SARS-CoV-2 receptor and entry genes are expressed by sustentacular cells in the human olfactory neuroepithelium

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Abstract

The recent emergence of the pathogenic SARS-CoV-2 initiated a worldwide health crisis. The entry of the virus into cells is mediated by the binding of the viral Spike protein to the angiotensin-converting enzyme-2 (ACE2), followed by its priming by the TMPRSS2 serine protease, both present on the cellular membrane of the target cells. In the respiratory tract, these targets are ciliated cells. Interestingly, various reports indicate an association between SARS-CoV-2 infection and anosmia, suggesting an alteration not restricted to the respiratory tissue, but that might also include the olfactory sensory epithelium. We explored this possibility by generating RNA-seq libraries from human neuroepithelium, in which we found significant expression of ACE2 and TMPRSS2. To determine whether specific cell types of this chemosensory tissue may coexpress both of the virus entry genes, we analyzed a scRNA-seq dataset. We determined that sustentacular cells, which are in direct contact with the external world and maintain the integrity of olfactory sensory neurons, represents a prime candidate for SARS-CoV-2 infection via the nose, and possibly for SARS-CoV-2-induced anosmia.

Introduction

A novel virus from the Coronaviridae family, termed SARS-CoV-2, which emerged in December 2019 in East Asia, is currently expanding on the planet. Its exact history is unknown, but its genomic sequence suggests that it was transmitted from bats to humans via an intermediate animal host[1]. It is now transmitted from human-to-human.[2] Infection by this RNA virus is associated with a severe respiratory syndrome called COVID-19, and is characterized by a significant mortality rate[1,3,4].

Entry of SARS-CoV-2 into target cells depends on the Spike protein (S), present on the virus capsid[5]. Viral attachment involves an interaction between S and the angiotensin-converting enzyme-2 (ACE2) on the surface of the target cell, and is followed by the priming of S by the cellular serine protease TMPRSS2, also attached to the cellular membrane, which eventually leads to the fusion between the cellular and the viral membranes[1,6]. Expectedly, the main targets of SARS-CoV-2, respiratory cells that line the respiratory airways and are infected by SARS-CoV-2, coexpress *ACE2* and *TMPRSS2*[7].

Multiple anecdotal reports, both from SARS-CoV-2-infected patients and medical staff, suggest an association between viral infection and alterations of olfactory perception, alterations ranging from mild perturbations to complete, although reversible anosmia[8-10]. The mammalian nasal cavity can be divided into two areas, the respiratory and the olfactory areas, that are anatomically, cellularly, and functionally different[11]. In humans, the respiratory part covers the major part of the nasal cavity. It includes the turbinates and is lined with a ciliated pseudostratified columnar epithelium. Its function is to humidify, cool or warm the incoming air, and to trap small particles before they get into the lungs. The second nasal area corresponds to the olfactory neuroepithelium. In our species, it is located in the very dorsal part of the cavity. There, it contacts chemicals entering the nose, an interaction which represents the first step in the process that leads to the identification of a given smell. This epithelium is pseudostratified, and includes Bowman's glands, olfactory sensory neurons, sustentacular cells, microvillar cells, globose and horizontal basal cells (cells that keep dividing during adult life and replenish the pool of sensory neurons)[12]. Each sensory neuron extends a dendrite that ends in multiple and long specialized ciliae, which are covered with odorant receptors and bath in the mucus that lines the nasal cavity. Olfactory sensory neuron dendrites are enwrapped inside specialized cells, the sustentacular cells[13], whose nuclei and cell bodies line the external layer of the thick neuroepithelium (although they remain attached to the basal lamina). The role played by the latter in maintaining the integrity and function of the neuroepithelium is critical, in a very similar way that Sertoli cells support germ cell development and survival. Indeed, the presence of various drugs (such as 3-methylindole[14], the anti-thyroid drug methimazole[15], or nickel sulfate[16]) to which sustentacular cells are quickly and very sensitive, leads to transient anosmia.

