



(51) International Patent Classification:

A61K 38/17 (2006.01) *A61P 31/14* (2006.01)
A61K 9/00 (2006.01) *C12N 15/01* (2006.01)
A61P 25/00 (2006.01) *A61K 31/70* (2006.01)
A61P 25/28 (2006.01)

SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

(21) International Application Number:

PCT/EP2024/057952

(22) International Filing Date:

25 March 2024 (25.03.2024)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

63/454,497 24 March 2023 (24.03.2023) US

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(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE,

(54) Title: COMPOSITIONS FOR PREVENTING AND/OR TREATING NEUROCOVID

(57) Abstract: The current invention relates to a pharmaceutical composition comprising a therapeutically active amount of a compound chosen from a mutant Wnt7 polypeptide or a fragment of a Wnt7 polypeptide, wherein said Wnt7 mutant polypeptide or fragment thereof and their use for the prevention, reduction in progression and/or treatment of neurologic problems associated with coronavirus infection in a subject.

WO 2024/200352 A1

COMPOSITIONS FOR PREVENTING AND/OR TREATING NEUROCOVID**FIELD OF THE INVENTION**

5 The present invention is directed to compositions and methods for treating or preventing cognitive and neuropsychiatric presentations of COVID-19 and Long COVID in subjects.

BACKGROUND

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Coronavirus disease-19 (COVID-19) is a worldwide health crisis. Now it is known that cognitive impairment ("brain fog"), executive function deficit, sensory/motor disorders, anxiety disorders, and fatigue are common cognitive and neuropsychiatric presentations of COVID-19 and Long COVID ("neuroCOVID"). The causes of
15 NeuroCOVID are thought to include cerebrovascular inflammation. Indeed, COVID-19 patients exhibit cerebrovascular abnormalities including vascular regression, basement membrane disruption, endothelial cell death, hypoxia/ischemia, BBB permeability, and leukocytic infiltration, accompanied by gliosis and loss of neurons and synapses.

20

Age is an influential factor in presentation of neurological components of Long COVID. Pediatric and young adult patients are more likely to recover from COVID-19 without ongoing symptoms, whereas individuals in middle-age and advanced age are highly susceptible to NeuroCOVID. Even mild SARS-CoV-2 infection can
25 precipitate ongoing neuroinflammation and neurodegeneration in susceptible age populations. For example, markers of endothelial inflammation and neuronal injury remain elevated in patient serum two months after mild COVID-19 in patients 36-65 years of age but are negligible in patients 18-35 years of age. After SARS-CoV-2 infection neuropsychiatric disorders are elevated including anxiety and post-
30 traumatic stress disorder in middle-aged individuals and a dramatic increase in dementia in the advanced ages.

WO2019/180204 and WO2023/094581 discuss the use of Wnt7 mutants in treating neurological disorders. Molecule K190A, a Wnt7 mutant where the lysine at position
35 159 is substituted for an alanine, was shown to be of particular interest in terms of activity. No link with NeuroCovid was however made at the time.

It is the aim of the current invention to provide compositions and methods for treating subjects having NeuroCOVID.

SUMMARY OF THE INVENTION

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The present invention and embodiments thereof serve to provide a solution to one or more of the above-mentioned problems. To this end, the present invention provides for a pharmaceutical composition for use according to claim 1. In particular, claim 1 discusses using Wnt7a mutant K190A for treating or preventing

10

NeuroCOVID.

Preferred embodiments of the composition are shown in any of claims 2 to 13.

15

In a second aspect, the present invention relates to a method of treatment or prophylaxis of NeuroCOVID according to claim 14.

In a third aspect, the present invention relates to a method of treatment or prophylaxis of indications caused by NeuroCOVID according to claim 15.

20

DESCRIPTION OF FIGURES

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The following description of the figures of specific embodiments of the invention is merely exemplary and is not intended to limit the present teachings, their application, or uses. Throughout the drawings, corresponding reference numerals indicate like or corresponding parts and features.

Figure 1 (A-E) presents a set of dot plots showing neurobehavioral and neuropsychiatric deficits in middle-aged mice with SARS-CoV-2 respiratory infection.

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Figure 2 (A-L) presents a set of microscopy images and bar graphs showing microglial/monocyte Iba1 immunoreactivity is increased in brain of older SARS-CoV-2 MA10 infected mice.

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Figure 3 (A-F) presents a set of immunofluorescent images and bar graphs showing advanced age exacerbates BBB permeability to T cells and blood macromolecules after respiratory SARS-CoV-2 infection.

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Figure 4 (A-H) presents the results of suppression of Wnt/ β -catenin signaling in brainstem endothelial cells and cerebrovascular reductions in middle-aged SARS-CoV-2 infected mice.

5 **Figure 5 (A-H)** presents a set of dot plots, flowcytometry histograms and microscopy images showing infection with SARS-CoV-2 increases brain endothelial cell β -catenin signaling in young adults but not middle-aged adults.

10 **Figure 6 (A-I)** presents the visualization of the data showing decreased cerebrovascular Wnt ligands, receptors, and transcriptional targets in aging. A-C) Real-time quantitative PCR for Wnt7a.

Figure 7 presents the visualization of the data showing activating Wnt/ β -catenin signaling prevents neurobehavioral signs of COVID.

15 **Figure 8 (A-J)** presents sets of immunohistochemistry and Immunofluorescent microscopy images showing association between sites of endothelial Wnt/ β -catenin activity, microglial nodules, and T cell perivascular infiltrates in COVID-19 patient brains.

20 **Figure 9 (A-D)** presents the visualization of the data showing the amounts of viral RNA present in brains of young and old C57Bl/6 mice after intranasal inoculation with SARS-CoV-2.

25 **Figure 10 (A-F)** presents a set of microscopy images and bar plots showing that age exacerbates astrocyte immunoreactivity for GFAP in mice inoculated with SARS-CoV-2.

30 **Figure 11 (A-H)** presents visualization of data showing Some BBB proteins are altered in older mice with SARS-CoV-2 infection.

Figure 12 (A-B) presents a set of microscopy images and an associated dot plot showing decreased laminin in brainstem of SARS-CoV-2 infected mice.

35 **Figure 13 (A-I and K-Q)** present visualization of the increased expression of Wnt/ β -catenin signaling in mice with brain endothelial-cell targeted SARS-CoV-2 infection.

Figure 14 (A-B) presents a set of microscopy images and an associated dot plot showing the Wnt mutant according to disclosure prevents fibrinogen extravasation in the brainstem of aged mice with mild respiratory SARS-CoV-2 infection.

5 DESCRIPTION

Both acute and chronic neurological problems in COVID-19 patients (hereinafter referred to as NeuroCOVID) have been documented. In a first aspect, the invention relates to a pharmaceutical composition for the prevention, reduction or treatment
10 of NeuroCOVID.

In an embodiment, a pharmaceutical composition is described comprising a therapeutically active amount of a Wnt7a mutant polypeptide or a fragment thereof wherein the lysine (K) residue at the position corresponding to position 159 in human
15 Wnt7a is substituted by an alanine (A) residue (K190A), or a nucleic acid encoding for said Wnt7 mutant polypeptide or fragment thereof for use in the reduction in progression and/or treatment of neurologic problems occurring after a coronavirus infection in a subject.

In another or further embodiment, a pharmaceutical composition is described comprising a therapeutically active amount of a Wnt7 mutant polypeptide or a fragment thereof wherein the lysine (K) residue at the position corresponding to position 159 in SEQ ID NO: 1 or SEQ ID NO: 2 is substituted by an alanine (A) residue, or a nucleic acid encoding for said Wnt7 mutant polypeptide or fragment
25 thereof for use in the reduction in progression and/or treatment of neurologic problems occurring after a coronavirus infection in a subject.

SEQ ID NO:1 and SEQ ID NO:2 correspond to the mature amino acid sequences of human or mouse Wnt7a and Wnt7b protein sequences respectively.

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The amino acid sequence corresponding to SEQ ID NO:1 (mature human Wnt7a) is:
LGASIIICNKIPGLAPRQRAICQSRPDAIIVIGEGSQMGLDECQFQFRNGRWNCALGERTVFG
KELKVGSR EAAFTYAIIAAGVAHAITA ACTQGNLSDCGCDKEKQGQYHRDEGWKWWGGCSA
DIRYGIGFAKVFDAREIKQNARTLMNLHNNEAGRKILEENMKLECKCHGVSGSCTTKTCWT
35 TLPQFRELGYVLKDKYNEAVHVEPVRASRNKRPTFLKIKKPLSYRKPM DTLVYIEKSPNYCEE
DPVTGSGVTQGRACNKTAPQASGCDLMCCGRGYNTHQYARVWQCNCCKFHWCCYVKCNTC
SERTEMYTCK

5

The amino acid sequence corresponding to SEQ ID NO:2 (mature human Wnt7b) is:
 LGANIICNKIPGLAPRQRAICQSRPDAIIVIGEGAQMGINECQYQFRFGRWNC SALGEKTVFG
 QELRVGSREAAFTYAITAAGVAHAVTAACSQGNLSNCGCDREKQGYYNQAEGWKWGGCSA
 DVRYGIDFSRRFVDAREIKKNARRLMNLHNNEAGRKVLEDRMQLECKCHGVSGSCTTKTCW
 5 TTLPKFREVGHLLKEKYNAAVQVEVVRASRLRQPTFLRIKQLRSYQKPMETDLVYIEKSPNYCE
 EDAATGSVGTQGRLCNRTSPGADGCDTMCCGRGYNTHQYTKVWQCNCKFHWCCFVKCNT
 CSERTEVFTCK

In the period of acute NeuroCOVID, patients may suffer from stroke, ischemic brain
 10 damage, changes in the mental state, inflammation of the brain, or brain damage
 due to toxins. More commonly known complaints are headache, abnormalities of
 smell and taste, neuropsychiatric illness, and dizziness. When these neurological
 problems last beyond the acute period, for 12 or more weeks from the onset of
 symptoms, the patient is said to have post-acute COVID-19 syndrome (PASC), also
 15 called Long Covid. Long NeuroCOVID may present as depression, insomnia, cognitive
 impairment, headache, and dizziness.

It has been also reported by NIH that coronavirus increases the risk of stroke,
 dementia, muscle and nerve damage, encephalitis, and vascular disorders as well as
 20 the other diseases and conditions occur within days to a few weeks after coronavirus
 infection which comprises: Multi-system inflammatory syndrome– which causes
 inflammation in the body's blood vessels, Transverse myelitis – an inflammation of
 the spinal cord, Guillain-Barré Syndrome (sometimes known as acute
 polyradiculoneuritis) – a rare neurological disorder which can range from brief
 25 weakness to nearly devastating paralysis, leaving the person unable to breathe
 independently, Dysautonomia – dysfunction of the autonomic nerve system, which
 is involved with functions such a breathing, heart rate, and temperature control,
 Acute disseminating encephalomyelitis (ADEM) – an attack on the protective myelin
 covering of nerve fibers in the brain and spinal cord, Acute necrotizing hemorrhagic
 30 encephalopathy – a rare type of brain disease that causes lesions in certain parts of
 the brain and bleeding (hemorrhage) that can cause tissue death (necrosis), Facial
 nerve palsies (lack of function of a facial nerve) such as Bell's Palsy, Parkinson's
 disease-like symptoms have been reported in a few individuals who had no family
 history or early signs of the disease.

35

Furthermore, the neurologic problems associated with coronavirus infection can lead
 to neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and
 amyotrophic lateral sclerosis.

In embodiments, the compound according to the disclosure has neurocognitive and psychomotor protection in mice infected with coronavirus.

