

1 **Fatal neuroinvasion of SARS-CoV-2 in K18-hACE2 mice is partially dependent on**
2 **hACE2 expression**

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27 **Running Title:** Spatiotemporal analysis of SARS-CoV-2 and *hACE2* in K18-hACE2
28 mice

29

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31 *in situ* hybridization, viral pathogenesis

32 **SUMMARY**

33 COVID-19 is a respiratory disease caused by SARS-CoV-2, a betacoronavirus. Here,
34 we show that in a widely used transgenic mouse model of COVID-19, lethality is
35 invariably associated with viral neuroinvasion and the ensuing neuronal disease, while
36 lung inflammation remains moderate.

37 **ABSTRACT**

38 Animal models recapitulating the distinctive features of severe COVID-19 are critical to
39 enhance our understanding of SARS-CoV-2 pathogenesis. Transgenic mice expressing
40 human angiotensin-converting enzyme 2 (hACE2) under the cytokeratin 18 promoter
41 (K18-hACE2) represent a lethal model of SARS-CoV-2 infection. However, the cause(s)
42 and mechanisms of lethality in this mouse model remain unclear. Here, we evaluated
43 the spatiotemporal dynamics of SARS-CoV-2 infection for up to 14 days post-infection.
44 Despite infection and moderate inflammation in the lungs, lethality was invariably
45 associated with viral neuroinvasion and neuronal damage (including spinal motor
46 neurons). Neuroinvasion occurred following virus transport through the olfactory
47 neuroepithelium in a manner that was only partially dependent on hACE2. Interestingly,
48 SARS-CoV-2 tropism was overall neither widespread among nor restricted to only
49 ACE2-expressing cells. Although our work incites caution in the utility of the K18-hACE2
50 model to study global aspects of SARS-CoV-2 pathogenesis, it underscores this model
51 as a unique platform for exploring the mechanisms of SARS-CoV-2 neuropathogenesis.

52 INTRODUCTION

53 The world is experiencing the devastating effects of the Coronavirus Disease 2019
54 (COVID-19) pandemic, a highly contagious viral respiratory disease caused by the
55 newly emerged betacoronavirus, Severe Acute Respiratory Syndrome Coronavirus-2
56 (SARS-CoV-2) (Andersen et al., 2020; Coronaviridae Study Group of the International
57 Committee on Taxonomy of, 2020; Wang et al., 2020a). The initial index case was
58 reported at a seafood market in Wuhan, Hubei Province, China in late 2019 (Andersen
59 et al., 2020). While still under investigation, it has been postulated that the progenitor of
60 SARS-CoV-2 may have originated from horseshoe bats (*Rhinolophus affinis*) or
61 Malayan pangolins (*Manis javanica*) that, following spill over into humans, acquired the
62 genomic features leading to adaptation and human-to-human transmission (Andersen et
63 al., 2020). SARS-CoV-2 has a high transmissibility rate and, to date, it has infected
64 nearly 84.5 million people with over 1.8 million fatalities (Johns Hopkins University &
65 Medicine, 2020). COVID-19 causes respiratory disease of variable severity, ranging
66 from mild to severe, with the development of acute respiratory distress syndrome
67 (ARDS) requiring intensive care and mechanical ventilation (Goyal et al., 2020;
68 Tenforde et al., 2020; Wang et al., 2020a; Wang et al., 2020b). Numerous comorbidities
69 including hypertension, obesity and diabetes, among others, are affiliated with an
70 increased risk of developing severe COVID-19 (Goyal et al., 2020; Simonnet et al.,
71 2020; Tartof et al., 2020; Team, 2020; Tenforde et al., 2020). Furthermore, a proportion
72 of infected patients develop poorly understood neurological signs and/or symptoms
73 mostly associated with the loss of smell and taste (anosmia and ageusia), headache,
74 dizziness, encephalopathy (delirium), and ischemic injury (stroke), among other
75 uncommon symptoms (DosSantos et al., 2020; Eliezer et al., 2020; Ellul et al., 2020;

76 Goyal et al., 2020; Lee et al., 2020; Liu et al., 2020; Solomon et al., 2020; Walker et al.,
77 2020; Wang et al., 2020b). Recently, SARS-CoV-2 RNA and antigen has been reported
78 in the brain of COVID-19 patients and the olfactory mucosa postulated as a port of entry
79 (Meinhardt et al., 2020). COVID-19 has severely challenged health care systems
80 around the globe, with the urgent need for medical countermeasures including the
81 development of efficacious vaccines and therapeutics.

82 Animal models permissive to SARS-CoV-2 that could serve as suitable models to
83 understand the pathogenesis of COVID-19 and as preclinical models for the evaluation
84 of vaccine and therapeutic targets are critically needed (Johansen et al., 2020;
85 McNamara et al., 2020; Munoz-Fontela et al., 2020). While various animal models
86 (mice, hamsters, non-human primates, ferrets, minks, dogs, and cats) have been
87 evaluated to date (Gaudreault et al., 2020; Imai et al., 2020; Meekins et al., 2020;
88 Munoz-Fontela et al., 2020; Rockx et al., 2020; Shi et al., 2020; Shuai et al., 2020; Sia
89 et al., 2020; Winkler et al., 2020), none faithfully recapitulates all of the pathological
90 features of COVID-19. The main limitation in the development of suitable murine models
91 of COVID-19 is related to the virus entry mechanism: SARS-CoV-2 binds to target cells
92 via interaction between the viral spike protein (S) and the host angiotensin-converting
93 enzyme 2 (ACE2), considered to be the major host entry receptor (Hoffmann et al.,
94 2020). The low binding affinity between the S protein and murine ACE2 (mACE2)
95 renders conventional mouse strains naturally resistant to infection, posing a challenge in
96 the development of murine models of COVID-19 (Conceicao et al., 2020; Damas et al.,
97 2020; Dinnon et al., 2020). These difficulties have been circumvented by the
98 development of transgenic murine models that express human ACE2 (hACE2) under

99 different promoters including cytomegalovirus (CMV), hepatocyte nuclear factor-
100 3/forkhead homologue 4 (HFH4), and cytokeratin 18 (K18) (Jiang et al., 2020; McCray
101 et al., 2007; Rathnasinghe et al., 2020; Winkler et al., 2020; Zheng et al., 2020). The
102 transgenic murine model expressing hACE2 under a K18 promoter (namely K18-
103 hACE2) was developed by McCray et al in 2007 to study SARS-CoV (McCray et al.,
104 2007), which shares the same host receptor as SARS-CoV-2 (Li et al., 2003).

105 **SARS-CoV-2 infection of K18-hACE2 mice results in up to 100% lethality similar to what**
106 has been reported for SARS-CoV (McCray et al., 2007; Winkler et al., 2020; Zheng et
107 al., 2020). Lethality was reported to be associated with lung inflammation and impaired
108 **respiratory function**, suggesting that this model can recapitulate features of the
109 respiratory disease observed in severe cases of COVID-19 (Rathnasinghe et al., 2020;
110 Winkler et al., 2020; Zheng et al., 2020). **While several studies have reported SARS-**
111 **CoV-2 neuroinvasion and neurological signs of disease in infected K18-hACE2 mice**
112 (Golden et al., 2020; Zheng et al., 2020), the contribution of CNS disease in the
113 lethality of the model and the mechanism by which neuroinvasion occurs, remain
114 unclear.

115 Under K18 regulation, the expression of hACE2 is reported to be limited mainly to
116 airway epithelial cells and enterocytes lining the colonic mucosa, to a lower degree
117 within kidney, liver, spleen and small intestine, and a minor level of expression in the
118 brain (McCray et al., 2007). However, the cellular distribution of ACE2, and particularly
119 hACE2, in tissues of K18-hACE2 mice remains largely undetermined. We hypothesized
120 that the nature, severity, and outcome of disease in K18-hACE2 mouse model is not
121 solely dictated by the expression and tissue distribution of hACE2 and that increased

122 lethality in this model is related to neuroinvasion. To investigate this hypothesis, we
123 undertook a comprehensive spatiotemporal analysis of histologic changes, cellular
124 distribution and abundance of viral antigen and RNA along with detailed analysis of the
125 distribution of hACE2 and its correlation with SARS-CoV-2 tropism.

126 Although SARS-CoV-2 antigen and RNA could be detected in ACE2-expressing cells
127 such as olfactory neuroepithelium (ONE) and alveolar type 2 (AT2) cells, we found that
128 SARS-CoV-2 primarily infected neurons and alveolar type 1 (AT1) cells lacking ACE2
129 expression. The lethality of the K18-hACE2 model was entirely due to viral
130 neuroinvasion, which occurred through the olfactory neuroepithelium. This process was
131 accompanied by clinically detectable neurological manifestations, and was partially
132 driven by ACE2-independent mechanisms. Altogether, this study expands the current
133 knowledge on the K18-hACE2 murine model to study COVID-19. Our findings will help
134 refine its utilization for providing a relevant understanding of the molecular mechanisms
135 driving neuropathogenesis and pulmonary pathology.

136

137 RESULTS

138 **SARS-CoV-2 is invariably fatal in infected K18-hACE2 mice with evidence of**
139 **neuroinvasion.** K18-hACE2 mice inoculated intranasally with SARS-CoV-2 (1×10^6
140 plaque-forming units [PFU]; $n=47$ [$n=25$ male and $n=12$ female]) began losing weight as
141 early as 2-3 days post-infection (dpi) irrespective of sex, with maximum weight loss
142 occurring at 7 dpi ($17.7\% \pm 7.8\%$ in male mice, $21.8\% \pm 3.1\%$ in female, and combined
143 $18.9\% \pm 6.9\%$; Figure 1A). Trends in weight loss were associated with an increase in

144 clinical scores and a decline in core body temperature, both of which became most
145 pronounced near the time of death (Figure 1B-C). SARS-CoV-2-infected K18-hACE2
146 mice exhibited neurological signs by 6-7 dpi, characterized by tremors, proprioceptive
147 defects, abnormal gait and imbalance. The majority of the animals were either
148 euthanized at this time or were found dead in their cage (93%; 28/30 [Figure 1D]). At
149 the time of death (7-10 dpi), the median clinical score was 3 (interquartile range = 2)
150 and the mean body temperature 30.6 ± 3.7 °C. Two male mice survived to the end of
151 the 14-day observation period. One of these animals had a maximum clinical score of
152 3/5 at 10 dpi and lost a maximum of 6% body weight, while the other displayed no
153 clinical signs or appreciable weight loss over the observation period. Furthermore,
154 neither of the two male survivors were hypothermic at any point in time, a feature that
155 was consistently observed in animals that succumbed to disease.

156 When compared with C57BL/6J mice, which were concomitantly infected with SARS-
157 CoV-2, the K18-hACE2 mice had several dark red, slightly depressed foci of variable
158 size (2-3 mm), scattered throughout the lung lobes at 2 and 4 dpi. By 7 dpi, these foci
159 grew into large coalescing, dark red depressions encompassing around 30% of the
160 pulmonary parenchyma, leading to the abnormal pulmonary buoyancy (i.e. tissues sank
161 when placed in formalin). Additionally, the mice euthanized due to clinical deterioration
162 frequently had a markedly dilated urinary bladder filled with concentrated urine. The
163 mice euthanized at 14 dpi (n=2) had sporadic dark red pinpoint foci in the pulmonary
164 parenchyma.

165 Lethality was associated with significant viral load in the lung and brain of the K18-
166 hACE2 mice (Figure 1E), as previously reported (Golden et al., 2020; Rathnasinghe et

167 al., 2020; Winkler et al., 2020; Zheng et al., 2020). In the lung, viral RNA was detectable
168 at the earliest experimental timepoint (2 dpi), with a mean of 6.42×10^7 RNA copies/mg,
169 peaked at 4 dpi (mean of 5.8×10^8 RNA copies/mg) and remained high at 7 dpi (mean of
170 3.71×10^7 RNA copies/mg). In contrast, viral RNA in the brain was low at 2 dpi (mean of
171 2.46×10^5 RNA copies/mg) but dramatically increased at 7 dpi (4-log increase)
172 representing the highest viral RNA load during the course of the study, with a mean of
173 1.27×10^9 RNA copies/mg. A small amount of viral RNA was detected in the serum
174 ($< 10^6$ RNA copies/ml). However, incubation of SARS-CoV-2 permissive cell lines (i.e.
175 Vero E6 cells) with serum samples did not result in any detectable productive infection
176 *in vitro*, confirming that K18-hACE2 are not viremic during infection and that small
177 amounts of naked viral RNA might be circulating in the bloodstream following release
178 from infected tissues (data not shown). **Altogether, these data illustrate that lethality was**
179 **associated with increasing viral load in the brain until time of death, in contrast to lung**
180 **viral load.**

181

182 **Transient SARS-CoV-2 infection in the nasal cavity of K18-hACE2 mice.** We next
183 performed detailed histologic analysis of various tissues to uncover the mechanism of
184 lethality in K18-hACE2 mice. For this, we first focused on the spatial and temporal
185 dynamics of SARS-CoV-2 infection in the upper respiratory tract and analyzed the
186 anterior/rostral nasal cavity and olfactory neuroepithelium for disease-associated
187 lesions. To do so, we performed a thorough sequential histologic analysis combined
188 with immunohistochemistry (IHC) and RNAscope® *in situ* hybridization (ISH) in order to

189 determine the cellular localization and abundance of SARS-CoV-2 antigen and RNA,
190 using an anti-spike monoclonal antibody and an S-specific RNA probe, respectively.
191 At 2 dpi, the anterior/rostral nasal cavity was characterized by mild, multifocal
192 neutrophilic inflammation (rhinitis) in all infected K18-hACE2 mice. In the rostral and
193 intermediate turbinates, histologic changes were characterized by segmental
194 degeneration and necrosis of the lining transitional and respiratory epithelium, with cell
195 rounding, loss of cilia and sloughing, along with scattered intracytoplasmic SARS-CoV-2
196 antigen and RNA (Figure 2). Nasal passages were partially filled with small amounts of
197 cellular debris, degenerate neutrophils and small numbers of erythrocytes. The lamina
198 propria underlying affected areas was infiltrated by low to mild numbers of neutrophils
199 and fewer lymphocytes (Figure 2). SARS-CoV-2 antigen and RNA were detected within
200 the epithelium lining the mucosa of rostral and intermediate turbinates. At 4 dpi,
201 epithelial degeneration and necrosis in the rostral and intermediate turbinates were no
202 longer observed, and histologic changes were consistent with mild, multifocal
203 lymphocytic rhinitis with rare clusters of few neutrophils within the lamina propria (Figure
204 2). Nasal passages were clear of exudate. The abundance of SARS-CoV-2 antigen and
205 RNA decreased, and was mostly restricted to rare positive cells in the respiratory
206 epithelium (Figure 2, Table 1 and Table S1). By 7 dpi, the nasal passages (including the
207 ONE) were histologically within normal limits and no SARS-CoV-2 antigen or RNA were
208 detectable in the rostral and intermediate turbinates (Figure S1).