Whether the apparent olfactory dysfunction associated with SARS-CoV-2 infection results from a general inflammation of the nasal cavity or from a more direct perturbation of the olfactory neuroepithelium is unknown. In any case, it is critical to determine whether this virus disposes of a niche to replicate just under the cribriform

plate of the ethmoid bone, a structure with large holes through which olfactory neuron axonal projections directly contact the olfactory bulb and offer a gateway to the brain.

We asked whether specific cells present in the human olfactory neuroepithelium may represent targets to SARS-CoV-2, by looking at the molecular players involved in infection. The coexpression of both *ACE2* and *TMPRSS2* in the same cell types would certainly make them prime candidates for infection. To explore this question, we first collected and analyzed the transcriptome of human olfactory biopsies from sensory olfactory tissue and from nasal respiratory epithelia. We found expression of both *ACE2* and *TMPRSS2* in nasal respiratory tissue, as expected, but also in olfactory neuroepithelia. We then used a publicly available human scRNA-seq dataset to precisely identify the cell types expressing these two genes. We found coexpression of both *ACE2* and *TMPRSS2* in olfactory horizontal basal cells, microvillar cells, Bowman's glands, but more importantly - because they are numerous and in direct contact with the outside world - in olfactory sustentacular cells. Finally, we investigated by using publicly available mouse and human brain scRNA-seq data, the presence of cells in the central nervous system; we found no cell type expressing both *ACE2* and *TMPRSS2,* or their orthologs, in the brain.

Results

With the aim of exploring the potential expression of *ACE2* and *TMPRSS2* in olfactory sensory tissue, we collected biopsies via nasal endoscopic surgery from 4 adult patients. Samples of both nasal respiratory epithelium and olfactory sensory epithelium were harvested. RNA was extracted, libraries generated, and sequenced.

The number of transcripts were evaluated for olfactory-specific genes (Figure 1 A-G), including *CNGA2* and *ANO2*, both encoding critical elements of the olfactory transduction cascade, *OMP*, corresponding to a specific marker of mature olfactory sensory neurons, three odorant receptor genes, *OR2AG1*, *OR5AN1* and *OR2T10*, and a marker of olfactory sustentacular cells (*ERMN*). As expected, olfactory epithelium markers displayed a significant difference in transcript amounts between both tissues (Figure 1A-G, T test on pooled scaled mean expression: p-value=5e-15). The presence of potential *ACE2* and *TMPRSS2* transcripts was then evaluated. We observed a mean of 0.8 and 63.7 TPMs in the respiratory epithelium samples for *ACE2* and *TMPRSS2* respectively (Figure 1 G,H), reflecting the presence of ciliated cells which represent targets of SARS-CoV-2. We found a mean of 0.6 and 76.1 TPMs in the sensory neuroepithelium samples for *ACE2* and *TMPRSS2* respectively (Figure 1H,I), indicating the presence in this tissue of cells that may either express both genes, or of a mix of cells that express either *TMPRSS2* or *ACE2*.

