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SARS-CoV-2 impact on the Central Nervous System: Are astrocytes and microglia main players or merely bystanders?

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Abstract:

With confirmed COVID-19 cases surpassing the 8.5 million mark around the globe, there is an imperative need to deepen the efforts from the international scientific community to gain comprehensive understanding of SARS-CoV-2. Although the main clinical manifestations are associated with respiratory or intestinal symptoms, reports of specific and non-specific neurological signs and symptoms, both at presentation or during the course of the acute phase, are increasing. Approximately 25-40% of the patients present neurological symptoms. The etiology of these neurological manifestations remains obscure, and probably involves several direct pathways, not excluding the direct entry of the virus to the Central Nervous System (CNS) through the olfactory epithelium, circumventricular organs, or disrupted blood-brain barrier (BBB). Furthermore, neuroinflammation might occur in response to the strong systemic cytokine storm described for COVID-19, or due to dysregulation of the CNS angiotensin system. Descriptions of neurological manifestations in patients in the previous coronavirus (CoV) outbreaks have been numerous for the SARS-CoV and lesser for MERS-CoV. Strong evidence from patients and experimental models suggests that some human variants of CoV have the ability to reach the CNS and that neurons, astrocytes and/or microglia can be target cells for CoV. A growing body of evidence shows that astrocytes and microglia have a major role in neuroinflammation, responding to local CNS inflammation and/or to dysbalanced peripheral inflammation. This is another potential mechanism for SARS-CoV-2 damage to the CNS. In this comprehensive review we will summarize the known neurological manifestations of SARS-CoV-2, SARS-CoV and MERS-CoV, explore the potential role for astrocytes and microglia in the infection and neuroinflammation, and compare them with the previously described human and animal CoV that showed neurotropism. We also propose possible underlying mechanisms by focusing on our knowledge of glia, neurons, and their dynamic intricate communication with the immune system.

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Introduction

The coronavirus disease 2019 (COVID-19) originated in Wuhan, China in December 2019 and the etiological agent was named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). The outbreak quickly turned into a global pandemic with over 8.5 million reported cases around the globe, and over 450,000 deaths (WHO report COVID19). The SARS-CoV-2 outbreak is not the first coronavirus (CoV) epidemic to emerge in the 21st century: both the 2002 Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) and the 2012 Middle East Respiratory Syndrome Coronavirus (MERS-CoV) have been included in this classification. Compared to other CoV (HCoV-229E, HCoV-OC43, HCoV-NL63) that are pathogenic to humans but present only mild clinical symptoms, SARS-CoV-2 resembles both MERS-CoV and SARS-CoV in their potential to cause a more severe disease, and also in the fact that they are able to infect both the upper and lower respiratory tract (Lau et al., 2020b). Most COVID-19 patients present mild signs, or even show asymptomatic disease progression. The main clinical symptoms are fever, dry cough, fatigue, and dyspnea (Yang et al., 2020a). However, about 17.8% require intensive care due to acute respiratory distress and massive cytokine release syndrome (*cytokine storm*), and 27.7% of patients with severe disease also have preexisting comorbidities (Chen et al., 2020; Yang et al., 2020a).

A mounting body of evidence from SARS-CoV-2 affected patients is showing that some patients develop serious Central Nervous System (CNS) symptoms, neurological and neuroradiological alterations (Helms et al., 2020; Jasti et al., 2020; Mao et al., 2020; Pryce-Roberts et al., 2020; Romero-Sánchez et al., 2020; Roman et al., 2020; and several others) and, in some cases, viral antigens, viral genomic sequences or specific antibodies against SARS-CoV-2 have been detected in cerebrospinal fluid (CSF) (Benameur et al., 2020; Moriguchi et al., 2020; Wu et al., 2020).

Intensive research during the last two decades has demonstrated that CNS neurons would not be able to survive without the support of glial cells in matters of metabolic balance, energy supply, immune response, and even effective neurotransmission. We now

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recognize that astrocytes are complex, heterogenic glial cells that encompass several important and versatile functions which include: maintenance of CNS homeostasis, structural and metabolic support, modulation of synaptogenesis and synaptic transmission, participation in immune response in cooperation with microglia, regulation of blood flow, and maintenance of the blood-brain barrier (BBB) (reviewed in (Barres, 2008; Sofroniew and Vinters, 2010; Clarke and Barres, 2013; Ramos 2016). When astrocytes sense stimuli associated to injury or disease, including viral infection, they respond with a process called astrogliosis that profoundly alters astrocytic biology. Recent transcriptomic analyses of reactive astrocytes have identified a spectrum of possible activation profiles ranging between two extreme phenotypes named A1 and A2. While A1 reactive astrocytes upregulate pro-inflammatory genes that induce neurodegeneration, increase BBB permeability and recruit peripheral immune cells, the A2 phenotype has been shown to increase the expression of genes that induce neuromodulation, immune tolerance and to be supportive for neuronal survival (Liddelow et al., 2017; Burda and Sofroniew, 2017; Liddelow and Barres, 2017; Zamanian et al., 2012). Intensive research from our group and others has shown that polarization to the A1 pro-inflammatory-neurodegenerative phenotype is facilitated by astroglial exposure to pathogen associated molecular patterns (PAMP) and to microglial-derived pro-inflammatory cytokines, and that microglial cooperation is essential to induce the A1 astroglial phenotype (Liddelow et al., 2017; Rosciszewski et al., 2018; 2019).

Previous experiences with SARS-CoV and MERS-CoV, as well as experimental data on neurotropic CoVs, have shown that CoV can reach the CNS and infect it. We here performed a comprehensive review on the CNS cell types affected by CoV infections with a special focus on the role of astrocytes and microglia in these conditions. To our best knowledge this is the first compilation of data concerning the astroglial and microglial role in these infections, comparing MERS-CoV, SARS-CoV, neurotropic CoVs and SARS-CoV-2.

Middle-East Respiratory Syndrome (MERS):

Middle-East Respiratory Syndrome coronavirus (MERS-CoV) has been identified as a cause of severe respiratory infection in humans since the 2012 outbreak, being the second highly pathogenic CoV to rapidly spread into human populations. The number of laboratory-confirmed cases reported by the WHO until January 2020 was of 2519 cases globally, with a mortality rate of 34.3% (WHO, 2019b). Although the MERS-CoV infection was asymptomatic in some patients, many cases present pneumonia and severe symptoms that can result in multiorgan failure and death (WHO, 2013).

