID-19 patients widened (see reviews, e.g., in refs 13 and 14). The degree of severity covered a wide range of clinical presentations that were not restricted to the respiratory tract or the pulmonary symptoms and signs. Patients with comorbidities such as hypertension, cardiovascular diseases, or diabetes were found to be more prone to develop severe forms of the disease and multiple organ dysfunction.^{12,15,16}

Early reports of sensory dysfunction such as hyposmia^{17,18} and other forms of dysosmias and dysgeusias^{18–24} were the

initial indications of nervous system involvement in COVID-19. These sensory dysfunctions were also observed in patients who recovered from COVID-19 and at later stages presented anosmia, rarely hyposmia.²⁵ Life-threatening neurological presentations, such as stroke, were and remain exceptional findings.²⁶

Reports of neurological complications of COVID-19 became more frequent as the number of hospitalized patients increased. These included cases of peripheral neuropathies and neuromuscular pathologies such as rhabdomyolysis or Guillain-Barré syndrome, to severe CNS complications such as encephalitis, encephalopathy, necrotizing hemorrhagic encephalopathy, some forms of epilepsy, or stroke (reviewed in refs 27–30). The incidence of some of these neurological clinical pictures appears to be relatively high: Mao and co-workers¹⁸ reported that 36.4% of COVID-19 patients showed neurological symptoms, with specific but mild symptoms such as dysgeusias or dysosmias early on in the course of the disease. The reader is referred to several other reviews on the clinical and neurological manifestations of COVID-19 that have recently appeared.^{14,24,29,31–39}

The occurrence of neurological manifestations in a viral infectious disease such as COVID-19 poses several interesting issues on the pathogenesis of this clinical entity. Since the first step of the viral infection—binding to a target host cell-surface molecule—mimics a ligand–receptor interaction, the question arises as to which tissues are the ports of entry and in which cells the receptors are located; second, which are the routes followed by the virions surpassing these first barriers to reach the CNS and produce neurological manifestations. This short review addresses these issues: the possible neural or non-neural routes and mechanisms that the SARS-CoV-2 virus could follow to reach and infect the CNS, the presence and distribution of the counterpart cell-host receptor for the SARS-CoV-2 (the membrane-bound metalloprotease angiotensin-converting enzyme 2, ACE2), as analyzed by immunohistochemical or more recently by mRNA transcriptomics, and finally a discussion of hypothetical new routes that the virus could follow to reach the CNS from its enteric entry point.

■ THE NASAL MUCOSA

The nasal cavity can be divided into three regions: the squamous region and the respiratory and the sensory olfactory mucosae.

The squamous region, the most external portion, is lined with stratified keratinized squamous epithelium and is therefore unlikely to be a site of viral infection under normal conditions. Most of the nasal cavity corresponds to the respiratory region, which is no longer a squamous multilayered epithelium but a non-keratinized pseudostratified epithelium lined with basal cells, ciliated and nonciliated columnar cells, secretory cells, and goblet cells (Figure 1).^{40,41} Basal cells are in contact with the basal membrane and have the inherent capacity to differentiate into the other types in the nasal mucosa. Columnar cells possess cilia and numerous microvilli at their apical surface and are “sealed” with tight junctions at the border between their apical zone and the uppermost section of the basolateral membrane. The goblet cells secrete mucin, a key component of the mucus layer. The respiratory region of the nasal cavity (Figure 1) is innervated by the trigeminal nerve, which stems from the pons in the brainstem⁴² and constitutes therefore a potential pathway for the centripetal routing of an infective agent.