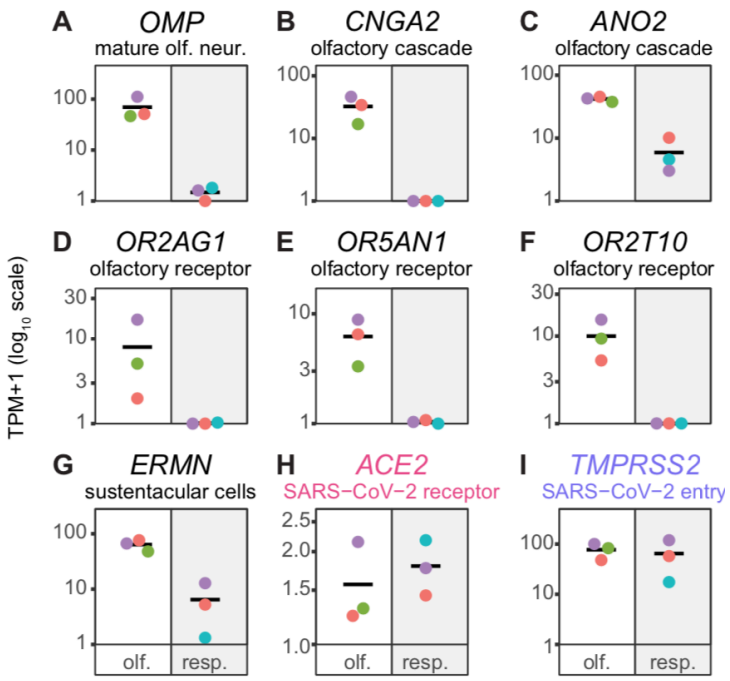

Figure 1

***ACE2* and *TMPRSS2* expression in olfactory and respiratory nasal epithelia.** (**A-G**) Transcript quantifications corresponding to olfactory sensory epithelium markers and (**H** and **I**) SARS-CoV-2 entry

proteins in olfactory and respiratory biopsies. A different color was attributed to the data of each patient (n=4). Olfactory and respiratory sample data points are shown on a white and grey background respectively.

To identify putative viral targets transcribing both *TMPRSS2* and *ACE2* in the neuroepithelium, we took advantage of a very recently published dataset reported by Durante et al[17]. This dataset contains the transcriptome of 28'726 single cells, collected during nasal endoscopic surgery of 4 adult patients. Prior to any cell type analysis, we monitored the existence of cells that would transcribe both *TMPRSS2* and *ACE2* (Figure 2A). We then performed an aggregate analysis of the 28'726 single cells, and generated a UMAP dimensionality reduction plot, which allowed to display the clustering of the 26 different cell types reported in the original publication[17], including olfactory, respiratory and immune cells (Figure 2B). We then identified all cells expressing *ACE2*, *TMPRSS2, ERMN* (an olfactory sustentacular cell marker) *and GSTA2* (a marker of respiratory ciliated and epithelial cells) (Figure 2C), or those coexpressing both *TMPRSS2* and *ACE2* (Figure 2D). Olfactory sensory neurons showed little or no expression of either *TMPRSS2* or *ACE2* (Figure 2C). In contrast, and expectedly, a clear cluster of cells corresponding to respiratory ciliated cells (Figure 2C,D), expressed both *TMPRSS2* and *ACE2*. More interestingly, individual cells pertaining to the olfactory neuroepithelium, including sustentacular cells (Figure 2C), did also express both *TMPRSS2* and *ACE2*. To better evaluate the coexpression levels of *TMPRSS2* and *ACE2* in the different cellular populations, we plotted the mean expression of *TMPRSS2* and *ACE2* corresponding to each of these subpopulations (Figure 2 E). Sustentacular cells and ciliated respiratory cells exhibited the highest levels of expression of both genes. Populations containing olfactory horizontal cells, microvillar cells and Bowman's glands also showed transcription of both gene, although at lower levels. Finally, to better evaluate the different expression levels inside the two cell types expressing the highest levels of both *TMPRSS2* and *ACE2*, we plotted the distribution of transcripts across all cells of both cell types (Figure 2 F,G).

Respiratory cells are thus not the only ones in contact with the outside world that exhibit the molecular keys involved in SARS-CoV-2 entry in the nose. Sustentacular cells, which are localized at a porous boundary between the central nervous system and the olfactory cavity, share the same characteristics.