There are a number of reports of MERS-CoV patients showing neurological symptoms (Arabi et al., 2015; Kim et al., 2017) although the overall number of reports showing neurological manifestations in MERS patients is low compared with the present knowledge on SARS-CoV-2. However, MERS-CoV belongs to lineage C of the *Betacoronavirus genus*, whose species are known to be potentially neuroinvasive (Desforges et al., 2014). Reported neurological symptoms in MERS-CoV infection mostly come from case reports, and findings include neuropathy, delirium, and seizures (Saad et al., 2014; Arabi et al., 2015; Algahtani et al., 2016; Kim et al., 2017). However, the number of reports is quite low probably due to the significant comorbidities and the severe critical condition of complicated MERS-CoV patients.

In spite of their similarity, MERS-CoV does not share with SARS-CoV and SARS-CoV-2 the mechanism to gain access to cells. MERS-CoV utilizes exopeptidase, dipeptidyl peptidase 4 (DPP4) as a receptor to enter host cells. Human DPP4 expression is a requirement to make non-susceptible species, like rodents, become susceptible to MERS-CoV infection (van Doremalen and Munster, 2015). MERS-CoV has also shown increased mortality in obese patients and the finding has been related to increased expression of DPP4 in obese people (Al-Hameed, 2017).

MERS-CoV has the potential ability to enter microglia, astrocytes and neurons, considering that all these cell types express DPP4 (Elkjaer et al., 2019; Kiraly et al., 2018). Among these CNS cells, GFAP+ astrocytes seem to show the largest DPP4 basal expression, and

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inflammation significantly increased the expression of the viral receptor (Kiraly et al, 2018). Moreover, DPP4 was also found in brain microvasculature (Kenny and Bourne, 1991; Zeng et al., 2019).

Studies in transgenic mice expressing human DPP4 (hDPP4) or adenoviral-based expression of hDPP4 in mice have shown that intranasal exposure to MERS-CoV resulted in illness and high mortality rates (reviewed in van Doremalen and Munster, 2015). In these mice, viral RNA was detected in several organs including lungs and brain. While dramatic lesions were found in the lungs of these hDPP4-expressing mice, no gross pathological changes were observed in the brain; however viral antigen expression was found in brain microglia, astrocytes, and neuronal cells, together with increased pro-inflammatory cytokines and chemokines (Tao et al., 2016; Agrawal et al., 2015; Zhao et al., 2015). Indeed, Tao and colleagues (2015) were able to recover viral particles from brain tissue and detected viral antigen expression in neurons and glia of the hDPP4 mice. *In silico* modeling of common marmoset DPP4 showed high similarity with hDPP4 and, as was predicted, this resulted in high susceptibility of marmosets to MERS-CoV (Raj et al., 2013; Farazano et al., 2014). Infected marmosets showed severe illness, and viral RNA was detected in all tested organs including lungs and brain (van Doremalen and Munster, 2014). There is, however, another report using transgenic hDPP4 mice that found no evidence of MERS-CoV viral infection in the brain, although these authors used a human DPP4 promoter that rendered negative hDPP4 expression in neurons and glia (Iwata-Yoshikawa et al., 2019).

Among the MERS-CoV patient case reports describing neurological symptoms (Saad et al., 2014; Arabi et al., 2015; Algahtani et al., 2016; Kim et al., 2017), it is interesting to note that MRI findings obtained by Arabi and colleagues (Arabi et al., 2015) were rather supportive of primary viral neuropathology resembling an acute disseminated encephalomyelitis (ADEM) (Marin and Callen, 2013). The study lacks, however, a confirmatory test for brain infection, such as could have been the positive detection of MERS-CoV in brain tissue or CSF (Arabi et al., 2015).

Taken together, the published evidence shows that we still lack confirmatory studies demonstrating the presence of MERS-CoV viral particles in the CNS of human patients, however the brain imaging findings (Arabi et al., 2015), together with clinical findings (Saad et al., 2014; Arabi et al., 2015; Algahtani et al., 2016; Kim et al., 2017), are highly indicative of a direct effect of MERS-CoV on the CNS. Experiments using hDPP4 expression in animals have shown that MERS-CoV has the ability of infecting and proliferating in microglia, astrocytes or even in neurons, (Tao et al., 2016; Agrawal et al., 2015; Zhao et al., 2015), but this seems to be highly dependent on DPP4 expression in these cell types (Iwata-Yoshikawa et al., 2019).

Severe Acute Respiratory Syndrome (SARS):

The first reported outbreak of a human CoV producing Severe Acute Respiratory Syndrome (SARS), named SARS-CoV, emerged in November 2002 in Guangdong Province, China. Since then, until the mysterious disappearance of SARS-CoV cases six months later, the outbreak resulted in 8098 SARS-CoV cases, with 774 deaths, a 9.56% mortality rate (WHO, 2004). As both the present SARS-CoV-2 and MERS-CoV, SARS-CoV belongs to the *Coronavirinae* subfamily, genera betacoronaviruses (Ng Kee Kwong et al., 2020).

SARS-CoV patients usually referred high fever associated with chills, headache, muscular pain and sometimes diarrhea. Most patients develop respiratory symptoms and pneumonia, with 10 to 20% of the patients requiring mechanical ventilation.

Several case reports showing neurological symptoms were documented for SARS-CoV. Among the neurological symptoms, the most abundant were those showing an effect on the CNS like seizures, dysphoria, vomiting and deliria, (Hung et al., 2003; Lau et al., 2004; Tsai et al., 2005; Xu et al., 2005) as well as stroke (Umapathi et al., 2004; Xu et al., 2005). In spite of the low number of patients showing neurological clinical presentation, the finding of viral RNA in both CSF (Hung et al., 2003; Lau et al., 2004) as well as in autopsied human brain tissue (Xu et al., 2005), reflects the potential neurotropism of SARS-CoV.

Xu and colleagues (2005) have shown that viral SARS-CoV proteins were expressed in the brain of a SARS-CoV patient that presented severe neurological symptoms. In addition, the authors also recovered infective viral particles from autopsy brain preparations of this patient. Brain sections showed an intense inflammation with CD68+ macrophages infiltration, neuronal necrosis, diffuse brain edema and reactive gliosis. Moreover, viral proteins were detected by immunohistochemistry in brain neurons and astrocytes (Xu et al., 2005).

