SARS-CoV-2 infects neurons and induces neuroinflammation in a non-human primate model of COVID-19

Graphical abstract

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In brief
Beckman et al. show that SARS-CoV-2 proteins are found in olfactory areas of the brains of rhesus macaques at 7 days post infection. In addition to direct neuronal infection, extensive neuroinflammation and vascular disruptions are observed, and these effects are exacerbated in aged, diabetic animals.

Highlights
- In macaques, SARS-CoV-2 is found in olfactory brain areas at 7 days post infection
- Neurons are initially the primary target of SARS-CoV-2 productive infection
- Neurocovid is accompanied by robust neuroinflammation and vascular disruption
- SARS-CoV-2 brain pathology is worsened by aging and diabetes in infected monkeys
SARS-CoV-2 infects neurons and induces neuroinflammation in a non-human primate model of COVID-19

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SUMMARY

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the etiologic agent of coronavirus disease 2019 (COVID-19), can induce a plethora of neurological complications in some patients. However, it is still under debate whether SARS-CoV-2 directly infects the brain or whether CNS sequelae result from systemic inflammatory responses triggered in the periphery. By using high-resolution microscopy, we investigated whether SARS-CoV-2 reaches the brain and how viral neurotropism can be modulated by aging in a non-human primate model of COVID-19. Seven days after infection, SARS-CoV-2 was detected in the olfactory cortex and interconnected regions and was accompanied by robust neuroinflammation and neuronal damage exacerbated in aged, diabetic animals. Our study provides an initial framework for identifying the molecular and cellular mechanisms underlying SARS-CoV-2 neurological complications, which will be essential to reducing both the short- and long-term burden of COVID-19.

INTRODUCTION

Coronaviruses, members of the subfamily Orthocoronaviridae, are enveloped single-stranded RNA viruses known to infect a wide range of bird and mammalian species, including humans. In 2019, the newly emerged coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) became a significant public health concern, infecting over 96 million people in the United States alone and resulting in more than one million deaths, disproportionately affecting older individuals with preexisting comorbidities.1 In addition to respiratory and gastrointestinal symptoms commonly caused by other coronaviruses, SARS-CoV-2 infection is accompanied by a myriad of neurological presentations in up to 80% of hospitalized patients, in addition to reductions in gray-matter thickness and tissue contrast observed through in vivo imaging.2,3 Considering the high degree of homology between SARS-CoV-2 and other coronaviruses with demonstrated neurotropic potential, including SARS-CoV-1 and Middle Eastern respiratory syndrome coronavirus (MERS-CoV),1,4,4 there is a critical need to determine if a direct infection of the central nervous system by SARS-CoV-2 is the underlying mechanism for the neurological symptoms in coronavirus disease 2019 (COVID-19).

Direct examination of post-mortem brain samples from patients with COVID-19 has yielded contradictory results, with several studies reporting the positive detection of viral RNA in the CNS,5,8 although others found low or undetectable viral RNA levels9–11. These variations underscore the need for animal models that may allow us to probe SARS-CoV-2 behavior in a more controlled environment, with rhesus macaques (Macaca mulatta) showing great potential as a platform for scientific discovery in COVID-19. These animals have extensive similarities with human immunological responses and have already significantly contributed to defining the safety and effectiveness of SARS-CoV-2 vaccines, uniquely positioning them to help us understand the effects of COVID-19 in the CNS.9,12

Considering the above, the California National Primate Research Center (CNPRC) has launched an ongoing effort to comprehensively characterize SARS-CoV-2 infection in macaques, including its neurotropic potential.13–15 In this study, we aimed to establish whether SARS-CoV-2 can be detected in the brains of rhesus monkeys during the acute phase of the infection by employing high-resolution three-dimensional (3D)
confocal microscopy combined with extensive morphometric analyses using semi-automated object segmentation.16,17 Toward that goal, we intranasally and intratracheally inoculated young, healthy rhesus monkeys (4–6 years old; n = 4) with a high dose of SARS-CoV-2 (2.5 \times 10^9 plaque-forming units [PFUs]) and euthanized the animals at 7 days post infection (7 dpi)14 (Figure S1). Additionally, to better understand how age and comorbidities may contribute to CNS infection, we included an additional cohort of type II diabetic (T2D), aged monkeys (18–24 years old; n = 4) subject to the same experimental design. Non-infected, aged-matched animals without T2D (young n = 2; aged n = 2) and with T2D (young n = 1; aged n = 1) were included in the study as biological controls. Chronic illness and current treatments, as well as COVID-19 pathology developed by infected animals, are presented in Table S1 and Data S1. Notably, at the time of the brain analyses, COVID-19 clinical signs of infection were generally mild and did not require intervention (more details in Shaan Lakshmanappa et al.14 and Rompay et al.15). A summary of the antibodies used in this study can be found in Figure S1.

RESULTS

SARS-CoV-2 markers are found in olfactory areas in an age-dependent manner at 7 dpi

Initial immunohistochemical labeling of SARS-CoV-2 nucleocapsid (N) protein in the frontal lobe revealed substantial immunoreactivity to SARS-CoV-2 N, demonstrating the presence of viral proteins in the brain within 7 days of inoculation. For the purposes of this section, results are reported for the piriform cortex, as this cortical area showed the highest immunohistochemical signal in preliminary results. Cell-type analysis revealed that the N immunosignal colocalized with all three major cell types within the brain, neurons (NeuN+), astrocytes (GFAP+), and microglia (Iba1+), in both young and aged animals (Figure 1A and 1B). Volume quantification of intracellular N protein by cell type indicates that neurons showed the highest degree of intracellular N regardless of age, and aged animals displayed higher volumes compared with young animals across all three cell types, neurons (4.3% versus 13%; p = 0.0004), astrocytes (2.3% versus 4.2%; p = 0.5836), and microglia (0.5% versus 2.9%; p = 0.4034), although only neurons reached statistical significance (Figures 1C and S2). These results suggest a partial neurotropism for SARS-CoV-2 and an age-dependent increase in infection burden.

Following the preliminary establishment of SARS-CoV-2 entry into the CNS, we expanded the analysis to include different viral markers and additional brain areas. In addition to SARS-CoV-2 N, we were able to detect SARS-CoV-2 spike (Spk) protein and double-stranded RNA (dsRNA), an intermediary molecule in the replication cycle of SARS-CoV-2 that serves as a proxy for productive infection.18 Immunolabeling for these markers was found in multiple areas of the primary olfactory cortex, including the olfactory tubercle, the piriform cortex, and the olfactory pole of the entorhinal cortex, irrespective of age. In aged animals, SARS-CoV-2 viral markers were also observed in the orbitofrontal cortex (area 14), which is part of the secondary olfactory cortex. In contrast, only minimal labeling was observed outside the primary olfactory cortex in young animals (Figures 1D, 1E, and S3). This distribution pattern is coherent with the axonal spread of the virus from the nasal olfactory epithelium, a mechanism of entry previously described for other coronaviruses.19–21

Since the distribution pattern of SARS-CoV-2 Spk and, in particular, dsRNA was predominantly neuronal (Video S1), we proceeded to perform a regional morphometric analysis of these markers in neurons of the abovementioned areas. Across all sites, dsRNA immunoreactivity was detected exclusively within the soma, while Spk immunoreactivity was found predominantly within dendrites (Figure S2). Compared with aged control (CTR) animals, infection with SARS-CoV-2 in older animals was associated with a significant decrease in cell body volume (p = 0.0063) and a trend for reduced dendrite volume (p = 0.0571) (Figure S2). Across the primary olfactory cortex, the percentage of intraneuronal dsRNA was significantly higher in aged animals compared with young animals: piriform cortex (4.4% versus 7.1%, p = 0.0049), olfactory tubercle (1.4% versus 3.9%, p = 0.0091), and entorhinal cortex (0.08% versus 3.4%, p = 0.0008). No statistical difference was observed in the orbitofrontal cortex (0.1% versus 0.5%, p = 0.9453), likely due to the very low overall signal observed in both young and aged animals (Figure 1F). While a similar pattern of higher intraneuronal localization was also observed for Spk, higher intragroup variability led to a statistically significant difference between young and aged animals exclusively in the entorhinal cortex (0.7% versus 3%; p = 0.0081) (Figure 1G).