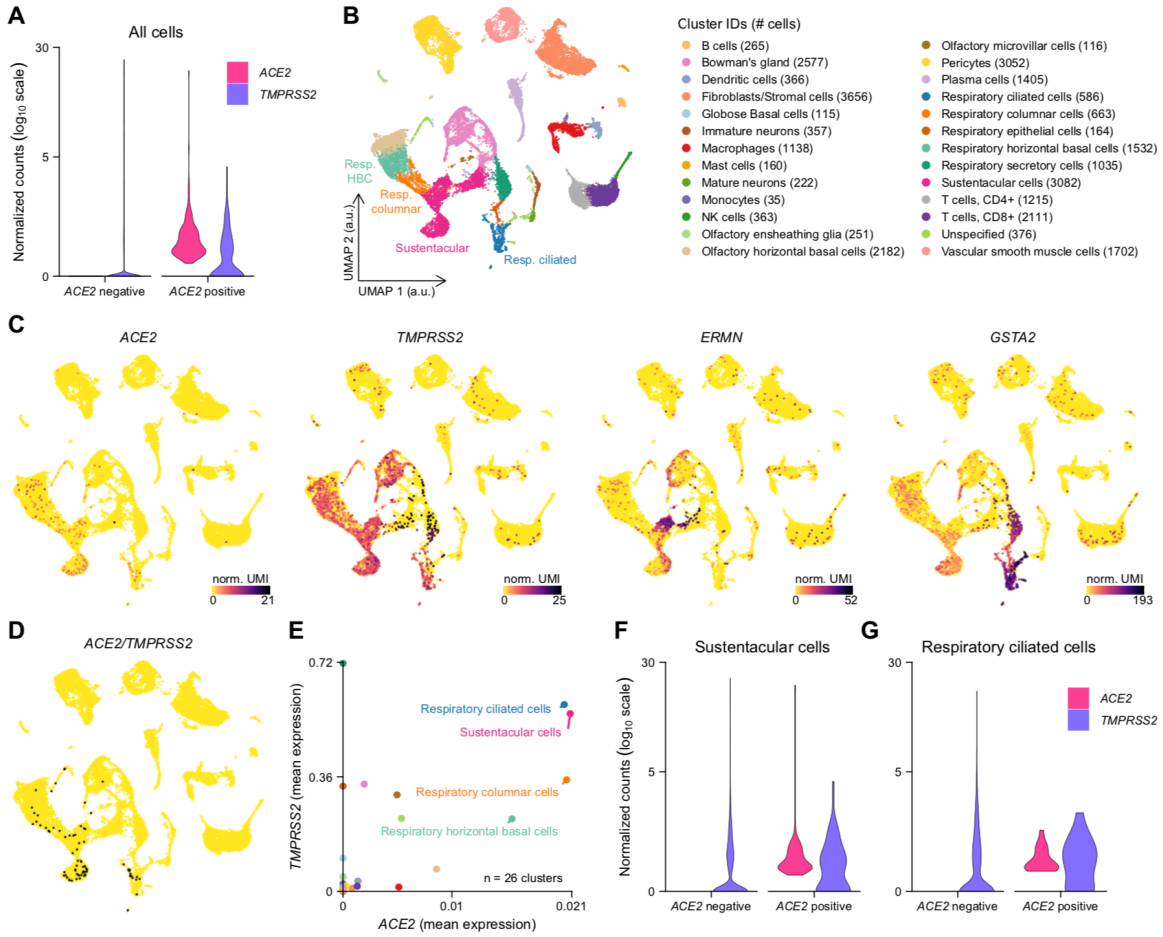

Figure 2

Co-expression of *ACE2* and *TMPRSS2* in sustentacular and respiratory ciliated cells. (**A**) Violin plots displaying *ACE2* and *TMPRSS2* normalized expression levels in all *ACE2* positive and *ACE2* negative cells. (**B**) Visualization of the clustering results reported in Durante et al.[17] on the UMAP plot. (**C**) Representation of *ACE2*, *TMPRSS2*, *ERMN* (a sustentacular cell gene marker) and *GSTA2* (a respiratory ciliated cell gene marker) normalized expression levels on the UMAP plot. (**D**) *ACE2* and *TMPRSS2* co-expressing cells, highlighted in black on the UMAP plot. (**E**) Coexpression of *ACE2* and *TMPRSS2* in the different cell clusters (means of *ACE2* and *TMPRSS2* normalized expression levels are plotted). (**F-G**) Violin plots displaying *ACE2* and *TMPRSS2* expression levels in *ACE2* positive and *ACE2* negative sustentacular cells (E) and respiratory ciliated cells (**G**). Color gradient scale in panel C and y-axis scale in panels A, F and G are in $\log_{10}$ scale.