In a series of patients that died from SARS-CoV infection, Ding and colleagues (2004) systematically analyzed the SARS-CoV presence in different organs and they found that the virus was present not only in the respiratory organs but also in isolated cerebral cortical neurons (Ding et al., 2004). SARS-CoV particles in cortical and hypothalamic neurons as well as important signs of neuronal degeneration and brain edema were also detected by Gu and colleagues (2005) using in a series of patients that died from SARS.

Interestingly, SARS-CoV isolated from human patients was shown to be able to infect mice (Glass et al., 2004; Subbarao et al., 2004), ferrets, domestic cats, and various species of monkeys (Martina et al., 2003; Haagmans et al., 2004). After intranasal inoculation, SARS-CoV infection in mice was initially present in the respiratory tract, then reaching different organs, including the brain (Glass et al., 2004). Mice brains showed a large increase of cells with SARS-CoV sequences and authors could also isolate infective viral particles from these mice brains (Glass et al., 2004), in a striking similarity to the human reported cases (Ding et al., 2004; Xu et al., 2005). In SARS-CoV infected mice, viral positive cells were predominantly observed in brain hippocampus in the pyramidal cell layer of the hippocampal CA1 region and in the dentate gyrus (Glass et al., 2004). In addition, evidence of neuroinflammation was confirmed by the elevated cytokine expression in the brains of SARS-CoV infected mice (Glass et al., 2004).

SARS-CoV was the first human CoV reported to bind to human angiotensin-converting enzyme 2 (ACE2) to infect host cells through interaction with SARS-CoV spike protein (Li et al., 2003; Prabakaran et al., 2004; Wang et al., 2004). The ACE2 expression profile was characterized in detail by Hamming and colleagues (2004) using human biopsies

(Hamming et al., 2004). As expected, they found ACE2 expression in the lung alveolar epithelial cells and enterocytes of the small intestine, but they also found that ACE2 was present in arterial and venous endothelial cells and arterial smooth muscle cells in all of the organs studied. Specifically in the brain, the authors found that ACE2 is expressed in endothelium and vascular smooth muscle cells (Hamming et al., 2004) while others later reported ACE2 expression in neurons and glia (Gallagher et al., 2006; Matsushita et al., 2010; Gowrisankar and Clark, 2016; Xu et al., 2017). At least on theoretical grounds, both glial cells and neurons, in addition to brain endothelium express ACE2 and are thus susceptible of interacting with SARS-CoV spike protein.

SARS-CoV outbreak stopped suddenly, therefore studies of human brains from SARS-CoV infected patients with CNS symptoms are lacking and most studies used the approach of humanizing the ACE2 in mice. For example, McCray and colleagues (2007) developed transgenic mice that express the human ACE2 receptor under the control of the cytokeratin 18 promoter (K18-*hACE2* mice). Intranasal infection of K18-*hACE2* mice with SARS-CoV resulted in a fatal disease, directly related to the number of copies of hACE2 transgene and hACE2 mRNA levels (McGray et al., 2007). Histopathological analysis of these animals showed extensive virus replication in the lungs, but also in the brain (McGray et al., 2007; Tseng et al., 2007).

Netland and colleagues (2008) performed experiments analyzing the temporal dynamics of brain infection following intranasal SARS-CoV administration in hACE2 mice. The authors showed that SARS-CoV N protein was detectable and surprisingly widespread in brain regions, and subsequent viral clearance was associated with neuronal loss in these areas (Netland et al., 2008). Time course studies showed a pattern of viral infection that was strongly suggestive of an entry via the olfactory nerve, with subsequent transneuronal spreading (Netland et al., 2008). Netland and colleagues (2008) did not detect reactive astrogliosis in SARS-CoV infected hACE2 mice. This is surprising when considering that this apparent astroglial unresponsiveness to viral infection is accompanied by an increased number of Iba1+ microgliocytes and IL-6 overexpression, a cytokine that is mainly produced by astrocytes and microglia. Moreover, overexpression of a plethora of pro-

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inflammatory cytokines was observed in the brain of SARS-CoV infected mice, most notably IL-6, IFN-γ, CCL2, and CCL12 (McGray et al., 2007; Tseng et al., 2007; Glass et al., 2004) and also in cases reported in humans (Xu et al., 2005). We now know that astrocytes are highly sensitive to increased pro-inflammatory mediators, and that this stimulus polarize astrocytes to the pro-inflammatory and neurodegenerative phenotype named A1 (Liddelow and Barres, 2017).

Taken together, the accumulated evidence from patients that died from severe SARS with CNS compromise as well as the experimental data from wild type or transgenic hACE2 animal models, show that SARS-CoV is able to effectively reach and infect CNS cells, express viral proteins and produce neurodegeneration. The hypotheses about the pathways utilized by SARS-CoV to reach the brain are essentially: i) the olfactory bulb/trans-synaptic pathway, or ii) the hematogenous spread from heavily infected lungs and airways. This latter alternative is facilitated by systemic inflammation that increases BBB permeability and astroglial/microglial pro-inflammatory response due to circulating cytokines and/or due to local viral replication in the CNS.

When analyzing the potential glial role in SARS-CoV infection, in addition to the possibility that the virus replicates in the glial cells, we should also consider the glial role in the strong neuroinflammatory response that can be induced by viral invasion or that can be attributed to the increased peripheral cytokine levels. Astrocytes and microglia are sensitive to peripheral cytokines and their activation and pro-inflammatory polarization could also increase BBB permeability. Pro-inflammatory astrocytes seem to have a main role in SARS-CoV infection by recruiting peripheral macrophages and lymphocytes to the brain parenchyma, thus increasing edema and cytotoxicity in these conditions (Xu et al., 2005). Specifically, an increased expression of IFN-γ was found in glial cells of a patient that developed severe CNS invasion by SARS-CoV (Xu et al., 2005). In addition, the possibility that SARS-CoV infection alters the brain microvasculature is likely, either by a direct effect on the endothelium and glial cells due to viral invasion, or secondary to the strong inflammatory response elicited in either human or animal hosts (Xu et al., 2005; Glass et al., 2004).