The olfactory region is located cephalically to the respiratory region, in and beneath the roof of each nasal cavity, and is lined with a specialized type of pseudostratified monolayer of epithelial cells, harboring (a) olfactory sensory (receptor) neurons, the first cells in the chain of olfactory sensory function, with an exquisite variety of odor receptors expressed at their apical (dendritic) cilia; (b) sustentacular (“supporting”) cells, which keep the electrolytic balance required for olfactory function, and as we will see constitute a possible gateway for SARS-CoV-2 entry into the CNS; (c) microvillar cells as in the respiratory region; (d) globose basal cells, which are stem cells that differentiate into olfactory neurons to replace those physiologically lost by aging, and (e) horizontal basal cells, also of stem lineage, that actively generate multiple mature cells in the olfactory epithelium, including the olfactory neuronal and non-neuronal cells.⁴³ Olfactory stem cells generate support cells in the absence of cell division.⁴⁴ The Bowman gland cells also contribute to mucus secretion and homeostatic electrolyte balance. Two major local effects of infection with human CoVs on the nasal mucosa are the disruption of the ciliated epithelium and ciliary dyskinesia, which affect mucociliary clearance and contribute to the pulmonary disease.⁴⁵

Recent single-cell RNA-Seq profiling analysis explored the cellular composition, distribution, and transcriptional heterogeneity of normal human airway mucosa obtained by bronchoscopic aspiration along the respiratory tract.⁴⁶ This resulted in an atlas (in the framework of the Human Cell Atlas, <https://www.humancellatlas.org/>) showing a rather stable cell type-specific gene expression all along the respiratory tract, but exhibiting differential gene expression between otherwise identical cell types from the nasal epithelium down to the 12th division of the tracheobronchial airway epithelium. In addition, rare pulmonary neuroendocrine and brush cells and ionocytes were found to derive from a common population of precursor cells.⁴⁶ The same authors explored in more detail the differentiation of the mucociliary system in another single-cell RNA-Seq transcriptomics study on cell cultures of human mucosal cells.⁴⁷ They found that goblet cells can be precursors of multiciliated cells and that a subgroup of multiciliated cells expressed the *DEU1* gene, a hallmark of massive centriole amplification at these cells.

Some of the RNA-Seq studies indicate that the transmembrane proteases purported to be the cell-host receptors for SARS-CoV-2 (see section below) are present in the respiratory epithelial cells,^{3,48} whereas other studies show only

low level expression of these two proteases in the human mucosal epithelial cells.⁴⁹ This latter study identified 194 nonolfactory genes. The human genome contains 857 olfactory receptor genes, of which roughly half are pseudogenes.

■ HUMAN VIRAL INFECTIONS OF THE NASAL MUCOSA

Rhinitis and rhinosinusitis (sometimes with concomitant polyposis), the predominant causes of olfactory impairment, were already the most common chronic medical conditions almost two decades ago, affecting in the USA alone about 32 million individuals, with permanent dysfunction predominantly observed in the elderly.⁵⁰ Acute viral rhinitis is the most common cause of nontraumatic olfactory dysfunction; traumatic causes are usually associated with head injuries causing tearing or severing of olfactory neuron axons at the cribriform plate. CoVs have been known to be infective agents of the nasal mucosa for many decades, and verification was provided by early experimental clinical studies. An example of this was the reported elevated olfactory thresholds to 1-butanol among volunteers who had been inoculated with one of the human CoVs responsible for the common cold, HCoV-229E, and who developed viral rhinitis relative to uninfected control subjects.⁵¹

Immunohistochemical studies have documented the alterations in olfactory epithelial cells as sequelae of various types of olfactory disorders following viral infections.⁵² The disability of the upper respiratory tract associated with viral diseases gave rise to a nosological entity, the postviral olfactory dysfunction (PVOD); rhinoviruses, belonging to a genus of the *Picornaviridae* family and etiological agent among other viruses of the common cold, are the most frequently isolated species, together with CoVs, parainfluenza virus Epstein–Barr virus, adenoviruses, enteroviruses, and respiratory syncytial viruses.^{53,54} Unlike these other viral rhinitides, the olfactory disability observed in several clinical cases of COVID-19 does not usually present rhinorrhea.²⁰