Taken together, our data indicate that SARS-CoV-2 is neurotropic and can be detected in olfactory areas of the macaque brain within 7 dpi. Viral proteins are found in both neuronal and glial cells, but productive infection at this time point is predominantly neuronal. Aged T2D animals displayed increased viral load across all areas examined, in addition to more pronounced cellular alterations in response to the virus.

SARS-CoV-2 infection leads to neuroinflammation that is exacerbated in aged animals

Although neurotropic viruses have developed mechanisms to escape host immune surveillance and facilitate CNS entry,22 local inflammation is still a factor contributing further to neuronal damage and death and may offer an addressable therapeutic target. Therefore, we investigated neuroinflammation in our rhesus model of COVID-19. Informed by the results described above, we focused our analysis on the piriform cortex, which consistently displayed the highest SARS-CoV-2 Spk and dsRNA levels.

We detected an increase in the number of astrocytes between young, infected animals and their age-matched, non-infected controls (p = 0.0581). Notably, a significant increase in the GFAP population was observed comparing young and aged infected animals (p = 0.011), suggesting either proliferation or translocation of astrocytes to the piriform cortex. In addition, a robust increase in astrocytic 3D cell volume was also observed when comparing young and aged infected animals (p = 0.0009) (Figures 2A, 2B, S2, and S4). A similar pattern of activation was detected for microglia, as measured by the fraction of Iba1+ cells coexpressing the major histocompatibility complex (MHC) class II surface receptor HLA-DR (p = 0.0344).
In addition, microglial proliferation/translocation in response to infection was significantly increased in aged, infected animals when compared with the young, infected group (p = 0.0049) (Figure 2D). These results suggest a robust inflammatory process in response to SARS-CoV-2 infection that mobilizes both astrocytic and microglial cascades (Figure S4). Notably, investigation of the piriform cortex of diabetic young and aged control animals detected inflammatory alterations associated with diabetes only in the aged animals (Figures S4 and S5).

(Figures 2C–2E, S4, and S5). In addition, microglial proliferation/translocation in response to infection was significantly increased in aged, infected animals when compared with the young, infected group (p = 0.0049) (Figure 2D). These results suggest a robust inflammatory process in response to SARS-CoV-2 infection that mobilizes both astrocytic and microglial cascades (Figure S4). Notably, investigation of the piriform cortex of diabetic young and aged control animals detected inflammatory alterations associated with diabetes only in the aged animals (Figures S4 and S5).

With normal aging, microglia acquire a hypersensitive state that can quickly shift to an inflammatory profile upon viral infection, increasing the risk of developing neurodegenerative diseases.23,24 Several lines of evidence indicate a similar shift takes place in the animal model described in this work: (1) HLA-DR+ microglia were found associated with degraded myelin basic protein (dgMBP) at the expense of regular MBP expression, potentially leading to white-matter injury (Figures 2F and S6); (2) HLA-DR+ microglia were found in contact with neurons expressing MHC class I, a viral antigen substantially enhanced in

**Figure 1. SARS-CoV2 preferentially infects neurons and spreads faster throughout the olfactory cortex of aged, infected monkeys**

(A and B) Quantitative quadruple staining combining markers for neuron (NeuN), microglia (Iba1), and astrocytes (GFAP) with SARS-CoV2 nucleocapsid protein (Npnt) was performed in the primary olfactory cortex of young and aged infected monkeys.

(C) Internalized Npnt volume was calculated in 3D and divided by the total 3D volume obtained for each infected cell type analyzed.

(D) The neurotropic potential of SARS-CoV2 was investigated in the primary olfactory cortex (blue; piriform cortex [PC], olfactory tubercle [OT], and entorhinal cortex [EC]) and the prefrontal secondary olfactory region, the orbitofrontal cortex (OFC; green).

(E) Representative micrographs and 3D volume reconstruction show spike (Spk) protein (purple) and dsRNA (red) expression across several olfactory regions. (F and G) Quantification of the intraneuronal levels of dsRNA (F) and Spk protein (G) demonstrates that SARS-CoV-2 spreads faster in aged animals compared with young, infected controls.

Scale bar, 50 μm. **p < 0.01, ***p < 0.001, two-way ANOVA, Sidak’s post hoc test. Numerical data are represented as mean ± SEM.

See also Figures S2 and S3 and Video S1.
Figure 2. The highly connected olfactory cortex shows a robust inflammatory response following SARS-CoV-2 infection

(A and B) Unbiased 3D microscopic quantification shows a significant increase in astrocyte recruitment (GFAP+) following SARS-CoV-2 infection.

(C and E) In addition, a robust increase in general (Iba1) and activated (HLA-DR) microglia markers are also associated with aging.

(F) HLA-DR+ reactive microglia were also found associated with degraded myelin basic protein (dgMBP), resulting in reduced normal myelin basic protein (MBP) expression in aged-infected animals in comparison with aged controls.

(G) To further analyze neuron-microglia dynamics across the experimental groups, we combined general markers for neurons (pan-NF) and microglia (Iba1) with the post-synaptic marker PSD95 in the primary olfactory region.

(H-L) Analysis of PSD95 puncta density (H), microglial total cell volume (I), pan-NF microglial engulfment (J), microglial soma volume (K), and PSD95 microglial engulfment (L) demonstrates a robust phagocytic response observed in the brain following SARS-CoV-2 infection, especially in the older infected population. Scale bars, 200 μm (A–F), 10 μm, and 5 μm (3D) (G). *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA, Tukey’s post hoc test. Numerical data are represented as mean ± SEM.

See also Figures S4–S7 and Video S1.
Figure 3. SARS-CoV-2 infection induces blood vessel disruption, reactive neutrophil recruitment, and ACE2 receptor downregulation

(A–D) Airyscan super-resolution microscopy was applied to quantify ACE2 and Spk protein expression and binding within olfactory neurons (pan-NF maker) across the different experimental groups.

(E) ACE2 expression is substantially reduced in the neurons of aged, infected animals compared with young, infected controls.

(F) In addition, around 15%–40% of the remaining receptors colocalize with Spk protein in both infected groups. Multiple structural abnormalities in the BBB were observed across the olfactory cortex of infected monkeys.

(G) Neurons (NeuN) and astrocytes (GFAP) present high levels of ACE2 expression and are also the major cell types expressing Spk protein.

(H) Notably, disrupted blood vessels are associated with activated neutrophils (MPO) and robust recruitment of reactive astrocytes.