SARS-CoV, which genome is closely related to the one of SARS-CoV-2 and whose effects on human tissues appear similar to those observed with SARS-CoV-2, has been observed in human brains[18-20]. Could SARS-CoV-2 replicate in the olfactory epithelium and could the infection spread to the brain? To explore the potential receptivity to SARS-CoV-2 infection of the various cell types in the brain, we evaluated, again, the potential coexpression of *ACE2* and *TMPRSS2* in neuronal and non-neuronal cell types in the central nervous system. We took advantage of the availability of three available datasets[21-23], corresponding to a collection of human brain neuron types and to two broader collections of mouse brain cell types. In the Tasic et al. dataset[23], which is restricted to human neurons, we found no expression of either *ACE2* or *TMPRSS2* (data not shown). In the first collection of mouse cell types

published by Zeisel et al[21], that include non-neuronal cells, we found a very limited number of cell types that express *Ace2* (Figure 3A). These cells are related to pericytes and coexpress the mural cell marker *Rgs5*. They express relatively low levels of *Ace2* transcripts, with a maximum of 0.28 normalized UMI counts in the pericytes cluster PER3, compared to 7.39 for *Rgs5*, a pericyte marker (Figure 3 B). *Tmprss2*, whose transcripts were barely detected, is expressed by even less cell types, with a maximum of 0.04 normalized UMI counts in Purkinje cells (not shown in the figure). These observations were confirmed by the second mouse dataset[22], in which mural cells were the main cell type that expressed *Ace2* (Figure 3 C). None of these cell populations coexpressed *Tmprss2*. The brain therefore does not appear a very promising target for the virus, even in the case of an infection/replication niche located in close proximity.