■ CELLULAR TOPOGRAPHY OF RECEPTOR MOLECULES FOR SARS-COV-2

Both SARS-CoV and SARS-CoV-2 exhibit marked tropism for cells that harbor ACE2 at their plasmalemma, predominantly in cells lining the oral and nasal cavities, upper respiratory tract, and bronchoalveolar cells.^{55–57} Other receptors and coreceptor have been postulated for SARS-CoV-2, such as CD147, also known as Basigin or EMMPRIN, a transmembrane glycoprotein belonging to the immunoglobulin superfamily.⁵⁸ The transmembrane serine proteases TMRSS2 and TMPRSS4 play a key role in facilitating SARS-CoV-2 spike fusogenic activity on the host-cell plasma membrane, thus promoting the entry of the virus into the cell,⁵⁹ acting as “co-receptors” of ACE2.

Why have the nasal and oral mucosae acquired such relevance in the context of COVID-19? At first glance, the contribution of the two mucosae does not appear to lend support to their possible role as viral reservoirs or massive sources of virions for secondary infections. The nasal mucosae cover a surface of ~150–160 cm² (refs 40 and 60) and the oral cavity ~215 cm² (ref 61). The tongue expresses much higher amounts of ACE2 than the rest of the oral mucosa.⁵⁶ In contrast, the total surface of the intestinal mucosae (~250 m²) or the upper respiratory tract and the pulmonary alveolar region (118 ± 22 m² and 91 ± 18 m² in male and female individuals, respectively (Colebatch and Ng 1992)) are several orders of magnitude larger. Despite their

relatively small surface, however, the nasal mucosae are of particular importance because of their anatomical vicinity and connections to the forebrain via the shortest of the cranial nerves, the olfactory nerve.

The oral and nasal mucosae became relevant within the context of the current pandemic following the observation of clinical symptoms in COVID-19 associated with alterations in the sensory systems of odor and taste, namely, dysosmias and disgeusias.^{21,23,32} Knowledge of the distribution, absolute number, and surface density of ACE2 viral receptor molecules constitutes the first step toward establishing which cells exhibit higher tropism for the viruses, are at greater risk of being infected, and ultimately offer better chances for the viruses to use them as entry points and routes to infecting other organs. The tropism of the virus for certain cells over others also points not only to them being acute targets for gaining entry into the organism but also to their preferential exploitation as reservoirs, as is the case with viruses that reemerge after long latencies. It became important to discriminate whether these sensory disturbances were a manifestation of a peripheral affectation of the mucosae or a more serious neurological complication involving the CNS. It was soon hypothesized that the olfactory epithelium was the likely site of enhanced binding of SARS-CoV-2, correlating this with the clinical olfactory dysfunction observed in some COVID-19 patients, and the possibility was suggested that the olfactory receptor neurons were the site of origin of subsequent brain infection by the virus.⁶² Although the early clinical data did not reveal a high incidence of severe neurological complications in COVID-19, such as encephalopathies or encephalitis,^{27,29} as the pandemic progressed over time so did the casuistic involving the CNS.³⁵

In parallel, experimental data began to emerge. Using a mouse animal model, the predominant expression of ACE2 and TMPRSS2 in the sustentacular cells of the olfactory epithelium was demonstrated, thus suggesting that non-neuronal cells could be responsible for the olfactory impairment.⁶³ This was followed by experimental demonstration that this was indeed the case: massive damage of the olfactory epithelium was observed as early as 2 days after nasal instillation of SARS-CoV-2 in golden Syrian hamsters, with a substantial loss of cilia.⁶⁴ The injured cells were primarily the sustentacular cells.

As is the case with other cell-surface receptors, the distribution and local density of ACE2 play a determinant role in the efficacy of the binding step, be it ligand or virion, especially if ACE2 forms supramolecular aggregates that enhance the chances of successful hits by the viral particle. Clustered ACE2 molecules in complex with MERS-CoV have been experimentally observed.⁶⁵

Transcriptomic RNA-Seq analyses have shown that respiratory epithelial cells express ACE2 and TMPRSS2, the transmembrane serine protease SS 2 required for viral S protein activation, albeit in variable amounts.^{3,48,49,59} The situation differs in the olfactory region of the nasal mucosa, where only TMPRSS2 appears to be present in both immature olfactory neurons and non-neuronal cells in mice.^{66,67} In the latter study, essentially all (98.9%) olfactory receptor genes were found to be expressed in mature olfactory sensory neurons.