(I) Altered BBB was also confirmed using aquaporin 4 (AQ4), a water channel expressed in astrocytic endfeet and involved in edema formation.

(legend continued on next page)
neurons from diabetic older animals, but worsened following SARS-CoV-2 infection (Figure S5); and (3) there is a higher frequency of microglia with abnormal morphology (e.g., truncated processes, round soma) in infected animals, and these microglia are often found adjacent to neurons with morphological changes, suggesting profound impact suggestive of neurodegenerative processes (Figures 2G, S7, and S8; Video S1).

Considering these findings, we hypothesized that SARS-CoV-2 neuroinflammation might lead to synaptic and cellular damage. Consistent with that idea, we observed a significant decrease in the density of PSD95+ puncta in infected, aged animals compared with young, infected ones (p = 0.0081) (Figure 2H). The total microglia cellular volume from aged, infected animals was also increased when compared with microglia from young, infected ones (p = 0.0015), as well as when compared with aged, non-infected controls (p = 0.0023) (Figure 2I). Notably, aged, infected microglia also presented an increase in the total volume of internalized pan-Neurofilament (pan-NF) fragments (young, infected versus aged, infected: p = 0.0003, aged, CTR versus aged, infected: p = 0.0019) (Figure 2J). Increased microglial soma volume (young infected versus aged infected: p = 0.0011, aged, CTR versus aged, infected: p = 0.0029) (Figure 2K) was also detected and correlated with selective microglia-engulfed PSD95 (young, infected versus aged, infected: p = 0.0005, aged, CTR versus aged, infected: p = 0.0037) (Figure 2L), suggesting a direct effect of activated microglia over the reduction in synaptic boutons (Figure 2I). A summary of the 3D analysis protocol used in this study can be found in Figure S8.

In summary, SARS-CoV-2 infection leads to a fast cascade of inflammatory events driven by astrocytic and microglial responses. In both cases, aged animals showed comparatively higher levels of inflammation than infected young animals. In addition, a significant increase in microglia density and synaptic engulfment were observed exclusively in older, infected animals, suggesting a compounding effect of aging and antiviral response. Notably, synaptic pruning and myelin degradation mediated by microglia may underlie some of the neurological deficits observed in patients with COVID-19.

**DISCUSSION**

In the present study, we have investigated the presence of SARS-CoV-2-related proteins in the brains of rhesus monkeys at 7 days post inoculation to see the neurotropic potential of SARS-CoV-2 at this early point in the course of the infection in a non-human primate. The overwhelming predominance of productive neuronal infection, combined with the spatially restricted distribution of viral proteins to the olfactory circuit, suggests the fast transneuronal spread of SARS-CoV-2 along corticocortical pathways, leading to its dissemination within the CNS via the olfactory connectome (summarized in Figure 4). This observation is in good agreement with previous reports that have demonstrated a similar progression pattern in laboratory animals for other human coronaviruses, such as HCoV-OC43 and HCoV-229E, and it closely matches the areas of decreased gray-matter thickness in human patients with COVID-19.

Furthermore, the impact on olfactory pathways is consistent with the persistent anosmia observed in some patients with COVID-19. We cannot exclude, however, the possibility that the virus reaches the CNS through other pathways (e.g., vascular breakdown, translocation of infected immune cells) at later stages of the infection.

The results found in this work also indicate that the presence of SARS-CoV-2-related proteins within the olfactory pathway is accompanied by extensive microglial and astrocytic changes typically associated with neuroinflammatory responses, including alterations in cellular volume, morphology, and populational density, in addition to abnormal blood vessels and infiltration of activated neutrophils. Our observations are remarkably similar to those of Rutkai et al., who found morphological changes in microglia consistent with activation, evidence of astrogliosis, and vascular homeostasis disruptions at 28 dpi. Combined, these works suggest that neuroinflammatory changes happen early during the course of the disease and can remain in place for an extended period of time, even if viral proteins are eliminated after the acute stage of the disease.
Importantly, in aged animals, neuroinflammation was accompanied by synaptic engulfment and myelin degradation in areas exhibiting a high density of HLA-DR+ microglia, suggesting that demyelinating lesions and synaptic loss could be early deleterious effects resulting from an exacerbated neuroinflammatory response that can persist for several weeks. This process may explain why some patients with COVID-19 experience neurological symptoms, even in the absence of severe respiratory disease, and it underscores the potential for anti-neuroinflammatory agents in the control of COVID-19-related neurological sequelae.

The importance of the more widespread and severe neuronal infection by SARS-CoV-2 and the corresponding increased neuroinflammatory response observed in aged macaques with T2D and other comorbidities should not be understated. Older patients with diabetes (and other age-related comorbidities) are the most vulnerable population regarding COVID-19 severity and lethality, reinforcing the need to represent this complex group of individuals in translational animal models.28 Moreover, several early reports indicate that older patients experience neurological symptoms with increased severity and frequency, including complex neurological presentations unique to this population.29 Our work suggests that viral infection and senescence/comorbidities lead to synergistic damage to central nervous function, helping explain the uniqueness of this group regarding neurologic complications in COVID-19. We have also identified dsRNA and SARS-CoV-2 proteins in the entorhinal cortex of our experimental model of aged macaques, a cortical region particularly vulnerable to tauopathy associated with Alzheimer’s disease (AD). Considering the short period of time investigated in this work, it is likely that SARS-CoV-2 eventually reaches the same temporal and frontal cortical fields that are affected in AD, with yet unknown consequences. More studies will be necessary to understand the temporal course of the infection, as well as its implications for long-term neurological sequelae and potentially dementia.

Limitations of this study
Some limitations of this study should be acknowledged. This study employs a group of aged animals (18–24 years old) with T2D combined with other comorbidities, an experimental group designed to mimic the clinical complexity of older human patients infected with SARS-CoV-2. Data obtained from these animals should be interpreted with care as there is an intrinsic challenge to dissociating the effects of aging, T2D, and other comorbidities. The limited supply of spontaneous T2D animals in our colony has constrained our ability to include more T2D controls, forcing us to compare data between aged, diabetic and aged, non-diabetic animals. Nevertheless, our diabetic controls have shown consistent results with those obtained in non-diabetic controls. The overall number of animals was also constrained as a whole due to cost and infrastructure limitations associated with keeping rhesus monkeys in A-BSL3 condition with complete veterinary care, even for a short period of time. To improve the chances of viral detection within the olfactory cortex 7 days after infection, we used a high dose of SARS-CoV-2 and inoculated the virus intranasally and intratracheally, another important caveat of this study. In addition, the olfactory epithelium and olfactory bulbs of these animals were unavailable.
at the time of this study, precluding us from drawing definitive conclusions about the route SARS-CoV-2 uses to access the brain, despite substantial evidence for an olfactory entry.

**AUTHOR CONTRIBUTIONS**

Conceptualization, S.S.I. and J.H.M.; investigation, D.B., A.B., G.B.D., S.O., K.K.A.V.R., S.S.I., and J.H.M.; supervision, J.H.M.; writing – original draft, D.B., G.B.D., and J.H.M.; writing – review & editing, D.B., G.B.D., K.K.A.V.R., S.S.I., and J.H.M.