- 5 It should be understood that all indications, neural problems listed above, and other neurological problems associated with a coronavirus infection fall within the definition of the term "neurologic problems" according to this disclosure.

10 In the context of the current description, "Coronaviruses" refer to a large family of viruses that usually cause mild to moderate upper-respiratory tract illnesses in humans. In embodiments, coronaviruses comprises SARS-CoV, MERS-CoV, SARS-CoV-2 virus, HCoV-NL63, HCoV-229E, HCoV-OC43, or HKU1. SARS-CoV-2, which emerged in 2019 and causes coronavirus disease 2019 (COVID-19).

- 15 In embodiments the compound is able to influence the permeability of the blood-brain barrier (BBB) in subjects.

In an embodiment, the compound disclosed is able to influence the permeability of the blood-brain barrier (BBB) in subjects infected with coronavirus, in particular
20 reduce the transcellular permeability, thereby increasing the BBB impermeability. The BBB selectively restricts permeability of CNS blood vessels to macromolecules and immune cells from the blood. BBB damage occurs from loss of endothelial tight junctions, which normally suppress inter-cellular diffusion, and from increased rates of vesicular traffic across the endothelial cytoplasm. The Wnt/ β -catenin signaling
25 pathway in cerebrovascular endothelium is required for early life induction of BBB properties.

In an embodiment, the compound disclosed is able to support the impermeability of the blood-brain barrier (BBB) in subjects. This is particularly relevant for subjects
30 exhibiting disrupted BBB properties due to coronavirus infection, leading to an -at least partly- dysfunctional BBB.

In an embodiment the compound increases the BBB impermeability and BBB integrity in virally-induced BBB permeability.

35

In an embodiment the compound increases the BBB impermeability and BBB integrity in age worsened BBB permeability.

In embodiments, the compound suppresses BBB transcytosis protein Caveolin-1. By suppressing Caveolin-1 the BBB impermeability is increased, thereby restoring, or maintaining the BBB functionality and integrity. Caveolin-1 is a signaling and scaffolding molecule that can promote transcellular BBB permeability. Caveolin-1 increases in cerebrovascular aging, where it contributes to proinflammatory age-related BBB permeability. In embodiments the compound is able to suppress Caveolin-1 thus restoring the BBB functionality and preventing neuroinflammation. In embodiments the compound is able to repair Caveolin-1-dependent transcellular BBB permeability.

10

In embodiment, the compound is able to prevent or reduce blood-brain barrier leakage in subjects. In particular embodiments, the compound is able to prevent or reduce blood-brain barrier leakage caused by SARS-CoV-2 infection.

15 In an embodiment, the compound is able to repair endothelial tight-junctional defects thus increasing the BBB impermeability, thereby restoring the BBB functionality.

In an embodiment, the compound is able to reduce vesicular transport thus increasing the BBB impermeability, thereby restoring the BBB functionality.

20

In an embodiment, the compound is able to restore the paracellular permeability and/or the transcellular permeability of the BBB, thereby increasing the BBB impermeability.

25

In embodiments the compound is a nucleic acid, preferably RNA or DNA.

In particular embodiments, the mutant Wnt7a protein, the nucleic acid encoding the mutant Wnt7a protein, or the nucleic acid expression cassette comprising the nucleic acid as taught herein is used in gene therapy.

30

Accordingly, also provided herein is a method for gene therapy, in particular central and/or peripheral nervous system-directed gene therapy, in a subject in need of said gene therapy comprising: introducing in the subject, in particular in the central and/or peripheral nervous system of the subject, a nucleic acid expression cassette or a vector as described herein; and expressing a therapeutically effective amount of the mutant Wnt7a protein encoded by the nucleic acid as taught herein in the subject, in particular the central and/or peripheral nervous system of the subject.

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In particular embodiments, the mutant Wnt7a protein or the nucleic acid encoding the mutant Wnt7a protein as taught herein is used in mRNA therapy.

- 5 Accordingly, also provided herein is a method for RNA therapy, preferably mRNA therapy, in particular central and/or peripheral nervous system-directed mRNA therapy, in a subject in need of said mRNA therapy comprising: introducing in the subject, in particular in the central and/or peripheral nervous system of the subject, a nucleic acid encoding the mutant Wnt7a protein as taught therein; and expressing
10 a therapeutically effective amount of the mutant Wnt7a protein encoded by the nucleic acid as taught herein in the subject, in particular the central and/or peripheral nervous system of the subject.

- An advantage of the use of RNAs therapy, it is generally believed that RNAs do not
15 integrate into the genome and therefore do not have the risk of insertional mutagenesis.

- Any other well-known methods of introducing nucleic acids into animal cells may be used herein. At the simplest, the nucleic acid can be directly injected into the target
20 cell / target tissue. Other methods include fusion of the recipient cell with bacterial protoplasts containing the nucleic acid, the use of compositions like calcium chloride, rubidium chloride, lithium chloride, calcium phosphate, DEAE dextran, cationic lipids or liposomes or methods like receptor-mediated endocytosis, biolistic particle bombardment ("gene gun" method), infection with viral vectors (i.e. derived from
25 lentivirus, adeno-associated virus (AAV), adenovirus, retrovirus or antiviruses), electroporation, and the like. Other techniques or methods which are suitable for delivering nucleic acid (NA) molecules to target cells include the continuous delivery of an NA molecule from poly (lactic-Co-Glycolic Acid) polymeric microspheres or the direct injection of protected (stabilized) NA molecule(s) into micropumps delivering
30 the product. Another possibility is the use of implantable drug-releasing biodegradable microspheres. Also envisaged is encapsulation of NA or providing NA in various types of liposomes (immunoliposomes, PEGylated (immuno) liposomes), cationic lipids and polymers, nanoparticles or dendrimers, poly (lactic-Co-Glycolic Acid) polymeric microspheres, implantable drug-releasing biodegradable
35 microspheres, etc.; and co-injection of NA with protective agent like the nuclease inhibitor aurointricarboxylic acid. It shall be clear that also a combination of different above-mentioned delivery modes or methods may be used.

In particular embodiments, the compound is provided in carriers, such as liposomes, lipid nanoparticles, nanostructured lipid carriers, nanoemulsions, polymer nanoparticles, polymer micelle or dendrimers. In a preferred embodiment, said carriers are liposomes or lipid nanoparticles (LNPs).

5

In an embodiment, said carriers are lipid-based carriers. Said lipid-based carriers comprise one or more lipids. The one or more lipids can be in solid and/or liquid form. Said lipid-based carriers may be LNPs, lipoplexes, liposomes, phospholipid micelles, solid lipid nanoparticles, nanostructured lipid carriers or nano-emulsions.

10 Lipid-based carriers useful according to the invention include, for example, cationic lipids, liposomes, in particular cationic liposomes, and micelles, and nanoparticles. Cationic lipids may form complexes with negatively charged nucleic acids. Any cationic lipid may be used according to the invention. Liposomes are phospholipid and cholesterol self-assembled bilayer membranes that enclose an aqueous core,
15 where hydrophilic molecules can be incorporated. Hydrophobic compounds can also be incorporated in the lipid bilayer. Liposomes can be classified in (i) small unilamellar vesicles (SUVs); (ii) large unilamellar vesicles (LUVs) and (iii) multilamellar vesicles (MLVs), according to their size and lamellarity. Solid lipid nanoparticles (SLNs) have a spherical shape with an average diameter of 10–1000
20 nm. They are used as a colloidal NP drug delivery system in which lipid drug carrier solidifies at room temperature as well as at body temperature. Different solid lipids can be exploited to produce SLNs, such as, tripalmitin, cetyl alcohol, cetyl palmitate, glyceryl monostearate, trimyristin, tristearin, stearic acid, etc. SLNs comprise of solid lipid, such as triglycerides, fatty acids, waxes, partial glycerides, and polyethylene glycosylated lipid; emulsifiers, such as polysorbates, poloxamer and lecithin; and
25 water. Nanostructured lipid carriers (NLC), comprise a blend of solid and liquid lipids which results in a partially crystallized lipid system and many have advantages such as enhanced drug loading capacity, drug release modulation flexibility and improved stability.

30

In an embodiment, said lipid-based carrier is a lipid nanoparticle. Solid lipid nanoparticles (SLNs, sLNPs), or lipid nanoparticles (LNPs), are nanoparticles composed of lipids that are suited to be used as a drug delivery vehicle for drug compounds, especially polynucleotides such as RNA or DNA.

35

In embodiments the compound is formulated in a viral vector.

In embodiments the pharmaceutical composition disclosed is combined with a second therapy.

5 In embodiments the pharmaceutical composition is administered parenterally, preferably intravenously or intrathecally.

The dosage or amount of the compound as taught herein, optionally in combination with one or more other active compounds to be administered, depends on the individual case and is, as is customary, to be adapted to the individual circumstances
10 to achieve an optimum effect. Thus, the unit dose and regimen depend on the nature and the severity of the disorder to be treated, and also on factors such as the species of the subject, the sex, age, body weight, general health, diet, mode and time of administration, immune status, and individual responsiveness of the human or animal to be treated, efficacy, metabolic stability and duration of action of the
15 compounds used, on whether the therapy is acute or chronic or prophylactic, or on whether other active compounds are administered in addition to the compound described in any of the embodiments above. In order to optimize therapeutic efficacy, the compound as taught herein can be first administered at different dosing regimens. Typically, levels of the compound in a tissue can be monitored using
20 appropriate screening assays as part of a clinical testing procedure, e.g., to determine the efficacy of a given treatment regimen. The frequency of dosing is within the skills and clinical judgement of medical practitioners (e.g., doctors, veterinarians or nurses). Typically, the administration regime is established by clinical trials which may establish optimal administration parameters. However, the
25 practitioner may vary such administration regimes according to the one or more of the aforementioned factors, e.g., subject's age, health, weight, sex and medical status. The frequency of dosing can be varied depending on whether the treatment is prophylactic or therapeutic.

30 In embodiments the subject receiving the composition is human or an animal.

In a second aspect the disclosure relates to a method of treatment or prophylaxis of neurologic problems occurring after a coronavirus infection, comprising administration of a therapeutically active amount of a Wnt7 mutant polypeptide,
35 preferably human Wnt7, or a fragment thereof wherein the lysine (K) residue at the position corresponding to position 159 in SEQ ID NO: 1 or SEQ ID NO: 2 is substituted by an alanine (A) residue, or a nucleic acid encoding for said Wnt7 mutant polypeptide or fragment thereof a patient in need thereof.

Further embodiments are as described above.

5 In another aspect the disclosure relates to a method of treatment or prophylaxis of
neurologic indications caused by an infection with coronavirus comprising
administration of a therapeutically active amount of a Wnt7 mutant polypeptide or a
fragment thereof wherein the lysine (K) residue at the position corresponding to
position 159 in human Wnt7, preferably Wnt7a, is substituted by an alanine (A)
10 residue, or a nucleic acid encoding for said Wnt7 mutant polypeptide or fragment
thereof to a patient in need thereof.

Further embodiments are as described above.

