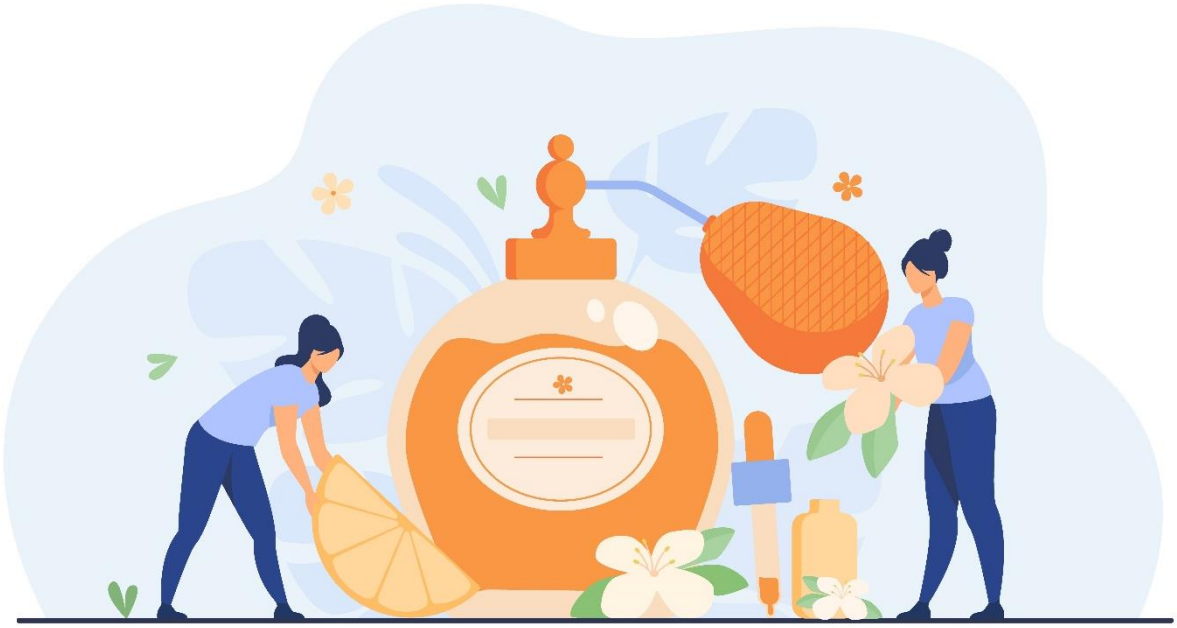
Pathophysiology of olfactory dysfunction as a result of COVID-19.



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Abstract

Olfactory dysfunction is a symptom in approximately 80% of COVID-19 patients. 25% of them suffer from smell loss for more than a month. Disordered smell can cause discomfort related to personal hygiene, loss of interest in or enjoyment of food and drinks and risks such as not being able to detect fire or inedible food. Ultimately, these are factors that contribute to symptoms of depression and nutritional issues which often results in reduced quality of life. COVID-19 is a novel disease, and the underlying mechanism of the olfactory dysfunction accompanying it is still unknown. In this review, the current hypotheses about the pathophysiology on a cellular level will be elucidated. These include swelling of the olfactory system, viral entry pathways, infection of olfactory epithelium cells, the involvement of the olfactory bulb, inflammation reaction, serotonin and tryptophan involvement, viral deposition, self-defense mechanisms, regeneration, and olfactory receptors. As a result of a wide variety of possible causes, difficulties comparing between studies and the intricate interplay between many tissues, immune system, and the neuronal system, drawing conclusions is still premature. Comparison issues are due to differing animal models and techniques, and small sample sizes. From current research, it seems most likely that non-neuronal cells in the olfactory epithelium are prone to infection with SARS-CoV-2. This seems due to the presence of ACE2 receptors alongside other entry molecules such as TMPRSS2, cathepsin L, furin and neuropilin-1. The disbalance that follows in combination with inflammation reactions, cause the olfactory sensory neurons to lose their ability to function properly. This in turn leads to disordered smell and disbalance in the olfactory bulb. Severe infection of the olfactory epithelium and its precursor and stem cells seems to be the determining factor in prolonged loss of smell and slow regeneration. Besides this main hypothesis, there are alternative hypotheses that are not being investigated to the same degree. These are not proven untrue yet, so should be examined still. In conclusion, more and especially more structured research into these mechanisms is needed to draw reliable conclusions about the underlying mechanisms of olfactory dysfunction due to COVID-19.

Keywords: COVID-19, olfaction, smell loss, pathophysiology, anosmia.