**DECLARATION OF INTERESTS**

The authors declare that they have no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Rabbit polyclonal anti-amyloid oligomer | Sigma-Aldrich | Cat#AB9234; RRID: AB_11214948 |
| Rabbit polyclonal anti-human ACE2 | Abcam | Cat#ab15348; RRID: AB_301861 |
| Goat polyclonal anti-ACE2 | R&D Systems | Cat#AF933; RRID: AB_355722 |
| Guinea pig polyclonal anti-Aquaporin 4 | Synaptic Systems | Cat#429004; RRID: AB_2802156 |
| Rat monoclonal anti-human CD66accd (clone YTH71.3) | Bio-Rad | Cat#MCA1147G; RRID: AB_2077339 |
| Rat monoclonal anti-mouse CD8a (clone 53-6-7) | Invitrogen | Cat#14-0081-82; RRID: AB_467087 |
| Rabbit monoclonal anti-Claudin-5 (clone EPR7583) | Abcam | Cat#fab131259; RRID: AB_11157940 |
| Goat polyclonal anti-Collagen Type IV | Sigma-Aldrich | Cat#AB769; RRID: AB_92262 |
| Rabbit polyclonal anti-Myelin basic protein, degraded | Us Biological | Cat#M9758-04; RRID: N/A |
| Mouse monoclonal anti-dsRNA (clone J2) | SciCons | Cat#10010200; RRID: AB_2651015 |
| Chicken polyclonal anti-GFAP | Abcam | Cat#ab4674; RRID: AB_304558 |
| Rabbit polyclonal anti-Iba1 | Fujifilm Wako | Cat#019-19741; RRID: AB_839504 |
| Mouse monoclonal anti-Iba1 (clone NCNP27) | Fujifilm Wako | Cat#013-27593; RRID: N/A |
| Guinea pig polyclonal anti-MAP2 | Synaptic Systems | Cat#188004; RRID: AB_2138181 |
| Rabbit monoclonal anti-human HLA-E (clone JF10-38) | Novus Bio | Cat#NPB2-66946; RRID: N/A |
| Mouse monoclonal anti-human HLA-DR (clone LN3) | BioLegend | Cat#327002; RRID: AB_893582 |
| Mouse monoclonal anti-human HLA-DR (clone LN3) | Invitrogen | Cat#MA5-11966; RRID: AB_10979984 |
| Human monoclonal anti-myelin basic protein (clone IGX3421) | Abcam | Cat#fab209328; RRID: AB_2818988 |
| Mouse monoclonal anti-human myeloperoxidase (clone 2C7) | Bio-Rad | Cat#MCA1757; RRID: AB_2146467 |
| Guinea pig polyclonal anti-NeuN | Synaptic Systems | Cat#266004; RRID: AB_2619988 |
| Mouse monoclonal anti-NeuN (clone A60) | Sigma-Aldrich | Cat#MAB377; RRID: AB_2298772 |
| Mouse monoclonal anti-Neurofilament H (clone SM132) | BioLegend | Cat#801701; RRID: AB_2564642 |
| Chicken polyclonal anti-Neurofilament heavy polypeptide | Abcam | Cat#fab4680; RRID: AB_304560 |
| Mouse monoclonal anti-Neurofilament 70 kDa (clone DA2) | Sigma-Aldrich | Cat#MAB1615; RRID: AB_30485 |
| Rabbit polyclonal anti-Olig-2 | Sigma-Aldrich | Cat#AB9610; RRID: AB_570666 |
| Mouse monoclonal anti-human Pan-Neuronal Neurofilament (clone TNJ-312) | Creative Diagnostics | Cat#DMAB7133; RRID: AB_2391764 |
| Guinea pig polyclonal anti-Parvalbumin | Synaptic Systems | Cat#195004; RRID: AB_2156476 |
| Goat polyclonal anti-PSD95 | Abcam | Cat#fab12093; RRID: AB_298846 |
| Mouse monoclonal anti-SARS/SARS-CoV-2 Nucleocapsid protein (clone E16C) | Invitrogen | Cat#MA1-7403; RRID: AB_1018420 |
| Rabbit polyclonal anti-SARS-CoV-2 Spike Protein | Abcam | Cat#fab272504; RRID: AB_2847845 |

(Continued on next page)
### RESOURCE AVAILABILITY

#### Lead contact
Additional information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, John H. Morrison (jhmorrison@ucdavis.edu).

#### Materials availability
This study did not generate new unique reagents.

#### Data and code availability
Microscopy data reported in this paper will be shared by the lead contact upon request, including original unmodified photomicrographs (.czi) and Imaris three-dimensional reconstruction files (.ims). This paper does not report any original code.

### EXPERIMENTAL MODEL AND SUBJECT DETAILS

#### Ethics statement
The number of animals and all animal procedures performed in this work were approved by the Institutional Animal Care and Use Committee at the University of California, Davis (IACUC Protocol #21735). Animals were maintained in accordance with the American...
Association for Accreditation of Laboratory Animal Care guidelines and the 2011 Guide for the Care and Use of Laboratory Animals. No effort was spared to minimize animal suffering in the course of the experiments reported here.

Animals

Fourteen colony-bred Indian-origin rhesus macaques (Macaca mulatta) of both sexes were employed in this study. Animals were divided into two main age ranges: young adults (4–12 years old, n = 7) and aged subjects (18–24 years old, n = 7). The young adult group was subdivided into three subgroups: non-infected non-diabetic (n = 2), non-infected type 2 diabetic (n = 1), and infected non-type 2 diabetic (n = 4). The aged adult group was also subdivided into three groups: non-infected non-diabetic (n = 2), non-infected type 2 diabetic (n = 1), and infected type 2 diabetic (n = 4). Diabetic animals were identified based on glycosylated hemoglobin values and continued to receive insulin therapy in addition to oral glucose control medications during the study period. Both sex distribution and the assignment of animals to each group were driven partially by colony constraints, particularly in regard to spontaneous type 2 diabetic animals, which are rare occurrences in the CNPRC colony. Detailed information for each animal’s chronic illness, as well as COVID-19 developed pathology, are provided in Table S1 and Data S1.

Prior to the study initiation, animals were confirmed to be seronegative for SARS-CoV-2 and were kept in a special barrier room. Shortly before inoculation, animals were transferred to an animal biosafety level 3 (ABSL-3) facility, where they were kept individually in indoor stainless-steel cages (Lab Product, Inc.) following national standards of sizing. Animals were exposed to a 12:12-h light/dark cycle, controlled temperature between 64 and 84°F, and controlled humidity between 30 and 70%. Animals had ad libitum access to water and received commercial chow (high protein diet; Ralston Purina Co.) and fresh produce supplements.