Other human CoV that show neurotropism:

Coronaviruses are widely known as human pathogens causing respiratory tract infections. In addition to SARS-CoV, MERS-CoV and SARS-CoV-2, other human CoV (HCoV) have been described in the last decades, including HCoV-229E, HCoV-NL63, HCoV-OC43 and HCoV-HKU1 (Zlateva et al., 2013; Gaunt et al., 2010; Desforges et al., 2019).

Although HCoV are usually restricted to the airways in immunocompetent patients, several studies showed evidence of neurotropic and neuroinvasive capacities of some HCoV strains (reviewed in Desforges et al., 2014; 2019). Early findings from Arbour and colleagues (2000) have shown a high prevalence of HCoV-229E and HCoV-OC43 in human brain autopsy samples of patients affected with multiple sclerosis and other neurological diseases (Arbour et al., 2000). In addition, neurological symptoms have been described in association with both HCoV-HKU1 and HCoV-NL63 infections (Severance et al., 2011).

Experimental studies have shown that HCoV strains have the ability of replicating in glial cells *in vitro*. In fact, the HCoV-229E and HCoV-OC43 have shown to infect glial and neuronal cell lines as well as primary human astrocytic cultures (Arbour et al., 1999a; Arbour et al., 1999b; Bonavia et al., 1997; Jacomy et al., 2006). *In vivo*, HCoV-OC43 is naturally neuroinvasive and promotes chronic encephalitis in mice, with clustering of microglial cells and neuronal loss (Jacomy et al., 2006).

Taken together, the present data point out to a neuroinvasive potential of HCoV-OC43 and HCoV-229E, where astrocytes, microglia and/or neurons could be the cell targets of the infection (Hwang and Bergmann, 2018; Malone et al., 2006). Again, the potential routes for HCoV are the hematogenous spread during viremia or the retrograde neuronal route (revised in Desforges et al., 2020).

In general terms, microglia and astrocytes play a critical role in RNA virus infection (Soung and Klein, 2018). The HIV is another RNA virus of great importance in public health where neurotropism, and especially the glial role in infection and chronicity, is being rapidly unveiled. HIV-1 exploits the *Trojan Horse Strategy* to reach the CNS parenchyma, where infected T-cells and monocytes cross the BBB, transferring infection to microglia and CNS

perivascular macrophages (Gonzalez-Scarano and Martin-Garcia 2005; Elbirt et al. 2015). While macrophages and microglia exhibit productive infection and can release viral particles, astrocytes become infected but they show restricted HIV-1 replication and non-productive infection (reviewed in Churchill and Nath, 2013). However, this is a *double-edged sword*; on the one hand, non-productive astroglial infection reduces brain damage, that would be otherwise a devastating CNS disorder, but on the other hand it allows for HIV-1 to latently persist in the brain parenchyma even in successfully treated patients with low viral load (reviewed in Pandey and Seth, 2019). Moreover, in patients with HIV-associated dementia, more than 19% of astrocytes have HIV sequences and the abundance is higher in astrocytes which are in close proximity to macrophages, especially at the perivascular regions (Churchill et al., 2009; reviewed in Pandey and Seth, 2019). In addition it is not clear how viral persistence affects the normal homeostatic functions of astrocytes that are essential for the CNS functionality.

Animal CoV that show neurotropism:

HCoV are molecularly and structurally related to several neuroinvasive animals CoV. Specifically MHV (mouse hepatitis virus) has been extensively studied, and both the John Howard Muller MHV (JHM-MHV) and the MHV-A59 strain, when inoculated intracranially or intranasally, are neurovirulent (Bender and Weiss, 2010; Cowley and Weiss, 2010). In general terms, after nasal inoculation, neurotropic strains of MHV reach the CNS through a transneuronal route up to the olfactory bulbs, from where viral particles spread into the brain parenchyma (Perlman et al., 1990; revised in Cowley and Weiss, 2010).

In rats, MHV infection promotes a primary stage of neuronal and glial infection, severe inflammation and destructive lesions mainly in the gray matter, followed by a secondary stage of demyelination with chronic inflammation, astroglial loss and axonal pathology (Nagashima et al., 1978, 1979; Zimprich et al., 1991).

Since the JHM-MHV strain is highly lethal in mice, a larger number of studies have been performed using the sublethal glia tropic variant of the JHM-MHV, designated v2.2-1

(Savarin and Bergmann, 2018). Both sub-lethal MHV-59 and JHM-MHV v2.2-1 viruses are able to produce an acute encephalomyelitis which turns into a persistent infection causing demyelinating lesions with presence of viral RNA, but absence of infective viral particles (Savarin and Bergmann, 2018). Infection of Lewis rats with JHM-MHV CoV induces viral particle incorporation by astrocytes and reactive astrogliosis at the surroundings of demyelinated lesion plates (Barac-Latas et al., 1997).

The murine JMH-MHV strain was shown to infect astrocytes *in vitro* and *in vivo* and astrocytes apparently constitute a viral reservoir in asymptomatic MHV-JHM-infected mice (Perlman and Ries, 1987). These early reports are consistent with the hypothesis that astrocytes can not only be hosts of the CoV but can also behave as reservoirs of CoV in the brain parenchyma. In culture, MHV infects astrocytes and microglia promoting a pro-inflammatory conversion of both cell types evidenced by an increase in pro-inflammatory cytokines such as TNFα, IL-1β and IL-6. Such phenomenon has been also evidenced *in vivo* during encephalitis and demyelination of spinal cord in mice models (Sun et al., 1995; Gonzales et al., 2004; Li et al., 2004).

The less aggressive JHM-MHV variant v2.2-1 also infects microglia, astrocytes, and oligodendrocytes. Here, major histocompatibility class (MHC) I and II complexes are upregulated in microglial cells and astrocytes in response to IFNγ, while oligodendrocytes may serve as a reservoir for viral persistence (Malone et al., 2006; Suzumura et al., 1986).