Layman's summary

Loss or change of the ability to smell is a symptom in about 80% of COVID-19 patients. Approximately 25% of these people suffer from it for more than a month. Smell loss can cause problems such as insecurities about personal hygiene, loss of interest in food and drinks and risks such as not detecting fire or inedible food. These are factors that contribute to symptoms of depression and nutritional issues which often results in reduced quality of life. COVID-19 is still a novel disease and because it is so complex, the underlying mechanism is still unknown. In this review, the current hypotheses about the causes of smell loss and cellular mechanisms behind it will be discussed. We will discuss the possible swelling of the olfactory system, viral entry possibilities, which cells are infected, how the olfactory bulb is affected, inflammatory reactions, olfactory receptors, and alternative hypotheses. There are many possible mechanisms that can cause the smell loss in COVID-19 patients. As well as multiple hypotheses, there are multiple animal models possible to do research with, such as mice and hamsters, with the possibility of genetic modifications. Besides, there are numerous ways to measure infection, inflammation, expression of receptors and signs of cell death. Taken together, because of the differences between studies, it is difficult to draw reliable conclusions from the research done until now. Current studies show that it is most likely that the neurons in the nose mucosa and in the olfactory bulb are not infected themselves. Rather, it seems like their surrounding, supporting cells are infected with SARS-CoV-2. They are infected because of the entry molecules, such as ACE2, TMPRSS2, cathepsin L, furin and neuropilin-1, they possess. As the surrounding cells give support to the olfactory neurons in terms of ions, metabolism and nutrients, the neurons are not in balance when their supporting cells are infected. This leads to the loss or change of ability to smell. In turn, this can lead to changes further along in the nervous system. How long the smell dysfunction lasts, seems to depend on whether the stem cells of the nasal epithelium are infected as well. This means that the recovery of the olfactory mucosa would take longer. Besides this main hypothesis, there are other alternative hypotheses, concerning viral deposition, the serotonin/tryptophan pathway, and a possible self-defense mechanism. These do not get as much attention, but as they are not ruled out yet, these should be investigated as well. In conclusion, more and especially more structured research into these mechanisms is needed to draw reliable conclusions about the underlying mechanisms of olfactory dysfunction due to COVID-19.

Introduction

Loss of the ability to smell is a common symptom of COVID-19 (1). At first, it was assumed nasal obstruction caused the anosmia, as occurs in common cold. Contradictory, in COVID-19, patients rarely report any nasal obstruction or rhinorrhea alongside their olfactory dysfunction (2). Approximately 80% of people who were recruited from European hospitals while COVID-19 positive, suffered from olfactory dysfunction (3,4). Though, research into the prevalence of olfactory dysfunction is diverging, due to differences in subjective and objective measurements and researched population (5). Olfactory dysfunction is a very diverse phenomenon, in terms of symptomology, onset and duration. Olfactory dysfunction can be presented in many ways. In COVID-19, people reported anosmia (absent olfaction), parosmia (altered olfaction), hyper- and hyposmia (increased or reduced olfaction) and phantosmia (imagined odors). Changed ability to smell, in contradiction to absent smell, suggests changes but not the total dysfunction or absence of olfactory sensory neurons (OSNs). People suffering from anosmia often pass through different stages of altered ability to smell before the ability to smell returns to normal. Besides type of dysfunction, the duration of the dysfunction differs greatly between patients. In a large study from Spain, approximately 80% noticed disordered smell (6). Approximately 70% of these patients recovered within 4 weeks after onset. From patients with COVID-19, approximately 25% suffer from disordered smell for more than a month. For some people the dysfunction even carries on for many months (7).

Literature about smell loss shows that the dysfunction of this primary sense can lead to serious problems in everyday life. The most common given reasons as to why smell dysfunction negatively impact life are related to personal hygiene, loss of interest in food and drinks, enjoyment of food and drinks and risks (8). Among these risks are not being able to detect fire or inedible foods. Taken together, these factors result in more symptoms of depression and nutritional issues, which contribute to less quality of life (9). This stresses the importance of not only saving and curing as many people as possible during this pandemic, but also helping people cope with or solve their smell dysfunction. In this review, the current hypotheses about the pathophysiology on a cellular level will be discussed. Research method is stated in Appendix 1. To be able to go into details about the mechanisms in healthy olfactory function, an introduction to the anatomy and functioning of this system will follow.

Olfactory system

The first interaction between odors and people takes place in the nose. The nose consists of two cavities split by the nasal septum. In these cavities, air flows through the nasopharynx, after which the air continues to the lungs for gas exchange. In the cavities of the nose, three conchae are present, which add the needed humidity and lead the air towards the olfactory epithelium in an optimal way to be able to bind odorants to the receptors (10). Receptors on olfactory sensory neurons bind to odorant molecules carried by air. Through the nasal mucosa present, the molecules in the air will have the first contact with sensory cells. The olfactory epithelium (OE) is built up from two layers, which are the olfactory mucosa and the lamina propria (11). At the olfactory mucosa multiple types of cells are present (Figure 1). The first being the olfactory receptors neurons (OSNs). These cells stretch over the epithelium as bilateral neurons (12). Their dendrites stick into the nasal mucosa with cilia on the end of the dendrite, the olfactory knob. This is where odorous molecules bind, which releases signals into the neurons. At the other end of the neuron, the axon passes through the cribriform plate, after which the neuron communicates with the olfactory bulb. Of these OSNs, multiple types exist, due to the different affinity for certain molecules and to which cells they project. In the olfactory epithelium, there are mature and immature OSNs. Immature OSNs will mature to become functional OSNs when needed and stimulated by the environment in the olfactory epithelium. Basal cells are located at the distinction between the olfactory mucosa and the lamina propria and are believed to be stem and precursor cells. Globose basal cells have many stem cells properties whereas horizontal basal are progenitors which are able differentiate into any needed cell in the olfactory epithelium when needed (13,14).