Virus inoculation

Animals assigned to infected subgroups were inoculated with a total of 2.5mL (2.5 x 10⁶ PFU) of SARS-CoV-2 2019-nCoV/USA-WA1/2020 (NR-52352, Lot/Batch #70033952; BEI Resources). Two milliliters of viral stock were delivered intratracheally via an 8 Fr feeding tube, and 0.25mL were administered directly into each nostril. The high inoculum dose used in this study has resulted in peak levels of replication within 1–2 days after inoculation as previously described.¹³–¹⁵

Clinical observations

A veterinarian blinded to animal group assignments was responsible for daily cage-side clinical monitoring, including records of responsiveness, discharge, respiratory rate and character, evidence of coughing and sneezing, appetite, and stool quality. Additional clinical assessments, such as rectal temperature, respiration, spO2, heart rate, and skin turgor were performed when animals had to be sedated for procedures. Animals were sedated with ketamine HCl (10mg/kg IM) and midazolam (0.25–0.5mg/kg IM) administered after assessment to facilitate sampling, as needed. A summary of clinical findings is presented in Table S1 and Data S1, and in-depth clinical assessment scores for these animals can be found in.¹³–¹⁵

METHOD DETAILS

Euthanasia and sample collection

On day 7 post-infection, animals were euthanized with an excess of pentobarbital and a full necropsy was performed under BSL-3 conditions. Following a full craniotomy, brains were removed, cut into 6mm blocks, and immersion-fixed in 10% formalin for 72 h at 4°C. Following fixation, brain blocks were cut into 50μm coronal sections using a vibratome and stored in PBS with 0.1% Azide to prevent microbial and fungal growth (Figure S1).

Immunohistochemistry

Free-floating 50μm-thick sections were treated for IHC as previously described.¹⁶,¹⁷ Briefly, sections were treated with antigen retrieval solution (Cat. #S1700; Wako) at 60°C for 30 min and then washed with PBS. Sections were then incubated in a permeabilizing blocking solution (5% bovine serum albumin, 5% normal goat serum, 5% normal donkey serum, 0.3% Triton X-100 in PBS) at room temperature for 2 h with gentle agitation. Detection of targets was performed by incubating sections in a cocktail of primary antibodies for 48 h at 4°C under agitation. The tissue was then washed with PBS and incubated with Alexa Fluor-conjugated secondary antibodies (1:500, Invitrogen) for 2 h at room temperature under agitation. After additional rinses in PBS, sections were counterstained with 0.5μg/mL of DAPI (Cat D1306; Invitrogen) and treated with autofluorescence eliminator reagent (Cat. #2160; EMD Millipore) prior to being mounted on slides with ProLong Gold Antifade Mountant (Cat. #P36930; Invitrogen).

Primary antibody cocktails were comprised of antibodies generated in different species/cell lines or different isotypes of mouse IgG. In some instances, multiple antibodies for the same target were used to expand the number of antibody combinations that could be employed. Regardless, comparative quantitative analyses were always performed with the same antibody, ensuring consistency within analyses. A list of primary antibodies used in this study and their respective dilutions can be found in Figure S1.

Microscopy and image analysis

All images were acquired in 3D (z stack between 25 and 50μm depending on the experiment) using an LSM800 confocal laser scanning head equipped with two GaAsP photomultiplier tubes mounted on an Axiosmager Z1 upright microscope (Carl Zeiss). For viral
particle detection and quantification, super-resolution microscopy was achieved using an Airyscan (Carl Zeiss) 32-channel area detector to collect pinhole-plane images at every scan position, resulting in a significantly reduced signal-to-noise ratio. All samples were coded, and image acquisition and analysis were performed by researchers blinded to the experimental groups. Photomicrographs were assembled into figures using the Photoshop 2022 software (Adobe) and illustration were prepared using the Illustrator 2022 software (Adobe).

**SARS-CoV-2 intracellular N protein volume**

To identify the profile of infected cells, a quadruple staining combining the neuronal marker NeuN, the astrocytic marker GFAP, the microglial marker Iba1, and the SARS-CoV-2 N protein was performed targeting layers I-III of the piriform cortex of young and aged animals. Additional analysis was performed in the same region of non-infected age-matched controls to ensure the specificity of the viral staining observed. Four young and four aged, infected animals were analyzed. Twenty randomly chosen fields were acquired with a 63X objective and deconvolved after Airyscan acquisition. Images were then exported and analyzed in 3D using the Imaris software 9.8 (Bitplane). Internalized N protein volume was then calculated in 3D and divided by the total 3D volume obtained for each infected cell type analyzed.

**Intraneuronal dsRNA and spike volumes**

A quadruple staining for DAPI, the neuronal marker MAP2, and viral markers spike (Spk) protein and dsRNA was performed in the piriform cortex, olfactory tubercle, rostromedial entorhinal cortex (also known as the olfactory part), and medial orbitofrontal cortex (a14). These areas were selected based on preliminary distribution studies of viral proteins and the well-established connectivity between these areas through the olfactory circuit. For each of the olfactory regions analyzed, 10–15 63X Airyscan images were obtained and deconvoluted for 3D volume analysis in Imaris. The total internalized dsRNA or spike viral volumes were divided by the total volume of MAP2 to obtain the percentage of total viral internalized particles.

**SARS-CoV-2 induced neuroinflammation**

To investigate microglia and astrocyte local recruitment to the cortical layers of primary olfactory regions, all 14 animals included in this study were analyzed. Two combinations of 4 antibodies were used for this analysis: DAPI + NeuN + Iba1+GFAP and DAPI + NeuN + Iba1+HLA-DR. ROI selection – First, using a 5X objective to acquire images exclusively on the DAPI channel, a tiled image of the entire section was acquired. Using the 5X tiled image, three ROIs were selected based on anatomical landmarks to be imaged with a 20X objective for later quantification. For example, in the piriform cortex: ROI-1 was centered around the frontal lobe piriform cortex, ROI-2 was centered around the junction of the frontal and temporal lobes, and ROI-3 was centered around the temporal lobe piriform. Great care was taken to ensure these three ROIs were anatomically consistent between all sections imaged. All images were exported coded and analyzed by a second researcher blind to the experimental groups in Imaris. The total number of microglia, astrocytes, and neurons was quantified in 3D for each z stack taken.

**3D microglial volume and engulfment activity**

For microglial engulfment analysis in the primary olfactory cortex, 20 microglia selected in a blinded fashion were selected from the piriform cortex of each animal. For each microglia, a z stack obtained with a 63X objective was collected, and the image was exported to the Imaris software to create a 3D volume surface rendering of each z stack. Engulfment quantification was done similarly to that previously described by us and others. Briefly, images were acquired and exported in 3D, where PSD95 puncta, a synaptic marker observed in excitatory synapses, was 3D surface rendered using the same parameters for all the animals. To measure the percentage of engulfment, the volume of internalized puncta (μm³) was divided by the total volume of microglial cells (μm³).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All analyses were performed in GraphPad Prism 9 (GraphPad), and datasets were assessed for normality parameters prior to significance determination. Values are expressed as means ± standard error of the mean in the text and in the figures. Statistical tests and p values are indicated in the main text or in the figure legend.
SARS-CoV-2 infects neurons and induces neuroinflammation in a non-human primate model of COVID-19

Danielle Beckman, Alyssa Bonillas, Giovanne B. Diniz, Sean Ott, Jamin W. Roh, Sonny R. Elizaldi, Brian A. Schmidt, Rebecca L. Sammak, Koen K.A. Van Rompay, Smita S. Iyer, and John H. Morrison
A Methodological design

SARS-CoV-2 Inoculation
Intranasal & Intratracheal
2019 Variant

Histological Processing
Microtomy
Immunohistochemistry (IF)