209 The ONE was histologically unremarkable at all timepoints despite the variable amounts
210 of viral antigen and RNA detected during the course of the study. At 2 dpi, multifocal
211 clusters of cells within the ONE contained abundant SARS-CoV-2 antigen and RNA

212 (Figure 2, Table 1 and Table S1), becoming gradually more rare at 4 and 7 dpi. No
213 SARS-CoV-2 antigen or RNA were detected in the ONE by 14 dpi (Table 1, Table S1,
214 and Figure S1).

215

216 **Moderate interstitial pneumonia in K18-hACE2 mice following SARS-CoV-2**

217 **infection.** To evaluate the effects of SARS-CoV-2 infection in the lower respiratory
218 tract of K18-hACE2 mice, we performed similar analysis to that described above. In the
219 lower respiratory tract, histologic alterations in the pulmonary parenchyma mainly
220 involved the alveoli, interstitium and vasculature. Pathologic alterations in the lungs
221 were overall characterized by variable degree of interstitial pneumonia and variable
222 abundance of SARS-CoV-2 antigen and RNA, which were strictly dependent on the
223 experimental timepoint.

224 At 2 dpi, mild perivascular and peribronchiolar inflammation, consisting primarily of
225 lymphocytes and lesser numbers of histiocytes, and occasional perivascular edema
226 were evident (Figure 3). Pulmonary vessels were frequently reactive and lined by a
227 plump endothelium with large numbers of marginating mononuclear leukocytes and few
228 neutrophils (Figure 3). Multifocal to coalescing areas of the pulmonary parenchyma,
229 mostly associated with a bronchopulmonary segment, were composed of alveolar septa
230 mildly expanded by lymphocytes, histiocytes and fewer neutrophils along with scattered
231 air spaces containing low numbers of neutrophils and mild numbers of histiocytes and
232 lymphocytes (Figure 3). SARS-CoV-2 antigen and RNA were multifocally detected
233 within the pulmonary parenchyma, and localized in alveolar type (AT) 1 and fewer AT2
234 pneumocytes predominantly within and adjacent to areas of interstitial pneumonia as

235 demonstrated by singleplex and multiplex IHC, and RNAscope[®] ISH (Figure 3 and
236 Figure S2).

237 At 4 dpi, peak in viral antigen and RNA abundance was reached (correlating with the
238 highest viral RNA load as determined by RT-qPCR) along with a higher number of
239 infiltrating lymphocytes (Figure 3 and Figure S2). SARS-CoV-2 cellular tropism did not
240 differ from that described at 2 dpi.

241 At 7 dpi, there was an increase in the severity of the interstitial pneumonia, which
242 affected up to ~30% of the parenchyma (Figure 3). Multifocal bronchopulmonary
243 segments were composed of alveolar septa expanded by numerous mononuclear cells
244 and fewer neutrophils that extend into the alveolar spaces, occasional septal necrotic
245 debris, and mild proliferation of AT2 cells (Figure 3). In some animals, the affected
246 alveoli were multifocally denuded to completely disrupted by necrosis, and filled with
247 necrotic cell debris and alveolar edema. As for earlier timepoints, mild perivascular and
248 peribronchiolar cuffing of lymphocytes and histiocytes was a common feature. While
249 bronchioles were mostly unaffected, sporadically they were partially lined by an
250 attenuated epithelium and contain rare, individualized mononuclear cells within their
251 lumina. SARS-CoV-2 antigen and RNA were still abundant (Figure 3, Table 1, Figure
252 S2, and Table S1), albeit to a lower extent than at 4 dpi suggesting progressive
253 resolution of viral infection by the host consistent with the RT-qPCR data (Figure 1E).
254 Interestingly, viral antigen was mostly distributed in AT1 and AT2 cells within
255 histologically normal areas of lung adjacent to areas of intense inflammation, while
256 areas of subacute interstitial pneumonia and mild AT2 hyperplasia were characterized
257 by scant to absent SARS-CoV-2 S immunoreactivity (Figure 3, Table 1 and Table S1).

258 Finally, multifocal areas of similar interstitial pneumonia with sporadic aggregates of
259 interstitial lymphocytes and more prominent AT2 hyperplasia were identified in the two
260 male survivors at 14 dpi (Figure 3). No SARS-CoV-2 antigen or RNA was detected at
261 this time point (Figure 3 and Figure S2).

262 Of note, no evidence of SARS-CoV-2 infection in bronchiolar epithelium and pulmonary
263 vasculature was seen at any time during the course of the study (Figure 3, Table 1,
264 Figure S2, and Table S1). Similarly, hyaline membranes, vascular thrombosis, and
265 syncytial cells were not observed at any time point across all animals; which strikingly
266 contrasts with the human patient (Martines et al., 2020) and non-human primate studies
267 (Aid et al., 2020; Blair et al., 2020). Hemorrhage and pulmonary edema were only rarely
268 observed and of minimal-to-moderate severity. In one animal (7 dpi), there was
269 localized flooding of bronchioles by degenerate neutrophils and cellular debris mixed
270 with birefringent foreign material consistent with aspiration pneumonia, a reported rare
271 complication in K18 hACE2 mice infected with SARS-CoV-1 that is ultimately attributed
272 to pharyngeal and laryngeal dysfunction secondary to central nervous system (CNS)
273 disease (McCray et al., 2007).

274 Altogether, our data shows evidence of a significant but moderate interstitial
275 pneumonia in infected K18-hACE2 mice. The histopathological features and the
276 moderate extent of the pneumonia observed in the lung of K18-hACE2 mice therefore
277 contrast with those observed in severe cases of COVID-19 in humans, and suggest that
278 the lethality observed in this model might be independent of lung inflammatory
279 mechanisms.

280

281 **Effective control of SARS-CoV-2 infection in the lower respiratory tract is**
282 **associated with T-cell and macrophage infiltration.** Subsequently, we aimed to
283 further investigate SARS-CoV-2 tropism in the lower respiratory tract of K18-hACE2
284 mice, as well as the dynamics of the host response in this tissue upon infection. We first
285 performed multiplex IHC to probe the localization of SARS-CoV-2 antigen in AT1
286 pneumocytes (cell marker: receptor for advanced glycation end-products [RAGE]), AT2
287 pneumocytes (cell marker: surfactant protein C [SPC]), and endothelial cells (cell
288 marker: CD31). We found that SARS-CoV-2 showed preferential tropism for RAGE+
289 AT1 pneumocytes, as well as scattered SPC+ AT2 pneumocytes, but not for CD31+
290 endothelial cells (Figure 4A).

291 Next, we optimized and applied a 5-plex IHC to characterize the inflammatory response
292 in the lungs of K18-hACE2 mice infected with SARS-CoV-2. At 2 dpi, the affected areas
293 of the pulmonary parenchyma were characterized by abundant viral antigen
294 accompanied by an early host response predominantly mediated by Iba-1+
295 macrophages and low numbers of CD3+CD8- T lymphocytes (presumably CD4+ T
296 lymphocytes, Figure 4C). At 7 dpi, the parenchyma was infiltrated by CD3+CD8+ and
297 CD3+CD8- T lymphocytes and abundant Iba-1+ macrophages with a reduction in the
298 number of SARS-CoV-2-infected cells, the latter mostly restricted to areas of the
299 parenchyma with less intense inflammatory response (Figure 4D). At 14 dpi, the
300 inflammatory response was characterized by multifocal interstitial aggregates of CD19+
301 B lymphocytes, abundant Iba-1+ macrophages, moderate numbers of CD3+CD8- T
302 lymphocytes, (scattered throughout the interstitium as well as associated with CD19+ B
303 lymphocyte aggregates) and a reduction in the number of infiltrating CD3+CD8+ T

304 lymphocytes with undetectable SARS-CoV-2 antigen (Figure 4E). Altogether, our data
305 showed that control of SARS-CoV-2 in the lung of K18-hACE2 mice is associated with
306 moderate interstitial pneumonia characterized by a strong local lymphocytic and
307 histiocytic host response. Effective immune control and moderate inflammation once
308 again suggest that lethality observed in K18-hACE2 mice is attributable to lung-
309 independent disease.

310 **Severe SARS-CoV-2 neuroinvasion and neurological damage in K18-hACE2 mice.**

311 Pursuing our hypothesis that the lethality of the K18-hACE2 mice is associated with
312 brain neuroinvasion, we analyzed sagittal sections of the brain including representation
313 of the cribriform plate and olfactory bulb at different timepoints post-infection (2, 4, 6-7
314 and 14 dpi) for histologic changes and viral antigen distribution (Figures 5 and 6).
315 Histologic alterations in the brain were first observed as early as 6 dpi but mostly at 7
316 dpi, and consisted of mild, multifocal neuronal spongiosis primarily throughout the
317 cerebral cortical layers of the somatomotor, somatosensory and visual areas (Figure 5).
318 Additionally, multifocal blood vessels were delimited by delicate cuffs of few
319 lymphocytes with mild numbers of reactive glial cells in the adjacent neuroparenchyma.
320 At 7 dpi, histologic alterations worsened in severity and became more widespread with
321 involvement of the olfactory bulb, cerebral cortex (most predominantly somatosensory
322 and somatomotor areas), hippocampus (mainly CA1 region), brainstem (thalamus),
323 midbrain and the dentate nucleus ventral to the cerebellum (Figure 5). At the level of the
324 olfactory bulb, the glomerular, external plexiform, and mitral cell layers were
325 characterized by moderate to marked neuropil vacuolization (spongiosis) with
326 occasional vacuoles containing intralesional cell debris. Elsewhere, the grey and white

327 matter within affected areas of the neuroparenchyma were extensively and
328 predominantly characterized by marked **spongiosis** with frequent clear vacuoles
329 containing intralesional cell debris, and multifocal shrunken, angular, hypereosinophilic
330 and pyknotic neuronal bodies with loss of Nissl substance/chromatolysis (neuronal
331 degeneration and necrosis, Figure 5) and occasionally delimited by multiple glial cells
332 (satellitosis). There were multifocal, delicate perivascular cuffs composed of
333 lymphocytes and the adjacent neuroparenchyma had an increase in the number of
334 reactive glial cells (gliosis). Notably, the cerebellum (cortical layers and associated white
335 matter of the cerebellar folia) was spared of histologic changes.

336 Neuronal changes correlated with abundant neuronal immunoreactivity for SARS-CoV-2
337 S antigen and viral RNA, with exclusive localization within the perikaryon and neuronal
338 processes (Figure 6). **SARS-CoV-2 antigen and RNA had a widespread distribution**
339 **throughout the brain in roughly 85% (11/13) of infected K18-hACE2 mice at 7 dpi,**
340 including neuronal bodies within the cerebral cortex, CA1, CA2 and CA3 regions of the
341 hippocampus, anterior olfactory nucleus, caudoputamen, nucleus accumbens, thalamic
342 nuclei including hypothalamus, midbrain, pons and medulla oblongata nuclei (Figure 6).
343 Few vestibulocochlear nerve fascicles showed immunoreactivity for viral antigen; while
344 no viral S antigen or RNA was detected in areas spared of histological changes
345 including the cerebellar cortex and white matter, optic nerve and retina, and the spiral
346 ganglion of the inner ear (albeit the eye and inner ear were not present in the majority of
347 sections examined). SARS-CoV-2 S antigen and RNA preceded histological findings
348 with rare viral antigen and RNA detected as early as 4 dpi in mitral and inner nuclear

349 neurons of the olfactory bulb, as well as small clusters of neurons within the anterior
350 olfactory nucleus and orbital area of the cerebral cortex (Figure 6).

351 Multiplex IHC for co-detection of SARS-CoV-2 antigen, Iba-1+ and GFAP+ glial cells
352 was performed at 7 dpi, timepoint at which widespread viral antigen and RNA along with
353 histological lesions were observed. Multiple IHC revealed that the gliosis in the brain of
354 infected mice was attributed to abundant GFAP+ astrocytes as well as Iba-1+ microglial
355 cells, which did not colocalize with SARS-CoV-2 S protein expression (Figure 7). Iba-1+
356 microglial cells with broad cytoplasmic ramifications were seen to be tightly associated
357 with SARS-CoV-2+ neuronal bodies, suggestive of an active host response against
358 infection (Figure 7).

359 We evaluated the extent of the CNS involvement in infected K18-hACE2 mice at 7 dpi
360 by additionally examining the cervicothoracic and lumbosacral segments of the spinal
361 cord. In 9/11 infected K18-hACE2 mice that showed widespread viral antigen in the
362 brain, the spinal cord also had mild-to-moderate viral antigen that predominated within
363 the cervicothoracic segments (Figure S3, Table 1 and Table S1). Neuronal changes
364 were similar to those noted in affected areas of the brain; however, gliosis and
365 perivascular cuffing were sparse.

366 Finally, Luxol Fast Blue was utilized to assess myelin loss (demyelination) following
367 SARS-CoV-2 invasion in the brain and spinal cord at 7 dpi. No evidence of
368 demyelination was noted.

369 Taken together, our data show that SARS-CoV-2 infection of K18-hACE2 results in
370 severe neuronal invasion of the CNS, likely via retrograde transport through the

371 olfactory bulb originating from axonal processes traversing the ONE. Viral
372 neuroinvasion resulted in extensive cytopathic effect in neurons, comprising not only the
373 brain but also the spinal cord.

374

375 **ACE2 expression and distribution does not fully reflect SARS-CoV-2 tissue**
376 **tropism in K18-hACE2 mice.** To further explore the mechanism driving lethal SARS-
377 CoV-2 infection in K18-hACE2 mice, we first investigated the tissue and cellular
378 distribution of the ACE2 receptor in both C57BL/6J and K18-hACE2 mice by IHC using
379 a cross-reactive anti-ACE2 antibody (cross-reactive to hACE2 and mACE2) (Table S2).
380 In the lower respiratory tract (lungs), ACE2 was ubiquitously expressed along the apical
381 membrane of bronchiolar epithelium and, less commonly, in rare and scattered AT2
382 pneumocytes (Figure 8). No ACE2 expression was found in AT1 pneumocytes.
383 Strikingly, no differences in the distribution and abundance of ACE2 expression were
384 identified between uninfected C57BL/6J, sham-inoculated K18-hACE2, and terminal (7
385 dpi) K18-hACE2 mice inoculated with SARS-CoV-2 (Figure 8).