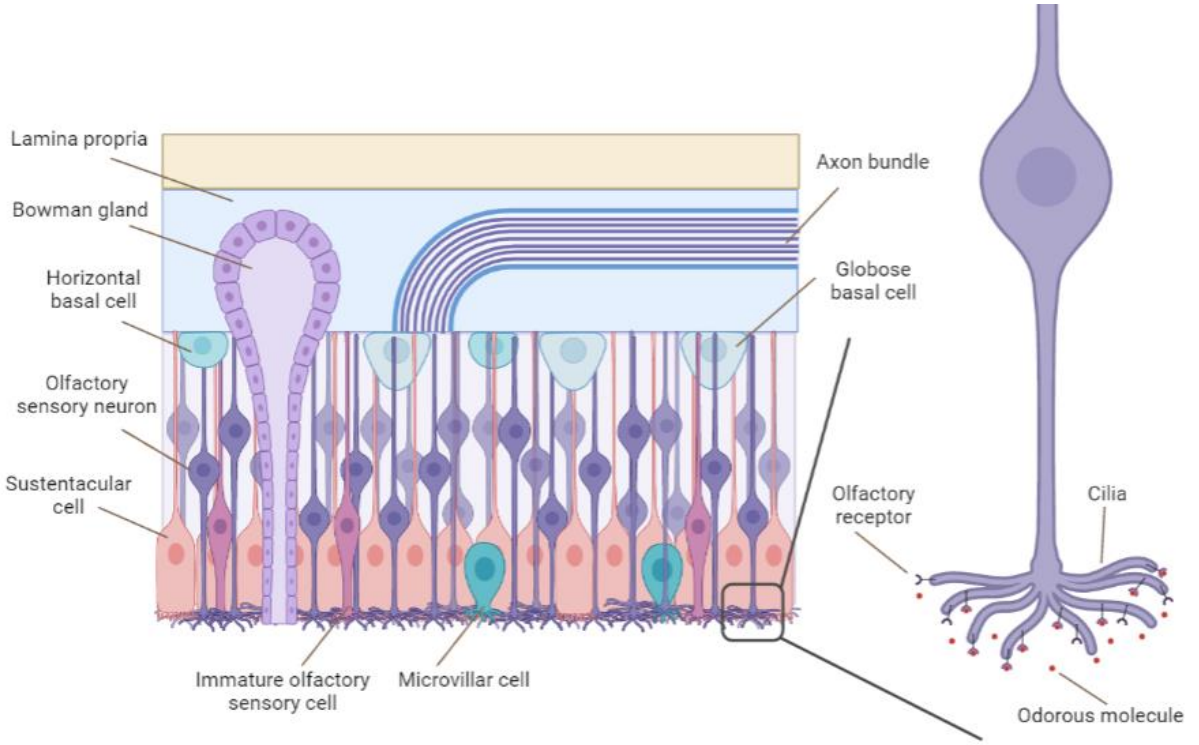

Figure 1: Overview of cell types present in the olfactory epithelium. Shown are the Bowman gland and its duct, basal cells (horizontal and globose), olfactory sensory neuron (immature and mature), sustentacular cells, microvillar cells and the axon bundles from the sensory neurons. Zoomed in on the olfactory sensory neuron, the cilia, olfactory receptors, and odorous molecules are visible. Created with BioRender.com.

Besides these main sensory components of the olfactory system, there are multiple cells that support and aid their function. Sustentacular cells give the OSNs metabolic, secretory, and phagocytic support (15). These supporting cells are covered in microvilli to observe the environment. The sustentacular cells form a barrier between olfactory mucus and the epithelium by tight junctions they form with the OSNs. Microvillar cells are located near the epithelial surface and are small cells covered with microvilli (16). Their function is still quite unknown, although it is thought that they might direct the regeneration and proliferation of cells in the epithelium or that they are a second group of cells able to bind to odors but evolved differently than the OSNs. Bowman glands in the lamina propria contribute the ions and fluid needed to be able to interact with the odor molecules (17,18). Their ducts extend through the olfactory epithelium and deposit their mucus there.

Through the lamina propria, bundles of OSN axons pass and go through the cribriform plate (Figure 2). The olfactory bulb is located at the front of the brain, below the frontal cortex, where the axons of the OSNs enter the bulbus and make their way to the glomerular layer, where they form the connection between the OSN axons and the second-order neurons. These second-order neurons are the tufted and mitral cells, together called the principal cells. Besides these principal cells, the olfactory bulb also contains several types of intrinsic cells or interneurons and granular cells. These granular cells and interneurons connect to tufted and mitral cells to combine their information and give feedback. Principal cells transfer their information via synaptic connections in the anterior olfactory nucleus. The lateral olfactory tract projects the information directly to the olfactory cortex. From there, reciprocal as well as extrinsic connections are made further in the brain to regulate the ability to smell and to process and beware of smell.