15 **EXAMPLES AND DESCRIPTION OF FIGURES**

With as a goal illustrating better the properties of the invention the following
presents, as an example and limiting in no way other potential applications, a
description of a number of preferred applications of the method for examining the
state of the grout used in a mechanical connection based on the invention, wherein:

20 **Figure 1** shows neurobehavioral and neuropsychiatric deficits in middle-aged mice
with SARS-COV-2 respiratory infection. A) Novel object recognition memory task
with 24h intertest interval. Young healthy mice preferentially attend to novel object
(Discrimination Index >50% indicated by dotted horizontal line). Young mice
25 infected with SARS-CoV-2, middle-aged healthy mice, and middle-aged mice
infected with SARS-CoV-2 do not prefer the novel object (n=5-8 per group) B) No
significant differences in total exploration time between groups (n=5-8 per group).
C) Decreased velocity in the open field in the 12-month-old SARS-COV-2 mice (n=8-
9 per group). D) 12-month-old infected mice have significantly increased latency in
30 the pole descent task, a complex motor coordination task involving
brainstem/thalamic connectivity (n=5 per group). E) Increased duration of each bout
of spontaneous grooming in the open field in the 12-month old SARS-CoV-2 infected
mice (n=8-9 per group). I) Fewer spontaneous grooming bouts initiated in the open
field in the 12-month-old SARS-CoV-2 infected mice (n=8-9 per group). *p<0.05,
35 **p<0.01, ***p<0.001, ****p<0.0001, one-way ANOVA and Sidak's multiple
comparisons test.

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Figure 2 presents a set of microscopy images and bar graphs showing microglial/monocyte Iba1 immunoreactivity is increased in brains of older SARS-CoV-2 MA10 infected mice. A-F) Representative confocal micrographs of microglia (Iba1+, green, DAPI, blue) in the brainstem reticular formation white matter (A-B), olfactory bulb glomerular layer (C-D), and hippocampal CA1 (E-F). Note microglial nodules with multiple nuclei in the brainstem of aged infected mice (arrowhead). B) Microglial (Iba1+) area quantification in the reticular formation of the brainstem of 2-month-old and 12-month-old mice 4 days after nasal inoculation with vehicle (saline) or SARS-CoV-2 MA-10. D) Microglial (Iba1+) area quantification in the olfactory bulb glomerular layer of 2-month-old and 12-month-old mice 4 days after nasal inoculation with vehicle (saline) or SARS-CoV-2 MA-10. F) Microglial (Iba1+) area quantification in the CA1 region of the hippocampus of 2-month-old and 12-month-old mice 4 days after nasal inoculation with vehicle (saline) or SARS-CoV-2 MA-10. G) Microglia (Iba1+, green) near to a blood vessel in the gray matter have normal appearance in vehicle treated mice. H) A blood vessel in the gray matter of an aged SARS-COV-2 infected mouse at 4DPI is invested with intense Iba1+ cells. I-J) Iba1 immunoreactivity in healthy aged brainstem. K-L) Ameboid morphology of Iba1+ cells in brainstem of 12-month-old infected mice. 4th Vent indicates fourth ventricle. One-way ANOVA with Sidak's multiple comparison test, *p<0.05, **p<0.01, ***p<0.001.

Figure 3 presents a set of immunofluorescent images and bar graphs showing advanced age exacerbates BBB permeability to T cells and blood macromolecules after respiratory SARS-CoV-2 infection. 2-month old and 12-month old mice were euthanized 4 days after respiratory infection with SARS-CoV-2. A) Immunofluorescent imaging of T cells (CD3, green) near blood vessels (Glut1, red) in the brainstem reticular formation of sagittal brain sections. B) Quantification of CD3+ T cells in the indicated neuroanatomic regions of 3-5 mice per group. Two-way ANOVA with Tukey's multiple comparison test. C) Immunofluorescent imaging of the blood protein fibrinogen (green) in brainstem reticular formation. D) Quantitation of fibrinogen mean fluorescent intensity (MFI) in brainstem reticular formation of 6-7 mice per group. One-way ANOVA and Sidak's multiple comparisons test. E) Western blot detection of fibrinogen in brain. F) Quantitation of fibrinogen β -chain bands (~58kDa) versus actin.

35

Figure 4 presents the results of suppression of Wnt/ β -catenin signaling in brainstem endothelial cells and cerebrovascular reductions in middle-aged SARS-CoV-2 infected mice A) Isolation of endothelial cells from the brainstem by microvessel

isolation and CD31 magnetic bead enrichment. B) Heat map of all brainstem endothelial cell DEG in 12-month-old vehicle treated mice and 12-month old mice at 4 days post inoculation with SARS-CoV-2. C) Volcano plot of brainstem endothelial cell differentially expressed genes between 12-month-old SARS-CoV-2 infected mice and 12-month-old vehicle treated mice. Annotated genes include representative mediators of viral response, cell adhesion, extracellular matrix, apoptosis, and the Wnt signaling pathway. (D) Gene ontology overrepresentation analysis of the differentially expressed genes between 12-month-old SARS-CoV-2 infected mice and 12-month-old vehicle treated mice, with non-redundant modules shown. Highly upregulated pathways include viral response. Highly downregulated pathways relate to regulation of extracellular matrix, immune regulation, and Wnt/ β -catenin signaling. E) Immunostaining for cerebrovascular membrane Collagen IV (red) and Glut1 (green) in brainstem tissue sections from 2-month-old and 12-month-old mice treated with vehicle or inoculated with SARS-CoV-2. White arrows indicate vascular basement membrane cuffs lacking Glut1+ endothelial cells. F) Scatter plot of Collagen IV positive vessel diameters in brainstem. Lines indicate mean and standard deviation. Kruskal-Wallis and Dunn's multiple comparison test. G) Scatter plot indicates percent of vessels with diameter less than 3 micron. One-way ANOVA and Sidak's multiple comparison test. H) Scatter plot depicting the number of string vessels (Collagen IV+ sleeves lacking Glut1+ endothelial cells) divided by total vessel density. One-way ANOVA and Sidak's multiple comparisons test.

Figure 5 presents a set of dot plots, flowcytometry histograms and microscopy images showing infection with SARS-CoV-2 increases brain endothelial cell β -catenin signaling in young adults but not middle-aged adults. A) Brainstem endothelial cells acutely isolated from 2-month-old adults inoculated with SARS-CoV-2 MA10 have increased intracellular content of the β -catenin transcriptional target Lef1. B) Brainstem endothelial cells acutely isolated from 12-month-old mice inoculated with SARS-CoV-2 MA10 do not significantly change Lef1 content. C) Quantitation of brainstem endothelial cell Lef1 content in young adult and aged mice inoculated with vehicle or SARS-CoV-2 MA10. D) TCF/Lef1:GFP transcriptional reporter mice were subjected to SARS-CoV-2 infection directed to brain endothelial cells using adeno-associated vector AAVBR1:human ACE2 followed by SARS-CoV-2 administration with intravenous (IV) or intranasal (IN) delivery. TCF/Lef:GFP expression was measured by immunostaining with Glut1 (red) as a marker of endothelial cells. Arrowheads indicate brain endothelial cells that are positive for TCF/Lef:GFP. E) Quantitation of brain endothelial cells that are positive for TCF/Lef1:GFP in brainstem, cerebellum, hippocampus, and olfactory bulb of mice treated with vehicle and mice treated with

IV or with IN SARS-CoV-2 inoculation. F) Flow cytometry histograms for intracellular IRF7 in brainstem endothelial cells of mice from the indicated treatment groups (healthy, AAV vector only ("vehicle"), AAV vector plus SARS-CoV-2 IN). Negative control indicates omission of the indicated fluorescent antibody. G) Quantitation of flow cytometry data demonstrating that brainstem endothelial cell intracellular IRF7, an indication of innate immune interferon activation, is induced in response to SARS-CoV-2 infection but not in response to AAV vector alone. H) Quantification of flow cytometry data demonstrating that brainstem endothelial cell expression of MHC-I is increased in response to SARS-CoV-2 infection but not in response to AAV vector alone.

Figure 6 presents the visualization of the data showing decreased cerebrovascular Wnt ligands, receptors, and transcriptional targets in aging. A-C) Real-time quantitative PCR for Wnt7a (A), Wnt3 (B), and the Wnt/ β -catenin transcriptional target Apccdd1 (C) in brain homogenate of SARS-CoV-2 infected mice, expressed as $\Delta\Delta Ct$ values normalized to GAPDH. Canonical Wnts and Apccdd1 are decreased during aging. N=6-7 per group. * $p < 0.05$, ** $p < 0.01$, one-way ANOVA and Sidak's multiple comparisons test. D) Acute isolation strategy for brainstem endothelial cells by microvessel isolation and CD31 magnetic bead enrichment. (E) Clustered heatmap of differentially expressed genes (FDR < 0.05) in 12-month-old versus 2-month-old healthy BECs (Z-score) and (F) gene ontology overrepresentation analysis of the differentially expressed genes. (G) Volcano plot of differentially expressed genes with the most differential genes labeled along with representative mediators in blood-brain barrier function, cell adhesion, extracellular matrix organization, and angiogenesis. (H) Clustered heatmap of Wnt pathway genes differentially expressed between healthy old and young BECs (Z-score). (I) The expression of Wnt pathway genes in single BECs during healthy aging in the Tabula Muris Senis atlas. 18-month-old and 24-month-old mRNA levels are shown relative to 3-month-old mouse BECs.

Figure 7 presents the visualization of the data showing activating Wnt/ β -catenin signaling prevents neurobehavioral signs of COVID. A) Pharmacologic activation of Wnt/ β -catenin signaling before and during SARS-CoV-2 infection with GSK3 β inhibitor 6BIO prevents cognitive impairment in 2-month-old mice in the novel object recognition assay with 24-hour intertest interval. B) Cerebrovascular-targeted engineered Wnt7a prevents cognitive impairment in 12-month-old mice in the novel object recognition assay with 12-hour intertest interval. C) Cerebrovascular-targeted engineered Wnt7a improves motor coordination in the pole descent task in 12-month-old mice. D) Cerebrovascular-targeted engineered Wnt7a decreases falls in

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12-month-old mice. One-way ANOVA with Sidak's multiple comparison test (A), unpaired t-test (B-C), or Chi-square with Fisher's exact test (D). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5 **Figure 8** presents sets of immunohistochemistry and Immunofluorescent microscopy images showing association between sites of endothelial Wnt/ β -catenin activity, microglial nodules, and T cell perivascular infiltrates in COVID-19 patient brains. A-C) Regions of perivascular microglial activation in the pons are identified by immunohistochemistry for Iba1 (A-B) and CD68 (C). D-E) Immunofluorescent
10 staining for the Wnt/ β -catenin transcriptional coactivator Lef1 (green) and the brain endothelial cell marker Glut1 (red) in regions of perivascular microglial activation in the pons. F-G) Foci of microglial activation and T cell infiltration are indicated by immunostaining for Iba1 (F) and CD3(G). H-J) In immunofluorescent images, regions of leukocytic infiltration have Lef1+ vascular cells.

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Figure 9 presents the visualization of the data showing the amounts of viral RNA present in brains of young and old C57Bl/6 mice after intranasal inoculation with SARS-CoV-2. A) Log scale scatterplot with median SARS-CoV-2 viral RNA detected by RT-PCR from the indicated tissues at 4 days post inoculation (DPI). No significant
20 difference in SARS-CoV-2 viral genomes/tissue in young adult versus middle-aged adult. N=14 2-month-old SARS-COV-2, n=11 12-month-old SARS-COV-2, n=9 2-month-old vehicle, n=8 12-month-old vehicle. ANOVA and non-parametric Kruskal-Wallis multiple comparisons test. Dotted line indicates limit of detection. B) Body weight percent change in 2-month-old adult vehicle treated, 2-month-old SARS-
25 COV-2 inoculated, 12-month-old adult vehicle treated, and 12-month-old adult SARS-COV-2 inoculated mice. $P < 0.001$, $p < 0.0001$, repeated-measures ANOVA with Sidak's multiple comparison test. C) Heat map depicting log2 fold change in expression of the indicated chemokines and cytokines in the blood of 2-month-old and 12-month-old mice 4 days after inoculation with vehicle or with SARS-CoV-2 by
30 multiplex; n=3-4 per group. D) Scatter plots depicting absolute values of cytokines and chemokines compared by two-way ANOVA and Dunnett's multiple comparison test, n=3-4 per group.