Data Analysis
Confocal Microscopy
3D Reconstruction
Morphological Analyses

Day 7
Euthanasia
Brain Harvesting
Fixation & Postfixation

Day 10+

B Major cell types and targets

SARS-CoV-2 Virus
Spine protein
Nucleocapsid
dsRNA

Neuron
NeuN
MAP2
NF-L
MHC I
NF-H
Ace2

Microglia
Iba1
MHC Class II (HLA-DR)
Aqueporin 4

Astrocyte
GFAP
CD66a

Neutrophil
MPO
CD66a

Myelin
MBP
dgMBP

Brain vasculature
Claudin-5
Collagen IV

Synaptic markers
PSD95
Synaptophysin

3D space

C List of primary antibodies used in this study

| Target Protein                              | Species   | Clonality   | Isotype | Dilution            | Vendor and product code | #RRID    |
|---------------------------------------------|-----------|-------------|---------|---------------------|-------------------------|----------|
| Angiotensin-Converting Enzyme 2 (ACE2)      | Rabbit    | Polyclonal  | IgG     | 1:100-1:400         | Abcam ab15348           | AB_301861|
| Angiotensin-Converting Enzyme 2 (ACE2)      | Goat      | Polyclonal  | IgG     | 1:50-1:100          | R&D Systems AF933       | AB_355722|
| Aquaporin 4 (AQP4)                          | Guinea Pig| Polyclonal  | IgG     | 1:500               | Synaptic Systems 429004 | AB_2802156|
| Cluster of Differentiation 66a (CD66a)      | Rat       | Monoclonal  | IgG2a   | 1:500               | Bio-Rad MCA1147G        | AB_323396|
| Claudin-5                                   | Rabbit    | Monoclonal  | IgG     | 1:500               | Abcam ab131259          | AB_11157940|
| Collagen Type IV (Collagen IV)              | Goat      | Polyclonal  | IgG     | 1:100               | Millipore AB769         | AB_11210995|
| Degraded Myelin Basic Protein (dgMBP)       | Rabbit    | Polyclonal  | IgG     | 1:500               | US Biological M9758-04  | -        |
| Double-stranded RNA (dsRNA)                 | Mouse     | Monoclonal  | IgG2a   | 1:400               | SciCons 10010200        | AB_2651015|
| Glial Fibrillary Acidic Protein (GFAP)      | Chicken   | Polyclonal  | IgY     | 1:1000              | Abcam ab4674            | AB_304558|
| Ionized calcium binding adaptor molecule 1  | Rabbit    | Polyclonal  | IgG     | 1:500               | Fujifilm Wako 019-19741 | AB_839504|
| Ionized calcium binding adaptor molecule 1  | Mouse     | Monoclonal  | IgG2b   | 1:500               | Fujifilm Wako NCNP24    | AB_281160|
| Microtubule-Associated Protein 2 (MAP2)     | Guinea Pig| Polyclonal  | IgG     | 1:1000              | Synaptic Systems 188004 | AB_2138181|
| Major Histocompatibility Complex Class I (HLA-E) | Rabbit | Monoclonal  | IgG     | 1:200               | Novus Bio NBP2-66946    | AB_2809803|
| Major Histocompatibility Complex Class II (HLA-DR) | Mouse | Monoclonal  | IgG2b   | 1:200               | BioLegend 327002        | AB_893582|
| Major Histocompatibility Complex Class II (HLA-DR) | Mouse | Monoclonal  | IgG2b   | 1:200               | Thermo Fisher MA5-11966 | AB_10979984|
| Myelin Basic Protein (MBP)                  | Human     | Monoclonal  | IgG1    | 1:500               | Abcam ab209328          | AB_2818988|
| Myeloperoxidase (MPO)                       | Mouse     | Monoclonal  | IgG1    | 1:500               | Bio-Rad MCA1757         | AB_2146467|
| Neuronal Nuclear Protein (NeuN)             | Guinea Pig| Polyclonal  | IgG     | 1:500               | Synaptic Systems 266004 | AB_2619988|
| Neuronal Nuclear Protein (NeuN)             | Mouse     | Monoclonal  | IgG1    | 1:500               | Millipore MAB377        | AB_2298772|
| Neurofilament- Heavy (NF-H)                 | Mouse     | Monoclonal  | IgG1    | 1:200               | BioLegend 801701        | AB_2715852|
| Neurofilament- Heavy (NF-H)                 | Chicken   | Polyclonal  | IgY     | 1:500               | Abcam ab4680            | AB_304560|
| Neurofilament- Light (NF-L)                 | Mouse     | Monoclonal  | IgG1    | 1:500               | Millipore MAB1615       | AB_94285|
| Pan-Neurofilament (Pan-NF)                  | Mouse     | Monoclonal  | IgG1/IgM| 1:400               | Creative Diagnostic DMAB7133 | AB_2391764|
| Postsynaptic Density Protein (PSD95)        | Goat      | Polyclonal  | IgG     | 1:500               | Abcam ab12093           | AB_298846|
| SARS-CoV-2 Nucleocapsid (N) protein         | Mouse     | Monoclonal  | IgG2b   | 1:100               | Thermo Fisher MA1-7403  | AB_1018420|
| SARS-CoV-2 Spike Protein                    | Rabbit    | Polyclonal  | IgG     | 1:500               | Abcam ab272504          | AB_2847845|
| SARS-CoV-2 Spike Protein                    | Human     | Monoclonal  | IgG1    | 1:100               | Thermo Fisher 703958    | AB_2866477|
| Synaptophysin 1                             | Guinea Pig| Polyclonal  | IgG     | 1:500               | Synaptic Systems 101004 | AB_1210382|