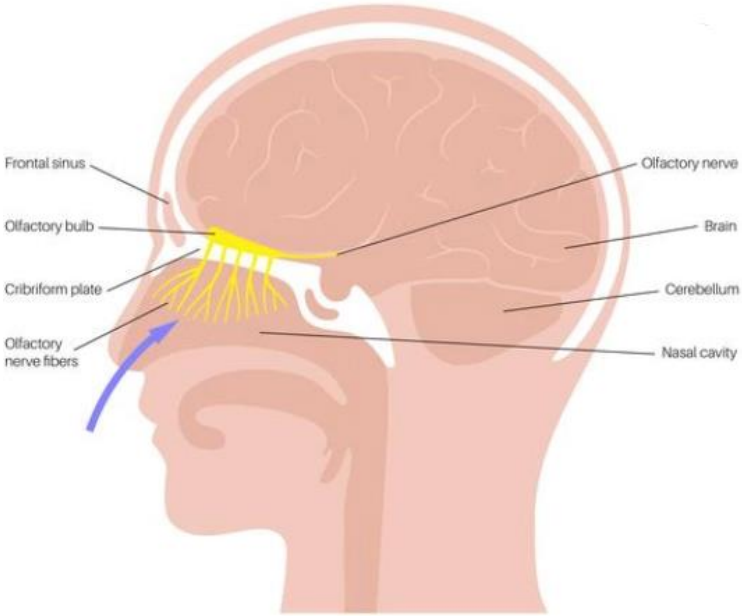

Figure 2: Overview of the olfactory system. On the left, the frontal sinus, olfactory bulb, cribriform plate, and olfactory nerve fibers are shown. To the right, the olfactory nerve from the olfactory bulb, the brain, the cerebellum, and the nasal cavity are annotated. Air enters the nasal cavity, where it encounters the olfactory sensory neurons. These are in contact with the olfactory bulb via the nerve fibers. Then the information is transferred in the olfactory bulb after which this is in contact with other brain parts via the olfactory nerve. Designed by Freepik.

Current hypotheses

As nasal obstruction and swelling of the OE do not explain the extent of olfactory dysfunction present in COVID-19, other hypotheses arise, focusing on viral entry pathways, infection of the OE and inflammatory reactions (2,19).

Animal models

To be able to investigate such an unpredictable disease, animal models are needed. In this case, for COVID-19, the animal models are used to investigate interventions but also to discover how the disease works, as olfactory epithelium and brain regions linked to olfaction are not easily accessible in humans. Often in biomedical research, mice and rats are preferred animal models because of the extensive knowledge on their behavior and genes and how easy they are to handle, keep and breed. When assessing animal models for a new disease the focus must be on the similarities in the disease progression in humans and the animals, to be able to draw the most meaningful conclusions possible.

Specific entry molecules bind to the spike protein (S-protein) of the virus, after which the virus can fuse with the host cell and infects it. Research into the entry molecules of SARS-CoV-2, is mainly focused on the ACE2 receptor. This is a receptor on the membrane of many types of human cells. Mice do also express an ACE protein, but murine ACE protein does not bind effectively to the S-protein of SARS-CoV-2 (20). There are studies which do make use of normal wild-type mice (21). As these do not express ACE2 receptors in the same way that humans do, it is difficult to translate the results of these animal experiments to humans (20). As well as wild-type mice, mouse strains manipulated to express of the human ACE2 receptor are used (22,23). This results in a good model for disease progression and interventions, but their use in studies for viral entry localization and infection localization could be debated because of their inherent altered expression of ACE2. Results from these studies should be interpreted carefully.

The animal model that is most often used in the studies discussed below and seems the closest to human anatomy while still ethically and easily available, is the model of golden Syrian hamsters. These animals express ACE2 receptors in a way similar to humans, where it binds to the S-protein of SARS-CoV-2 and aids viral entry. Disease course was thoroughly examined in this model and found to be similar to human patients, which gives confidence in the same mechanisms being present (24–27).

Entry molecules

For SARS-CoV-2 to be able to infect cells, proteases of the host cells need to activate the virus's S-protein before it can enter the cell. The ACE2 receptor is the main receptor in humans that binds to this S-protein after activation, although before that, more proteins are involved in this activation process. For example, transmembrane serine protease 2 (TMPRSS2) and endosomal cysteine protease cathepsin L are activators of the S-protein via two separate pathways (28,29). In studies done in human cell cultures, it was shown that TMPRSS2 is an important factor for priming of the spike-protein on the virus membrane (29,30). Likewise, neuropilin-1 (NRP-1) and furin have been proven to have big impact on the viral ability to enter by priming the S-protein (31,32). Furin acts as a cleavage protein of the S-protein on the virus membrane. Then, the S-protein is split into S1 and S2 parts, of which S2 then binds to neuropilin-1 receptors. NRP-1 receptors are membrane bound coreceptors for a tyrosine kinase receptor, by which entry is enabled. As well, cathepsin L is shown to be needed for virus entry by fusing the membranes of the endosomes of the host cells with the virus membrane (28,30) Lastly, two

pore channel subtype 2 and phosphatidylinositol 3-phosphate 5-kinase are shown to play an important role in endocytosis of the host cells endosome and the virus membrane (28).