Figure 10 presents a set of microscopy images and bar plots showing that age exacerbates astrocyte immunoreactivity for GFAP in mice inoculated with SARS-CoV-2. Percent area astrocyte immunoreactivity for GFAP (green) was identified by immunostaining in midline sagittal brain sections of 2-month-old and 12-month-old mice 4 days after inoculation with vehicle or SARS-CoV-2. A, D) Brainstem reticular
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formation white matter. B, E) Olfactory bulb. Quantification reflects olfactory glomerular layer only. C, F) Hippocampus. Quantification reflects CA1 stratum radiatum. Graphs shows averages of at least three sections per mouse, n=3-4 mice per group. **p<0.01, ***, p<0.01, one-way ANOVA and Sidak's multiple comparisons test.

Figure 11 presents visualization of data showing Some BBB proteins are altered in older mice with SARS-CoV-2 infection. Immunostaining for Caveolin-1 (green) and DAPI nuclear stain (blue) in brainstem reticular formation of 2-month-old healthy, 2-month-old SARS-CoV-2, 12-month-old healthy, and 12-month-old SARS-CoV-2 infected mice at 4DPI. Caveolin-1 mean fluorescence intensity in brainstem is significantly increased by age and infection. One-way ANOVA and Sidak's multiple comparisons test, n=3 per group. Western blot for transcellular and paracellular BBB proteins in brainstem homogenate from young and middle-aged mice inoculated with SARS-CoV-2 or vehicle. No significant differences in Caveolin-1 protein by Western blot. SARS-CoV-2 inoculation decreases brainstem ZO-1 protein quantity in all ages by Western blot, p<0.05. No significant differences in tight junction proteins Occludin and Claudin-5 by Western blot. Western blot data reflects n=5 mice per group, assessed by one-way ANOVA and Sidak's multiple comparisons test.

Figure 12 presents a set of microscopy images and dot plot showing decreased laminin in brainstem of SARS-CoV-2 infected mice. Brainstem tissue sections from 2-month-old and 12-month old mice treated with vehicle or inoculated with SARS-CoV-2 (MA10) 4 days prior to euthanasia were subjected to immunostaining for the cerebrovascular basement membrane protein laminin (green). Nuclei were counterstained with DAPI (blue). Note increased density of nuclei in the area around inflamed vessels in the SARS-CoV-2 infected mice. Scatterplot depicts the area of brainstem reticular formation immunopositive for laminin staining. One-way ANOVA, Sidak's multiple comparisons test.

Figure 13 present visualization of the increased expression of Wnt/ β -catenin signaling in mice with brain endothelial-cell targeted SARS-CoV-2 infection. A-D) AAVBR1:mCherry 2×10^{11} viral genomes/mouse transduces brain endothelial cells in cortex (A), olfactory bulb (B), and hippocampus (C). Choroid plexus ependymal cells (D) are not transduced. E-F) Transduction of brain endothelial cells with mCherry reporter 2 weeks after intravenous administration of brain endothelial-cell tropic AAVBR1:mCherry at 2×10^9 , 2×10^{10} (E), or 2×10^{11} (F) viral genomes. G) Quantification of brain endothelial cells (Glut-1, green) positive for mCherry 2 weeks

after indicated doses of AAVBR1:mCherry. H) Quantification of human ACE2 (hACE2) mRNA in brain, olfactory bulb, and lung of mice treated with AAVBR1:hACE2. Real time QPCR specific to hACE2 was used to differentiate between transduced human ACE2 and endogenous mouse ACE2. AAVBR1:hACE2 transduced endothelial cells in brain and olfactory bulb but not lung. I) Real time QPCR of interferon-inducible CXCL10 in brains of mice infected intravenously (IV) or intranasally (IN) with SARS-CoV-2 (WA1 strain) after transduction with AAVBR1:hACE2, but not in mice that received AAVBR1:hACE2 alone. No significant induction of Wnt7a or Wnt7b by real time QPCR in brains of mice transduced with AAVBR1:hACE2 and inoculated with SARS-CoV-2. K) Schematic cartoon of TCF/Lef:GFP fluorescent reporter construct for Wnt/ β -catenin transcriptional activity. L) Neuroanatomic regions of analysis. M-N) Immunofluorescent images of TCF/Lef:GFP (green) and immunostaining with Glut1 (red; endothelial cells) in hippocampus (M) and cerebellum (N). Single channel images and merged higher magnification images are shown. White arrowheads indicate TCF/Lef:GFP+ endothelial cells whereas empty arrowheads indicate TCF/Lef:GFP- endothelial cells. O) Dot plot depicting quantification of the percent of endothelial cells that are positive for TCF/Lef1:GFP fluorescence in cerebellum and hippocampus. One-way ANOVA and Sidak's multiple comparison test. P) Real time qPCR data displayed as delta-delta Ct values for Wnt7a and Wnt7b in brain homogenate from mice with brain endothelial cell-targeted SARS-CoV-2 infection or the indicated controls. Q) Flow cytometry for MHC-II expression on endothelial cells isolated from mice with brain endothelial cell-targeted SARS-CoV-2 infection or the indicated controls. Neg control indicates omission of the fluorescently conjugated MHC-II antibody.

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Figure 14 Wnt7aK190A prevents fibrinogen extravasation in the brainstem of aged mice with mild respiratory SARS-CoV-2 infection. A-B) Fibrinogen immunoreactivity is increased in the brainstem of 12-month-old mice 6 days after mild respiratory infection with SARS-CoV-2 strain MA10. Blood vessels are visualized with immunoreactivity for Glut1; nuclei are detected with DAPI. Mice that received AAV-PHP.eB-GFP prior to SARS-CoV-2 infection have abundant perivascular fibrinogen in the brainstem (A). Mice that received AAV-PHP.eB-Wnt7aK190A-GFP prior to SARS-CoV-2 infection have less fibrinogen in the brainstem. C) Graph depicting mean and standard error of the mean for % area fibrinogen immunoreactivity in the brainstem. Each dot represents one mouse. N=6 AAV-PHP.eB-Wnt7aK190A-GFP; N=3 AAV-PHP.eB-Wnt7aK190A-GFP Unpaired student t-test, p=0.06.

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The present invention will now be further exemplified with reference to the following examples. The present invention is in no way limited to the given examples or to the embodiments presented in the figures.

5 **Example 1:**

Since the pandemic, much knowledge has been gained as to the underlying changes leading to Long NeuroCOVID.

- 10 Age-related declines in cerebrovascular function and blood-brain barrier (BBB) integrity could increase susceptibility to NeuroCOVID. The BBB selectively restricts permeability of CNS blood vessels to macromolecules and immune cells from the blood. BBB damage occurs from loss of endothelial tight junctions, which normally suppress inter-cellular diffusion, and from increased rates of vesicular traffic across
- 15 the endothelial cytoplasm. The Wnt/ β -catenin signaling pathway in cerebrovascular endothelium is required for early life induction of BBB properties . We and others have shown that Wnt/ β -catenin promotes adult BBB maintenance and repair. The selective regulation of BBB vesicular trafficking becomes progressively impaired during aging, decreasing CNS bioavailability of growth factors and contributing to
- 20 neuroinflammation. The biological processes governing increased BBB leakage in advanced age are incompletely understood. We hypothesized that decreased cerebrovascular Wnt/ β -catenin in aging worsens NeuroCOVID by deregulating brain endothelial cells and the BBB.
- 25 To test age-dependent mechanisms of NeuroCOVID, we intranasally inoculated 2-month-old and 12-month-old C57Bl/6 mice with a strain of SARS-CoV-2 adapted for pathogenicity in mice. This leads to viral replication in the lung. We find that intranasal inoculation with SARS-CoV-2 causes dissemination of viral RNA into the brain, cerebrovascular inflammation, T cell infiltration, and BBB leakage.
- 30 Importantly, age worsened virally-induced BBB permeability, neuroinflammation, and psychomotor impairment. Advanced age and infection downregulated Wnt/ β -catenin pathway ligands, receptors, and cofactors known to promote BBB integrity. Experimental strategies to bolster cerebrovascular Wnt/ β -catenin signaling effectively prevented SARS-CoV-2-induced cognitive and motor coordination
- 35 impairments. Thus, age strongly influences severity of BBB disruption, neuroinflammation and neuropsychiatric presentation in SARS-CoV-2 infected mice by suppressing brain endothelial cell Wnt/ β -catenin signaling.

Materials and Methods

Mouse models:

Male C57Bl/6 mice purchased from Jackson laboratories at 8 weeks of age or at 12 months of age were housed on site in a specific pathogen free barrier suite for at least 7 days prior to initiation of experiments. Mice were transferred to the Animal BioSafety Level 3 facilities at least 2 days prior to inoculation. Mice were maintained on standard light-dark cycles with ad libitum food and water in micro-isolation cages. Cages holding 4-5 mice were randomized to either SARS-CoV-2 inoculation or vehicle (saline) inoculation groups. Mouse-adapted SARS-CoV-2 (MA10) was provided by Ralph Baric (University of North Carolina, Chapel Hill, North Carolina, USA). SARS-CoV-2 (MA10) was propagated and titered on Vero-E6 cells (ATCC, CRL1586). Mice were anesthetized with isoflurane and challenged via intranasal inoculation with 1×10^4 foci-forming units (FFU SARS-CoV-2 MA10). Lungs or brains were isolated from mice at the indicated time post infection. Each morning mice were assessed for body condition score including body weight, coat condition, posture, and qualitative inspection of respiratory rate and behavior. At onset of clinical signs, mice were further assessed in a battery of assays for physical and neurocognitive function before euthanasia, as described below.

Euthanasia, tissue collection, and brain section immunohistochemistry:

Mice were perfused with ice-cold saline under deep isoflurane anesthesia. Brains were cut in sagittal sections. Left brains were dissected into olfactory bulb, forebrain, brainstem, cerebellum, and spinal cord, and homogenized in either Buffer RLT for RNA isolation or in RIPA buffer for Western blotting. Right brains were immersion fixed in 4% paraformaldehyde for 48 hours, processed and paraffin embedded. Antigen retrieval was conducted with sodium citrate buffer for 30 minutes at 95 degrees. Sections were blocked and permeabilized with 10% bovine serum albumin and 0.2% Triton-X 100 in phosphate buffered saline. Primary antibodies for immunostaining included Collagen IV (Abcam ab236640), CD3 (Abcam ab16669), Glut1 (Abcam ab40084), Fibrinogen (LS Bio LS-C150799-1), Caveolin-1 (Invitrogen PA5-17447), GFAP (Millipore c115516), and Iba1 (Abcam ab178847). Secondary antibodies were conjugated to Alexa fluorophores. Microscopy was conducted using Zeiss LSM710 or Leica DMI8 microscopes. Quantification was performed using FIJI software (NIH).

Western blotting:

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Mechanically homogenized brainstem samples were incubated for 60 minutes at room temperature in 2x RIPA buffer containing 0.2% NP-40, 0.2% SDS, and 2% Triton X-100 to inactivate SARS-CoV-2. Samples were then quantified with BCA assay and loaded onto 12% acrylamide gels, transferred onto PVDF membranes, blocked with LiCOR Intercept Buffer, incubated with primary antibodies including Occludin (Invitrogen 71-1500), ZO-1 (Invitrogen 33-9100), Caveolin-1 (Invitrogen PA5-17447), β -actin (Abcam ab6276), incubated with LiCOR far-red conjugated secondary antibodies, and detected with LiCOR Odyssey CLX.