#RRID – Research Resource Identifier
Figure S1. Methodological overview of the study, Related to STAR Methods. (A) Schematic representation of the study methodology. Four young healthy (3.5-6 Y.O) and four aged (18-22 Y.O) rhesus monkeys were infected intranasally and intratracheally with SARS-CoV-2 (2019-nCoV/USA-WA1/2020; BEI Resources) at high dose (2.5x10^6 plaque-forming units [PFU]) and euthanized one week later. In addition, non-infected brains from diabetic and non-diabetic animals were processed in a similar way for the same microscopy analysis. (B) Major targets investigated in this study and the markers used to identify them. (C) List of primary antibodies used in this study.
Figure S2. Complementary volumetric analyses of neuronal morphology, infection tropism, and neuro-inflammation, Related to Figure 01. (A-F) In young (A, B) and aged (C, D) infected animals, individual and combined channels are shown in the XY and XZ planes. In (B) and (D), detail of the original images and the 3D volume reconstructions used to identify and quantify the cell types infected and the total internalized viral volume. In comparison with young, infected animals, aged-infected monkeys present a significant increase in astrocyte hypertrophy in comparison with the other cell types (E, ***p=0.0009). In contrast, SARS-CoV2 nucleocapsid protein was detected in higher quantities in neurons and significantly in higher levels in aged, infected animals (F, **p=0.0069). Two-way ANOVA, Sidak’s post hoc test. (G, H) To better understand the extensive neuronal cell damage observed in the primary olfactory cortex following SARS-CoV-2 infection, we developed a protocol to calculate how the virus affects the cell body and dendrites separately. In the cell body of neurons within layers I-III of the piriform cortex, dsRNA vesicles were found associated with degraded neuron-specific microtubule stabilizer MAP2, and a reduced cell body volume was observed in aged, infected animals in comparison with age-matched controls (G, **p=0.0032). Dendritic beading, an early marker of neuronal injury and swelling, was observed within neuronal dendrites expressing high levels of spike protein and is correlated with decreased dendritic total volume (H, *p= 0.0571). Unpaired t-test. Data are represented as mean ± SEM. Scale bar: 50µm (A-D), 5µm (G, H).
Figure S3. Viral RNA and SARS-CoV2 spike protein in the olfactory cortex of infected animals, Related to Figure 01. (A-D) Individual and merged representative micrographs and 3D volume reconstruction of olfactory regions of young, infected animals: piriform (A), olfactory tubercle (B), entorhinal cortex (C), and orbitofrontal cortex (D). Double-stranded RNA and SARS-CoV2 spike protein are strongly detected in primary but not secondary olfactory regions of young, infected monkeys. (E-H) Individual and merged representative micrographs and 3D volume reconstruction of olfactory regions of aged, infected animals: piriform (E), olfactory tubercle (F), entorhinal cortex (G), and orbitofrontal cortex (H). In comparison to young animals, aged-infected animals present considerably more neuronal infection and SARS-CoV2 viral protein spread reaching the OFC, presumably through neuronal connections. Scale bar: 50µm.
Figure S4. Glial and vascular disruption induced by SARS-CoV-2 infection, Related to Figure 02. (A-D) Each panel shows representative micrographs and 3D volume reconstructions of the experimental groups: (A) young control, (B) young infected, (C) aged control, and (D) aged infected monkeys. The individual and merged micrographs show astrocytes (GFAP) and microglia (Iba1) interaction with neurons (NeuN). Detailed 3D reconstruction analysis highlights the glia morphological profile change induced by the SARS-CoV2 infection in the brain. (E-H) Individual channels and merged images of the piriform cortex from young diabetic CTR (E), young SARS-CoV2 infected (F), aged diabetic CTR (G), and aged diabetic infected (H) monkeys. Morphological alterations in astrocytes (GFAP) and microglia (Iba1) were associated with the diabetes condition but worsened following SARS-CoV2 infection. (I-P) The human leukocyte antigen (HLA, type D.R.) is a major histocompatibility complex class II (MHCII) receptor expressed by the microglial population upon disturbances in the immunological homeostasis in the CNS, participating in the recruitment of CNS-infiltrating T cells. As shown in the representative individual and merged images of each group (I, K, M, O), there is an increased expression of this receptor in the aged-infected animals in comparison with the other groups. Notably, a heat-map representation of the microglia volume profile observed in the olfactory region (J, L, N, P) shows a progressive increase in the number of individual cells presenting
altered volume, a marker of cellular activation and neuronal damage (heat-map shows a range of microglia volume distribution from 50 µm³/blue to 2000 µm³/red). Scale bar: 100µm (A-D photomicrographs; I-P), 50µm (A-D 3D volumes), 45µm (E-H).
Figure S5. SARS-CoV-2 neuroinfection is associated with MHC Class I and II upregulation and neuron-microglia interaction disruption, Related to Figure 02. (A-D) Representative images from young diabetic CTR (A), young SARS-CoV2 infected (B), aged diabetic CTR (C), and aged diabetic infected (D) monkeys highlight the increased microglial expression of HLA-DR observed in both SARS-CoV2 infected groups. Increased HLA-DR expression was also observed in the aged diabetic CTR animal but was worsened by the viral infection. (E-H) Using pan NF as a neuronal marker and Iba1 to label microglia, we applied Airyscan super-resolution microscopy to investigate how diabetes and SARS-CoV2 infection alter neuron-microglia dynamics. In young diabetic CTR (E) and young infected (F) animals, no substantial alterations were found in the primary olfactory cortex. On the other hand, a more activated microglial profile was observed in the aged diabetic CTR (G) and was notably worsened by SARS-CoV2 infection, as shown in the representative micrograph from an aged diabetic infected animal (H). (I-L) In aged control monkeys, HLA-DR is usually found in low quantities surrounding intact blood vessels, in physiological balance with the neuronal and glial population. On the other hand, MHC1 is not generally expressed in healthy adult brains, and no expression was detected in the aged CTR brain (I). Following the viral infection in aged animals, microglia cells expressing the MHC class II receptor HLA-DR are recruited locally and found in association with altered blood vessels and MHC1+ neurons (J). Both classes of proteins are essential for the recognition of cytotoxic T cells and markers of T cell infiltration in the brain. To further understand the role of the antigen presentation by MHC proteins in the brain following SARS-CoV-2 infection in young and aged infected animals, we further analyzed the expression of both adaptive immunity markers in the olfactory cortex using 3D volumetric reconstruction. As shown in representative images in (K) and (L), in both the XY and XZ planes, neurons MHC1+ and microglia HLA-DR+ in association with disruptive blood vessels are found in both infected groups, but with higher frequency in the aged, infected animals. Scale bar: 20µm (A-D), 10µm (E-L photomicrographs), 5µm (I-L 3D Volumes).
Figure S6. Increased expression of degraded myelin and HLA-DR expression found in SARS-CoV2 infected animals, Related to Figure 02. A combination of antibodies targeting normal (MBP, white) and degraded myelin (dgMBP, green) were used with HLA-DR (microglia and blood vessels) and DAPI. Three-dimensional analyses in the piriform cortex show increased expression of dgMBP at the expense of the normal MBP, associated with increased HLA-DR expression (A, B). Comparison of the piriform cortex from young and aged infected animals highlights an increased presence of dgMBP, associated with increased expression of HLA-DR (C, D). Scale bar: 50µm.
Figure S7. SARS-CoV-2 presence in the brain distorts regular neuron-microglia interaction, Related to Figure 02. Multilabel fluorescence microscopy was performed targeting several neural proteins involved in synaptic transmission and neuron-glia interaction. For this analysis, adjacent stereological sections (every 50 µm) of the primary olfactory cortex from infected monkeys were used. As shown in (A), clusters of microglia (Iba1) are actively interacting with dendrites (neurofilament light, NF-L), and the presence of presynaptic marker synaptophysin (SYP) and postsynaptic marker (PSD95), were observed in abundance within reactive microglia. Interestingly, as demonstrated in (B), neurons expressing the SARS-CoV-2 nucleocapsid protein (N ptn) still present a more preserved neurofilament-heavy (NF-H) structure. (C) Utilizing pan NF marker combined with Iba1, we were able to detect entire neurons expressing N ptn being engulfed by reactive microglia. The reactive profile of the microglia interacting with neurons was confirmed by combining microglia general marker Iba1 and activated microglia marker HLA-DR (D). Scale bar: 50 µm.
Aged Control

Aged Infected

Physiological

Reactive

Surveillance

Excessive synaptic pruning

Increased phagocytic activity

Inflammation

Spine loss

Neuronal death

D1. 3D Confocal Multilabel Image Acquisition

D2. 3D vol. creation

D3. 3D colocalization and volume analyses
Figure S8. Overview of the super-resolution Airyscan microscopy and 3D Segmentation employed to examine cellular morphology, neuron-microglia interactions, and PSD95 puncta engulfment, Related to STAR Methods. (A-C) Compared with age-matched controls (A), high-resolution microscopy analysis of the primary olfactory cortex of infected animals presents abnormal microglia and fragmented/dying neuron interaction (B). Full detailed 3D reconstruction of microglia shows dynamic changes in this cell type profile, closely related to neuroinflammatory process and neurodegeneration (C). (D) To investigate the microglial engulfment of synaptic markers and neuronal proteins, micrographs were first acquired using a 63x objective in a confocal microscope (Step 1). Next, images were exported to Imaris software, and a 3D volume surface was created for each marker analyzed (Step 2). The cell body (yellow) and total volumes (green) for each microglia were calculated, as well as the 3D puncta volume of PSD95 (Step 3). Engulfment activity was measured by quantifying the volume of internalized PSD 95 ($\mu \text{m}^3$) divided by the total volume of the involved microglia cell ($\mu \text{m}^3$). Scale bar: 5µm (A-C), 50µm (D).
Table S1. Information regarding the animals used in this study, including chronic illness and treatments, Related to the STAR methods.