As well as studies into which receptors and proteins play an important role in virus entry and therefore infection, studies have been done into the expression of these proteins in the animal models used for COVID-19 and humans. First, it is shown that the amount of ACE2 receptors present in the animals enlarges with age (33,34). If this is true as well in humans, this might cause older individuals to be more prone to contracting COVID-19 and more severe disease course (35). Many studies focus on the expression of ACE2 receptors (36–38). In mice, their ACE receptor was found in sustentacular cells mainly, but the distribution over the whole OE was non-homogenous (21). In humans as well as in mice, the sustentacular cells, basal cells, Bowman glands and vascular pericytes of the OE express ACE2. In the olfactory bulb the expression of ACE2 receptors is not clear, as some find none, and some find ACE2 in the nonneuronal cells. Different techniques were used to execute these experiments. Besides ACE2, TMPRSS2 is investigated. It is shown to be expressed in sustentacular cells and Bowman glands in human tissue (37). As well as ACE2 and TMPRSS2, the presence of furin is shown in the supporting cells and bowman glands of mice and human samples (39). In the olfactory bulb, no cells express ACE2, TMPRSS2 and furin simultaneously. Shown in another study is that NRP-1 is expressed mostly in the basal cells and aids viral entry in human tissue (40). As well, the suspicion is raised that cofactors play a more important role than currently assumed, because of the extremely low ACE2 protein levels in the OE cells (41). It could be, that there are viral entry routes possible without the involvement of ACE2 altogether.

To summarize, ACE2, TMPRSS2, Furin and NRP-1 together form a cascade of reactions which allows the S-protein of the corona virus to be spliced, after which a part gets bound, and the other part acts as entry molecule which makes the connection happen between the host cell and the virus. This is what causes the virus to be able to enter host cells and infect them. These studies show that these entry proteins are predominantly present in non-neuronal cells in the OE and olfactory bulb, which suggests that only these, and not the neuronal cells, can get infected with SARS-CoV-2.

Cell infection

Results from previously discussed research indicates that non-neuronal cells in the OE are prone to infection, based on the entry receptor localization. Conclusions on which of the cells in the OE and olfactory bulb are actually infected by the virus, are still unclear. Studies in golden Syrian hamsters and humanized ACE2 mice show infection of sustentacular cells, basal cells, and cells of the Bowman glands (42–45). Severe reduction of OE thickness is seen as a result of the infection. Some studies in golden Syrian hamsters additionally show infection of OSNs (42,43). This points to the direct infection of majority of the cells in the OE, including neurons. Contradictory, other studies in golden Syrian hamsters and humanized ACE2 mice show that the active infection of OSNs is highly unlikely (44,45). There, only a few infected immature OSNs were seen. Concluded from these findings is that neuronal cells are unlikely to be directly infected. Nevertheless, Bryche et al. show that when the neuronal cells are not infected themselves, the OSN present cilia loss (45). Ye et al. found that the olfactory receptors and the olfactory binding proteins were severely downregulated during the infection (44). Both observations show the reaction in the OSNs in response to their aiding surroundings being affected. This abovementioned study, as well as the study from Dias de Melo et al., qualified olfactory dysfunction (43). Their mice and hamsters underwent exercises of searching for odorous foods and they found a significant difference between COVID-19 negative and positive animals, which shows their lack of ability to smell. This strengthens their studies, as the ability to smell is coupled to the infection this way.

The inflammatory response of cells in the OE being infected is extensive. First, in human nasal tissue, an increase in TNF-α was found (46). This is a factor that enhances inflammation reaction. Furthermore, an increase in the excretion of IL-6 was shown in human blood samples, which can affect olfaction peripherally (47,48). It was shown that an abundance of factors from the inflammatory response, IL-1β, IL-6, TNF-α, MIP1-α, RANTES and IP-10 and immune cell infiltration, were present in the nasal cavity of golden Syrian hamsters (42,43). Possibly, this points to the inflamed surroundings of the OSNs influencing their functioning, without direct infection or damage.

Besides, the changes seen in the olfactory sensory neurons can be due to less expression of the olfactory receptors (49,50). Nuclear architecture disruption is a possible reaction to the disorganization of the olfactory epithelium, inflammation, and the lacking support of the surrounding cells. Transcriptional changes in the olfactory sensory cells were seen in human and hamster samples. Downregulation of genes such as Atf5, adcy3, omp, gfy, lhx2, and gng13 repress the expression of the olfactory receptors. Besides, downregulation of genes concerning the cAMP second messenger pathway and modulatory pathways from the olfactory system were observed. The infection of surrounding cells, the lacking support from that and the cytokines present possibly cause the OSNs to malfunction, without them being infected themselves.

It is unclear whether OSNs can get directly infected by SARS-CoV-2, because of the lack of ACE2 receptors and the lack of finding infected cells in most studies. Though, in some studies in golden Syrian hamsters, infection of the OSNs was found (42,43). An explanation for the discrepancy would be a different way of infection. The two proposed theories for this are (i) the close connection between the OSNs and the sustentacular cells and (ii) ACE independent viral entry and infection of the OSNs. As discussed by Sadeghipour and Mathias, exosomes that deliver virus particles from one cell to the other play a role in various viruses, which could also be the case for SARS-CoV-2 (51). Then, it would not be needed for the cell to have entry receptors for the virus, but to have the ability to take up an

exosome. A hypothesis including close proximity between an infected cell and the OSNs was also stated by Ye et al., where the only immature OSNs that got infected, were positioned directly next to a sustentacular cell, which get infected by SARS-CoV-2 easily (44). ACE independent viral entry is proposed as a possibility as well, because of the additional entry molecules that are found (52). Mayi et al. propose the involvement of NRP-1 as the other entry pathway for SARS-CoV-2, as also suggested by Cantuti et al. (40). NRP-1 is found in almost all cells in the olfactory epithelium, even in the olfactory neurons and in the olfactory bulb. Mayi et al. showed that in mice, the use of this entry pathway leads to representation of infection of the neurons of the olfactory bulb.