10 Whole brain RNA Isolation and RT-qPCR:

Tissues were homogenized in Buffer RLT. Tissues were then transferred to the BSL2 laboratory. RNA was extracted from tissue homogenate using a Zymo Research Quick-RNA 96 Kit (R1052). Viral genomes were quantified via quantitative RT-PCR with the N1 Primer/Probe Kit from Integrated DNA Technologies (IDT, 10006713). We generated cDNA using High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems) following the manufacturer's specifications. cDNA was probed with Power SYBR™ Green PCR Master Mix (Applied Biosystems) RT-qPCR.

Primer sequences are listed in table 1:

GAPDH	AACTTTGGCATTGTGGAAGG (SEQ ID: 3)	ACACATTGGGGGTAGGAACA (SEQ ID: 4)
Cav1	GACGCGCACACCAAGGAGATT (SEQ ID: 5)	CTGACCGGGTTGGTTTTGAT (SEQ ID: 6)
Cldn5	GCTTCCCGGTCAAGTACTCT (SEQ ID: 7)	CCTCCCGCCCTTAGACATAG (SEQ ID: 8)
Ocln	AGTGTGGATGACTTCAGGCA (SEQ ID: 9)	TCATAGTGGTCAGGGTCCGT (SEQ ID: 10)
Wnt3	CTCGGCGCTGCTTCTAATG (SEQ ID: 11)	CTTCACACCTTCTGCTACGC (SEQ ID: 12)
Wnt5a	TCTCCTTCGCCCAGGTTGTT (SEQ ID: 13)	CCTGTCTTCGCACCTTCTCCA (SEQ ID: 14)
Wnt5b	GCTGCTGACTGACGCCAACT (SEQ ID: 15)	GCACTCTCTGATGCCCGTCTT (SEQ ID: 16)
Wnt7a	CGCCAAGGTCTTCGTGGATG (SEQ ID: 17)	CGGCCTCGTTGTATTTGTCCT (SEQ ID: 18)
Wnt7b	TTTCTCGTCGCTTTGTGGATG (SEQ ID: 19)	GCCTGACACACCGTGACACTT (SEQ ID: 20)
Apcdd1	CCCACATTCACCATCTACGC (SEQ ID: 21)	CACGCAGCCATTGGTATGTG (SEQ ID: 22)

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Table 1: Primer list for selected genes.

For each primer pair, a no-template control was included, and each sample was run in triplicate. Samples were tested in 384-well plates using ViiA 7 Real-Time PCR System (Applied Biosystems). The conditions were set to 50°C for 2 min, 95°C for 2 min and 40 cycles of 95°C for 15 s, and 60°C for 1 min at QuantStudio 5 Real-Time

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PCR System (Applied Biosystems). The experimental results indicating 90%–100% efficiency was analyzed by using the comparative Ct (ddCt) method to detect the relative expression of the target gene. RNA quantifications were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by subtracting the average Ct value with that of GAPDH for each sample. The ddCt value was calculated by subtracting the dCt value for the gene from that of the GAPDH. Finally, the relative copy number was determined as 2^{-ddCt} . The results were analyzed and plotted as the relative expression of the target by using GraphPad

10 Prism software:

Endothelial cell isolation from brainstem and cortex

Brainstem and cortex were isolated from MA10 and vehicle infected mice at 4 DPI. Minced tissue was homogenized by passing through 21G syringe before and after digestion with papain (Worthington LK003178) and DNase (Worthington LK003172) for 15 min in a 37°C water bath. Bulk myelin was separated from microvessels by centrifugation through 25% BSA gradient. Red blood cells were then removed from microvessels with ACK RBC lysis buffer. Resulting microvessels were further dissociated with collagenase/dispase (Millipore Sigma 10269638001) and DNase (Worthington LK003172) for 1 h in 37°C water bath and passed through 100 µm cell strainer (PluriSelect USA 43-10100-60). Brainstem dissociated microvascular cells were stained for flow cytometry analysis or were additionally processed with myelin removal beads (Miltenyi 130-069-731) and selected with CD31 microbeads (Miltenyi 130-097-418) on magnetic columns (Miltenyl 130-042-401) to generate single-cell suspensions with >95% CD31+ and >90% viability. RNA was then isolated from the purified CD31+ endothelial cells using Qiagen RNeasy Micro Kit (Qiagen 74004) according to manufacturer's specifications. cDNA library preparation was conducted by the University of Chicago Genomics Facility with Oligo-dT at 30 million clusters/sample.

30 RNA sequencing and analysis:

Fastq files were quality checked using FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) prior to downstream analysis. Reads were aligned using STAR (version 2.7.6a) against the GRCm38 (mm10) genome provided by Ensembl. Count tables were generated using featureCounts (Subread release 2.0.1). Differential expression analysis was performed between testing groups using Deseq2. Clusterprofiler was used for overrepresentation analysis of the differentially expressed genes against the gene

ontology database. Differential expression visualizations were created using the seaborn and sanbomics python packages.

Single-cell transcriptomes of aged and young non-myeloid brain cells were obtained from Tabula Muris Senis (A single-cell transcriptomic atlas characterizes ageing tissues in the mouse, 2020). Differential expression between brain endothelial cells from 3-month-old, 18-month-old, and 24-month-old mice was preformed using the rank_genes_groups function from Scanpy using default parameters.

Flow cytometry:

To determine β -catenin transcriptional activity, dissociated microvascular cells were sequentially incubated with viability indicator, FC block, and surface stains. Cells were fixed and permeabilized overnight, stained with intracellular antibodies, and analyzed using a Cytoflex cytometer. For Lef1 analysis, cells were gated for viable CD31+ singlets. Reagents included Zombie Violet Fixable viability stain 1:200 (Biolegend 423113); anti-mouse CD16/CD32 Fc Block 1:200 (Biolegend 101301); CD31 Rat anti-Mouse, PE 1:100 (BD Biosciences 561073); Caveolin-1 Rabbit anti-mouse, Alexa Fluor 647 1:200 (Cell Signaling 31411); LEF1 Rabbit anti-mouse, Alexa Fluor 488 1:50 (Cell Signaling 8490S), and transcription factor fixation and permeabilization buffer (Biolegend 421401).

Multiplex Chemokine/Cytokine Analysis:

Plasma samples from 2- and 12-month-old mice inoculated with vehicle or with SARS-CoV-2 were processed on a 0.45 μ m MultiScreenHTS IP Filter Plate (Millipore, Cat# MSIPS4W10) using the Bio-Plex Pro Mouse Chemokine Panel 31-Plex kit (Bio-Rad, Cat# 12009159). Samples were ran using the Luminex MAGPIX® instrument and cytokine levels were acquired with the xMAP system software, where sample concentrations were extrapolated from Bio-Plex Pro Mouse Chemokine Standard values (Bio-Rad, Cat# 12002796).

Behavioral Assays:

Behavior tasks were conducted between 8-11 AM in a dark biosafety cabinet laminar flow hood in the BSL3 facility. Open field was conducted by filming mice for ten minutes with an overhead camera in white plastic bins 13 inches x 19 inches (Ikea) with pebbled floor. Motility was automatically computed using Noldus EthoVision XT software. Velocity, distance traveled, time spent in the center, time spent in the edges were read for the first 10 minutes of the records as well as the relative time spent in the center for open field analysis. The frequency and duration of grooming was independently recorded by two blinded observers following the specific

behavioral pattern as described. The number of attempts, cumulative duration, and average duration of each grooming was calculated and plotted. For the pole assay, the rod of a buret support stand (1/2 inch diameter, 18 inches length) mounted on a metal base covered with clean corn cob bedding in a test cage. The latency to descend the pole and dismount onto the bedding was measured. For the composite cerebellar ataxia phenotyping, we assigned up to 3 points each for abnormal performance in the ledge test, hindlimb clasping, gait, and kyphosis. For novel object recognition (NOR), we first tested a catalog of 10 objects for intrinsic preference. Objects were similar in size (1-2 inches wide, 3-4 inches tall), visually interesting, without smell, and made of easily cleaned non-porous materials, for example 25 ml suspension flasks filled with pebbles, 50 ml conical tubes filled with corncob bedding, and red shotglasses. For familiarization session, we placed individual mice in a dark open field containing two suspension flasks and allowed 10 minutes exploration. Behavior was filmed with an overhead mounted wide-angle webcam (Logitech C920S HD Webcam). Intersession intervals of 14-hours or 24-hours were used as indicated. For the testing session, mice were reintroduced into the field containing one suspension flask and one novel object, and filmed for 10 minutes. Objects and field were cleaned with ethanol and dried in between mice. Videos were coded and independently scored by two blinded scientists for duration of exploration of each object. The statistical differences were calculated by using one-way ANOVA in GraphPad.

Wnt studies:

AAV-PHP.eB-Wnt7aK190A-GFP and AAV-PHP.eB-GFP were administered to 12-month-old male C57Bl/6 mice by retroorbital injection of 2×10^{11} viral genomes in 25 μ l of PBS three weeks prior to inoculation with SARS-CoV-2 MA10 (1×10^4 FFU intranasal). Mice were euthanized at 5 days post inoculation. 6BIO, (2'Z,3'E)-6-Bromoindirubin-3'-oxime (Sigma B1686) at 1mg/kg or vehicle (5% DMSO, 95% saline) was administered by intranasal delivery twice daily in 25 μ l volume for 7 days, starting 2 days before SARS-CoV-2 MA10 (1×10^4 FFU intranasal) inoculation.

Results

SARS-CoV-2 Viral measurements

To establish a mouse model with which to interrogate CNS consequences of respiratory SARS-CoV-2 infection, we inoculated C57Bl/6 mice with SARS-CoV-2 MA10 by intranasal route. We compared infection in mice of two ages: young adult (2 months, n=14) and middle aged (12 months, n=11). We asked whether altered

disease outcomes could be related to increased viral burden in the brain or in the lung. We therefore conducted RT-qPCR for SARS-CoV-2 viral RNA. Age did not significantly influence viral RNA burden in the lung at 4DPI (Fig 9). Geometric means for viral RNA in brain regions ranged from 11 to 349 viral genomes/mg tissue and did not significantly differ between brain region (olfactory bulb, forebrain, cerebellum, brainstem, spinal cord) or between ages (Fig 9A non-parametric two-way ANOVA). Viral RNA was below limit of detection in blood, heart, kidney, liver, and spleen (n=5 per group, data not shown), indicating that accumulation of viral RNA is specific to the lung and brain. Thus, moderately advanced age does not increase SARS-CoV-2 RNA in the lung or brain in C57Bl/6 mice 4 days after nasal inoculation. We also assessed body weight as an indicator of overall health status. We found that 2-month-old adults maintained their weight while 12-month-old mice decreased body weight by an average of 10% at four days post inoculation (4DPI) (Fig 9B). This result is similar to reports in 12-month-old BALBC mice. Expression of cytokines and chemokines in blood was similar in young adult and middle-aged mice with SARS-CoV-2 infection, as measured by multiplex ELISA (Fig 9C-D). Thus, SARS-CoV-2 causes age-dependent weight loss in C57Bl/6 mice, but age is not an important factor in determining circulating cytokines or viral load in the brain. SARS-CoV-2 infection causes cognitive impairment and neuropsychiatric abnormalities

Because COVID-19 and Long COVID are associated with cognitive and neuropsychiatric decline, we assayed cognitive and neuropsychiatric behaviors in aged SARS-CoV-2 infected mice. We first conducted novel object recognition (NOR), a well-established assay for learning and memory (Fig 1A). We conducted familiarization at 3DPI and NOR at 4DPI (24h inter-session interval). Mice that spend more than 50% of their exploration time with the novel object are considered to "remember" the familiar object and prefer the novel object. As expected, 2-month-old healthy mice preferred the novel object (Fig 1A-B). Importantly, we found that 2-month-old mice infected with SARS-CoV-2 were impaired in this assay of learning and memory (Fig 1A). 2-month-old vehicle treated mice attended to the novel object 61.2% of the time whereas infected 2-month-old mice attended to the novel object 48.4% of the time (Fig 1A-B). NOR performance significantly decreased with age: using the 24-hour intertest interval, healthy 12-month-old did not favor the novel object more time than accounted for by random chance (50%) (12-month vehicle-treated mice, mean 51.5%). To avoid potential confounding effects of inactivity, exclusion criteria were failure to explore objects for at least 0.5% of the trial time (Fig. 1B). Two infected mice were excluded from the NOR assay. Importantly,

however, no significant differences were noted in the overall duration of object exploration between the infected and vehicle-treated mice (Fig 1B). These results establish that respiratory SARS-CoV-2 infection decreased cognitive performance.