After intranasal exposure in mice, MHV-A59 produces acute hepatitis, meningitis, and encephalitis followed by a chronic phase of inflammatory demyelinating disease. It is clear that the acute phase involves active viral replication in liver and brain, while in the chronic phase viral RNA persist in the brain, but without evidence of viral replication or viral antigen expression (Lavi and Cong, 2020). Infection with MHV-A59 induces upregulation of several pro-inflammatory cytokines in astrocytes as well as the interferons IFNα, IFNβ and IFNγ (Lavi and Cong, 2020). On the other hand, the same authors showed that in microgliocytes, MHV-59 induces the expression of IL6 and of all the members of the IFN and TNF family. Interestingly, the non-encephalitic MHV strain (MHV-2), that does not produce acute or chronic encephalitis, failed to induce cytokine expression in astroglial or

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microglial cultures (Lavi and Cong, 2020). These important findings reinforce the concept that polarization of reactive astrocytes and microglia to the pro-inflammatory phenotype is a key event in determining neural detrimental effects of neurotropic CoV infections.

However, the switch to a pro-inflammatory phenotype seems not to be the unique astroglial and microglial response to neurotropic virus, and especially to neurotropic CoV. Astrocytes and microglia are the CNS resident cells most prominently involved in the innate immunity responses, as they are able to respond to PAMP that interact with pattern recognition receptors (PRR) highly expressed in microglia but also expressed in lower level in astrocytes (Farina et al., 2007; Russo and McGavern, 2015; Hwang and Bergmann, 2018; Rosciszewski et al., 2018; 2019). Recent findings have shown that astrocytes mount a delayed but robust antiviral response to the neurotropic MHV-A59 CoV *in vivo* in the rodent encephalitis model (Hwang and Bergmann, 2018). In this model, astrocytes upregulated the IFN-α/β pathway to a greater extent than microglia. Concomitantly, the specific ablation of IFNAR in astrocytes using a transgenic mGFAPcre $IFNAR^{fl/fl}$ mice resulted in severe encephalomyelitis, increased neutrophil and decreased T-cell infiltration, uncontrolled viral spread in the CNS parenchyma and increased lethally in deficient mice (Hwang and Bergmann, 2018). As mentioned before, IFNγ, one of the type 2 interferons (IFN-II), is a key molecule in the response of glial cells to viral infection and it is produced by cells which are not brain residents. However, type 1 interferons (IFN-I) and the astroglial IFNAR also play a major role in responses to neurotropic viral infection, including CoV (Hwang and Bergmann, 2018; Malone et al., 2006; Owens et al., 2014).

In addition to the astroglial role in preventing CNS spreading of MHV-A59, microglia seems also to be required to control neurotropic CoV infection. A recently published article showed that depletion of microglia using PLX5622, an inhibitor of colony stimulating factor 1 receptor (CSF1R), in animals exposed to JHM-MHV increased mortality, impaired control of viral replication, altered CD4+ and CD8+ T-cell infiltration, increased demyelination and neuro-repair, (Mangale et al., 2020). However, microglia depletion may also impair astroglial response to viral CoV infections.

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406 The glial role in the response to neurotropic viruses seems not to be unique for CoV and
407 has been also shown for West Nile virus encephalitis in mice, where astrocytes mount an
408 IFN-I-dependent response to regulate BBB permeability and protection (Daniels et al.,
409 2017) and microglial deletion has also been detrimental (Funk and Klein, 2019; Seitz et al.,
410 2018). Microglia is also required to decrease mortality in Japanese encephalitis virus (JEV)
411 infected mice (Seitz et al., 2018) and Theiler's murine encephalomyelitis virus (TMEV)
412 (Sanchez et al., 2019a; Waltl et al., 2018).
413
414 **Severe Acute Respiratory Syndrome-2 (SARS-CoV-2):**
415
416 While the hallmarks of COVID-19 moderate clinical presentation are fever, respiratory
417 symptoms including dry cough and dyspnea and diarrhea, a more severe respiratory
418 condition termed acute respiratory distress syndrome (ARDS) can eventually develop, and
419 it includes the cytokine release syndrome (*cytokine storm)*. In these severe cases, SARS-
420 CoV-2 exits the respiratory tract niche, and propagates to other organs of the body
421 including brain (Gubernatorova et al., 2020). Indeed, the systemic infection is evidenced as
422 reduced number of blood immune cells, abnormal liver and metabolic functions (Fu et al.,
423 2020; Pan et al., 2020), and neurological symptoms (Asadi-Pooya and Simani, 2020; Helms
424 et al., 2020; Mao et al., 2020). Neurological manifestations of SARS-CoV-2 infection are just
425 starting to be reported and systematically recorded in detail, with several initiatives to
426 consolidate and systematically record clinical findings (Ferranese et al., 2020; Roman et al.,
427 2020; Romero-Sanchez et al., 2020). Between 25 and 40% of the SARS-CoV-2 patients
428 present neurological symptoms, these can go from mild symptoms including olfactory and
429 gustatory disorders, dizziness, headache, confusion, to a more severe cerebrovascular
430 disease, encephalitis, seizures or the Guillain-Barré syndrome (Asadi-Pooya and Simani,
431 2020; Mao et al., 2020). The presence of SARS-CoV-2 viral particles in the CNS has been
432 evidenced in some cases (Benameur et al., 2020; Moriguchi et al., 2020; Wu et al., 2020),
433 with reports showing SARS-CoV-2 presence in CSF (Finsterer and Stollberger, 2020), and a
434 recent report showing SARS-CoV-2 viral particles in neural and capillary endothelial cells of

frontal lobe brain sections of a COVID-19 patient (Paniz-Mondolfi et al., 2020). In several patients, SARS-CoV-2 infection has been associated with ischemic stroke, and this has been originally attributed to excessive inflammation, hypoxia, or diffuse intravascular coagulation (Lu et al., 2020a; Mao et al., 2020; Yang et al., 2020b).

SARS-CoV-2 is a single-stranded positive-sense RNA virus sharing 79% and 50% identity with SARS-CoV and MERS-CoV, respectively (Lu et al., 2020b). The spike (S) protein of CoV is known to engage cell surface receptors to facilitate viral entry into the cells. It has been determined, as seen for SARS-CoV that SARS-CoV-2 spike S protein also uses ACE2 as entry receptor (Hoffmann et al., 2020). Taking into account that SARS-CoV-2 shares the ACE-2-mediated mechanism of entrance to the cells, it is highly possible that these molecular mechanisms would also help explain the more severe forms of the disease, including the possibility of a disbalanced renin-angiotensin system, which alters vascular permeability and severe acute lung injury as was demonstrated for SARS-CoV (Imai et al., 2005; Kuba et al., 2005).