386 We therefore aimed at analyzing expression and distribution of *hACE2* mRNA using
387 RNAscope® ISH. Although no expression of *hACE2* mRNA was detected in the lungs of
388 non-transgenic C57BL/6J mice (Figure 9), expression of *hACE2* mRNA was detectable
389 but low in the lungs of K18-hACE2 mice, and mostly involved bronchiolar epithelial cells
390 with sporadic expression within few pneumocytes (Figure 9). In summary, these findings
391 indicate that *hACE2* expression in the lungs of K18-hACE2 is low, and that expression
392 of *hACE2* is not the sole host factor determinant of susceptibility to SARS-CoV-2. This
393 is clearly exemplified by the following: 1) certain cell types that, while expressing

394 *hACE2*, were non-permissive to SARS-CoV-2 infection throughout the experiment (i.e.
395 bronchiolar epithelial cells); and 2) the near diffuse infection of AT1 cells by 4 dpi
396 despite sparse to absent expression of *hACE2* in these cells. These observations
397 support evidence for an ACE2-independent viral entry mechanism playing a major role
398 in the pulmonary dissemination of K18-hACE2 mice.

399 In contrast to the lung, increased ACE2 expression was clearly evident in the nasal
400 cavity of K18-hACE2 mice compared to C57BL/6J mice. We assessed ACE2
401 expression on the rostral nasal epithelium, respiratory epithelium at the level of the
402 intermediate turbinates (Figure 8) as well as in the ONE and olfactory bulb (Figure 8).
403 Unlike C57BL/6J mice, in which ACE2 was undetectable within the nasal cavity, ACE2
404 was diffusely expressed within the apical membrane of transitional and respiratory
405 epithelium, and segmentally within the apical surface of the ONE in both sham-
406 inoculated and SARS-CoV-2-infected K18-hACE mice (Figure 8). While minimal to rare
407 expression of *hACE2* mRNA was identified in the neurons of the mitral layer of the
408 olfactory bulb, olfactory neuroepithelium and respiratory epithelium of rostral turbinates,
409 estimation of its abundance and distribution could not be fully assessed since the
410 decalcification procedure had a significant impact in the quality of cellular mRNA as
411 demonstrated by the low detection of the housekeeping mRNA, *Ppib* (data not shown).

412 In the brain of both C57BL/6J and K18-hACE2 mice, ACE2 immunoreactivity was
413 observed in the vascular endothelium lining blood vessels (Figure 8), as well as
414 ependymal and choroid plexus epithelium. In contrast, distribution of *hACE2* mRNA
415 expression involved clusters of neurons within the cerebral cortex, hippocampus,
416 midbrain, brainstem and Purkinje cells from the cerebellum, with no expression noted in

417 non-transgenic C57BL/6J mice (Figure 10 and Figure S4). There was no expression of
418 *hACE2* mRNA in vascular endothelial cells. Taken together, our data show a
419 discrepancy between ACE2 protein and RNA expression and distribution within the
420 CNS. This is partly attributable to the fact that the ACE2 antibody we utilized cross
421 reacts with both *hACE2* and *mACE2*, while the *ACE2* probe employed was human
422 specific. The absence of *hACE2* hybridization with simultaneous ACE2
423 immunoreactivity in the capillary endothelium supports the notion that ACE2 expression
424 in these cells is of murine origin. The absence of ACE2 immunoreactivity in neurons is
425 suggestive of a potential restriction in the translation (or post-translation) of the ACE2
426 receptor in these cells. This, in addition to the fact that Purkinje cells of the cerebellum
427 are non-permissive to SARS-CoV-2 infection in spite of the expression of *hACE2*
428 mRNA, also suggests that ACE2 is likely not the sole host factor associated with
429 neuroinvasion and that other ACE2-independent entry mechanisms contribute to
430 neuroinvasion and spread by SARS-CoV-2 in this murine model.

431

432 **Absence of infection and histologic lesions in extrapulmonary and extraneural**
433 **tissues despite ACE2 expression.** Other tissues examined included heart, kidney,
434 stomach, duodenum, jejunum, ileum, cecum, and colon. All of these were histologically
435 within normal limits (data not shown). No SARS-CoV-2 S antigen was detected in any of
436 these tissues at any time point (data not shown). ACE2 distribution was evaluated in
437 sections of the heart, stomach, small intestine and colon. While ACE2 expression was
438 limited to the capillary vascular endothelium in the heart and stomach, intense
439 expression was noted in the non-glandular mucosa of the stomach and apical surface of

440 enterocytes lining the small intestinal mucosa. Colonic enterocytes rarely expressed
441 ACE2 (Figure S5).

442

443 **DISCUSSION**

444 The K18-hACE2 transgenic mouse model has become a widespread laboratory animal
445 model suitable for studying SARS-CoV-2 pathogenesis as well as medical
446 countermeasures against COVID-19 (Johansen et al., 2020). The suitability of this
447 model relies on the common host entry receptor shared between SARS-CoV-1 and
448 SARS-CoV-2 (Hoffmann et al., 2020; Li et al., 2003), and transgenic mice expressing
449 hACE2 under the K18 promoter develop lethal clinical disease associated with
450 pulmonary pathology and neuroinvasion, with high viral titers (Golden et al., 2020;
451 McCray et al., 2007; Moreau et al., 2020; Oladunni et al., 2020; Rathnasinghe et al.,
452 2020; Winkler et al., 2020; Yinda et al., 2020; Zheng et al., 2020). In contrast, other
453 murine models of SARS-CoV-2 (e.g. adenovirus-transduced hACE2 mice and
454 transgenic mice expressing hACE2 under the HFH4 promoter) develop only mild
455 disease with limited and short-lived viral replication and pulmonary pathology, and low
456 to no lethality (Jiang et al., 2020; Rathnasinghe et al., 2020). While the K18-hACE2
457 murine model has been critical in shedding light on mechanisms of lung injury and
458 dysfunction, it fails to faithfully recapitulate several key histologic features of severe and
459 lethal cases of COVID-19 in humans, such as diffuse alveolar damage (DAD) with
460 hyaline membrane formation and multi-organ failure associated with hypercoagulability
461 and widespread microthrombi formation (Maiese et al., 2020; Martines et al., 2020).

462 In order to better understand the pathogenesis of SARS-CoV-2, well-characterized
463 animal models are critically needed (Munoz-Fontela et al., 2020). Even though the K18-
464 hACE2 murine model is currently under extensive use, several aspects associated with
465 the temporospatial dynamics of SARS-CoV-2 infection remain poorly characterized,
466 including the expression and cellular distribution of hACE2. We attempted to further
467 characterize pathological aspects related to viral pathogenesis in this unique murine
468 model, and hypothesized that the temporospatial distribution of SARS-CoV-2 and
469 pathological outcomes following infection in the K18-hACE2 murine model is partially
470 but not solely associated with hACE2 and that increased lethality in this model is related
471 to neuroinvasion. The study presented herein provides additional novel information
472 regarding the temporal and spatial aspects of SARS-CoV-2 infection in the K18-hACE2
473 mouse model with emphasis on pathological outcomes as well as a thorough and
474 methodical characterization of ACE2 expression in this transgenic mouse model, which
475 contributes to our understanding of this critical model used for preclinical evaluation of
476 vaccines and antiviral therapeutics. The results presented herein not only demonstrate
477 that lethality of this murine model is associated with neuroinvasion and subsequent
478 neuronal cytopathic effect, but that SARS-CoV-2 tropism is not solely restricted to
479 ACE2-expressing cells in K18-hACE2 mice. Thus, the neuropathogenic potential of
480 SARS-CoV-2 is dependent on other host factors.

481 In this study, we have utilized a large cohort of K18-hACE2 mice (n=50) in order to
482 sequentially evaluate SARS-CoV-2 tropism and pathological alterations, spatial and
483 temporal analysis of host factors including inflammatory response and ACE2/hACE2
484 expression, and survival curve analysis for a period of 14 dpi. Similarly to previous

485 studies, which only observed the outcome of SARS-CoV-2 for up to 7 dpi (Golden et al.,
486 2020; Moreau et al., 2020; Oladunni et al., 2020; Rathnasinghe et al., 2020; Winkler et
487 al., 2020), infected K18-hACE2 mice initially developed significant clinical disease with
488 marked weight loss and increased respiratory effort associated with the development of
489 interstitial pneumonia and high viral load as determined via IHC, ISH and RT-qPCR; an
490 unquestionable feature of this model. Survival curve analysis clearly demonstrated that
491 lethality in infected mice only occurs after 6 dpi and in the vast majority of mice (96.3%),
492 coincided with the initiation of neurologic signs and/or symptoms, neuronal cytopathic
493 effect, and abundance of viral S antigen and RNA in the CNS. These observations are a
494 clear indication of the rapid and fatal neuroinvasive nature of this model. Our study also
495 demonstrates that SARS-CoV-2 has a tropism for motor neurons within the spinal cord
496 (predominantly within the cervicothoracic segments), which was only observed in mice
497 with concurrent brain involvement suggesting a descending infection. This finding helps
498 rationalize the neurologic signs observed with this model including decreased
499 mobility/responsiveness and decreased urine voiding that was evident at necropsy,
500 reflective of severe urinary bladder dilation and accumulation of concentrated urine.
501 Given the spinal cord involvement, the latter is likely attributed to altered spinal reflexes
502 and/or decreased intervention of the detrusor muscle, which is required for normal
503 micturation. An additional striking clinical feature in infected K18-hACE2 mice at 7 dpi
504 was hypothermia (mean body temperature of 30.6 °C), which is likely a consequence of
505 hypothalamic and motor neuron dysfunction associated with SARS-CoV-2 neurotropism
506 and could serve as a clinical indicator of CNS involvement. Our results unequivocally
507 demonstrate that neuroinvasion represents the driving component of fatality in this

508 animal model compared to others such as Syrian hamsters, which display more severe
509 pulmonary disease and infection of the ONE, but no evidence of neuroinvasion (Bryche
510 et al., 2020). Furthermore, these animals invariably recover within 14 days following
511 intranasal infection with SARS-CoV-2 (Bryche et al., 2020; Imai et al., 2020; Osterrieder
512 et al., 2020; Rosenke et al., 2020; Sia et al., 2020). Very few infected K18-hACE2 mice
513 (2/30) from our cohort survived until the end of the study (14 dpi) and, while ameliorated
514 pulmonary lesions were noticed in these, they did not exhibit involvement of the CNS
515 further supporting the observation that mice suffering from SARS-CoV-2 neuroinvasion
516 invariably succumb at earlier time points post-infection. Both of these survivor mice
517 developed pulmonary interstitial aggregates of B lymphocytes that were distinct from
518 earlier time points, which could be suggestive of the development of protective adaptive
519 immunity. The normal histologic appearance of the CNS in surviving mice and lack of
520 any residual neurologic signs supports the notion that animals can fully recover from
521 infection, albeit we acknowledge that limited neuroinvasion in these animals could not
522 be disproven. Furthermore, we acknowledge that extensive neurobehavior testing,
523 which is beyond the expertise of the authors, would be required to rule out any long-
524 term sequelae in the rare instance of survivors. Overall, these findings are of
525 importance to researchers with a particular interest in studying SARS-CoV-2-associated
526 neuropathogenesis, as premature euthanasia due to other clinical features (i.e., weight
527 loss, ruffled fur, and/or respiratory distress) have the potential to precede CNS disease
528 and such terminal endpoints, if elected, may preclude evaluation of the effects of SARS-
529 CoV-2 in the CNS. Instead decreased responsiveness/mobility and tremors are
530 interpreted to reflect better clinical findings supportive of neuroinvasive disease.

531 To date, the precise mechanism(s) enabling neuroinvasion in the K18-hACE2 model is
532 poorly understood (Bryche et al., 2020; DosSantos et al., 2020; Ellul et al., 2020; Liu et
533 al., 2020; Solomon et al., 2020). Here, we determined that K18-hACE2 transgenic mice
534 show a significant upregulation in the expression of ACE2 in the nasal cavity
535 (particularly in the neuroepithelium) compared to wild-type C57BL/6J mice, in which
536 ACE2 expression is undetectable by IHC. This difference between K18-hACE2 and
537 C57BL/6J mice is clearly attributed to the expression of the *hACE2* transgene, and is a
538 key feature to the neuropathogenesis of this model. Interestingly, temporal analysis of
539 SARS-CoV-2 S antigen and RNA in the ONE of transgenic mice preceded and/or
540 occurred simultaneously with infection of neurons within the glomerular and mitral layers
541 of the olfactory bulb, indicative of possible viral retrograde transport through axonal
542 processes traversing the cribriform plate and allowing viral spread within cortical
543 neurons and beyond. Expression of *hACE2* within neurons in the CNS is overall low and
544 does not correlate with our immunohistochemical findings, where ACE2 protein was
545 restricted to capillary endothelium, ependymal and choroid epithelium with sparing of
546 neurons and their processes. These findings suggest the ACE2 expression in these
547 anatomical compartments could be attributed to *mACE2* or indicative of a post-
548 transcriptional event that could be limiting neuronal expression of *hACE2*. These along
549 with the fact that *hACE2* mRNA is not abundantly and equally expressed among
550 different neuronal populations and that Purkinje cells in the cerebellum express *hACE2*
551 mRNA, but are not permissive to SARS-CoV-2 infection, suggest that entry of SARS-
552 CoV-2 into neurons is likely mediated by other host receptors independent of ACE2.

553 Infection of brain organoids has been shown to be inhibited by the use of anti-ACE2
554 antibodies (Song et al., 2020). However, brain organoids do not recapitulate the
555 complexity of the entire CNS, and retrograde transport of viral particles into the CNS
556 can hardly be modeled *in vitro*. Altogether, this suggests that while ACE2 is assuredly
557 an important mediator of CNS neuroinvasion, *in vitro* platforms to study mechanisms of
558 SARS-CoV-2 neuroinvasion are limiting, and require the use of more complex
559 experimental systems. Recently, neuropilin-1, a transmembrane glycoprotein serving as
560 cell surface receptor for semaphorins and other ligands, was shown to serve as an
561 alternative receptor for SARS-CoV-2 mediating entry into the neuroepithelium and
562 neurons (Cantuti-Castelvetri et al., 2020). Even though we analyzed the expression of
563 neuropilin-1 in this study (data not shown), we observed ubiquitous expression in the
564 nasal passages, brain, kidneys, liver and lungs, precluding any definitive conclusions in
565 support or against these claims (Cantuti-Castelvetri et al., 2020).