5 We then asked whether infection impaired brainstem-related motor tasks. 2-month-old mice inoculated with SARS-CoV-2 trended toward hyperkinesia compared with uninfected 2-month-old mice (>1.5 -fold greater velocity in the open field, Fig 1C). In contrast, 12-month-old mice inoculated with SARS-CoV-2 moved more slowly (~ 2 -fold reduction in velocity) (Fig 1C). Open field locomotion is influenced by
10 voluntary and involuntary behaviors reflecting cerebral and brainstem circuitry. To gain more insight into the motor deficit, we measured bradykinesia and parkinsonism with the pole descent assay (Fig 1D). The pole-descent assay integrates complex motor circuits, and is disrupted in models of Parkinson's disease, other neuropsychiatric disorders, and after traumatic brain injury. 12-month-old mice
15 inoculated with SARS-COV-2 had significantly greater latency to descend the pole (mean 14.7 second descent) as compared to 12-month-old mice treated with vehicle (mean 7.6 second descent) or to 2-month-old mice inoculated with SARS-COV-2 (5.9 second descent) (Fig 1D). Interestingly, no defects were noted in a composite phenotypic battery for cerebellar ataxia incorporating ledge test, hindlimb clasping,
20 gait, or kyphosis (not shown). This suggests that cerebellum circuitry may be spared in SARS-CoV-2 infection in all ages. In summary, SARS-CoV-2 infection in middle-aged mice but not young adult mice results in severe bradykinesia, indicating that age strongly influences motor deficits in SARS-CoV-2 infection.

25 Neuropsychiatric disorders are epidemiologically but not mechanistically linked with COVID-19 and Long-COVID. Neurocognitive, psychiatric, and motor/sensory disorders are increased after COVID-19, especially in middle-aged and advanced-age adults. To gain insight into how SARS-CoV-2 infection might modulate neural control of behavior, we analyzed spontaneous grooming (Fig 1E-F). Self-grooming
30 in mice is an innate behavior coordinately regulated by cerebral modification of brainstem chains of motor activities. Grooming is altered in diverse models of neuropsychiatric disorders including anxiety disorder, obsessive-compulsive disorder, autism spectrum, Parkinson's disease, and Huntington's disease. We observed grooming changes in infected 12-month-old mice (Fig 1 E-F): 12-month-old mice inoculated with SARS-COV-2 nearly doubled grooming bout duration as
35 compared with 12-month-old vehicle treated mice or 2-month-old infected mice (Fig 1E-F). 2-month-old infected and uninfected mice had indistinguishable grooming duration. We then measured grooming initiations (Fig 1F). Again, we noted age-

dependent modification of grooming in infected mice. 12-month-old infected mice initiated grooming fewer times (mean 2.9) than did 2-month-old infected mice (mean 9.8) (Fig 1F). In summary, SARS-CoV-2 infection in middle-aged mice but not young adult mice prolonged repetitive stereotypical behavior.

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SARS-COV-2 acute gliosis:

Morphological microglial responses to infection is measured. Previous studies have documented microglial activation and monocyte/macrophage CNS infiltration in COVID-19. Indeed, macrophages infected with SARS-CoV-2 die by inflammatory
10 pyroptotic death. We assessed Iba1, a calcium binding protein expressed in microglia and macrophages which is profoundly upregulated in inflammation. We observed that SARS-COV-2 infection doubled Iba1 immunoreactivity in brainstem and olfactory bulb in age-matched comparisons (Fig 2A-D). Moreover, aged infected mice had significantly greater Iba1 immunoreactivity than young infected mice in both
15 brainstem and olfactory bulb (Fig 2A-D). Increased hippocampal CA1 Iba1 immunoreactivity in infected young adults may contribute to their impaired performance in the novel object recognition test (Figure 1A).

Moreover, microglial morphology changed in response to infection in the aged mice.
20 Microglia in infected brains had fewer complex processes and enlarged soma (Fig 2A). Microglial nodules containing multiple nuclei were present in ~70% of aged infected mouse brainstem sections (Figure 2A). Some vessels contained large, ameboid Iba1+ cells embedded within the vessel wall, giving the appearance of perivascular engraftment of peripheral monocytes (Fig 2G-H). Similarly, we
25 consistently found numerous small round Iba1+ profiles underlying the 4th ventricle in the pontine central gray (Fig 2I-L), similar to features reported in older COVID-19 encephalitis patients. Thus, microglia undergo morphologic evidence of reactivity in a region-specific manner in response to respiratory infection with SARS-COV-2 in aged mice.

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We then assessed immunoreactivity for the astrocyte intermediate filament protein GFAP, a sensitive indicator of neuroinflammation. Healthy aging was associated with 3-fold increased GFAP in brainstem and ~2-fold in hippocampus (Fig 10A-F). In young mice, SARS-COV-2 infection did not increase GFAP (Fig 10). In 12-month-old-
35 mice, infection further increased GFAP in brainstem, but not in olfactory bulb or hippocampus (Fig 10). Thus, we observed neuroanatomically specific patterns of astrocytic response to SARS-CoV-2 infection in aged mice.

SARS-CoV-2 induced cerebrovascular inflammation and T cell infiltration is exacerbated by age

We noted morphologic similarity between the perivascular hypercellularity after SARS-CoV-2 infection and after classic models for CNS autoimmune disease, in which T cell invasion of the CNS causes tissue destruction and motor/neuropsychiatric impairment. In neurotropic infections, CNS influx of virus-specific T cells is essential to control infection but also causes post-infectious cognitive dysfunction (Ai and Klein, 2020; Prasad and Lokensgard, 2019). We therefore asked whether SARS-CoV-2 respiratory infection increased T cell infiltration of the brain in older mice. Indeed, we observed ~60% increase in CD3+ T cells in the brains of 12-month-old SARS-CoV-2 infected mice as compared with 2-month-old infected mice (Fig 3A-B). We then assessed the neuroanatomic distribution of T cells in the CNS, to learn whether SARS-CoV-2 infection might cause inflammation in specifically vulnerable brain regions. Indeed, we observed a 4-fold increase in CD3+ T cell infiltration of the brainstem in SARS-CoV-2 infected 12-month-old mice as compared to younger infected mice ($p < 0.001$, two-way ANOVA and Tukey's multiple comparisons test; Figure 3A-B). We observed a ~1.5-fold increase in olfactory bulb T cells in 12-month-old SARS-CoV-2 infected mice compared with age-matched controls, and a non-significant trend when compared with 2-month-old SARS-CoV-2 infected mice. No significant differences were noted in T cell number in cortex, hippocampus, thalamus, or cerebellum between 2-month-old and 12-month-old infected mice (Fig 3B). Thus, we found that middle-age increases T cell CNS infiltration after SARS-CoV-2, with T cells in infected middle-aged mice primarily crossing the BBB in the brainstem and the olfactory bulb.

25

SARS-CoV-2 transcellular BBB leakage to blood proteins

We asked whether inflammation could be related to vascular leakage. For this, we measured CNS accumulation of fibrinogen, a multimer of the fibrin protein abundant in blood that is normally excluded from the brain by the actions of the BBB. Fibrinogen is highly pro-inflammatory, triggering demyelination, destruction of synapses, and cognitive impairment in neurological diseases. We found brainstem parenchymal area of fibrinogen immunoreactivity was greater in infected mice as compared to age-matched vehicle controls (Fig 3C-D). Two-month-old infected mice had approximately doubled fibrinogen area immunoreactivity in the brainstem reticular formation as compared with age-matched healthy mice. 12-month-old infected mice had ~ 3-fold greater fibrinogen area immunoreactivity in brainstem as compared with 12-month-old healthy mice. Infected 12-month-olds also had more fibrinogen area immunoreactivity than infected 2-month-olds. We then quantified

the β band of fibrinogen in Western blot of brainstem homogenate (Fig 3E-F). 12-month-old infected mice had more brainstem fibrinogen than age-matched vehicle. Thus, age exacerbates BBB leakage caused by SARS-CoV-2 infection.

BBB permeability occurs through two pathways: movement through the paracellular spaces in between adjacent endothelial cells through disrupted tight junctions, and through the cytoplasm of the endothelial cell (transcytosis). Caveolin-1 is a signaling and scaffolding molecule that can promote transcellular BBB permeability. Caveolin-1 increases in cerebrovascular aging, where it contributes to proinflammatory age-related BBB permeability. We assessed Caveolin-1 and tight junction proteins. We measured mean fluorescence intensity of Caveolin-1 immunostaining in brainstem sections (Fig 11 A-B). SARS-CoV-2 infection significantly upregulated Caveolin-1 in the 12-month-old mice. By Western blot, Caveolin-1 was not significantly changed (Fig 11C-D). We also investigated whether age and SARS-CoV-2 would downregulate tight junction proteins, potentially contributing to paracellular BBB leakage. By Western blot, SARS-CoV-2 infection decreased brainstem ZO-1, a protein stably linking junctional proteins to the cytoskeleton to restrict macromolecular permeability (Fig 11E-H). However, we did not observe significant differences in tight junction proteins Occludin or Claudin5 (Fig 11E-H). These results suggest that age exacerbates BBB leakage caused by SARS-CoV-2 infection.

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Brain endothelial cell transcriptomic changes in aged, infected mice

To interrogate biological processes which could underlie severe neuroinflammation in aged, infected mice, we conducted bulk RNAseq of microvascular endothelial cells acutely isolated from the brainstem of 12-month-old mice, 4 days after inoculation with SARS-CoV-2 or vehicle (Fig 4A). We identified 184 DE genes which were upregulated and 229 DE genes which were downregulated in brainstem ECs in aged COVID mice as compared with healthy aged mice (Fig 4B). DE genes clustered in gene ontology groups related to viral defense, Wnt signaling, cellular adhesion and extracellular matrix, angiogenesis and vascular morphogenesis, and Apoptosis (Fig 4C-D). Response to virus biological process were upregulated, including DEG in the type I interferon pathway components (Irf7, Ifit1, Ifit3, Ifit3b, Oas2). Proinflammatory LCN2 was markedly increased (Fig 4C). Downregulated suppressors of innate immune activity included the Rev-Erb alpha transcription factor (Nr1d1). Genes involved in antigen processing and presentation including Class I MHC (B2M), normally expressed at low levels in brain EC, were increased. This suggests that brain endothelial cells are immunologically activated in aged infected mice.