An RNA-Seq study found that goblet, basal, and ciliated cells in the respiratory region of the nasal mucosa (Figure 1) express high levels of the ACE2 and TMPRSS2 genes, together with genes involved in innate immune functions and antiviral genes (*IDO1*, *IRAK3*, *NOS2*, *TNFSF10*, *OAS1*, and *MX1*), suggesting that these cell types could serve as entry points for SARS-CoV-2 infection and, not less importantly, viral reservoirs for

dissemination. Highest expression was found in goblet (especially goblet 2 cells) and ciliated cells.⁶⁸ The TMPRSS2 gene was only expressed in a subset of ACE2+ cells, suggesting that the virus might use alternative pathways.⁶⁸ TMPRSS2- cells could instead use cathepsin B/L as a substitute membrane-bound enzyme co-opted as receptor.³ Also applying bulk and single-cell RNA-Seq methods, another study analyzed the cell types present in the olfactory epithelium and olfactory bulb that expressed the ACE2 and TMPRSS2 genes.⁶⁹ Remarkably, neither olfactory sensory neurons nor olfactory bulb neurons express the SARS-CoV-2 host-cell receptors. Instead, the gene coding for the ACE2 protein in the olfactory bulb were only found in the vascular pericytes, the cells involved in maintaining blood pressure regulation and the integrity of the BBB, as well as in olfactory support cells and stem cells. TMPRSS2 was not expressed in the olfactory bulb. The authors surmise that it is the infection of the non-neuronal cells that contributes to the olfactory dysfunction of COVID-19 patients. This opens up the possibility that once the epithelial cell barrier is surpassed, the virions enter local capillaries in the nasal submucosa and make their way into the capillary lumen via the pericytes, rich in ACE2, and the endothelial cell, also rich in ACE2,⁷⁰ to reach general circulation.

An in vitro study using organoids of human airway epithelium found that SARS-CoV-2 readily infected ciliated cells but not goblet cells.⁷¹ These findings contrast with those of another study in which ACE2+TMPRSS2+ gene coexpressing cells were found in nasal goblet secretory cells, AT2 alveolar cells, and ileal absorptive enterocytes. By treating primary human upper airway basal cells with distinct types of inflammatory cytokines, or infecting cells with human influenza virus, the authors further showed that the ACE2+ gene is stimulated by human interferon- α .⁷² In one of the most comprehensive analyses to date, RNA-Seq libraries compiled from ~29 000 single cells from human olfactory neuroepithelium found expression of ACE2 and TMPRSS2 genes in sustentacular cells, and expression of the ACE2 protein alone was observed in a subset of these cells.⁷³ Olfactory sensory neurons showed little or no expression of the two proteins.

A note of caution comes from a study drawing a comparison between the transcriptomic mRNA profile and the immunocytochemical protein expression profile of ACE2 in more than 150 cell types. The highest expression of ACE2 was observed in the intestinal tract, with brain showing no expression, and low or no expression in a subset of cells in the respiratory system.⁵⁷ The study also reanalyzed the transcriptomics profiles of 9 other studies in the literature, confirming both the high expression levels (>60%) in ileal enterocytes and the enrichment of ACE2 in AT2 cells in the pulmonary parenchyma.