566 Anosmia and ageusia (loss of smell and taste, respectively) represent the earliest and
567 most common but transient neurologic symptoms in people with COVID-19, being
568 reported in $\geq 50\%$ of cases (Eliezer et al., 2020; Ellul et al., 2020; Walker et al., 2020).
569 Hyposmia or anosmia has also been clearly characterized in K18-hACE2 mice,
570 occurring between 2-3 dpi, which was characterized through a series of unique
571 behavioral tests requiring a normal sense of smell (Zheng et al., 2020). Other neurologic
572 manifestations of COVID-19 are associated with acute cerebrovascular disease, with
573 cohort studies reporting strokes in 2–6% of hospitalized patients (Ellul et al., 2020;
574 Wang et al., 2020b). Long-term neurologic sequelae associated with COVID-19 or its
575 effect on neurodegenerative diseases remain unclear (Wang et al., 2020b). Very little is

576 known about the pathogenesis of these neurologic manifestations and whether they are
577 directly or indirectly associated with SARS-CoV-2. ACE2 expression has been
578 described in humans both in health and with chronic rhinosinusitis, with expression
579 noted in sustentacular cells of the ONE, but not within immature and mature olfactory
580 neurons (Chen et al., 2020). This observation led the authors to suggest that anosmia in
581 COVID-19 is likely attributable to an indirect effect of SARS-CoV-2 infection. However,
582 recent studies evaluated brain and nasal autopsies from patients who died of COVID-
583 19, in which SARS-CoV-2 antigen and RNA was detected in cells of neural origin within
584 the ONE and cortical neurons occasionally associated with locally ischemic regions
585 (Meinhardt et al., 2020; Song et al., 2020). These studies provide conclusive evidence
586 that the K18-hACE2 mice serves as a model with translational significance, even though
587 ischemic lesions have not been reported including those in our study, further justifying
588 its use to dissect the intricate mechanisms involved in SARS-CoV-2-mediated CNS
589 invasion. Even though SARS-CoV-2 infects sustentacular cells within the
590 neuroepithelium of Syrian hamsters (Bryche et al., 2020), the K18-hACE2 and a
591 CRISPR/Cas9 hACE2 knock-in mouse model (Sun et al., 2020) are the only models
592 that develop neuroinvasion with wild-type virus and, thus, will be particularly useful for
593 studying SARS-CoV-2 neuropathogenesis, particularly the mechanisms of viral
594 trafficking of into the CNS through the neuroepithelium.

595 Another important observation of the K18-hACE2 model is that SARS-CoV-2 tropism
596 extensively involves infection of ACE2 and *hACE2* negative cells, including certain
597 population of neurons and the vast majority of AT1 pneumocytes. Similarly, sole
598 expression of *hACE2* in some cell types (i.e., bronchiolar epithelial cells) clearly does

599 not render these cells susceptible to SARS-CoV-2 even following intranasal exposure,
600 and underscore that other undetermined host factors are most likely required to allow
601 viral entry. Therefore, this model is extremely relevant for investigating the role of
602 ACE2-independent entry.

603 In conclusion, this study provides a comprehensive spatiotemporal analysis of SARS-
604 CoV-2 infection in the K18-hACE2 transgenic murine model along with an analysis of
605 the contribution of ACE2 in the permissiveness of the model. Our work provides
606 evidence that SARS-CoV-2 exhibits a marked neurotropism, and that this process likely
607 occurs through mechanisms that are in part hACE2-independent. Lethal CNS invasion,
608 combined with the absence of several of the pulmonary hallmarks associated with
609 severe human COVID-19, therefore calls for attentive caution when utilizing the K18-
610 hACE2 mouse model to investigate certain aspects of SARS-CoV-2 pathogenesis. The
611 protective ability of anti-viral therapies and T-cell based vaccines against lethal
612 challenge in this model might indeed be then underestimated due to the increased
613 susceptibility to CNS invasion. Regardless, the K18-hACE2 mouse model represents a
614 promising model for understanding the mechanisms governing SARS-CoV-2
615 neuroinvasion, ACE2-independent virus entry, and evaluating potent and fast-acting
616 prophylactic countermeasures.

617

618 **MATERIALS AND METHODS**

619 **Biosafety.** All aspects of this study were approved by the Institutional Biosafety
620 Committee and the office of Environmental Health and Safety at Boston University prior

621 to study initiation. Work with SARS-CoV-2 was performed in a biosafety level-3
622 laboratory by personnel equipped with powered air-purifying respirators.

623 **Cells and viruses.** African green monkey kidney Vero E6 cells (ATCC[®] CRL-1586[™],
624 American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's
625 minimum essential medium (DMEM; Gibco, Carlsbad, CA [#11995-065]) containing
626 10% fetal bovine serum (FBS, ThermoFisher Scientific, Waltham, MA), 1X non-essential
627 amino acids (ThermoFisher Scientific), penicillin and streptomycin (100 U/ml and 100
628 µg/ml), and 0.25 µg/ml of amphotericin B (Gibco[®], Carlsbad, CA), and incubated at 37
629 °C and 5% CO₂ in a humidified incubator.

630 **SARS-CoV-2 isolate stock preparation and titration.** All replication-competent
631 SARS-CoV-2 experiments were performed in a biosafety level 3 laboratory (BSL-3) at
632 the Boston University' National Emerging Infectious Diseases Laboratories. 2019-
633 nCoV/USA-WA1/2020 isolate (NCBI accession number: MN985325) of SARS-CoV-2
634 was obtained from the Centers for Disease Control and Prevention (Atlanta, GA) and
635 BEI Resources (Manassas, VA). To generate the passage 1 (P1) virus stock, Vero E6
636 cells, pre-seeded the day before at a density of 10 million cells, were infected in T175
637 flasks with the master stock, diluted in 10 ml final volume of Opti-MEM (ThermoFisher
638 Scientific). Following virus adsorption to the cells at 37 °C for 1 h, 15 ml DMEM
639 containing 10% FBS and 1X penicillin/streptomycin was added to the flask. The next
640 day, media was removed, the cell monolayer was rinsed with 1X phosphate buffered
641 saline (PBS) pH 7.5 (ThermoFisher Scientific) and 25 ml of fresh DMEM containing 2%
642 FBS was added. Two days later, when the cytopathic effect of the virus was clearly
643 visible, culture medium was collected, filtered through a 0.2 µm filter, and stored at -80

644 °C. Our P2 working stock of the virus was prepared by infecting Vero E6 cells with the
645 P1 stock, at a multiplicity of infection (MOI) of 0.1. Cell culture media was harvested at 2
646 and 3 dpi, and after the last harvest, ultracentrifuged (Beckman Coulter Optima L-100k;
647 SW32 Ti rotor) for 2 h at 25,000 rpm (80,000 X g) over a 20% sucrose cushion (Sigma-
648 Aldrich, St. Louis, MO). Following centrifugation, the media and sucrose were discarded
649 and pellets were left to dry for 5 minutes at room temperature. Pellets were then
650 resuspended overnight at 4 °C in 500 µl of 1X PBS. The next day, concentrated virions
651 were aliquoted and stored at -80 °C.

652 The titer of our viral stock was determined by plaque assay. Vero E6 cells were seeded
653 into a 12-well plate at a density of 2.5×10^5 cells per well and infected the next day with
654 serial 10-fold dilutions of the virus stock for 1 h at 37 °C. Following virus adsorption, 1 ml
655 of overlay media, consisting of 2X DMEM supplemented with 4% FBS and mixed at a
656 1:1 ratio with 1.2% Avicel (DuPont; RC-581), was added in each well. Three days later,
657 the overlay medium was removed, the cell monolayer was washed with 1X PBS and
658 fixed for 30 minutes at room temperature with 4% paraformaldehyde (Sigma-Aldrich).
659 Fixed cells were then washed with 1X PBS and stained for 1h at room temperature with
660 0.1% crystal violet (Sigma-Aldrich) prepared in 10% ethanol/water. After rinsing with tap
661 water, the number of plaques were counted and the virus titer was calculated. The titer
662 of our P2 virus stock was 4×10^8 plaque forming units (PFU)/ml.

663 **Mice.** Mice were maintained in a facility accredited by the Association for the
664 Assessment and Accreditation of Laboratory Animal Care (AAALAC). All protocols were
665 approved by the Boston University Institutional Animal Care and Use Committee
666 (PROTO202000020). Heterozygous K18-hACE2 C57BL/6J mice of both sexes (strain:

667 2B6.Cg-Tg(K18-ACE2)2PrImn/J) were obtained from the Jackson Laboratory (Jax, Bar
668 Harbor, ME). Animals were group-housed by sex in Tecniplast green line individually
669 ventilated cages (Tecniplast, Buguggiate, Italy). Mice were maintained on a 12:12 light
670 cycle at 30-70% humidity and provided ad-libitum water and standard chow diets
671 (LabDiet, St. Louis, MO).

672 **Intranasal inoculation with SARS-CoV-2.** At 4 months of age, K18-hACE2 mice of
673 both sexes were intranasally inoculated with 1×10^6 PFU of SARS-CoV-2 in 50 μ l of
674 sterile 1X PBS (n=47 [n=25 male and n=12 female]), or sham inoculated with 50 μ l of
675 sterile 1X PBS (n=3; female). Inoculations were performed under 1-3% isoflurane
676 anesthesia. Animals were either enrolled in a 14-day survival curve study (n=33).
677 Animals were only euthanized early if they reached euthanasia criteria, or if the
678 experiment included predetermined euthanasia timepoints for tissue sampling (2, 4, or 7
679 dpi; n=15).

680 **Clinical monitoring.** Animals included in the 14-day survival curve study were
681 intraperitoneally implanted with an RFID temperature-monitoring microchip (Unified
682 Information Devices, Lake Villa, IL, USA) 48-72 hours prior to inoculation. An IACUC-
683 approved clinical scoring system was utilized to monitor disease progression and
684 establish humane endpoints (Table 2). Categories evaluated included body weight,
685 general appearance, responsiveness, respiration, and neurological signs for a
686 maximum score of 5. Animals were considered moribund and humanely euthanized in
687 the event of the following: a score of 4 or greater for 2 consecutive observation periods,
688 weight loss greater than or equal to 20%, severe respiratory distress, or lack of

689 responsiveness. Clinical signs and body temperature were recorded once per day for
690 the duration of the study.

691 **Tissue processing and viral RNA isolation.** Tissues were collected from mice and
692 stored in 600 μ l of RNA $later$ (Sigma-Aldrich; # R0901500ML) and stored at -80 °C. For
693 processing, 20 – 30 mg of tissue were placed into a 2 ml tube with 600 μ l of RLT buffer
694 with 1% β -mercaptoethanol and a 5 mm stainless steel bead (Qiagen, Valencia, CA;
695 #69989). Tissues were then dissociated using a Qiagen TissueLyser II (Qiagen) with
696 the following cycle parameters: 20 cycles/s for 2 min, 1 min wait, 20 cycles/s for 2 min.
697 Samples were centrifuged at 17,000 X g (13,000 rpm) for 10 minutes and supernatant
698 was transferred to a new 1.5 ml tube. Viral RNA isolation was performed using a Qiagen
699 RNeasy Plus Mini Kit (Qiagen; #74134), according to the manufacturer's instructions,
700 with an additional on-column DNase treatment (Qiagen; #79256). RNA was finally
701 eluted in 30 μ l of RNase/DNase-free water and stored at -80 °C until used.

702 **RNA isolation from serum.** Total viral RNA was isolated from serum using a Zymo
703 Research Corporation Quick-RNATM Viral Kit (Zymo Research, Tustin, CA; #R1040)
704 according to the manufacturer's instructions. RNA was eluted in 15 μ l of RNase/DNase-
705 free water and stored at -80 °C until used.

706 **SARS-CoV-2 E-specific reverse transcription quantitative polymerase chain
707 reaction (RT-qPCR).** Viral RNA was quantitated using single-step RT-quantitative real-
708 time PCR (Quanta qScript One-Step RT-qPCR Kit, QuantaBio, Beverly, MA; VWR;
709 #76047-082) with primers and TaqMan® probes targeting the SARS-CoV-2 E gene as
710 previously described (Corman et al., 2020). Briefly, a 20 μ l reaction mixture containing
711 10 μ l of Quanta qScriptTM XLT One-Step RT-qPCR ToughMix, 0.5 μ M Primer

712 E_Sarbeco_F1 (ACAGGTACGTTAATAGTTAATAGCGT), 0.5 μ M Primer
713 E_Sarbeco_R2 (ATATTGCAGCAGTACGCACACA), 0.25 μ M Probe E_Sarbeco_P1
714 (FAM-ACACTAGCCATCCTTACTGCGCTTCG-BHQ1), and 2 μ l of template RNA was
715 prepared. RT-qPCR was performed using an Applied Biosystems QuantStudio 3
716 (ThermoFisher Scientific) and the following cycling conditions: reverse transcription for
717 10 minutes at 55 $^{\circ}$ C, an activation step at 94 $^{\circ}$ C for 3 min followed by 45 cycles of
718 denaturation at 94 $^{\circ}$ C for 15 seconds and combined annealing/extension at 58 $^{\circ}$ C for 30
719 seconds. Ct values were determined using QuantStudioTM Design and Analysis software
720 V1.5.1 (ThermoFisher Scientific). For absolute quantitation of viral RNA, a 389 bp
721 fragment from the SARS-CoV-2 E gene was cloned onto pIDTBlue plasmid under an
722 SP6 promoter using NEB PCR cloning kit (New England Biosciences, Ipswich, MA).
723 The cloned fragment was then *in vitro* transcribed (mMessage mMachine SP6
724 transcription kit; ThermoFisher) to generate an RT-qPCR standard.

725 **Serum infectivity assay.** Vero E6 cells were plated in a 24-well plate at a density of
726 50,000 cells per well. To assess for infectious particles, 20 μ l of serum was added onto
727 cells with 250 μ l of DMEM supplemented with 2% FBS and 1% penicillin and
728 streptomycin and incubated for 6 hours at 37 $^{\circ}$ C. After incubation, serum was removed,
729 cells were washed twice with 1 ml of 1X PBS, and 1 ml of DMEM supplemented with
730 2% FBS 1% penicillin and streptomycin was added to each well. Media was collected
731 24- and 96-hours post infection for RT-qPCR analysis.

732 **Histology.** Animals were anesthetized with 1-3% isoflurane and euthanized with an
733 intraperitoneal overdose of ketamine and xylazine before harvest and fixation of tissues.
734 Lungs were insufflated with ~1.5mL of 1% low melting point agarose (Sigma-Aldrich)

735 diluted in 1X PBS using a 24-gauge catheter placed into the trachea. The skull cap was
736 removed and the animal decapitated and immersed in fixative. Additional tissues
737 harvested included the heart, kidneys, and representative sections of the
738 gastrointestinal tract, which included the duodenum, jejunum, ileum, cecum, and colon.
739 Tissues were inactivated in 10% neutral buffered formalin at a 20:1 fixative to tissue
740 ratio for a minimum of 72 hours before removal from BSL-3 in accordance with an
741 approved institutional standard operating procedure. Following fixation, the whole head
742 was decalcified in Immunocal™ Decalcifier (StatLab, McKinney, TX) for 7 days before
743 performing a mid-sagittal section dividing the two hemispheres into even sections.
744 Tissues were subsequently processed and embedded in paraffin following standard
745 histological procedures. Five-micron sections were obtained and stained with
746 hematoxylin and eosin or Luxol Fast Blue (myelin stain).