There are several hypotheses as to how could SARS-CoV-2 reach and affect the CNS. Neuronal retrograde dissemination has been observed in other CoV (see above), and taking into account the presence of the virus in nose swabs, hyposmia symptoms in some patients, and the expression of ACE2 in the nasal mucosa (Hou et al., 2020), a transneuronal pathway involving olfactory epithelium>olfactory bulb>olfactory nucleus in the pyriform cortex cannot be ruled out (**Figure 1**). Several preprint articles have identified the expression of ACE2 in olfactory mucosa cells of human origin (Baxter et al., 2020; Brann et al., 2020; Hikmet et al., 2020).

Another possible way for SARS-CoV-2 to enter the CNS is the hematogenous route during viremia in severe affected patients. SARS-CoV-2 could enter through the circumventricular organs, which lack BBB, or through altered BBB where endothelial cells and astroglial end-feet could have a major role (**Figure 1**). Endothelial cells express ACE2 and thus are potential targets to be infected by SARS-CoV-2 (Hamming et al., 2004; Feng et al., 2010). Although there is no data of SARS-CoV-2 presence in astrocytes, it is likely that astrocytes become infected having in mind the reports showing astrocytes infected and even being a

reservoir for several CoV as commented in the previous sections (Xu et al., 2005; Jacomy et al., 2006; Desforges et al., 2019; Sun et al., 1995; Gonzales et al., 2004; Li et al., 2004) (**Figure 2**).

In addition to astrocytes being potential targets for SARS-CoV-2, both astrocytes and microglia are highly sensitive to systemic pro-inflammatory cytokines (Perry et al., 2007; Teeling and Perry, 2009; Murta et al., 2015; Murta and Ferrari, 2016). Indeed, the massive release of inflammatory cytokines described for severe COVID-19 patients could be enough to destabilize the tight junctions of the BBB endothelial cells and astrocytes, thus facilitating viral entry (Li et al., 2015; Swanson and McGavern, 2015) (**Figure 2**). Furthermore, as explained in the above sections, astrocytes and microglia are part of the local neuroinflammatory response of the CNS, and express PRR, the cellular receptors needed to initiate and/or amplify innate immune responses within the CNS (Farina et al., 2007; Russo and McGavern, 2015; Hwang and Bergmann, 2018; Rosciszewski et al., 2018; 2019). As part of the BBB, astrocytes would rapidly receive the pro-inflammatory signals from endothelial cells, in addition to microglial-derived cytokines. Microglia has been demonstrated to closely interact with monocytes and lymphocytes in viral infections affecting the CNS (see for example Hwang and Bergmann, 2018; Mangale et al., 2020). Moreover, it has been shown that the presence of reactive pro-inflammatory microglia, the exposure to pro-inflammatory cytokines such as IL-1β, TNFα, IL-6, or the exposure to PAMP/DAMP can induce in astrocytes the polarization to the A1 phenotype, which facilitates neuroinflammation and neurodegeneration (Liddelow et al., 2017; Rosciszewski et al., 2018; 2019) (**Figure 2**). Astrocytes have also been shown to play a major role in the IFN response during viral infection with neurotropic CoV (Hwang and Bergmann, 2018).

Astrocytes also have a major role in synthesizing brain angiotensin (Stornetta et al., 1988; Sherrod et al., 2005), and its production is essential for maintaining BBB integrity (Wosik et al., 2007). Pro-inflammatory cytokines disrupt angiotensin II production by human astrocytes *in vitro* (Wosik et al., 2007) and thus astrocytic angiotensin production could be affected in SARS-CoV-2 patients, facilitating BBB disruption and exacerbating neuroinflammation. The CNS renin-angiotensin system is involved in critical biological

493 functions, including the control of cerebral blood flow, vascular repair, neuronal protection
494 and memory (Wosik et al., 2007).
495
496 **Conclusions on the role of astrocytes and microglia in SARS-CoV-2 infection:**
497
498 Recent reports suggest that SARS-CoV-2 presents neurotropism. However, to our present
499 knowledge there are no details available on how different brain cells types respond to the
500 infection (Cardona et al., 2020; Ng Kee Kwong et al., 2020). Our present review of the
501 published reports on MERS-CoV and SARS-CoV outbreaks, human CoV studies and
502 experimental data from animal models, may serve as a good starting point to delineate the
503 possible role of microglia and astrocyte responses to SARS-CoV-2.
504 One of the main questions that we attempted to explore was whether the published
505 evidence supports the hypothesis that astrocytes and microglia are directly related to
506 SARS-CoV-2 invasion to the CNS, whether these cells were subjected to productive
507 infection or were non-productive reservoirs of SARS-CoV-2. It is definitely early to answer
508 this question, having no evidence from histopathological data or single cell RNAseq of
509 human brains from SARS-CoV-2 patients. However, it can be speculated on the basis of
510 SARS-CoV findings and the research performed with human and animal CoV that showed
511 neurotropism, that this possibility may exist (**Figure 2**). It is well known that HIV-1 utilizes
512 astrocytes as reservoir, but this RNA virus is characterized by a rapid viremia and
513 lymphocyte infection, using these cells as *Trojan Horse* to enter the CNS, then infecting
514 microglia in a productive form and astrocytes in a non-productive form. This profuse acute
515 viremia and the use of lymphocytes to expand infection seem not to be features of SARS-
516 CoV-2, however, brain endothelial cells express ACE2 and may become infected and allow
517 productive infection at the BBB of the CNS. Could the astroglial end-feet in contact with
518 endothelial cells get infected with SARS-CoV-2 particles released from infected
519 endothelium? This is an open question, but the finding of SARS-CoV positive astrocytes in
520 human cases, together with the evidence that indicates that human HCoV-OC43 and

HCoV-229E strains and animal MHV strains do infect astrocytes may support necessary and important future investigations in this direction.