Heterogeneity in the expression of ACE2 missense mutants in different ethnic groups was recently reported,⁷⁴ providing another source of variability in risk among COVID-19 patients and the different susceptibility of certain organs to become targets of the disease. RNA-Seq data extracted from more than 4 million human cells by the Human Cell Atlas project identified subsets of respiratory epithelial cells in the nasal passages, airways, and alveoli coexpressing both ACE2 and the protease (ACE2+TMPRSS2+).⁷⁵ Coexpression in enterocytes, corneal epithelial cells, cardiomyocytes, heart pericytes, olfactory sustentacular cells, and renal epithelial cells may provide higher-susceptibility targets for the virus. Furthermore, some of these ACE2+TMPRSS2+ gene-expressing cells were found to share a gene expression program that mediates viral entry and

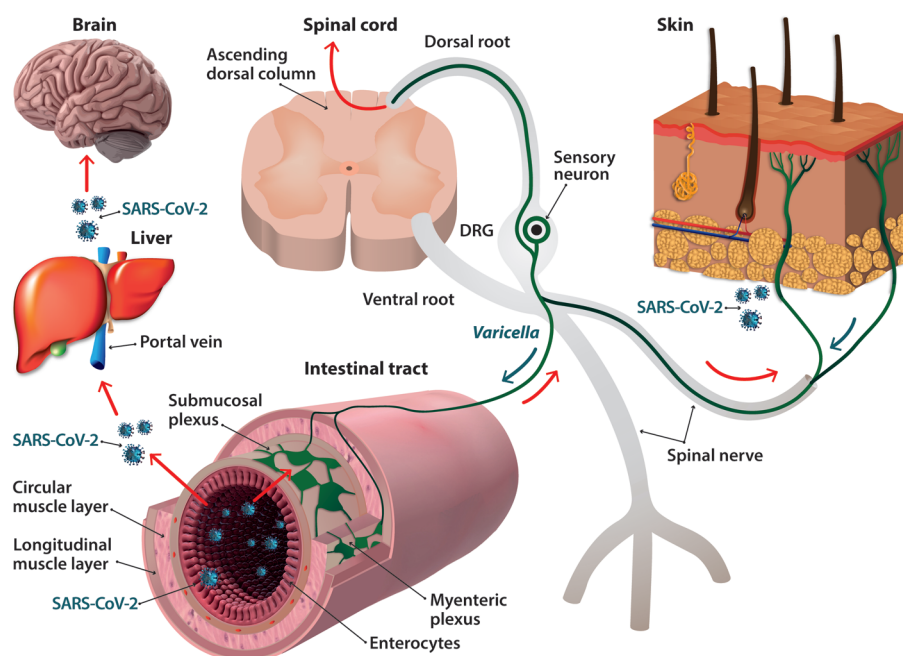


Figure 2. Hypothetical alternative SARS-CoV-2 routes in addition to the ones originating in the nasal mucosa (Figure 1). These routes stem from a common *entry point*: the enterocyte lining the intestinal lumen (bottom left corner of the diagram). Upon enterocyte or para-enterocyte (tight junction) infection, the virus may gain access to (a) the submucosal capillary network of the portal vein system (red arrows at the left bottom of the scheme) to reach the liver and subsequently the brain via a *hematogenous route*. (b) A second hematogenous route, following infection of inflammatory cells in the submucosal connective tissue to eventually reach the CNS via a “Trojan horse” mechanism has been observed with some CoVs in other human respiratory diseases.¹⁰¹ (c) I also suggest the possibility that SARS-CoV-2 may utilize a third neural route resulting from the infection of submucosal plexus and/or myenteric plexus neurons to deliver virions into the CNS via a neuron-to-neuron track¹⁰² following the dorsal root ganglion sensory neuron retrograde path (see main text).

immune functions, like genes coding for IL-6, its receptor IL-6R, and coreceptor IL-1R.⁷⁵ In children, T2 inflammation and interferon response to respiratory viruses upregulate the *ACE2* and *TMPRSS2* genes through other interleukins such as IL-13.⁷⁶ However, interferon differentially increases only *ACE2* in asthmatic patients,⁷⁷ highlighting the variability of interleukin regulation of gene expression.