747 **Immunohistochemistry and RNAscope® *in situ* hybridization.**

748 Immunohistochemistry (IHC) was performed using a Ventana BenchMark Discovery
749 Ultra autostainer (Roche Diagnostics, Indianapolis, IN) using tyramide signaling
750 amplification (TSA) technology. Specific IHC assay details including antibodies, antigen
751 retrieval, sequence of multiplex assays, and incubation periods are found in Table S2.
752 SARS-CoV-2 S was semiquantitatively scored as follows: 0, no viral antigen observed;
753 1, up to 5% positive cells per 400X field examined; 2, 5-25% positive cells per 400X
754 field examined; and 3, up to 50% positive cells per 400X field examined.

755 For SARS-CoV-2 RNAscope® ISH, an anti-sense probe targeting the spike (S;
756 nucleotide sequence: 21,563-25,384) of SARS-CoV-2, USA-WA1/2020 isolate
757 (GenBank accession number MN985325.1) was used as previously described

758 (Carossino et al., 2020; Gaudreault et al., 2020). The RNAscope[®] ISH assay was
759 performed using the RNAscope 2.5 LSx Reagent Kit (Advanced Cell Diagnostics,
760 Newark, CA) on the automated BOND RXm platform (Leica Biosystems, Buffalo Grove,
761 IL) as described previously (Meekins et al., 2020). Briefly, four-micron sections of
762 formalin-fixed paraffin-embedded tissue were subjected to automated baking and
763 deparaffinization followed by heat-induced epitope retrieval (HIER) using a ready-to-use
764 EDTA-based solution (pH 9.0; Leica Biosystems) at 100 °C for 15 min. Subsequently,
765 tissue sections were treated with a ready-to-use protease (RNAscope[®] 2.5 LSx
766 Protease) for 15 min at 40 °C followed by a ready-to-use hydrogen peroxide solution for
767 10 min at room temperature. Slides were then incubated with the ready-to-use probe
768 mixture for 2 h at 40 °C, and the signal amplified using a specific set of amplifiers
769 (AMP1 through AMP6 as recommended by the manufacturer). The signal was detected
770 using a Fast-Red solution for 10 minutes at room temperature. Slides were
771 counterstained with a ready-to-use hematoxylin for 5 min, followed by five washes with
772 1X BOND Wash Solution (Leica Biosystems) for bluing. Slides were finally rinsed in
773 deionized water, dried in a 60 °C oven for 30 min, and mounted with Ecomount[®]
774 (Biocare, Concord, CA, USA). A SARS-CoV-2-infected Vero E6 cell pellet was used as
775 a positive assay control. For all assays, an uninfected mouse was used as a negative
776 control.

777 For *hACE2* mRNA RNAscope[®] ISH, an anti-sense probe targeting *hACE2* (GenBank
778 accession number NM_021804.3; Cat. No. 848038) with no cross-reactivity to murine
779 *Ace2* was used in a similar manner as described above with the exception that AMP5
780 and AMP6 were incubated for 45 min and 30 min, respectively. Murine *peptidylprolyl*

781 *isomerase B (Ppib)* mRNA was used as a housekeeping gene to determine RNA quality
782 and a Vero E6 cell pellet was used as a positive assay control.

783 **Multispectral microscopy.** Fluorescently labeled slides were imaged using a Mantra
784 2.0™ Quantitative Pathology Workstation (Akoya Biosciences, Marlborough, MA). To
785 maximize signal-to-noise ratios, images were spectrally unmixed using a synthetic
786 library specific for the Opal fluorophores used for each assay and for 4',6-diamidino-2-
787 phenylindole (DAPI). Furthermore, an unstained section was used to create an
788 autofluorescence signature that was subsequently removed from whole-slide images
789 using InForm software version 2.4.8 (Akoya Biosciences).

790 **Statistical analysis.** Descriptive statistics and graphics as well as Kaplan-Meier
791 (survival) curves and statistical tests were performed using GraphPad Prism statistical
792 analysis software (GraphPad, San Diego, CA). Clinical parameters were analyzed using
793 a Student *t*-test. Significance levels were set at p -value<0.05 in all cases.

794

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805

806 **CONFLICT OF INTEREST STATEMENT**

807 The authors declare no conflicts of interest.

808

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810 and M. Bosmann designed the study; F. Douam, N Crossland, M. Saeed, M. Carossino,
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815

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1045

1046 **FIGURE LEGENDS**

1047 **Figure 1.** SARS-CoV-2 caused lethal disease in K18-hACE2 mice (n=33) inoculated
1048 intranasally with 1×10^6 plaque forming units (PFU). Body weight (A), clinical signs (B),
1049 temperature (C), and mortality (D) were monitored daily. Viral loads (genome copy
1050 numbers/mg or ml) were monitored in the brain, lungs (E) and serum (F) throughout the
1051 study. Mean genome copy numbers are depicted. The limit of detection (LOD) is shown
1052 with a dashed line.

1053 **Figure 2.** Temporal analysis of SARS-CoV-2 infection in the nasal cavity of K18-hACE2
1054 mice at 2 (A-D) and 4 (E-H) days following intranasal inoculation. Histological changes
1055 and viral antigen (brown) and RNA (red) distribution and abundance were assessed. At
1056 2 dpi, suppurative rhinitis in the rostral and intermediate turbinates (A, arrow) correlated
1057 with abundant intraepithelial SARS-CoV-2 antigen (C) and RNA (C, inset). Abundant
1058 viral antigen and RNA was detected in the olfactory neuroepithelium (ONE, D and inset)
1059 in the absence of histologic lesions (B). At 4 dpi, only sporadically infected cells were
1060 noted in the epithelium lining the turbinates and ONE (G and H, arrow and insets) in the
1061 absence of histologic lesions (E and F). H&E and Fast Red (viral RNA), 200X total
1062 magnification. Bar = 100 μ m.

1063 **Figure 3.** Temporal analysis of SARS-CoV-2 infection in the lungs of K18-hACE2 mice
1064 at 2 (D-F), 4 (G-I), 7 (J-L) and 14 (M-O) days following intranasal inoculation.
1065 Histological changes and viral RNA (red) distribution and abundance were assessed.
1066 Mild to moderate interstitial pneumonia was evident starting at 2 days post-infection
1067 (dpi) with frequently reactive blood vessels (E, arrow) and a peak in viral RNA at 4 days
1068 post-infection (I). At 7 and 14 days post-infection, there was evidence of alveolar type 2
1069 (AT2) cell hyperplasia (K and N, arrows), with viral RNA restricted to areas spared of
1070 inflammation (L). The pneumonia was significantly ameliorated by 14 dpi (M) with no
1071 viral RNA (O). H&E and Fast Red (viral RNA), 50X (A, D, G, J, and M; bar = 500 μ m)
1072 and 200X (B, C, E, F, H, I, K, L, M and N; bar = 100 μ m) total magnification.

1073 **Figure 4.** SARS-CoV-2 tropism and temporal immunoprofiling of the pulmonary host
1074 inflammatory response following SARS-CoV-2 intranasal inoculation in K18-hACE2. (A)
1075 SARS-CoV-2 (yellow) showed tropism for RAGE⁺ alveolar type 1 (AT1, magenta) and

1076 scattered SPC⁺ alveolar type 2 (AT2, red) pneumocytes (A1 and A2, arrowheads and
1077 arrows, respectively) but not for CD31⁺ endothelial cells (green). SARS-CoV-2 (orange)
1078 was abundant and localized within AT1 and AT2 pneumocytes, with a progressive
1079 increase in the number of CD3⁺CD8⁻ T cells (blue, presumed to be CD4⁺ T cells), Iba-
1080 1⁺ histiocytes (magenta), and CD8⁺ T cells (red) throughout the course of the
1081 experiment (2 days post-infection [dpi, C], 7 dpi [D] and 14 dpi [E]). SARS-CoV-2 S
1082 antigen was no longer detectable by 14 dpi, and numerous aggregates of CD19⁺ B cells
1083 (green) were noted (E). Multiplex fluorescent IHC, 200X total magnification. Bar = 50
1084 μm

1085 **Figure 5.** Temporal neuronal damage in K18-hACE2 mice following intranasal
1086 inoculation with SARS-CoV-2. No histologic changes were noted in the cerebrum or
1087 olfactory bulb until 6-7 days post-infection (dpi, A-D). SARS-CoV-2 antigen (brown) was
1088 evident as early as 4 dpi (F, arrow). At 6-7 dpi, mild (G, arrowheads) to marked (G,
1089 inset) spongiosis with neuronal degeneration and necrosis involving multiple areas
1090 within the cerebral cortex and elsewhere were noted. Similar changes were evident in
1091 the olfactory bulb, with occasional perivascular cuffs/gliosis (H, arrowhead) and
1092 abundant viral antigen (I, arrows). No histologic alterations or viral antigen was detected
1093 in survivor mice at 14 dpi (J-L). H&E and DAB (viral antigen), 100X (A, B, D, E, G, H, J,
1094 K; bar = 200 μm) and 200X (C, F, I, L; bar = 100 μm) total magnification.

1095 **Figure 6.** Invasion of SARS-CoV-2 into the central nervous system. Sagittal sections of
1096 the head were analyzed for viral antigen and RNA distribution. SARS-CoV-2 infected
1097 neurons within the mitral layer of the olfactory bulb (1, arrow) as well as small clusters
1098 of neuronal bodies within the cerebral cortex (2, SARS-CoV-2 RNA in inset) as early as

1099 4 days post-infection. At 7 days post-infection, SARS-CoV-2 antigen was widespread
1100 along the mitral layer of the olfactory bulb (1) and throughout the central nervous
1101 system (2, SARS-CoV-2 RNA in inset) with exception of the cerebellum. EPL, external
1102 plexiform layer; GCL, granular cell layer; GL, glomerular layer; ML, mitral layer. DAB
1103 (viral antigen) and Fast Red (viral RNA). 7.5X (bar = 2.5 mm) and 200X (bar = 100 μ m)
1104 total magnification.

1105 **Figure 7.** Analysis of SARS-CoV-2 tropism within the central nervous system at 7 days
1106 post-infection using multiplex immunohistochemistry. Abundant SARS-CoV-2 antigen
1107 (green) localized within cortical (A), hippocampal (B) and brainstem (C) neurons with
1108 abundant neighboring Iba-1+ microglia (orange) and clusters of GFAP+ astrocytes
1109 (gliosis, magenta). 40X total magnification. Bar = 50 μ m.

1110 **Figure 8.** Distribution of ACE2 in lungs, nasal cavity, brain and olfactory bulb of wild-
1111 type C57BL/6J and transgenic (mock and SARS-CoV-2-infected) K18-hACE2 mice was
1112 analyzed via immunohistochemistry using a cross-reactive anti-ACE2 antibody. In the
1113 lungs (A-C), ACE2 expression (brown) was mostly restricted to the apical membrane of
1114 bronchiolar epithelial cells with scattered positive pneumocytes (inset arrows). Nasal
1115 (rostral/intermediate turbinates [R/I]) and olfactory epithelium (ONE) were devoid of
1116 ACE2 in C57BL/6J mice (D) but expression was enhanced in K18-hACE2 mice with
1117 intense apical expression (E and F). ACE2 expression within the brain (G-I) and
1118 olfactory bulb (J-L) was restricted to capillary endothelium with no neuronal expression.
1119 DAB, 200X total magnification. Bar = 100 μ m.

1120 **Figure 9.** Expression and distribution of *hACE2* mRNA in the lungs of C57BL/6J and
1121 K18-hACE2 transgenic mice via RNAscope® ISH. While no expression of *hACE2* was

1122 noted in the lungs of wild-type C57BL/6J mice (A), *hACE2* was expressed in the
1123 bronchiolar epithelium (arrowheads) and sporadic pneumocytes (arrows) in transgenic
1124 K18-hACE2 mice (B and C), which correlated with immunohistochemical findings. Fast
1125 Red, 400X total magnification. Bar = 50 μ m.

1126 **Figure 10.** Expression and distribution of *hACE2* mRNA in the brain of K18-hACE2
1127 transgenic mice via RNAscope® ISH. *hACE2* was expressed in clusters of neurons
1128 within the cerebral cortex (A), hippocampus (B) as well as in other locations including
1129 Purkinje cells of the cerebellum. Fast Red, 400X total magnification. Bar = 50 μ m.

1130

1131 SUPPLEMENTARY FIGURES

1132 **Figure S1.** Histologic and viral antigen distribution in the nasal cavity of K18-hACE2
1133 mice following SARS-CoV-2 infection. No histologic lesions or antigen were detected at
1134 7 and 14 days post-infection (dpi). 200X total magnification. Bar = 100 μ m.

1135 **Figure S2.** Localization and abundance of SARS-CoV-2 RNA (red) and antigen (brown)
1136 in the lungs of infected K18-hACE2 mice as demonstrated by anti-Spike RNAscope® *in*
1137 *situ* hybridization and immunohistochemistry. 2 (A and B), 4 (C and D), 7 (E and F) and
1138 14 (G and H) days post-infection (dpi). 50X (bar = 500 μ m) and 100X (bar = 100 μ m)
1139 total magnification.

1140 **Figure S3.** Histological and immunohistochemical findings in the cervicothoracic spinal
1141 cord of SARS-CoV-2-infected K18-hACE2 mice at 7 days post-infection. Histologically
1142 (A), Multifocal neuronal bodies within the grey matter are shrunken, angular and
1143 hyperchromatic (neuronal degeneration and necrosis), and the neuroparenchyma has
1144 multiple clear spaces filled with small amounts of debris (spongiosis) with a slight

1145 increase in the number of glial cells (gliosis). H&E, 100X total magnification. (B)
1146 Abundant SARS-CoV-2 spike protein localized within the perikaryon and processes of
1147 motor neurons within the spinal cord. DAB, 200X total magnification. Bar = 100 μ m.

1148 **Figure S4.** Expression of *hACE2* mRNA (red) in the brain of C57BL/6J and K18-hACE2
1149 mice determined by RNAscope® ISH. While wild-type mice exhibit no expression (A),
1150 *hACE2* is expressed in clusters of neurons at multiple locations in K18-hACE2 mice (B).
1151 Fast Red, 400X total magnification. Bar = 50 μ m.

1152 **Figure S5.** Expression of ACE2 (red) in the enterocytes lining the small intestine of
1153 C57BL/6J and K18-hACE2 mice. Immunohistochemistry was performed using a cross-
1154 reactive anti-ACE2 antibody. In the stomach, expression was intense in the non-
1155 glandular mucosa and capillaries of the glandular mucosa (A-C, arrows). In the colon,
1156 scattered enterocytes express ACE2 (G-I, arrows). Fast Red, 100X (A-C; bar = 200 μ m)
1157 and 200X (D-I; bar = 100 μ m) total magnification.