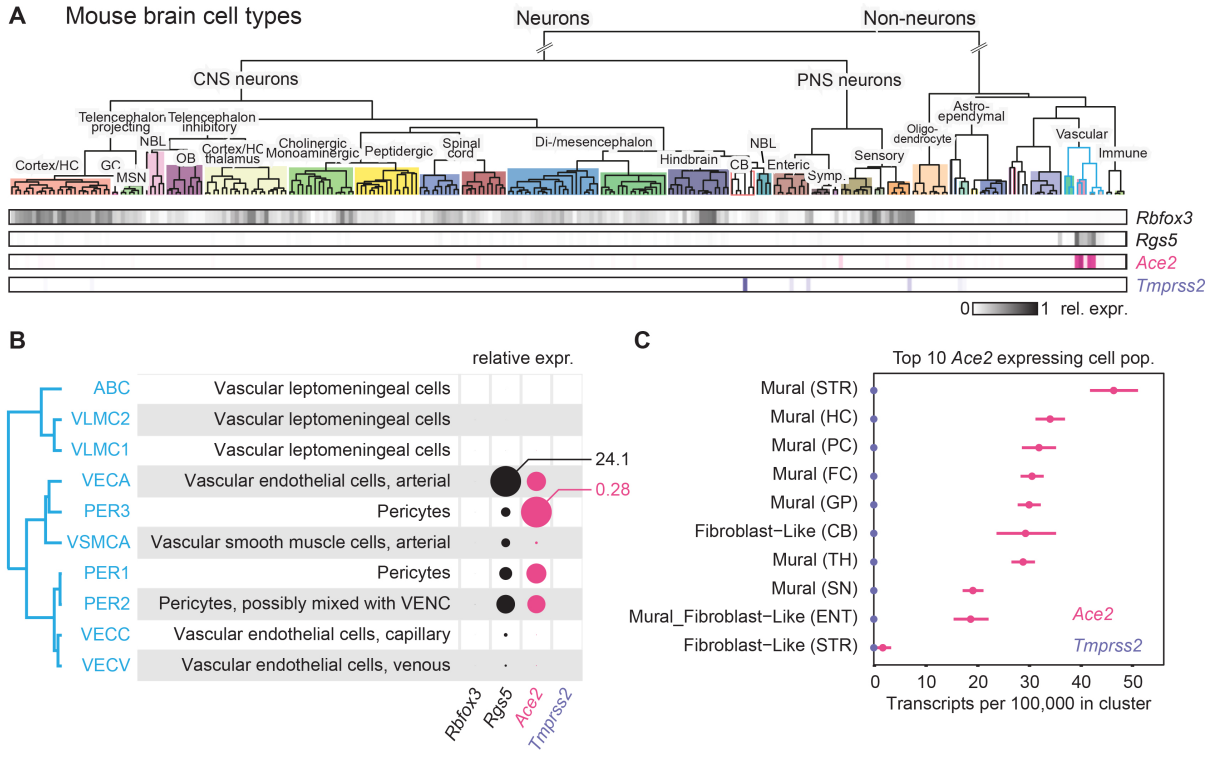

Figure 3

***Ace2* and *Tmprss2* expression in the mouse brain.** (**A**) Mouse brain cell type classification from Zeisel et al.[21] were visualized via http://mousebrain.org/. Rows with shaded boxes below the tree indicate the gene mean expression levels for each cell type, relative to its maximum. *Rbfox3*, encoding the neuronal marker NEUN, and *Rgs5,* a marker of pericyte cells*,* are shown in comparison with *Ace2* and *Tmprss2*. (**B**) Detail of the gene expression levels in pericyte-related cells (blue edges clade of the classification dendrogram in (**A**)). Scaled blue circles represent mean relative expression levels. Maximum values of mean normalized expression levels for *Rgs5* and *Ace2* are connected to the corresponding circles. (**C**) *Ace2* and *Tmprss2* expression levels in the top 10 *Ace2*-expressing cell populations from the http://dropviz.org/ dataset[22]. Abbreviations in parentheses denote the origin brain structure (STR: striatum, HC: hippocampus, PC: posterior cortex, FC: frontal cortex, GP: globus pallidus, CB: cerebellum, TH: thalamus, SN: substantia nigra, ENT: entopeduncular).

Discussion

By taking a combined approach, based both on our own RNA-seq data and on published scRNA-seq datasets, we demonstrate that olfactory sustentacular cells and other non-neuronal cell types in the olfactory neuroepithelium, but not olfactory sensory neurons, coexpress *TMPRSS2* and *ACE2*, the two key players in the binding and entry of the SARS-CoV-2 into human cells.

A fair question is to ask how common *TMPRSS2* and *ACE2* coexpression is in human cells. A first answer, although limited to the 26 nose cell types analyzed here, is provided in Figure 2 A: not often. We also looked at various other human cell types using available scRNA-seq datasets, corresponding to various organs (data not shown) and in particular to neuronal tissue (Figure 3), and found *TMPRSS2* and *ACE2* coexpression to be a very rare characteristic.

Naturally, much is still to be learned about the players involved in SARS-CoV-2 infection of human cells. Thus, although *ACE2* and *TMPRSS2* are currently believed to represent the major players during cell entry, other molecules, such as CD147 in particular, may also play a significant role [24].

How likely is it that the coexpression of *TMPRSS2* and *ACE2* in olfactory sustentacular cells is at the origin of the SARS-CoV-2-induced anosmia? And why are horizontal basal cells that also coexpress *TMPRSS2* and *ACE2* not prime candidates? First, given the slow rate of neuronal renewal in the main olfactory epithelium and the apparent rapid development of SARS-CoV-2-triggered anosmia, a perturbation of the stem cell pool constituted by the horizontal basal cells appears unlikely to represent an immediate disturbance to the system. Second, the significant amounts of *TMPRSS2* and *ACE2* transcripts we observed in sustentacular cells are in the same range as the one observed in respiratory ciliated cells, suggesting at least that sustentacular cells play in the same league in terms of SARS-CoV-2 receptors. Third, sustentacular cells are in direct contact with the olfactory cavity, and thus with anything that enters the nose. Finally, a known and critical role is played by sustentacular cells in the maintenance of the olfactory neuroepithelium integrity: if they die, the whole neuroepithelium disaggregates, leading to anosmia. Taken together, and despite the fact that one cannot exclude inflammation and the infection of other non-neuronal cell types in the olfactory neuroepithelium as an origin of the SARS-CoV-2-induced anosmia, the link between the viral molecular entry tools expressed by olfactory sustentacular cells and the chemosensory alteration appears quite credible. Naturally, a next step would be the study of the olfactory epithelium of a mouse model expressing the human *ACE2/TMPRSS2* in sustentacular cells, and nasal biopsies of human olfactory neuroepithelia just after SARS-CoV-2 infection.