What would be the risk of having astrocytes infected with SARS-CoV-2 in the CNS? This question has two very different outcomes. If the astrocytes undergo a productive infection, the destruction of the astroglial network that gives support to neurons, oligodendrocytes and other cells would have catastrophic effects in the CNS, together with a massive pro-inflammatory response due to cell death. The present clinical evidence of neurological symptoms in SARS-CoV-2 patients does not support this possibility. Much more likely is that a non-productive infection in astrocytes may turn astrocytes to a mild pro-inflammatory phenotype, causing a *malfunction* of standard astroglial activities such as metabolic support to neurons and oligodendrocytes, excitatory aminoacid uptake, balancing CNS homeostasis, ion buffering, maintenance of BBB integrity, crosstalk with microglia and other immune cells (**Figure 2**).

If astrocytes and microglia do not become infected, do these findings exclude astrocytes and microglia from having an important role in human SARS-CoV-2 infection? Surely not. Although SARS-CoV-2 replication in astrocytes may still seem controversial, our present knowledge on astrocytes and microglia, and more specifically, on their role in neuroinflammation in the injured brain as well as in viral encephalitis (Desforges et al., 2019; Chen et al., 2019; Soung and Klein, 2018; Das Sarma, 2014), indicates that it is likely that glial cells have an important role in initiating/expanding neuroinflammation in the SARS-CoV-2 infected brain (**Figure 2**). Astrocytes and microglia behave as innate immunity cells in the CNS, participating of neuroinflammation initiation and expansion (Liddelow et al., 2017; Rosciszewski et al., 2018; Yun et al., 2018; Joshi et al., 2019). Astrocytes can polarize to an A1 pro-inflammatory phenotype that facilitates neuroinflammation, neurodegeneration and BBB disruption, especially when they are exposed to PAMP or to pro-inflammatory cues derived from microglia (Liddelow et al., 2017; Rosciszewski et al., 2018; Yun et al., 2018; Joshi et al., 2019). The astroglial A1 phenotype seems to be induced by microglial activation, but is then sustained in an autonomous manner (Liddelow et al., 2017). Moreover, astroglial-microglial cooperation was also shown to be activated by

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DAMP (Damage-Associated Molecular Patterns) released by damaged neurons (Rosciszewski et al., 2019; Kumar, 2019). Considering the extensive neuronal death observed both in the previous SARS-CoV human cases and experimental models and the CNS and systemic exacerbated cytokine expression, it is tempting to hypothesize that CoV infection in the CNS would have the ability of polarizing astrocytes and microglia to the pro-inflammatory phenotype, thus worsening the clinical outcome and neurovirulence. It is important to note that this scenario does not require that SARS-CoV-2 infects the CNS, since endothelial cells may transfer the pro-inflammatory signals to the CNS parenchyma (**Figure 2**).

Last, but not least, is the possibility that SARS-CoV-2 infection profoundly alters the angiotensin brain network. As commented above, the disbalanced ACE2 receptor expression due to viral entrance to cells, together with a mild effect on astrocytes could render unknown consequences for the CNS in the SARS-CoV-2 infected patients (**Figure 2**).

In many patients, SARS-CoV-2 infections have been associated with ischemic stroke, and this has been originally attributed to excessive inflammation, hypoxia, or diffuse intravascular coagulation (Lu et al., 2020a; Mao et al., 2020; Yang et al., 2020b). This is just an example that, in the light of the data compiled here, a caution sign should be put on in this simplistic direct association of stroke and also to other CNS symptoms, excluding a primary effect of SARS-CoV-2 on the CNS.

The present SARS-CoV-2 outbreak has taken the world by surprise and has demanded for rapid implementation of measures that help contain the virus, while scientists all over the world race against the pandemic to find treatments and solutions. Taking into consideration the important neurological clinical manifestations stated above, and the role of the different CNS cell types, gaining as much knowledge as possible from COVID-19 impact in the nervous system is essential to improve our treatment strategies and therapies for this and future CoV outbreaks.

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Figure Legends:

Figure 1: SARS-CoV-2 potential entry routes to the CNS.

SARS-CoV-2 may reach CNS using two main routes: the olfactory epithelium or the hematogenous (blood) routes. When entering through the olfactory epithelium, viral particles would have access to the CNS using transneuronal/synaptic pathways. Following neuronal infection, viral particles released may infect microglia and astrocytes. In the hematogenous entry route, endothelial cells may become infected, followed by perivascular astrocytes and perivascular macrophages. Secondarily, viral particles may reach microglia and neurons. Also during viremia in severe affected patients, SARS-CoV-2 can reach choroid plexus and circumventricular organs, which lack of BBB, and subsequently entering to brain parenchyma. A third possibility of CNS infection may arise after BBB breakdown as a consequence of systemic inflammation and cytokine release. Increased BBB permeability could facilitate SARS-CoV-2 entrance to the CNS.

Figure 2: Potential astroglial roles in SARS-CoV-2 infection

Astrocytes are viral hosts. Perivascular astrocytes may incorporate viral particles by direct contact with infected endothelial cells. However, BBB breakdown may also lead to viral infection of non-perivascular astrocytes. In both situations, infected astrocytes may act as host cells giving non-productive infection with mild inflammatory response; or astrocytes may give rise to a productive infection, with destruction of astroglial network. This latter possibility is more unlikely based on clinical findings.

Astrocytes are not viral hosts. Astrocytes are not primary targets of viral infection, but they are responsive to pro-inflammatory signals from endothelial cells, macrophages, microglia and/or neurons. In such case, astrocytes may polarize to A1 pro-inflammatory – neurodegenerative phenotype expanding neuroinflammation and stopping being protective and supportive to neurons which also degenerate by lack of nutrients and neurotrofic factors.