1158

1159

1160

1161 **Table 1.** SARS-CoV-2 viral antigen abundance in tissues derived from SARS-CoV-2-infected K18-hACE2 mice. Median
 1162 scores are represented along with ranges between brackets when applicable.

DPI	AT1/AT2	Bronchioles	Rostral turbinates	Intermediate turbinates	ONE	Olf. bulb	Brain	Spinal cord (CT)	Spinal cord (LS)	GI*	Kidneys
Mock	0	0	0	0	0	0	0	0	0	0	0
2	2 (1-2)	0	1 (0-2)	2 (1-2)	1 (1-2)	0	0	0	0	0	0
4	2 (1-3)	0	0 (0-1)	0 (0-1)	1 (0-1)	0 (0-1)	0 (0-1)	0	0	0	0
6-8	2 (1-3)	0	0	0	1 (0-1)	1 (0-2)	3 (0-3)	1 (0-2)	0 (0-1)	0	0
14	0	0	0	0	0	0	0	0	0	0	0

1163 0, no SARS-CoV-2 antigen observed; 1, 0 to 5% of cells within a high magnification (400X) field are positive for viral
 1164 antigen; 2, 5 to 25% of cells within a high magnification (400X) field are positive for viral antigen; 3, >25 to <50% of cells
 1165 within a high magnification (400X) field are positive for viral antigen. NA, not available. AT1, alveolar type 1 pneumocytes;
 1166 AT2, alveolar type 2 pneumocytes; ONE, olfactory neuroepithelium; CT, cervicothoracic segment; LS, lumbosacral
 1167 segment; GI, gastrointestinal tract.

1168 *Sections examined included stomach, small intestine (duodenum, jejunum and ileum) and large intestine (cecum and
 1169 colon).

1170 **Table 2.** Clinical scoring system used for clinical monitoring of SARS-CoV-2-infected K18-hACE2 mice.

Category	Score = Criteria
Body weight	1 = 10-19% loss
Respiration	1 = rapid, shallow, increased effort
Appearance	1 = ruffled fur, hunched posture
Responsiveness	1 = low to moderate unresponsiveness
Neurologic signs	1 = tremors

1171

1172

Figure 1

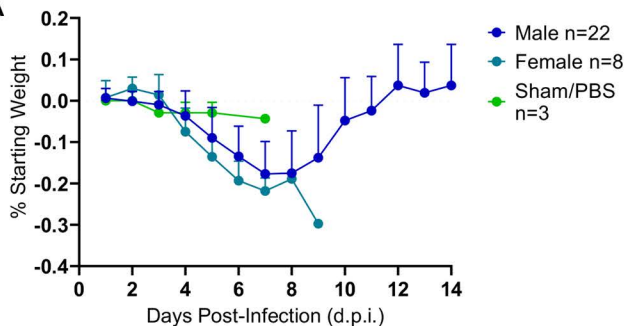
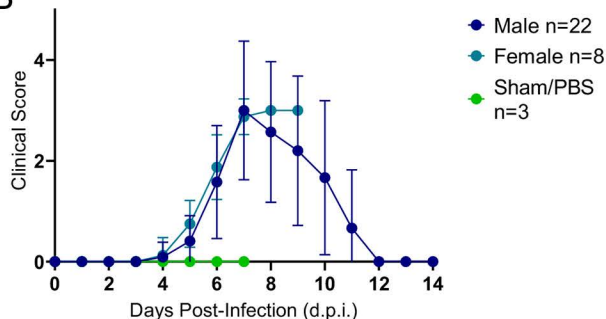
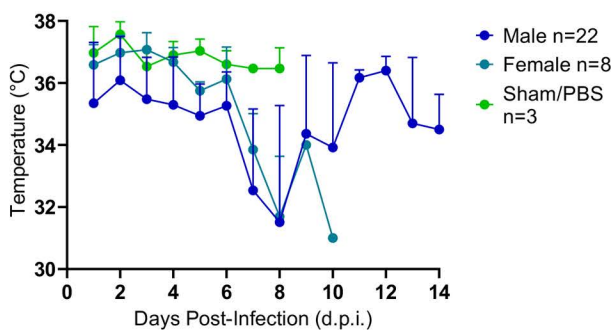
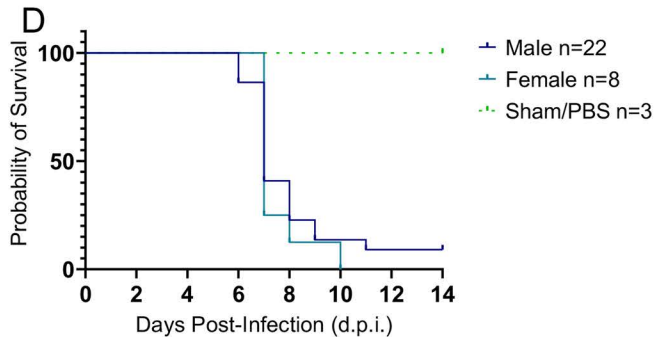
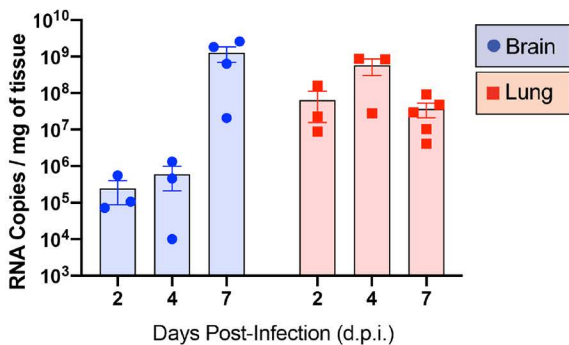
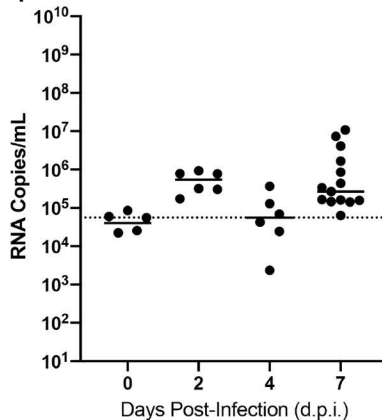
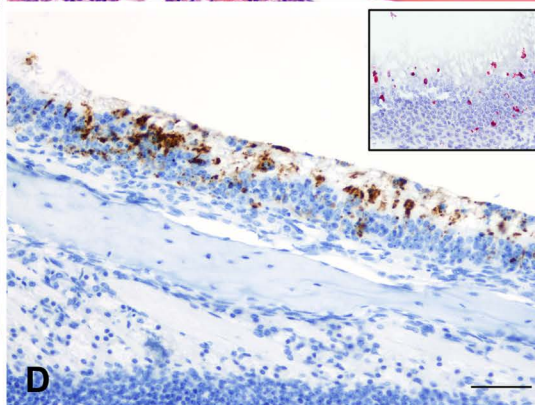
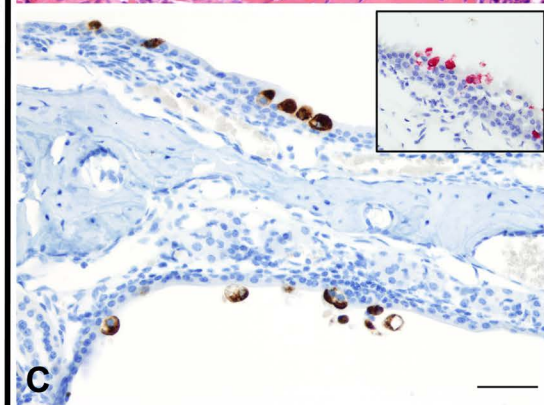
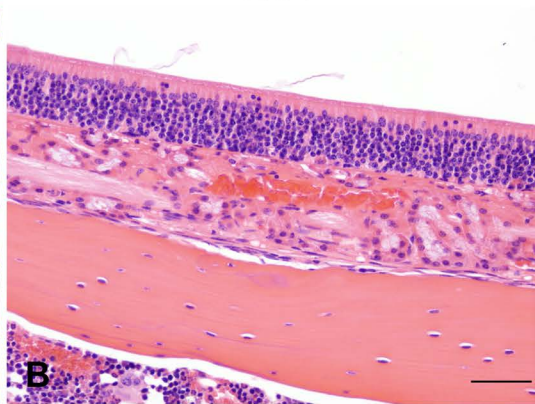
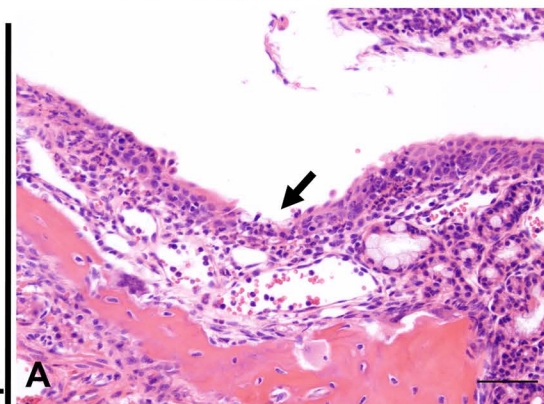
A**B****C****D****E****F**

Figure 2

Rostral/intermediate

ONE

2 dpi



4 dpi

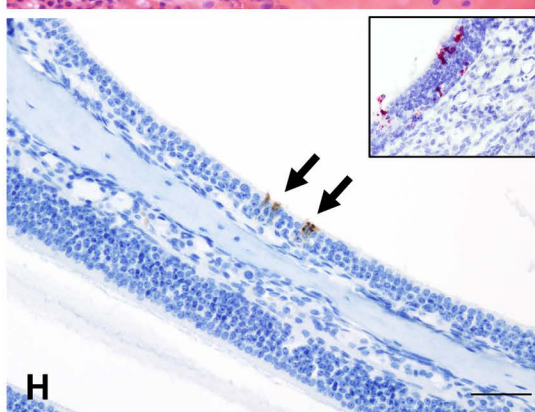
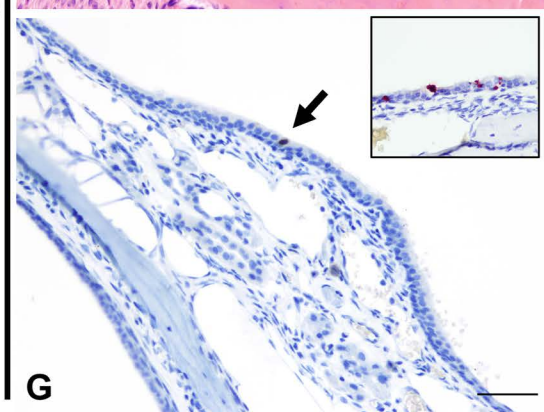
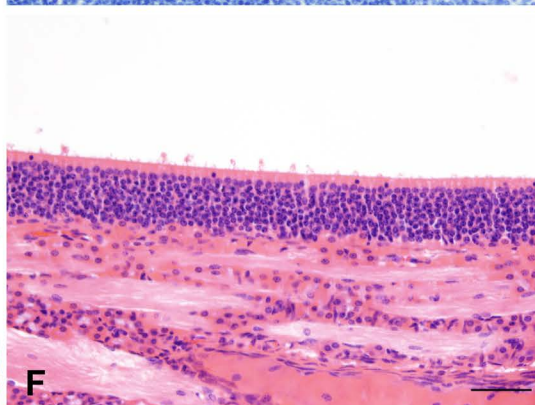
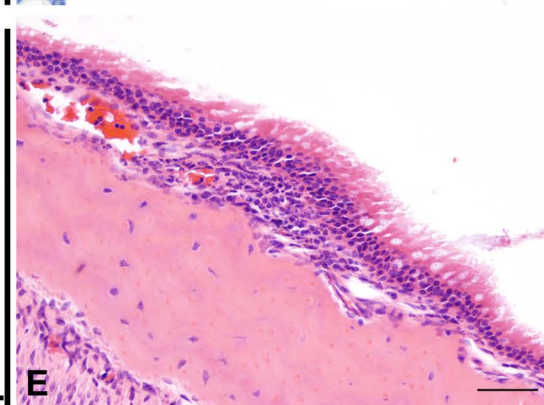