The idea that viruses may affect directly or indirectly the integrity and function of the sensory part of the olfactory system is not new. Viruses may indeed affect the neuroepithelium in different ways. They often alter specific cell types, including neurons. For example, olfactory sensory neurons and sustentacular cells were shown to be major portals of host entry of the Murid Herpesvirus-4 (MuHV-4)[25]. Again, the direct contact of this latter cell type with the respiratory tract, as well as its phagocytic activity, may explain its particular sensitivity to viral infection[26]. The cell type targeted

by viruses matters, in particular to fight the infection. For example, in the case of a MuHV-4 challenge, olfactory sensory neurons are not responsive to interferon treatment (IFNγ), while sustentacular cells are[27].

Finally, SARS-CoV-2-infection has not only been linked to the loss of a single chemosensory ability. Indeed, in addition to anosmia, ageusia - that is loss of taste - has been reported[8]. Whether these reports truly reflect taste anomalies, or rather olfactory perturbations that may drastically affect the flavor of food, is unclear. However, this potential double effect on two chemosensory systems that share nothing at the periphery may suggest a more central alteration, involving for example a direct infection of the brain by SARS-CoV-2. Again, such viral migration to the central nervous system, and in particular from the olfactory neuroepithelium, would not be a first. SARS-CoV particles (that is the virus responsible for the previous SARS epidemic in 2002) have in fact been found in the human brain[18-20]. Adding to this idea is the observation that in a transgenic mouse model expressing the human *ACE2* and infected with SARS-CoV, brain lesions were observed. Moreover, a retrospective case study on 214 COVID-19 patients reported neurological manifestations possibly correlated with the severity of the disease (with the confounding factor that old people are more likely to be very sick)[28]. However, in the present work we did not identify neuronal or non-neuronal cell types in the mouse or human brain that coexpressed the orthologs of the human *ACE2* and *TMPRSS2*. This lack of coexpression is also true for human olfactory sensory neurons, as shown in this work. Moreover, significant brain damage in humans seems quite unlikely or at least rare, since no specific cognitive or other brain-related deficits resulting directly from SARS-CoV-2 infection in the central nervous system were reported.

Materials and Methods

Human biopsies and bulk RNA-sequencing

After authorization from the University of Geneva ethics commission, human biopsies were collected at the Geneva University Hospital. Tissues of 4 individuals (3 males and 1 females) were resected during nasal cavity surgeries under general anesthesia. Biopsies consisted in small pieces of tissue of approximately 3 mm in diameter, from the respiratory or the sensory epithelia. Biopsies were snap frozen in liquid nitrogen immediately upon collection. For RNA extraction, tissues were placed in RLT Buffer (Qiagen) with beta-mercaptoethanol and lysed with stainless steel balls (5mm diameter, Schieritz and Huenstein AG) using a homogenizer (Big Prep, MB Biomedicals). Total RNA was isolated using RNeasy Mini Kit (Qiagen) following the manufacturer protocol. The quality and quantity of total RNA were evaluated with a Bioanalyzer (Agilent). Stranded cDNA libraries with suitable adapters for multiplexing were generated with Truseq RNA and DNA sample preparation kits (Illumina) following ribodepletion of the total RNA (200ng of total RNA per sample). Samples were multiplexed for sequencing in a HiSeq®2500 Sequencing system, generating 100bp single-end reads.

Bulk RNA-sequencing data analysis

RNA-seq reads were mapped onto the GRCh38 human genome assembly with STAR[29] version 2.7.0a using the Ensembl v99 gene annotation file (GTF). Mutlimapped reads were filtered out with the option --outFilterMultimapNmax set to 1. Gene expression was quantified using featureCounts[30] version 1.6.3 with the default parameter values and the aforementioned GTF file. TPM values were calculated for each gene within each sample to normalize for the sequencing depth. For Figure 1, olfactory cascade genes were selected for their known expression specificity in the olfactory neurosensory epithelium, and 3 OR genes were arbitrarily selected amongst the top 25 most expressed OR genes. ERMN was selected as a marker of sustentacular cells[13,31].