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We also observed alterations in several genes important in other neuroinflammatory diseases (Fig 4C). Pathways promoting BBB integrity were suppressed, e.g. Desert hedgehog and fibrillin-1 (Fbn1). PICALM, a regulator of vesicular endocytosis related to Caveolin-1, was increased. Extracellular matrix organization and positive regulation of cell adhesion were downregulated; concordantly, negative regulation of cell adhesion and negative regulation of cell-matrix adhesion were both upregulated. These included collagens (Col3a1, Col5a2), ECM proteases (Sulf2, MMP11), gap junctions (Cx40, Cx37[Gja4]), and integrins (Itgae). Upregulated genes included protease inhibitors Serpina3. DEG were important in leukocyte activation (Il12a, H2lyc2) and BBB infiltration (Itgae), and coagulation (F3). The transcription factor Sox9, which transcribes collagen protein-coding genes, was decreased. Apoptosis transcripts were elevated (FAS, EndoG, Xaf1, Bcl2). Given that COVID-19 causes cerebral hypoxia, it was also interesting to note significantly elevated transcripts for Rgs5, a hypoxia-linked pro-apoptotic gene in endothelial cells. Other upregulated DEG related to hypoxia included Hif3a and Higd1b. We also found MAL to be upregulated; MAL promotes secretion of extracellular vesicles from CNS EC and is proposed as a biomarker for BEC damage in multiple sclerosis.

SARS-CoV-2 induces age-dependent cerebrovascular remodeling

We then interrogated brainstem tissue sections for cerebrovascular structural changes. We conducted immunostaining for the vascular basement membrane proteins Collagen IV (Fig 4E) and laminin (Fig 12), and Glut-1 as a marker of BBB endothelial cells. We determined that the mean vessel width was decreased in young mice with SARS-CoV-2 infection, and was also decreased by age alone (Fig 4F). We further determined that with aging, vessels with very small size (<3 μ m diameter) became prevalent (Fig 4G). Laminin density was decreased by aging alone, and by infection (Fig 12). Infection in aged mice increased density of cerebrovascular basement membrane tubes without detectable interior endothelial cell staining ("string vessels"), suggestive of endothelial apoptosis and vascular regression (Fig 4H). This is consistent with previous reports of string vessels in COVID-19 patient brains and expands upon previous reports of SARS-CoV-2 infection in young adult rodents.

Wnt/ β -catenin pathway activity is induced by brain endothelial cell infection

We next tested the importance of suppression of the Wnt signaling pathway in BEC from aged, infected mouse brainstem. Wnt ligands Wnt7a/7b act on brain endothelial cells to suppress BBB permeability during late embryogenesis/early postnatal life. Similarly, Wnt ligands are required for the maintenance of BBB function in the adult.

We previously reported that cerebrovascular endothelial cell Wnt/ β -catenin signaling is induced by acute inflammation in patient tissue and in experimental models for multiple sclerosis and Huntington's disease. Data suggests that cerebrovascular endothelial cell Wnt/ β -catenin signaling is a protective response to inflammation and disease, because loss of the canonical Wnt/ β -catenin pathway specifically in brain endothelial cells induced dramatic Caveolin-1-dependent transcellular BBB permeability, increased T cell infiltration, angiogenesis, and neuroinflammation, whereas β -catenin activation normalized vascular pathology. We therefore investigated whether the increased BBB permeability in middle-aged mice infected with SARS-CoV-2 could be associated with Wnt/ β -catenin dysregulation.

We hypothesized that young adults infected with SARS-CoV-2 would increase cerebrovascular Wnt/ β -catenin signaling, thereby controlling cerebrovascular inflammation. We further hypothesized that age-related attenuation of the cerebrovascular Wnt/ β -catenin response would exacerbate SARS-CoV-2 cerebrovascular inflammation. Wnt7a and Wnt3 are canonical Wnt ligands that exert their BBB-stabilizing effects through β -catenin activation of TCF/Lef transcription factors. We therefore measured TCF/Lef1 in endothelial cells acutely isolated from the brainstem of young healthy, young infected, aged healthy, and aged infected mice (Fig 5A-C). We found that the mean fluorescence intensity of Lef1 ~ doubled in brainstem endothelial cells derived from young adult mice with SARS-CoV-2 infection as compared to healthy young adult mice (Fig 5A-C). In marked contrast, Lef1 levels were statistically similar in brain endothelial cells isolated from aged healthy and aged SARS-CoV-2 infected mice (Fig 5B-C). Thus, brainstem endothelial cells of young adults but not middle-aged adults increase BBB Wnt/ β -catenin transcription in response to respiratory infection with SARS-CoV-2.

Barrier forming cells of the cerebrovasculature, meninges, and choroid plexus are potential targets of SARS-CoV-2 infection and demonstrate inflammatory changes in COVID-19 infected individuals and animal models. We asked whether brain endothelial cell β -catenin pathway activity might be induced cell-autonomously by brain endothelial cell infection with SARS-CoV-2. To address this question, we used brain-endothelial cell trophic adeno-associated virus AAVBR1 to transduce brain endothelial cells with human ACE2 prior to inoculation with the ancestral SARS-CoV-2 WA-1 strain (which utilizes human but not mouse ACE2 as a receptor for viral entry)(Fig 5D). We confirmed by immunostaining of brain tissue sections that AAVBR1:mCherry transduced brain endothelial cells in multiple neuroanatomic regions, but not ependymal cells (Fig S5A-D). To establish optimum dosage, we delivered 2×10^9 , 2×10^{10} , or 2×10^{11} viral genomes of AAVBR1:mCherry (Fig

S5E-F). We determined that 2×10^{11} viral genomes yielded nearly complete transduction of brain endothelial cells as detected by microscopy for mCherry fluorescence (Fig S5G). We then showed that AAVBR1:hACE2 achieved transduction with high specificity for the brain as compared with the lung, by conducting real time

5 QPCR with primers specific to the human ACE2 gene that do not amplify the mouse Ace2 gene (Fig S5H). Data indicating successful SARS-CoV-2 infection of brain endothelial cells included significantly increased IRF7 intracellular staining by flow cytometry in brainstem endothelial cells isolated from mice treated with AAVBR1:hACE2 plus SARS-CoV-2 but not in mice treated with AAVBR1:hACE2 alone

10 (Fig 5G). Similarly, interferon-inducible chemokine CXCL10 was increased in brain homogenate from mice treated with AAVBR1:hACE2 plus SARS-CoV-2 (Fig S5I). We then characterized downstream indicators of inflammatory response by flow cytometry on acutely isolated brainstem endothelial cells. Indeed, MHC-I expression increased ~ 4 fold, and MHC-II increased ~ 30 -fold, in brain endothelial cells from

15 mice with brain endothelial-targeted SARS-CoV-2 infection (Fig 5H and Fig S5Q). In order to assess transcriptional activation of the Wnt/ β -catenin pathway in endothelial cells, we utilized the TCF/Lef:H2B:eGFP transgenic mouse line, in which TCF/Lef transcription produces eGFP with nuclear localization (Fig 13K). We delivered 2×10^{11} viral genomes of AAVBR1:hACE2 to 2-month-old TCF/Lef:H2B:eGFP mice

20 (Fig 5D). We then inoculated with SARS-CoV-2 WA-1 by intravenous route (to maximize brain endothelial cell infection) or by intranasal route (to mimic a respiratory route of infection). Two days after SARS-CoV-2 inoculation, mice were euthanized and brain tissue sections were prepared for histological analysis of GFP production in brain endothelial cells (Fig 5E and Fig S5L-O). Indeed, we found that

25 brain endothelial cell-targeted infection with SARS-CoV-2 significantly increased brain endothelial cell Wnt/ β -catenin transcription of the eGFP reporter in brainstem and olfactory bulb (Fig 5E-F). TCF/Lef1 activity was variable in cerebellum and hippocampus. Wnt/ β -catenin activation was similar after intranasal or intravenous inoculation with SARS-CoV-2 (Fig 5E-F). TCF/Lef1 activation in infected brain

30 endothelial cells appears to be ligand independent and cell autonomous, because brain EC-targeted infection did not change levels of canonical ligands Wnt7a/Wnt7b, which can be secreted by astrocytes in inflammation. These data indicate that in young adults, SARS-CoV-2 infection robustly activates brainstem endothelial cell Wnt/ β catenin activity and indicators of immune activation.

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Attenuated Wnt/ β -catenin signaling in brain endothelial cells in aging

Attenuation of the cerebrovascular Wnt/ β -catenin response in the aged could be due to decreased production of canonical Wnt ligands. Indeed, age decreased brain

mRNA for Wnt7a by ~50% and Wnt3 by ~80% (Fig 6A-B). No change was observed in Wnt7b (not shown). Age also decreased production of the Wnt transcriptional target Apcd1 (Fig 6C).

5 We then conducted bulk RNAseq analysis of endothelial cells acutely isolated from the brainstem of healthy 2-month-old and healthy 12-month-old mice (Fig 6 D-F). In GO overrepresentation analysis, downregulated categories were related to cell adhesion (cell-cell adhesion, cell-substrate adhesion, extracellular matrix assembly, and cell junction assembly) (Fig 6F). We noted significant DEG related to BBB
10 function including decreased Sparc, Serpina1, Serpina3, laminin alpha 1, laminin beta 1) (Fig 6G). Notch pathway transcripts Maml1 and Maml3 were decreased. We found indicated significant downregulation of Wnt receptors (e.g. Fzd4), transcriptional activators, and transcriptional targets of Wnt/ β -catenin pathway activity (e.g Wisp1/CCN4) (Fig 6H). Similarly, Fgfbp1 was downregulated in healthy
15 aging; Fgfbp1 is an activator of Wnt signaling that promotes BBB development. We confirmed and extended these findings by interrogating age-related transcriptional changes in brain endothelial cells in Wnt/ β -catenin pathway components in the Tabula Muris Senis database (Fig 6I) (A single-cell transcriptomic atlas characterizes ageing tissues in the mouse, 2020). Like our data, we found significant
20 downregulation of Fzd4, Sox17, Sox4, and Wisp1/CCN4 in brain endothelial cells of 18-month-old and 24-month-old mice as compared with 3-month-old mice. These data indicate that cerebrovascular aging decreases brainstem EC responsiveness to Wnt/ β -catenin pathway activation. Age-related declines in canonical Wnt ligands, and decreased brainstem EC pathway components required for response to ligands,
25 are likely to prevent infection-induced Wnt/ β -catenin responses. In summary, the Wnt/ β -catenin pathway is dysregulated in regions of neuroinflammation in middle-aged mice infected with SARS-CoV-2.

30 Activating Wnt/ β -catenin signaling prevents SARS-CoV-2-induced cognitive impairment

Finally, we asked whether experimental strategies to enhance Wnt/ β -catenin pathway activity might protect from disease, using pharmacological approaches and using cerebrovascular-targeted engineered Wnt ligands. Our first strategy was to use small molecules that inhibit the GSK3 β destruction complex, which degrades β -
35 catenin. We prophylactically treated 2-month-old mice with 6BIO, a GSK3- β inhibitor, to increase Wnt/ β -catenin pathway activity. 6BIO prevented the development of cognitive impairment in the novel object recognition task in 2-month old mice inoculated with SARS-CoV-2 (Fig 7A).