Figure 3

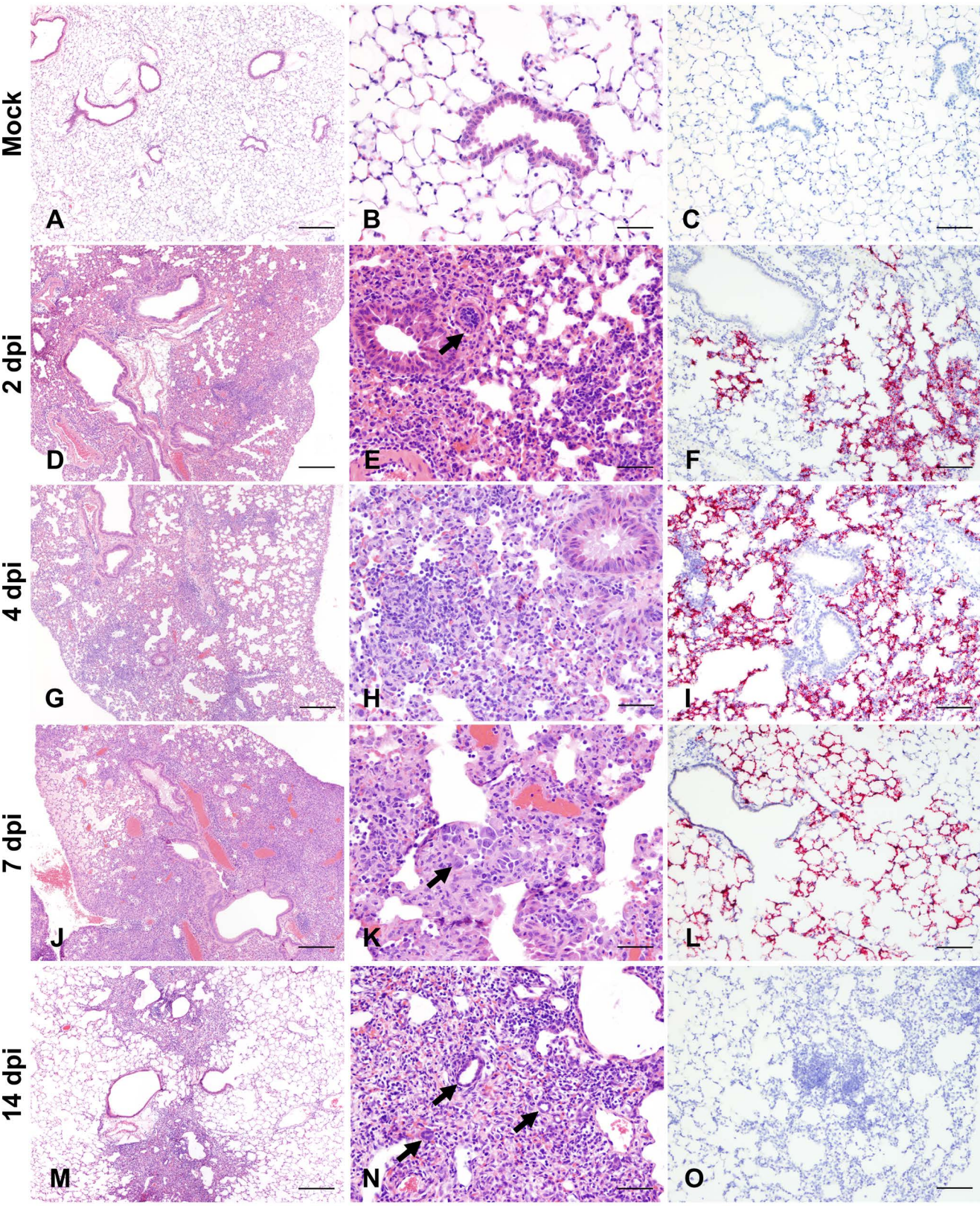


Figure 4

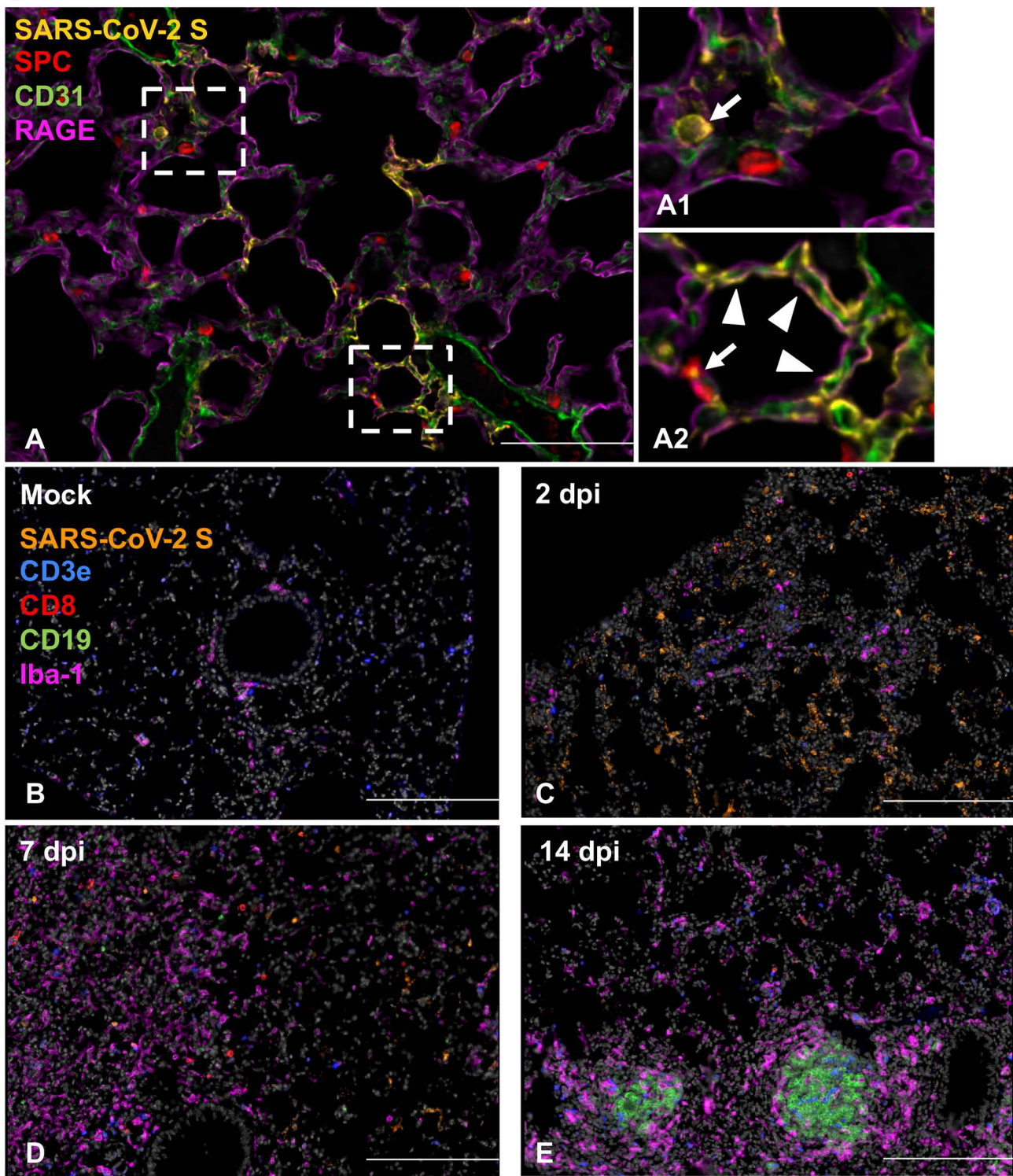


Figure 5

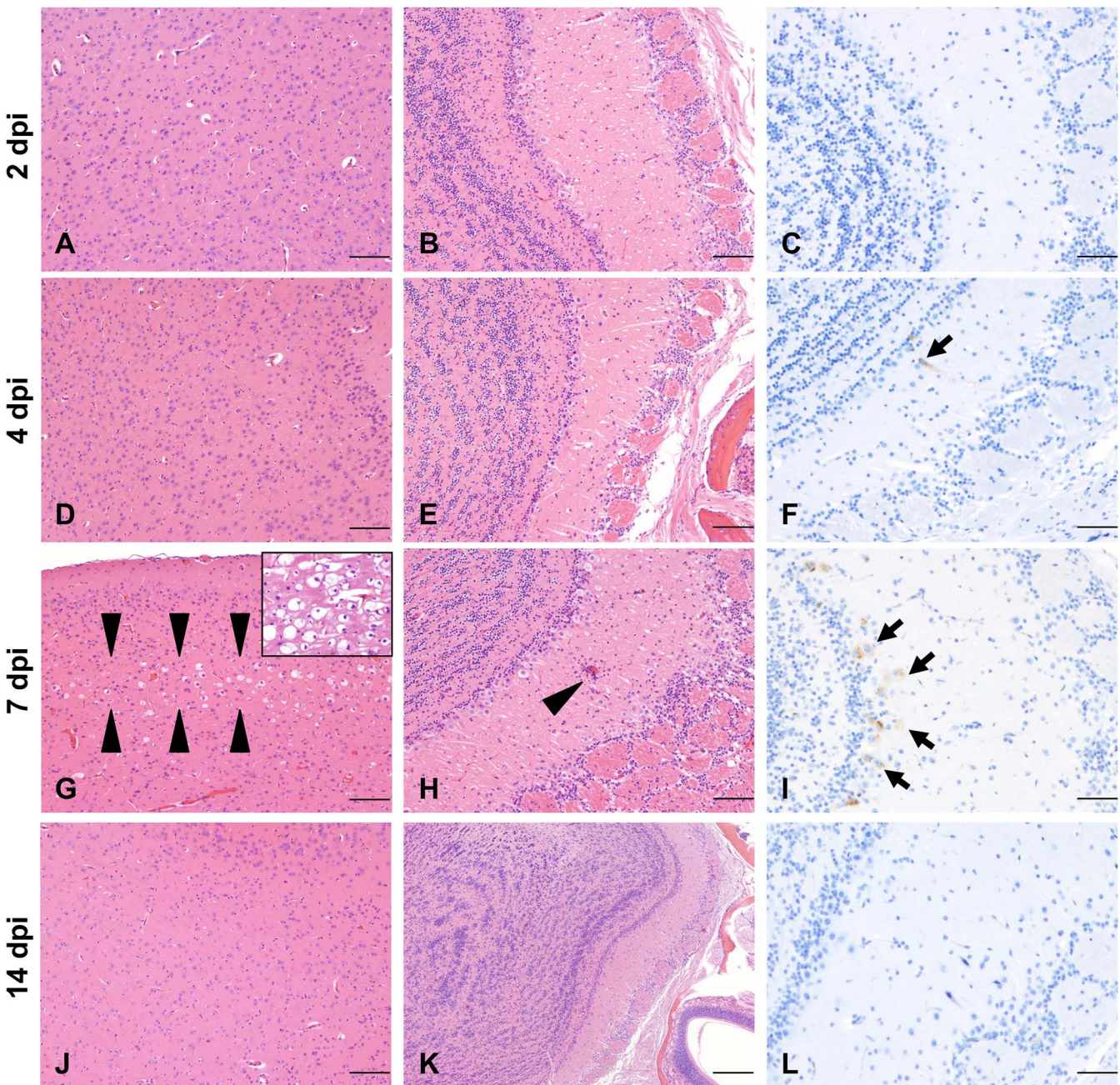


Figure 6

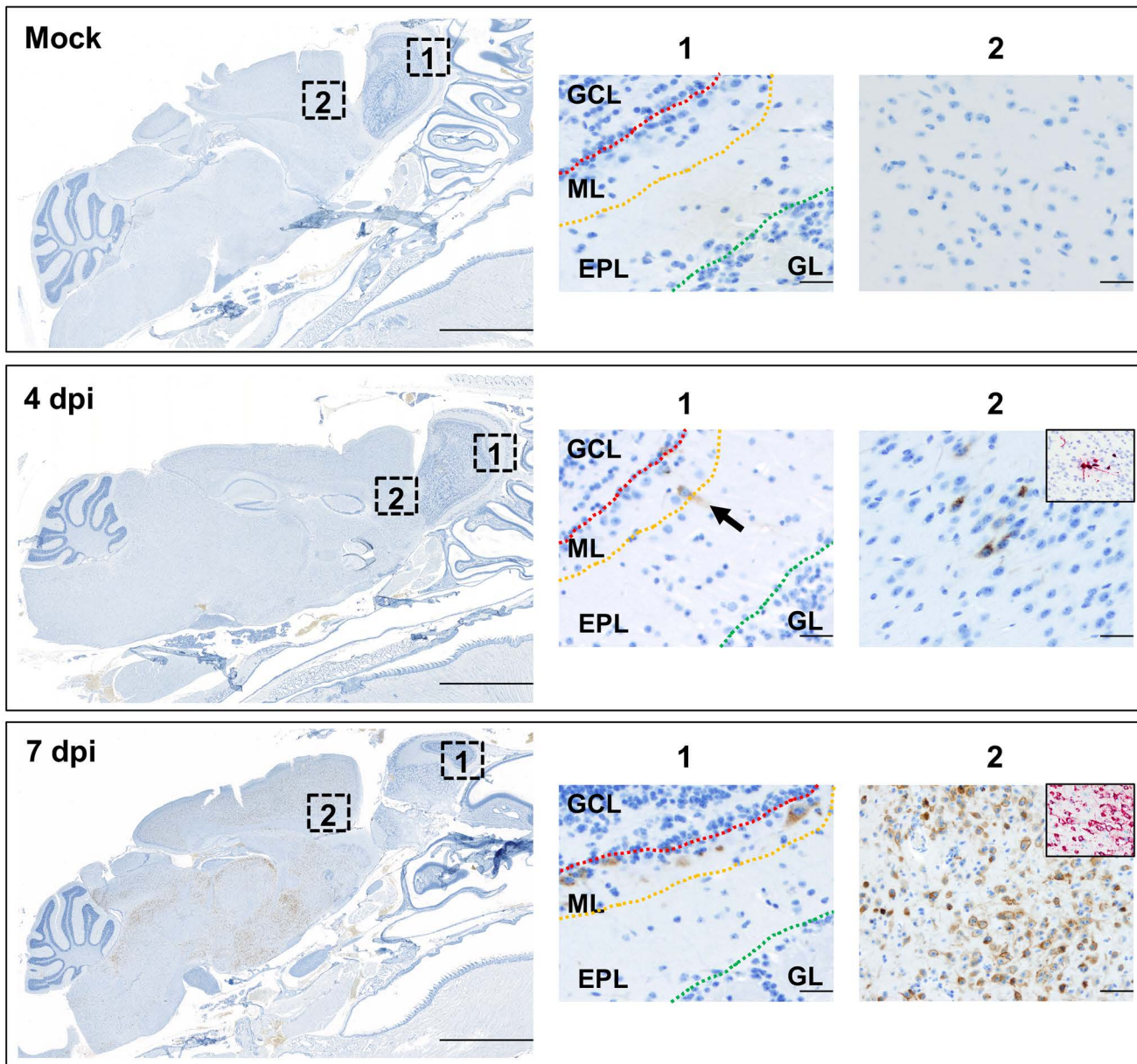
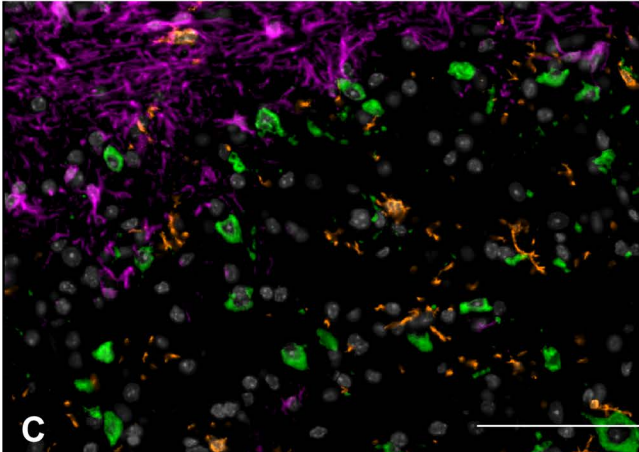
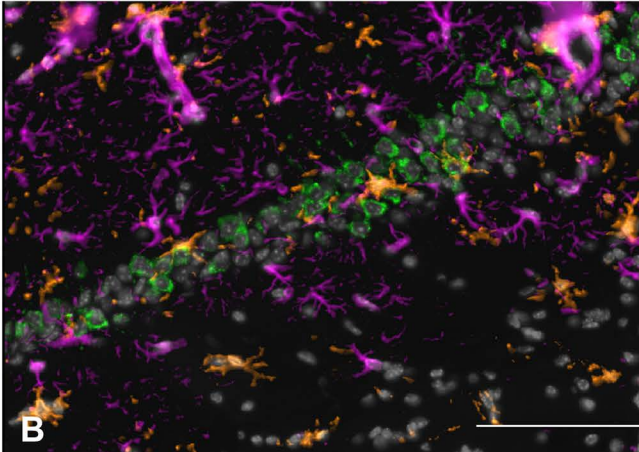
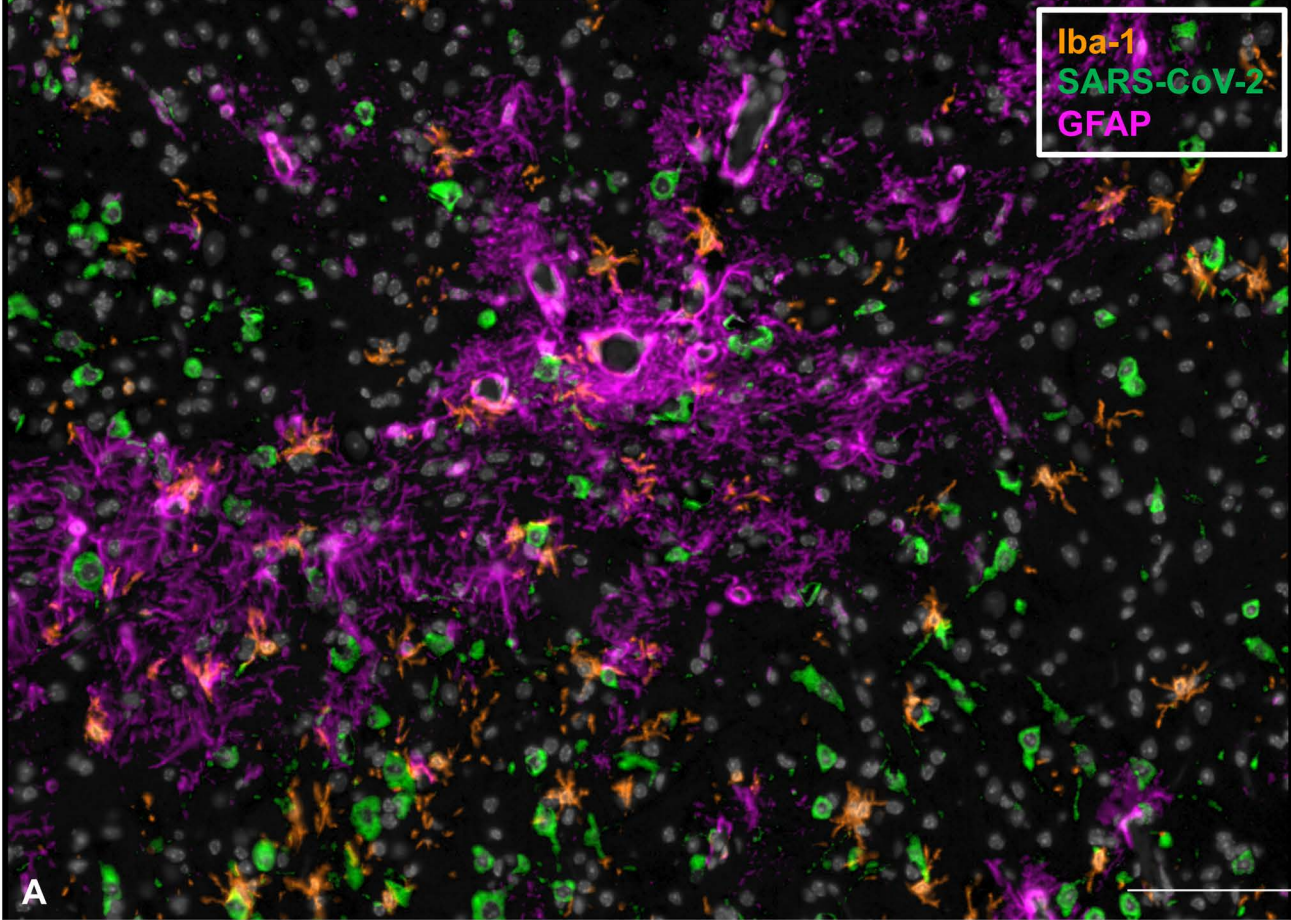