| Animal number | Sex   | Age | Chronic Illness                                                                 |
|---------------|-------|-----|---------------------------------------------------------------------------------|
| MMU-1         | Female| 6   | Endometriosis                                                                   |
| MMU-12        | Female| 4   | None                                                                            |
| MMU-13        | Male  | 12  | Diabetes, cystitis; Concurrent medication: Lantus (insulin glargine)             |
| MMU-6         | Female| 4   | None                                                                            |
| MMU-3         | Female| 5   | Endometriosis                                                                   |
| MMU-11        | Male  | 4   | None                                                                            |
| MMU-5         | Female| 6   | Endometriosis                                                                   |
| MMU-8         | Female| 19  | Cecal Adenocarcinoma                                                            |
| MMU-10        | Female| 21  | Hepatic Amyloidosis, Endometriosis                                               |
| MMU-14        | Male  | 23  | Diabetes, obesity and suspected tract infection; Concurrent medication: Tresiba (insulin degludec) |
| MMU-2         | Female| 20  | Diabetes, gastrointestinal adenocarcinoma; Concurrent medication: Tresiba (insulin degludec) |
| MMU-4         | Female| 24  | Diabetes, Hypertension; Concurrent medication: Lantus (insulin glargine), pioglitazone |
| MMU-7         | Female| 22  | Diabetes, endometriosis, diverticulosis; Concurrent medication: Tresiba (insulin degludec) |
| MMU-9         | Female| 18  | Diabetes, Hypertension; Concurrent medication: Tresiba (insulin degludec)        |

| Animal number | MMU-6   | MMU-3   | MMU-11  | MMU-5   | MMU-2   | MMU-4   | MMU-7   | MMU-9   |
|---------------|---------|---------|---------|---------|---------|---------|---------|---------|
| Days after Infection | Body Weight (kg) | Rectal temperature (°C) | Heart rate (bpm) | SpO2 |
| -2           | 5.72    | 100.4  | 144     | 97      |
| 0            | 5.04    | 99.7   | 164     | 97      |
| 2            | 6.46    | 100.2  | 136     | 97      |
| 4            | 5.98    | 101.1  | 165     | 99      |
| 6            | 6.14    | 101.6  | 121     | 100.1  |
| Days after Infection | Body Weight (kg) | Rectal temperature (°C) | Heart rate (bpm) | SpO2 |
| -2           | 8.13    | 100.1  | 176     | 97      |
| 0            | 8.04    | 99.9   | 151     | 99      |
| 2            | 7.56    | 101.1  | 181     | 99      |
| 4            | 7.45    | 103.2  | 166     | 97      |
| 6            | 7.88    | 102.6  | 159     | 98      |
# Data S1 - Detailed measurements related to COVID-19 development in infected monkeys

| Animal number | Days after infection | Symptoms | Respiratory character | Respiratory Rate (breaths/min) | Food consumption |
|---------------|----------------------|----------|-----------------------|-------------------------------|-----------------|
| MMU-6 Female 4 YO | Days after infection | None | None | WL | None |
| 1 | 1 | BAL | None | None | WL | None |
| 2 | 1 | BAL | None | None | WL | None |
| 3 | 1 | BAL | None | None | WL | None |
| 4 | 1 | BAL | None | None | WL | None |
| 5 | 1 | BAL | None | None | WL | None |
| 6 | 1 | BAL | None | None | WL | None |
| 7 | 1 | BAL | None | None | WL | None |
| 8 | 1 | BAL | None | None | WL | None |
| MMU-3 Female 5 YO | Days after infection | None | None | WL | None |
| 1 | 1 | BAL | None | None | WL | None |
| 2 | 1 | BAL | None | None | WL | None |
| 3 | 1 | BAL | None | None | WL | None |
| 4 | 1 | BAL | None | None | WL | None |
| 5 | 1 | BAL | None | None | WL | None |
| 6 | 1 | BAL | None | None | WL | None |
| 7 | 1 | BAL | None | None | WL | None |
| MMU-11 Female 4 YO | Days after infection | None | None | WL | None |
| 1 | 1 | BAL | None | None | WL | None |
| 2 | 1 | BAL | None | None | WL | None |
| 3 | 1 | BAL | None | None | WL | None |
| 4 | 1 | BAL | None | None | WL | None |
| 5 | 1 | BAL | None | None | WL | None |
| 6 | 1 | BAL | None | None | WL | None |
| 7 | 1 | BAL | None | None | WL | None |
| MMU-5 Female 6 YO | Days after infection | None | None | WL | None |
| 1 | 1 | BAL | None | None | WL | None |
| 2 | 1 | BAL | None | None | WL | None |
| 3 | 1 | BAL | None | None | WL | None |
| 4 | 1 | BAL | None | None | WL | None |
| 5 | 1 | BAL | None | None | WL | None |
| 6 | 1 | BAL | None | None | WL | None |
| 7 | 1 | BAL | None | None | WL | None |
| MMU-2 Female 20 YO | Days after infection | None | None | WL | None |
| 1 | 1 | BAL | None | None | WL | None |
| 2 | 1 | BAL | None | None | WL | None |
| 3 | 1 | BAL | None | None | WL | None |
| 4 | 1 | BAL | None | None | WL | None |
| 5 | 1 | BAL | None | None | WL | None |
| 6 | 1 | BAL | None | None | WL | None |
| 7 | 1 | BAL | None | None | WL | None |
| MMU-4 Female 24 YO | Days after infection | None | None | WL | None |
| 1 | 1 | BAL | None | None | WL | None |
| 2 | 1 | BAL | None | None | WL | None |
| 3 | 1 | BAL | None | None | WL | None |
| 4 | 1 | BAL | None | None | WL | None |
| 5 | 1 | BAL | None | None | WL | None |
| 6 | 1 | BAL | None | None | WL | None |
| 7 | 1 | BAL | None | None | WL | None |
| MMU-7 Female 22 YO | Days after infection | None | None | WL | None |
| 1 | 1 | BAL | None | None | WL | None |
| 2 | 1 | BAL | None | None | WL | None |
| 3 | 1 | BAL | None | None | WL | None |
| 4 | 1 | BAL | None | None | WL | None |
| 5 | 1 | BAL | None | None | WL | None |
| 6 | 1 | BAL | None | None | WL | None |
| 7 | 1 | BAL | None | None | WL | None |
| MMU-9 Female 18 YO | Days after infection | None | None | WL | None |
| 1 | 1 | BAL | None | None | WL | None |
| 2 | 1 | BAL | None | None | WL | None |
| 3 | 1 | BAL | None | None | WL | None |
| 4 | 1 | BAL | None | None | WL | None |
| 5 | 1 | BAL | None | None | WL | None |
| 6 | 1 | BAL | None | None | WL | None |
| 7 | 1 | BAL | None | None | WL | None |