The situation in the olfactory bulb is unclear. On 3D T2 FLAIR MRI scans it is shown that the olfactory bulb is affected by the changes of infection because the olfactory bulb shows up smaller (53). Multiple studies found ACE2 receptor to be present in the non-neural cells of the olfactory bulb (36,37,39,54). On the other hand, it is most likely not directly infected by SARS-CoV-2, as that was not found in the previously mentioned studies (42,45). Some studies found signs of apoptosis and inflammation, which shows that the olfactory bulb is affected by the changes induced by COVID-19 (42). As it seems unlikely, but it is unclear whether OSNs and the neurons in the olfactory bulb can get infected by SARS-CoV-2, the possibility exists that the retrograde infection of the central nervous system could take place via that pathway (55). In animal studies, it appears that via the retrograde pathway the central nervous system gets infected, but these are the humanized ACE2 mouse models, of which the tropism of the ACE2 receptors and therefore the virus is most likely not transferable to humans (56). Altogether, it is too early to conclude about the infection of the central nervous system. The symptoms of people experiencing neuronal complaints due to COVID-19 are wide ranged and cerebral spinal fluid studies do not give exclusive answers (57).

The olfactory neuronal system is special in its ability to regenerate its neurons. Basal cells in the olfactory epithelium seem less likely to be infected than sustentacular cells but were not ruled out (42). This may explain the difference in longevity of the olfactory dysfunction symptoms. The possible infection of basal cells may explain why in some people, the regeneration of the epithelium is relatively quick, whereas in some people, it takes months. The regeneration cycle of neurons in the OE takes between 28 and 35 days (58). Most patients recover from anosmia within 4 weeks, so this shows a discrepancy. It can be concluded that most people's olfactory dysfunction is not due to the lack of OSN's. The most likely hypothesis here is that the olfactory dysfunction has something to do with the infection of the sustentacular cells, by which the function of the OSN's is lessened. Then, within 4 weeks the olfactory function is restored in most cases due to the internal or environmental recovery of the OSN's. In case of more severe damage to the OSNs, recovery might take longer, as progenitors, immature OSNs or basal cells need to differentiate into functional mature OSNs. When the damage is substantial and the basal cells are infected and damaged as well, this recovery process will take even longer. Animal studies suggest that the ability to smell is fully reversible, based on the thickness of the OE, measured in histological images of golden Syrian hamster OEs (59). Another hypothesis states that if olfactory dysfunction causes smell deprivation for a prolonged period, the connections to and in the olfactory bulb will degenerate (60). When the olfactory function is then restored in the OE, the olfactory bulb cannot process the information, which will still lead to the inability to smell. This might take training or in general take longer to recover.

Taken together these studies suggest that supporting cells of the olfactory system such as the sustentacular cells, bowman glands and basal cells are most prone to be infected with SARS-CoV-2 as

these cells possess ACE2 receptors on their membranes. In most cases, OSNs do not seem to be able to get infected with the virus. Multiple mechanisms of indirect effect on the sensory neurons are possible. First, the inflammation of the support cells causes them to not be able to do their function of supporting through the metabolic aid, for example (61). Therefore, the sensory cells are not able to function properly, leading to smell loss. Second, the inflammatory response of the body and the cytokines that come with that may cause the olfactory sensory neurons to be disrupted, by which the normal functioning of sensory neurons is impaired (54). Third, the damage to sustentacular cells and bowman glands may lead to cell death or damage of the olfactory sensory neurons, which subsequently leads to olfactory dysfunction (62).

Alternative hypotheses

Viral deposition hypothesis

The theory concerning viral deposition states that the viral load in the olfactory epithelium is the decisive factor in whether someone experiences olfactory dysfunction (63). Airborne aerosol droplets are shown to deposit primarily on the OE, compared to other parts of the airways. This shows to disrupt of the olfactory epithelium. This, in principle, is logical, as more exposure to the virus leads to more local infection, which in turn leads to more damage to the local tissue. This then results in olfactory disfunction. This hypothesis has a lot of interplay with the main hypothesis about the OE infection and these hypotheses are therefore not mutually exclusive.

Self-defense mechanism hypothesis

In the previous discussed studies, the focus was on how the virus might have disrupted a subset of cells in the human olfactory epithelium. Le Bon et al. discuss a very different approach to the problem (64). What if the disruption of the olfactory epithelium is a self-defense mechanism? Evidence exists of this happening with other viruses as well (65). The theory states that, especially in young people who still possess a large ability to regenerate olfactory neurons, the olfactory cells go into apoptosis when the viral load in their cells crosses a certain threshold. Then, the subject experiences sudden olfactory dysfunction. The reason behind this self-destructive mechanism might be to protect the central nervous system. Infection of the central nervous system can be dangerous. The olfactory system is the only neuronal system with such direct contact between the outside world and a connection to the brain and because of its easy access from the outside, it is prone to damage. Besides, it is also the only neuronal system with the ability to regenerate their neurons. Taken together, it would be beneficial to have the scale tilted more towards self-destruction, but then simultaneously keeping the possibility of regeneration intact. This theory might explain why olfactory dysfunction is a symptom primarily seen in relatively young people with mild additional symptoms of COVID-19.