Figure 7



Iba-1
SARS-CoV-2
GFAP

Figure 8

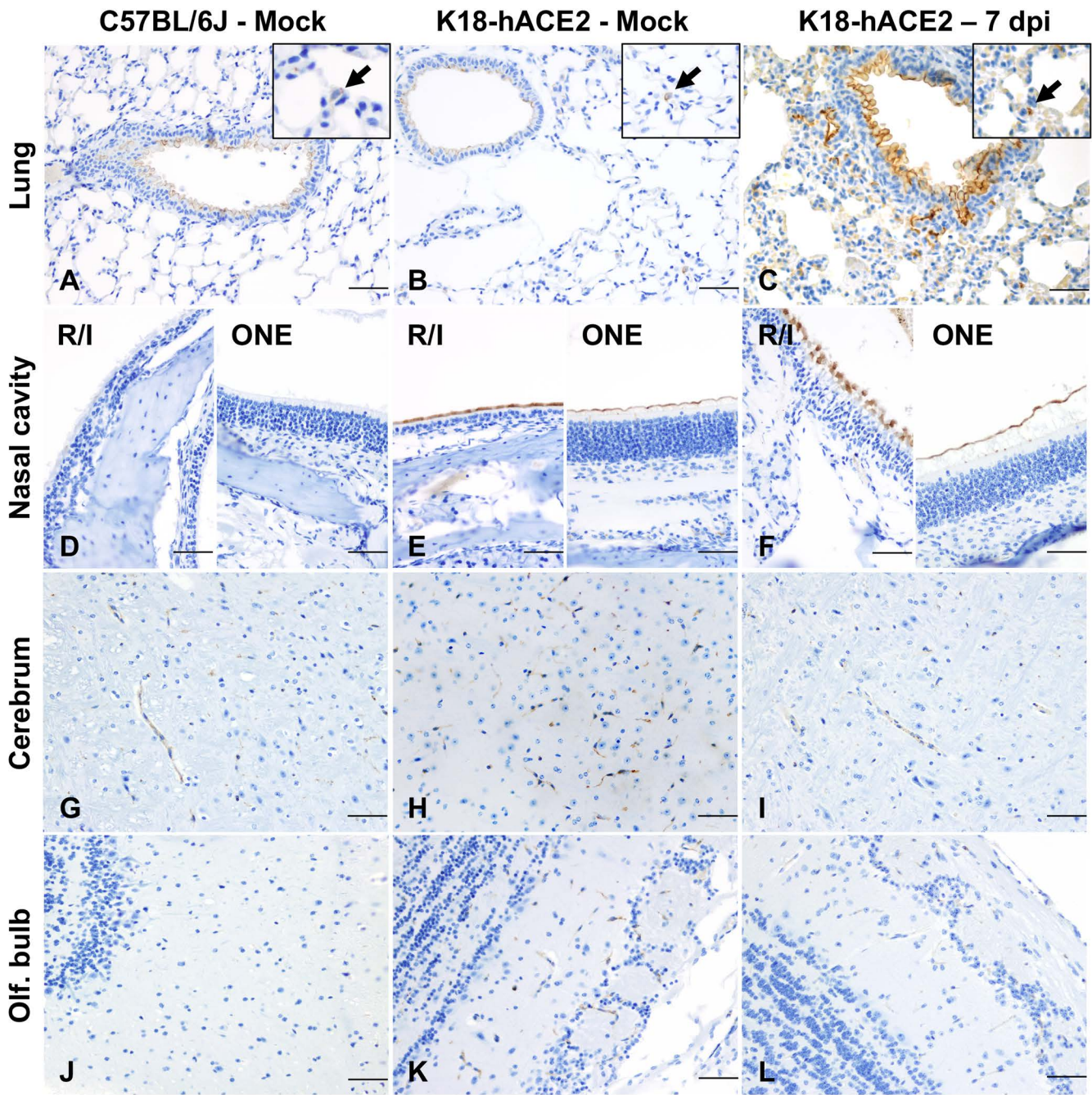
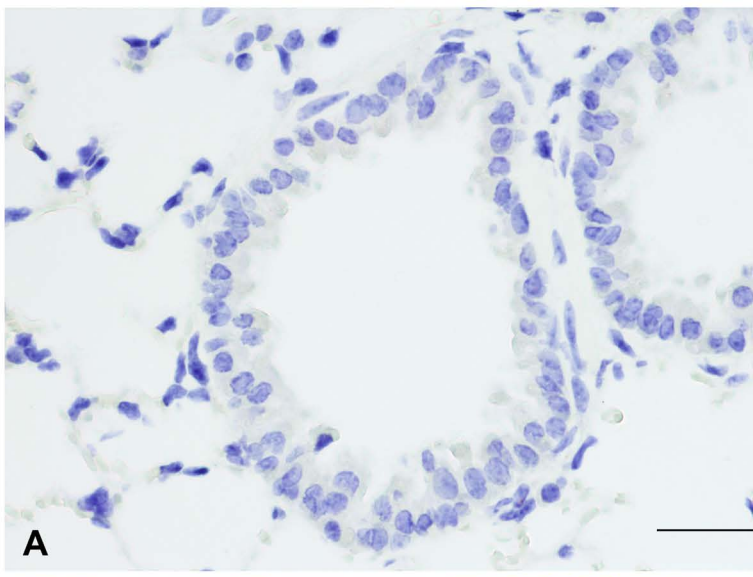
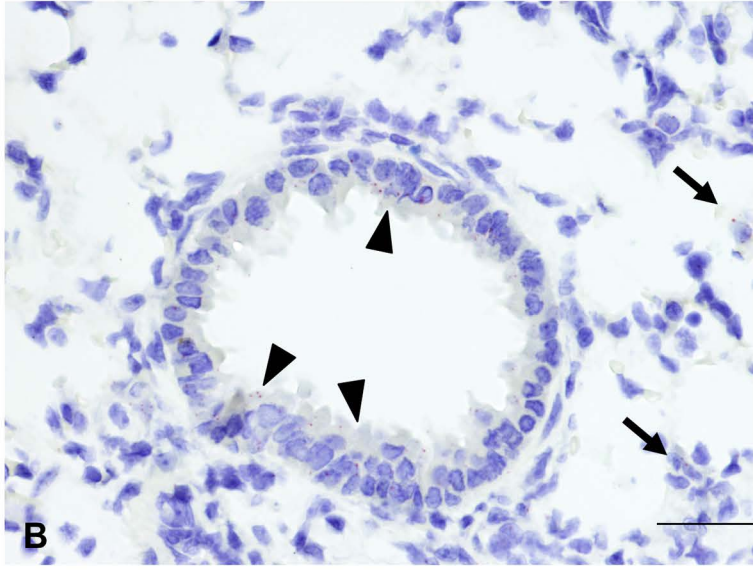


Figure 9

C57BL/6J



K18-hACE2 (4 dpi)



K18-hACE2 (7 dpi)

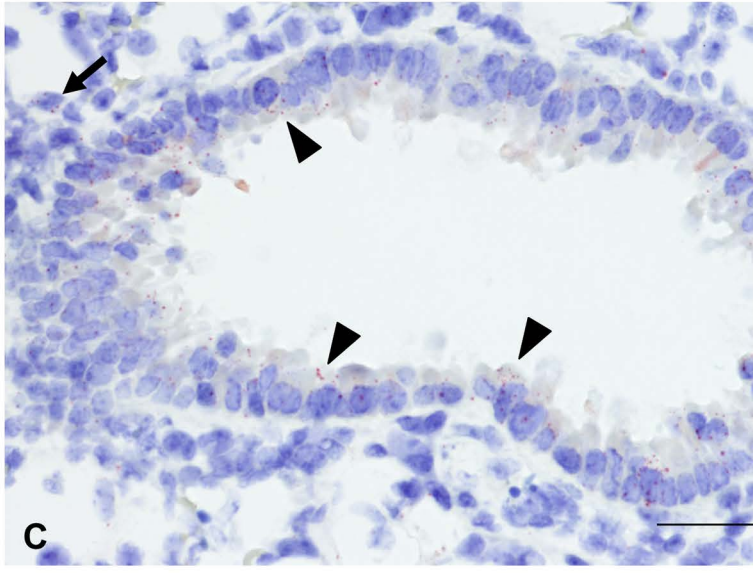
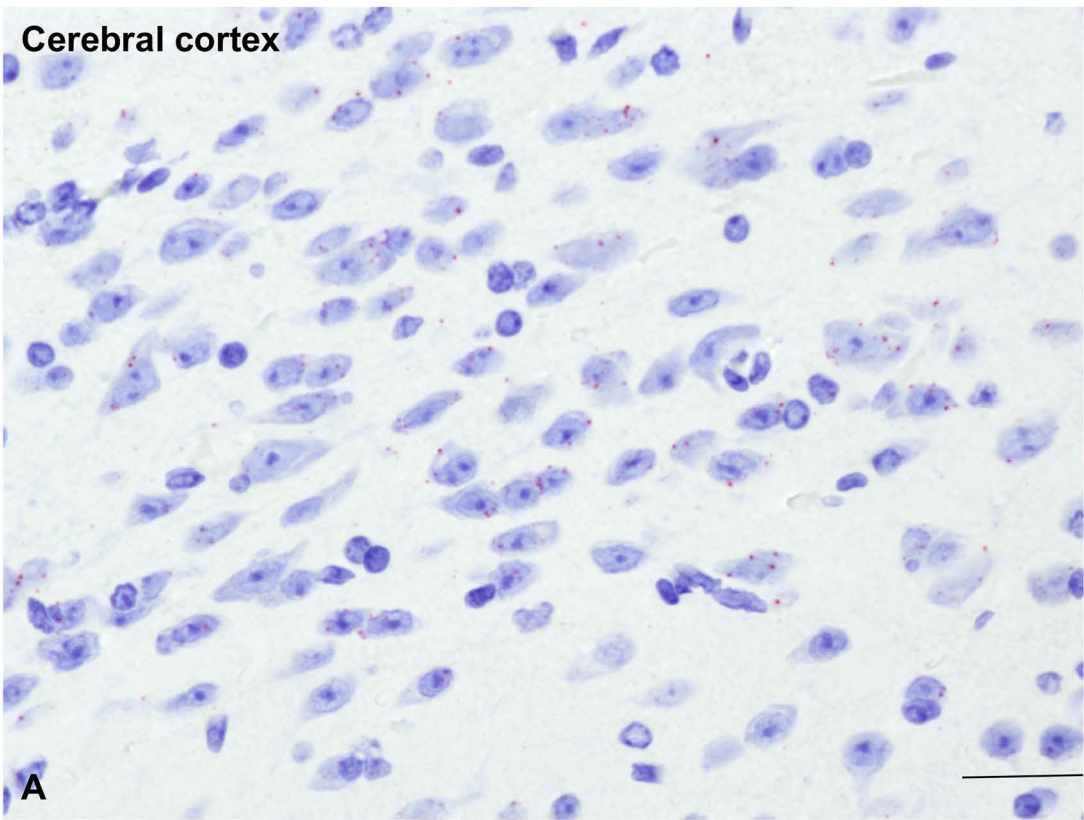


Figure 10

Cerebral cortex



Hippocampus