A recent immunocytochemical study found expression of *ACE2* in the motile cilia of the respiratory tract epithelia and demonstrated that among various factors, comorbidities modify the expression of the enzyme.⁷⁸ One such comorbidity is smoking, a habit that alters the morphology of cilia and mucociliary clearance.⁷⁹ Using reverse genetics, a green fluorescent protein (GFP)-reporter virus was used as a probe to investigate SARS-CoV-2 pathogenesis along the respiratory tract. The highest expression was found in ciliated epithelial cells of the nasal mucosa with decreasing expression along the lower tract, paralleled by a similar pattern in viral infectivity in cell cultures; in pulmonary tissue, ciliated cells and AT2 pneumocytes showed expression, though much lower than in nasal mucosa.⁸⁰ Mice placed for 5 months under regimes of exposure to cigarette smoke showed a dose-dependent increase in pulmonary *ACE2* levels, up to ~80% higher in animals subjected to the maximal dose; a similar dose-dependent expression was observed in humans, with an associated expansion of the number of *ACE2*-rich mucous-secreting goblet cells.⁸¹

One remarkable aspect of the COVID-19 pandemic is that right from its outbreak it triggered a wide variety of studies conducted at an accelerated pace and focused on different aspects of the disease, from the clinical to the more basic extremes of the research spectrum. The transcriptomics approaches discussed in this section reflect the contribution of

the latter, with the converging expertise of high-throughput next generation sequencing (NGS) RNA-Seq techniques, specialized *ad hoc* software technology, in silico analyses of large library data banks, and single-cell cytology approaches to map the static cellular transcriptome. The implementation of these approaches has led to the rapid identification of the phenotypic distribution of the host-cell receptor of SARS-CoV-2, the metalloenzyme *ACE2*, in epithelial mucosae, and the coreceptor protease *TMPRSS2*. A most interesting outcome is the heterogeneous distribution revealed by the single-cell analyses and not apparent (or averaged) in population-level studies, depicting subsets of epithelial cellular phenotypes. These transcriptional signatures may prove important in understanding the susceptibility of certain cells to infection, virion-mammalian cell interactions, the characteristics of the virion replication cycle in different cell types, and in devising prophylactic or therapeutic strategies for this and other viral diseases.

■ ACE2 IN THE CNS

ACE2 is a key element in the anti-inflammatory and hypotensive arm of the renin-angiotensin-aldosterone system (RAAS). An endogenous RAAS is operative in the CNS.^{82–84} This system, which has an important role in the brain, has two branches: the vasoconstrictor and pro-inflammatory renin-angiotensin [1-9]-angiotensin converting enzyme (ACE) branch and the vasodilator and anti-inflammatory *ACE2*-angiotensin [1-7]-Mas receptor arm. *ACE2* enzymatically modifies the vasoconstrictor peptides angiotensin II and angiotensin I into the vasodilator peptides, Ang [1-7] and Ang [1-9], respectively. Angiotensin [1-7] (Ang[1-7]) is the predominant form in various regions of the brain, including the hypothalamus and amygdala, as well as in

the medulla oblongata.⁸⁵ Ang[1-7] inhibits hypothalamic noradrenergic neurotransmission, reducing inflammation, oxidative stress, and neuronal apoptosis (see literature meta-analysis in ref 86).

Potential sites for SARS-CoV-2 recognition in brain can be inferred from the *ACE2* mRNA distribution in adult human brain. *ACE2* has been found to be highly expressed in the substantia nigra, choroid plexus, ventricles, middle temporal gyrus, posterior cingulate cortex, and olfactory bulb.⁷⁰ The protein is expressed in the cytoplasm of both neuronal and glial cells of human brain, in sympathetic tracts of the brainstem, and in the motor cortex.⁸² Transcriptomic analyses have found *ACE2* to be highly expressed in both excitatory and inhibitory neurons, astrocytes, oligodendrocytes, and endothelial cells.⁷⁰ Interestingly, *ACE2* has also been found in the brainstem cardiorespiratory nuclei,^{83,87} raising the possibility that the direct SARS-CoV-2 attack on these centers is responsible for the atypical form of the acute respiratory distress like-syndrome (ARDS) that characterizes some terminal forms of the disease, now redefined as ARDS-like syndrome in COVID-19 or “CARDS”.^{88,89}