Single-cell RNA-sequencing data analysis

Cell and gene filtering: The processed 10X Genomics output files of all four patients of the single-cell RNA-seq dataset reported in Durante et al.[17] were downloaded from the NCBI GEO database with the accession number GSE139522. The data was analyzed in R version 3.5.0 using the Seurat R package version 3.1.4[32-34] following the indications described in Durante et al.[17] and using custom scripts. Briefly, the 10X files were loaded into R using the Read10X function of Seurat. A single Seurat object for all four patients was created using the CreateSeuratObject function of Seurat. At this step, genes not expressed in at least 3 cells at a threshold of a minimum of 1 UMI count were excluded from the analysis. The percentage of mitochondrial counts was then calculated for each cell using the PercentageFeatureSet function of Seurat. Cells

were removed from the analysis if they had less than 400 detected UMI counts, expressed less than 100 or more than 6000 genes, and if their mitochondrial counts exceed 10% of their total counts. This filtering resulted in retaining 28,726 cells and 26,439 genes.

Dataset integration: The standard Seurat version 3 integration workflow was used to integrate the data from all patients, as described in Durante et al.[17] First, the data from each patient was separated using the SplitObject function of Seurat and their raw gene UMI counts were normalized by the total number of counts per cell, scaled to 10^4 and log-transformed using the NormalizeData function of Seurat (normalization.method = "LogNormalize"; scale.factor = 10000). The top 5000 variable genes were then selected using the variance stabilizing transformation method of the FindVariableFeatures function of Seurat (selection.method = "vst"; nfeatures = 5000). To assemble all datasets, integration anchor genes across the four datasets were identified using the first 30 dimensions of the canonical correlation analysis and 5000 genes with the FindIntegrationAnchors function of Seurat (reduction = "cca"; dims = 1:30; anchor.features = 5000). The IntegrateData function of Seurat was then used to integrate all four datasets into a single Seurat object.

UMAP plot generation: To generate a UMAP plot[35,36], the integrated data was scaled using the ScaleData function of Seurat and the first 30 principle components were computed using the RunPCA function of Seurat. These 30 principle components were then used as input to the RunUMAP function of Seurat. The clusters identified by Durante et al.[17] were displayed on the UMAP plot (Figure 2B) and were used for subsequent analyses.

Gene expression analysis and plotting: All gene expression analyses were performed using the actual normalized counts (not log-transformed) from the non-integrated assay of the Seurat object, but the scales of the axes in the plots are in $\log_{10}$ after adding a pseudocount of 1 (except for Figure 2E). *ACE2* and *TMPRSS2* co-expressing cells were identified as expressing at least 1 UMI count of both genes. Other R packages used for data analysis and plotting include Matrix version 1.2.14, reshape2 version 1.4.3, dplyr version 0.8.0.1, ggplot2 version 3.2.0, patchwork version 0.0.1, cowplot version 0.9.3, grid version 3.5.0; extrafont version 0.17, viridis version 0.5.1, RColorBrewer version 1.1.2 and lemon version 0.4.1.

Brain cell database mining

For mouse brain single-cell transcriptomes, the datasets of Zeisel et al.[21] (Figure 3 A and B) and Sauders et al.[22] (Figure 3 C) were consulted on their respective websites: http://mousebrain.org/ and http://dropviz.org/. For human brain cells, we searched for *ACE2* and *TMPRSS2* expression in the Allen Brain Atlas single-nucleus RNA-seq dataset of the human brain[23], available online at https://portal.brain-map.org/atlases-and-data/rnaseq.

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While in the process of submitting the present work, another article was published by Brann et al.[37] in BioRxiv on the same topic, that is on the existence of targets molecularly compatible with efficient SARS-CoV-2 infection in the olfactory neuroepithelium; it reports findings similar, although not identical, to ours.

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