Serotonin/tryptophan hypothesis

Besides hypotheses concerning pathophysiology of the olfactory epithelium, there are also hypotheses that combine all aspects of the symptom behind the scenes, like the interplay of COVID-19 with the production of serotonin (66). Serotonin is made from tryptophan, an essential amino acid. This amino acid is taken up from food, where the ACE2 receptor plays a key role as a chaperone protein in the amino acid transport across the membrane in the intestines. ACE2 is widely present in the gastro-intestinal tract. Deficiency of the ACE2 receptor leads to substantial reduction of serotonin in the body. When SARS-CoV-2 is bound to the ACE2 receptors, these are no longer available for the uptake of tryptophan, which leads to less ability to produce serotonin. COVID-19 patients are shown to have lowered levels of serotonin in their serum (67). Serotonin is known to be important for the functioning of the olfactory system via the neurons. In short, it increases activity of olfactory bulb glomeruli, and it stimulates mitral and tufted cells. In general, it could be stated that a lowering of serotonin causes less ability to smell. This concludes that SARS-CoV-2 could possibly influence the ability to smell via gastro-intestinal infection which lowers the uptake of tryptophan. This in turn lowers the production of serotonin, which can then not play their part in olfactory function. Little research is done into this hypothesis yet.

Discussion

In this review, the current hypotheses about the underlying mechanisms of olfactory dysfunction caused by COVID-19 were summarized. In this chapter, the used techniques, focus and pitfalls will be discussed.

The studies previously described in this review have very small sample sizes. These are still the beginning stages of research into this phenomenon. Probably, this is in many cases due to problems related to the COVID-19 pandemic itself such as lockdowns and a shortage of laboratory animals. Funding for emerging fields, which are very subjective to change and developments, is often challenging as well. The small samples sizes might account for some of the discrepancies between the studies. In future studies it would be very beneficial to work with more animals per condition and timepoint, to be able to draw more reliable conclusions. It might be argued from an ethical point of few that the less animals, the better. Although, it can be debated that using this few animals results in not sufficiently secure outcomes of the studies to be able to rely on their conclusions. Then, the use of the few animals is not sufficiently useful and therefore ethically debatable. So, in future research, the number of animals used should be weighed against the security of conclusions that can be made from the results from the experiments.

All research described above is aimed to ultimately be translated to the pathology of COVID-19 in humans, to be able to find therapeutic options for COVID-19. With some of these studies, the ability to be translated to humans and the interpretation of the results are debatable. Provided, fundamental research is often not translatable, which does not mean it is not useful, because future research building on fundamentals is needed for this translation. However, in some studies described above, the step towards translation to human science seems not plausible, due to animal models which do not seem fit for investigating COVID-19. It is most efficient to use animal models as close to human anatomy as possible. In mouse models, some obstacles occur. For example, wild-type mice, these do not express ACE in the same way as humans do (21). Their ACE receptor does not bind to the virus's S-protein, which indicates it is not a good model for SARS-CoV-2 infection. Next, as stated above, using mice with a humanized ACE2 receptor to look for entry sites and infection sites can be debated. When entry molecules, such as ACE2 receptors, are expressed in a modified manner in these animals, the changes made in this animal model, contribute largely to the outcome measures of the study. They state themselves, that there are inconsistencies due to tissue tropism of the ACE2 receptor in brain parts between studies both using humanized ACE2 receptors, due to differences in design. For example, a mouse model from the SARS-1 outbreak was tested and proved functional for SARS-CoV-2 studies (22). Here they expressed a human receptor on a keratin promotor because it had similar tropisms as the SARS virus. In another variant, they expressed the human ACE2 receptor on the promotor of the murine ACE receptor (23). Although the models seem similar, their design may differ. Humanized ACE2 models are good models for following the course of disease or to test medicine and vaccines, but not for investigating virus entry and identification of infected cells.

Besides this hurdle in interpreting results, it is not secured that the animals experience the smell dysfunction in addition to the visual changes of their OE. Many studies only use visual cues of sensory loss, such as the thinning of the OE or observed infiltration of inflammatory cells via microscopy. A good alternative is shown in Ye et al. and Dias de Melo et al., where the animals do tests with different food pellets and finding food exercises (43,44). Here, animals with and without infection are to find hidden food. When the ability to smell is changed or lost, the infected animals should take longer to

find the food or not find the food at all. This way, the visual ques of olfactory dysfunction can be coupled to the ability. With these two studies who show these tests to be successful, their conclusions can be interpreted with more security. When proven that olfactory loss is cellularly and behaviorally identical in the test animals and humans, these can be good control experiments.