The widespread distribution of the host-cell receptor for SARS-CoV-2 in the CNS can be correlated with the symptomatology of some of the neurological presentations and/or complications of COVID-19. Data from autopsies are still scarce. A most recent study of 32 COVID-19 autopsies showed SARS-CoV-2 RNA in the respiratory and cardiovascular regulatory centers in the medulla oblongata.⁹⁰ Further data are required to fully substantiate the direct attack on the CNS or its involvement as a cause of death, although some cases of severe encephalitis and encephalopathies as neurological complications have been reported.^{91–93}

■ ALTERNATIVE HYPOTHETICAL ROUTES THAT SARS-COV-2 MAY FOLLOW TO REACH THE CNS

Using the nasal mucosae as its *entry point* and primary infection site, SARS-CoV-2 can gain access to the brain parenchyma following the various short-path neural or hematogenous routes that link these mucosae to the anatomically adjacent forebrain across the cribriform plate, as analyzed previously (Figure 1), but additional viral *entry points* need be considered because of their much larger areas, abundance of SARS-CoV-2 receptors and coreceptors, and hence important virion replicative capacity. These are the respiratory system (tract epithelium-to-capillary or alveolar pneumocyte cell-to capillary) leading to virus passage to the pulmonary/general circulation (which will not be dealt with) and the gastrointestinal tract (Figure 2). In addition, I discuss here *hypothetical routes* relating to the latter.

The intestinal (predominantly the small intestine) lumen *entry point* is a monolayer of cylindrical epithelium with a predominant cell phenotype, the enterocyte, covering a surface close to 250 m² in contrast to the ~150 sq. cm of the nasal mucosae. Virus infection proceeds at the apical surface of the enterocyte, covered with microvilli that increase the absorptive area (relative to a flat surface) ~25 times and which concomitantly amplifies the coverage with *ACE2* receptor molecules. The gene coding for this receptor, *ACE2*, is expressed together with its coreceptor, *TMPRSS2* in absorptive enterocytes in human ileum⁷² together with *TMPRSS4*.⁵⁹

Although SARS-CoV-2 internalization is still not fully understood, other CoVs employ both endocytic and non-endocytic mechanisms.⁹⁴ MHV-2, for instance, is internalized by a clathrin-mediated, Eps15-independent mechanism.⁹⁵ SARS-

CoV-2 pseudovirions were recently shown to be internalized in mammalian cells *in vitro* mainly by an endocytic mechanism dependent on the protease cathepsin L, the lysosomal downstream effector pore channel subtype 2 (TPC2), and phosphatidylinositol-3,5-bisphosphate (PI(3,5)P2). The inositol lipid is synthesized by phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) in the early endosome, making this enzyme a potential drug target for SARS-CoV-2 inhibition.⁴⁸ An example of a nonendocytic internalization mechanism is the passage of virions between epithelial intercellular junctions.⁹⁶

Once inside the enterocyte, the virus undergoes its replicative cycle, and virion shedding across the enterocyte basal membrane puts SARS-CoV-2 in contact with the rich and extensive capillary network in the intestinal villi, only tens of microns from the epithelial lining. Pericytes⁶⁹ and endothelial cells, rich in *ACE2*,⁷⁰ offer new targets for the virion to reach the general circulation via the hepatic portal system. This first *hematogenous route* (a) is schematically portrayed in Figure 2. Evidence of gastrointestinal disease is observed in 45% of COVID-19 necropsies.⁹⁷ Adverse pre-existing endothelial conditions as observed in several comorbidities in COVID-19 patients and/or the effects of hyperimmune response syndrome on the endothelial cell bed (cytokine release syndrome)⁹⁸ may lay the ground for capillary dysregulation supporting SARS-CoV-2 infection of the CNS after defeating a weakened BBB (Figure 2).