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Potential CNS entry routes for SARS-CoV-2

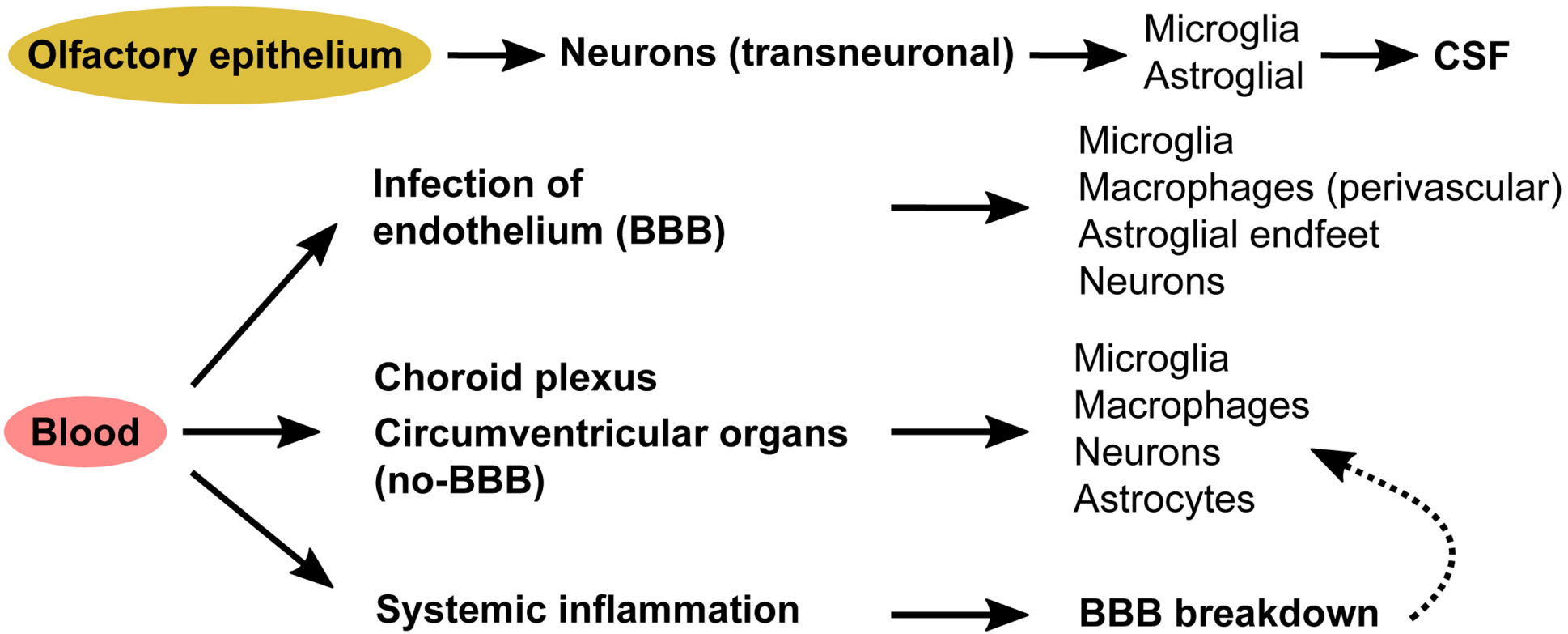

Figure 1

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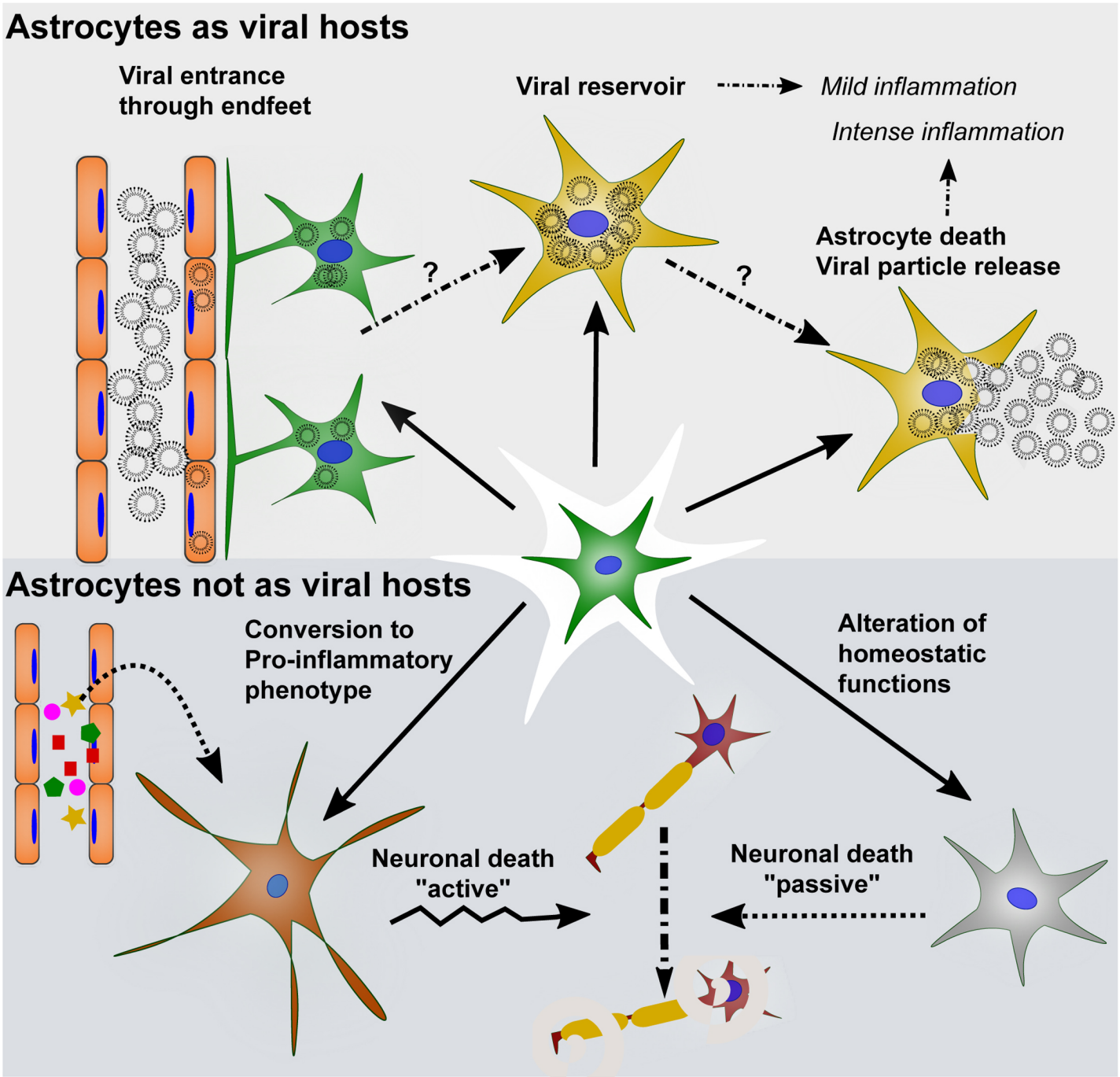

Figure 2