All current literature found about the development of animal models for the use of research into COVID-19, is focused on the entry protein ACE2. As stated above, ACE2 might not be the only pathway and might not be needed in every entry pathway. Therefore, for future research to be more complete, animal models including the alternative entry pathways including entry pathways using TMPRSS2, furin and NRP-1 could be investigated, for example. Similarly, when investigating which cells are infected in the OE, many studies focus on only a subset of cells present in the OE. This causes a skewed view of certain findings and their importance. Besides, some studies speak of groups of cells such as 'supporting cells of the OE', which makes it difficult to conclude from multiple studies, when others investigate more distinct cell types such as the sustentacular cells and microvillar cells separately.

Today, because of how new this disease is, and therefore its accompanied research field, research is widely spread in terms of outcome measures, procedures, animal models used, tests, etc. This causes difficulty comparing and analyzing results from studies. For example, when researching which cells are infected in the OE, different techniques were used. One study used the presence of viral RNA, where another study used the presence of antigens against the virus in the cells as a marker of infection. Another example is the presence of inflammation reaction, which is measured in both presence of immune cells, and inflammatory cytokines. ACE2 expression in cells can also be tested in many ways. In the articles discussed here, they used RNA sequencing in nasal samples and postmortem immunostaining. In these examples, both techniques seem legitimate and correct, but it is not clear whether these differences in techniques might also account for differences in outcomes. Even so, it is unclear whether when the same technique was used, the results would be the same as they are stated now.

Concluded, the use of different techniques and whether or not a cell type or pathway is investigated at all, makes for difficulties drawing conclusions from current research. This is enlarged by the small sample sizes and diverging directions of research.

Conclusion and future perspective

In conclusion, various hypotheses exist on the cellular mechanisms behind the olfactory loss often accompanying COVID-19. In the OE, ACE2 is expressed mostly in the supporting cells. Besides ACE2, TMPRSS2, cathepsin L, furin and NRP-1 are brought forward as virus entry aiding proteins. Although, it is still unclear whether the neurons in the OE can get infected directly, indirectly, or not at all, it is clear that the neurons are affected by the inflammation and lack of support. Down-regulation of olfactory receptors, less cilia, and signs of cell death were seen as a result. If it becomes clear that direct infection of the OSNs is occurring as well, this would lead to internal dysfunction of the OSNs simultaneous with the disruption caused by the infection in the surrounding. Besides the main hypothesis, where it seems like the neurons do not get infected but are affected by their surroundings, there are a few under-investigated other hypotheses. These include the involvement of the serotonin production, viral deposition, and self-defense mechanism.

Future research into the interplay between COVID-19 and the loss of the ability to smell should continue to focus on the working mechanisms and possible interventions. Smell training and therapies including medicine while or after disease period are investigated which if proven to be effective would offer major possibilities for people suffering extensively from this peculiar symptom of COVID-19. It seems there is a distinction between people who suffer from olfactory dysfunction, concerning duration. One group only suffers from this symptom simultaneous to other symptoms of COVID-19. The other, smaller, group, suffers for a long period of time, which suggests a different or more severe underlying mechanism, probably related to basal cell infection, olfactory sensory neuron damage or death and olfactory bulb degeneration. This should be the focus of studies into regeneration, intervention and be cause for prevention research.

Concerning animal experiments, it would be wise to continue with reasonable sized samples, of at least three animals per condition per time period, after which reliable conclusions can be drawn. To be able to compare between studies and translate to humans eventually, studies must include specific behavioral tests to prove (dys)ability of olfaction. Besides, animal models closest to human physiology should be considered extensively. In conclusion, I would suggest elaborate studies including golden Syrian hamsters where their olfaction is tested before, multiple times during and after disease period. Olfaction can be tested using search for favorable food exercises. When inducing COVID-19, MRI scans of nasal epithelium and olfactory bulb should be made. Besides, proteomics of the olfactory epithelium should be investigated, and immunostaining and subsequent microscopy should be used to investigate infection and transmission between cells. When such an elaborate study is carried out, more reliable conclusions could be drawn after which new study directions could be proposed.

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Appendix

Appendix 1: Search method

Literature search was conducted via PubMed, by a carefully chosen selection of articles found by search string conducted from the combinations of keyword groups stated below. All articles found to be relevant were read carefully whereafter a summary and discussion was written according to the critical thinking of the author. Research was done from the 27th of September till the 15th of October of 2021.

- Olfactory dysfunction, smell loss, anosmia, parosmia, phantosmia.
- COVID-19, SARS-CoV-2.
- Pathology, physiology, pathophysiology, mechanism, cellular mechanism.
- Sustentacular cells, support cells, olfactory neurons, olfactory sensory neuron, olfactory receptor neuron, olfactory basal cells, globose basal cells, horizontal basal cells, Bowman glands, olfactory bulb.
- Entry proteins, ACE2 receptor, angiotensin-converting enzyme 2, TMPRSS2, transmembrane protease serine 2, furin, neuropilin-1, NRP, cathepsin L, cathepsin B.
- Golden Syrian hamster, hACE2 mice, mice, rat, animal model.
- Tryptophan, serotonin, 5-TH.

Appendix 2: Abbreviation list

ACE2:	angiotensin-converting enzyme 2
COVID-19:	corona virus disease 2019
NRP1:	neuropilin-1
OE:	olfactory epithelium
OR:	olfactory receptor
OSN:	olfactory sensory neuron
SARS-CoV-2:	severe acute respiratory syndrome coronavirus 2
S-protein:	spike protein
TMPRSS2:	transmembrane protease serine 2