(b) Having reached the general circulation, SARS-CoV-2 may also employ a second hematogenous route—the so-called “Trojan horse” mechanism—known to be operative for several microbial agents that infect the brain parenchyma,⁹⁹ involving extravasation of inflammatory phagocytic cells (leukocytes, mostly monocytes and lymphocytes) into the meninges and cerebrospinal fluid. This route would follow essentially the same course as (a) above, except that it is a facilitated path, because the capillary network in the outer meningeal space is devoid of tight junctions. Furthermore, the meningeal lymphatic system serving the CNS provides additional pathways from the meningeal space to the brain parenchyma.¹⁰⁰

(c) A third hypothetical *neural route* is that SARS-CoV-2 virions, after surpassing the enteric epithelial wall, could directly infect neuronal cells of the submucosal or myenteric plexus and through neuron-to-neuron transport¹⁰² and also the sympathetic neuron of the dorsal root ganglion, as shown in Figure 2. The viruses would then be able to centripetally reach the CNS via the spinal cord, subsequently propagating either from neuron to neuron synaptically or crossing the blood–cerebrospinal fluid barrier and the choroid plexus, again bypassing the BBB using the Trojan horse mechanism described above. Other neurotropic viruses such as varicella zoster virus¹⁰³ employ the DRG sympathetic neuron bidirectionally to reach the CNS and the peripheral sensory nerve endings. Bulk RNA transcriptomic analyses have shown expression of the *ACE2* gene in human DRG neuronal cells^{104,105}. Human DRG neurons express *MRGPRD* and *Nppb* genes, the former of which is selectively expressed in a subset of nociceptive receptors that forms peripheral nerve endings in colon¹⁰⁶ or meninges¹⁰⁷ together with the *ACE2* gene.¹⁰⁵ The painful peripheral neuropathies observed in some COVID-19 patients could be associated with interferon-1-induced hyperexcitability of DRG neurons resulting from viral infection.¹⁰⁸ A similar exacerbated immune response may also account for the peripheral vascular inflammatory reactions observed in the multisystem Kawasaki-like syndrome that affects some COVID-19 patients,^{109,110} particularly children. Dermatological manifestations of COVID-

19 such as maculopapular exanthem present in ~36% of patients¹¹¹ could also progress from the DRG neuron to the skin in an anterograde fashion, as schematically shown in the bottom right portion of Figure 2 (red arrow). For all these reasons, the intestinal mucosa is proposed to be a preferred entry point, major viral reservoir, and favored starting point for neurotropic routes.

CONCLUDING REMARKS

The distribution and abundance of the ACE2 molecule in different cells dictate the tropism of the virus and probably the viral load in each target surface. In the first part of the review, I analyzed the “cellular cartography” of the receptor molecule in the various cell phenotypes of the nasal and intestinal mucosae based on RNA-Seq analysis. Although there is as yet no universal consensus, the topography and abundance of ACE2 and SARS-CoV-2 coreceptor protein, TMPRSS2, and experiments in animal models raise the possibility that the most likely site of peripheral lesion associated with the dysosmias in COVID-19 is the sustentacular cell, a non-neuronal epithelial cell. Effects on the CNS via the nasal mucosa cannot be discarded, and the ACE2 transcriptomics show a remarkably wide distribution of the receptor in many regions of the brain.

The second part of the review dissects hypothetical routes to the CNS and other targets stemming from a much larger receptive region, the apical plasmalemma of the intestinal epithelial cell, the enterocyte. The intestinal mucosa is proposed to be a preferred entry point, major viral reservoir, and favored starting point for neurotropic routes that could be used by SARS-CoV-2 upon binding to ACE2 and fusing to the enterocyte's apical membrane with the aid of TMPRSS2 and TMRSS4. Examples of the correlation between these postulated routes and some clinical manifestations of COVID-19 are provided.

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Author Contributions

I conceived and designed the study, searched the literature, interpreted the data, and wrote the manuscript. I conceived the illustrations and had help to produce them.

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