Interestingly, Reck and GPR124 coreceptors for canonical Wnt ligands were not differentially expressed in aging in our brainstem endothelial cell dataset, nor in the Tabula Muris Senis forebrain endothelial cell dataset. This suggested the possibility that co-receptor activation might overcome age-related cerebrovascular downregulation of Wnt/ β -catenin pathway activity. We therefore tested if cerebrovascular-targeted Wnt ligands stabilized for enhanced GPR124 agonism could prevent neurobehavioral impairment in aged mice. We administered AAV-PHP.eB:Wnt7aK190A, or AAV-PHP.eB:GFP (vector control), to 12-month old mice three weeks prior to SARS-CoV-2 inoculation. AAV-PHP.eB transduces astrocytes, neurons, and endothelial cells. We conducted neurobehavioral tests at 5 days post inoculation. Strikingly, AAV-PHP.eB:Wnt7aK190A significantly improved cognitive performance after SARS-CoV-2 infection in the novel object recognition task (Fig 7B). To assess motor coordination, we conducted the pole descent task for parkinsonism, as before. Mice that received AAV-PHP.eB:Wnt7aK190A prior to SARS-CoV-2 inoculation had shorter latency for descent (Fig 7C). Furthermore, we quantified the frequency of falls during the pole descent task. We found that mice that received AAV-PHP.eB:Wnt7aK190A prior to SARS-CoV-2 inoculation had significantly fewer falls (Fig 7D). In conclusion, strategies to enhance Wnt/ β -catenin signaling prevent cognitive and neurobehavioral deficits caused by SARS-CoV-2 infection.

Wnt mutant therapy reduces blood-brain barrier leakage caused by SARS-CoV-2 infection

The engineered Wnt7a agonist according to the disclosure has robust neurocognitive and psychomotor protection in mice. To assess whether Wnt restoration therapy could also reduce blood-brain barrier leakage caused by mild respiratory SARS-CoV-2 infection, we prepared formalin fixed paraffin embedded sections of brainstem and conducted immunostaining for fibrinogen, a blood protein typically excluded from the brain parenchyma by the actions of the blood-brain barrier. As expected, we observed abundant fibrinogen in the brainstem of mice that had received AAV-PHP.eB-GFP prior to SARS-CoV-2 infection. Importantly, fibrinogen accumulation was markedly reduced in the brainstem of mice that had received AAV-PHP.eB-Wnt7aK190A-GFP prior to SARS-CoV-2 infection.

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The present invention is in no way limited to the embodiments described in the examples and/or shown in the figures. On the contrary, methods according to the

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present invention may be realized in many different ways without departing from the scope of the invention.

CLAIMS

1. A pharmaceutical composition comprising a therapeutically active amount of a Wnt7a mutant polypeptide or a fragment thereof wherein the lysine (K) residue at the position corresponding to position 159 in Wnt7a is substituted by an alanine (A) residue (K190A), or a nucleic acid encoding for said Wnt7a mutant polypeptide or fragment thereof for use in the reduction in progression and/or treatment of neurologic problems occurring after a coronavirus infection in a subject.
2. Pharmaceutical composition comprising a therapeutically active amount of a Wnt7 mutant polypeptide or a fragment thereof wherein the lysine (K) residue at the position corresponding to position 159 in SEQ ID NO: 1 or SEQ ID NO: 2 is substituted by an alanine (A) residue, or a nucleic acid encoding for said Wnt7 mutant polypeptide or fragment thereof for use in the reduction in progression and/or treatment of neurologic problems occurring after a coronavirus infection in a subject.
3. The pharmaceutical composition for use according to claim 1 or 2 wherein the coronavirus is SARS-CoV, MERS-CoV, SARS-CoV-2 virus, HCoV-NL63, HCoV-229E, HCoV-OC43, or HKU1.
4. The pharmaceutical composition for use according to any of the claims 1 to 3 wherein said compound can influence the permeability of the blood-brain barrier (BBB) in subjects.
5. The pharmaceutical composition for use according to any of the claims 1 to 4 wherein said compound can influence the transcellular permeability of the blood-brain barrier (BBB) in subjects.
6. The pharmaceutical composition for use according to any of the previous claims, wherein said mutant Wnt7 polypeptide or fragment thereof suppresses BBB transcytosis protein Caveolin-1.
7. The pharmaceutical composition for use according to any of the previous claims, wherein said mutant can prevent or reduce blood-brain barrier leakage in subjects.
8. The pharmaceutical composition for use according to any of the previous claims, wherein said compound is a nucleic acid, preferably RNA or DNA.
9. The pharmaceutical composition according to any of the previous claims, wherein said compound is provided in a liposome or lipid nanoparticle.
10. The pharmaceutical composition for use according to any of the previous claims, wherein said compound is formulated in a viral vector.

11. The pharmaceutical composition for use according to any of the previous claims, wherein said composition is combined with a second therapy.
12. The pharmaceutical composition for use according to any of the previous claims, wherein said composition is administered parenterally, preferably intravenously or intrathecally.
13. The pharmaceutical composition for use according to any of the previous claims, wherein said subject is human or an animal.
14. A method of treatment or prophylaxis of neurologic problems occurring after a coronavirus infection, comprising administration of comprising a therapeutically active amount of a Wnt7 mutant polypeptide or a fragment thereof wherein the lysine (K) residue at the position corresponding to position 159 in human Wnt7, preferably Wnt7a, is substituted by an alanine (A) residue, or a nucleic acid encoding for said Wnt7 mutant polypeptide or fragment thereof a patient in need thereof.
15. A method of treatment or prophylaxis of neurologic indications caused by an infection with coronavirus comprising administration of comprising a therapeutically active amount of a Wnt7 mutant polypeptide or a fragment thereof wherein the lysine (K) residue at the position corresponding to position 159 in human Wnt7, preferably Wnt7a, is substituted by an alanine (A) residue, or a nucleic acid encoding for said Wnt7 mutant polypeptide or fragment thereof to a patient in need thereof.

FIGURES

Figure 1:

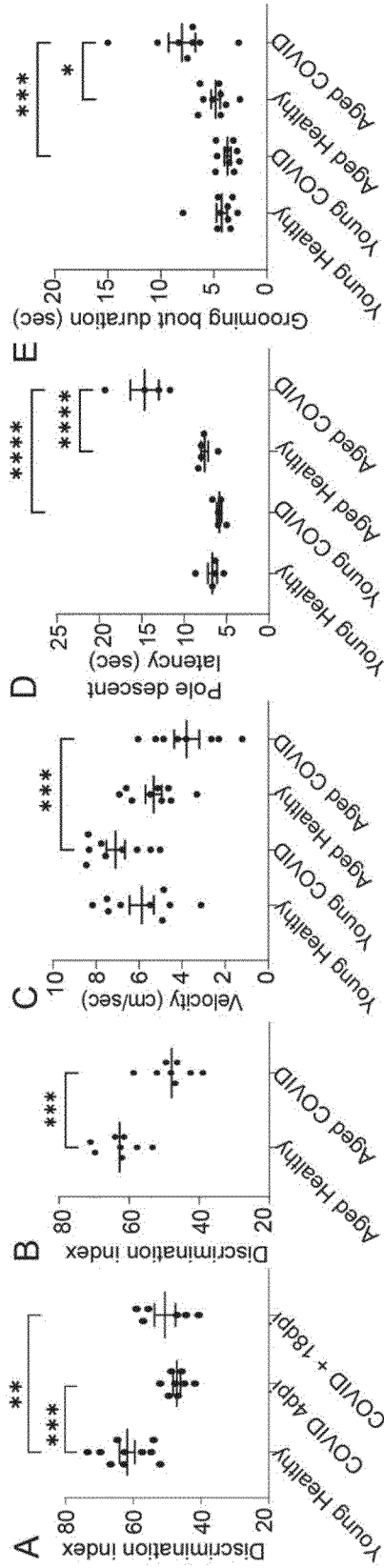


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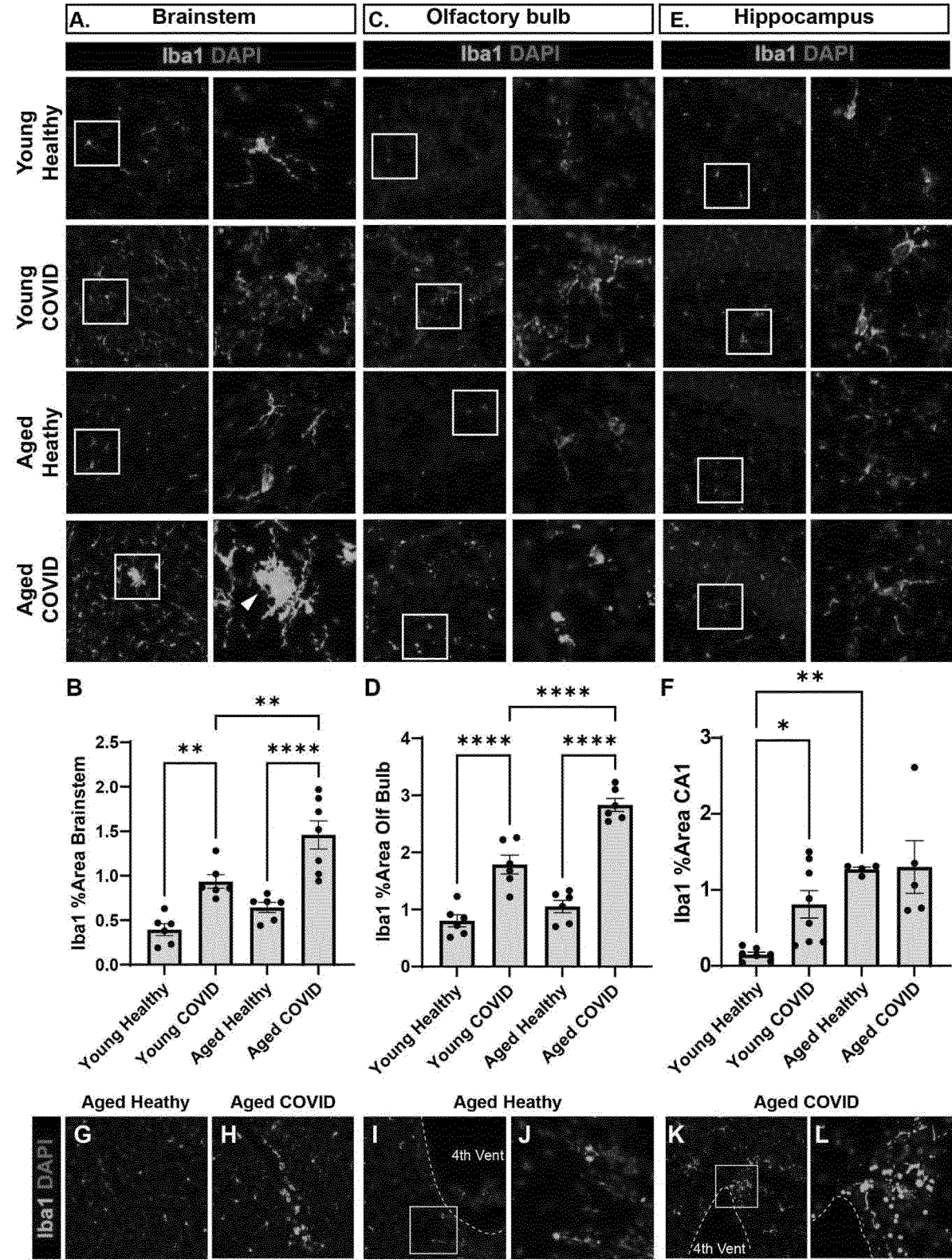


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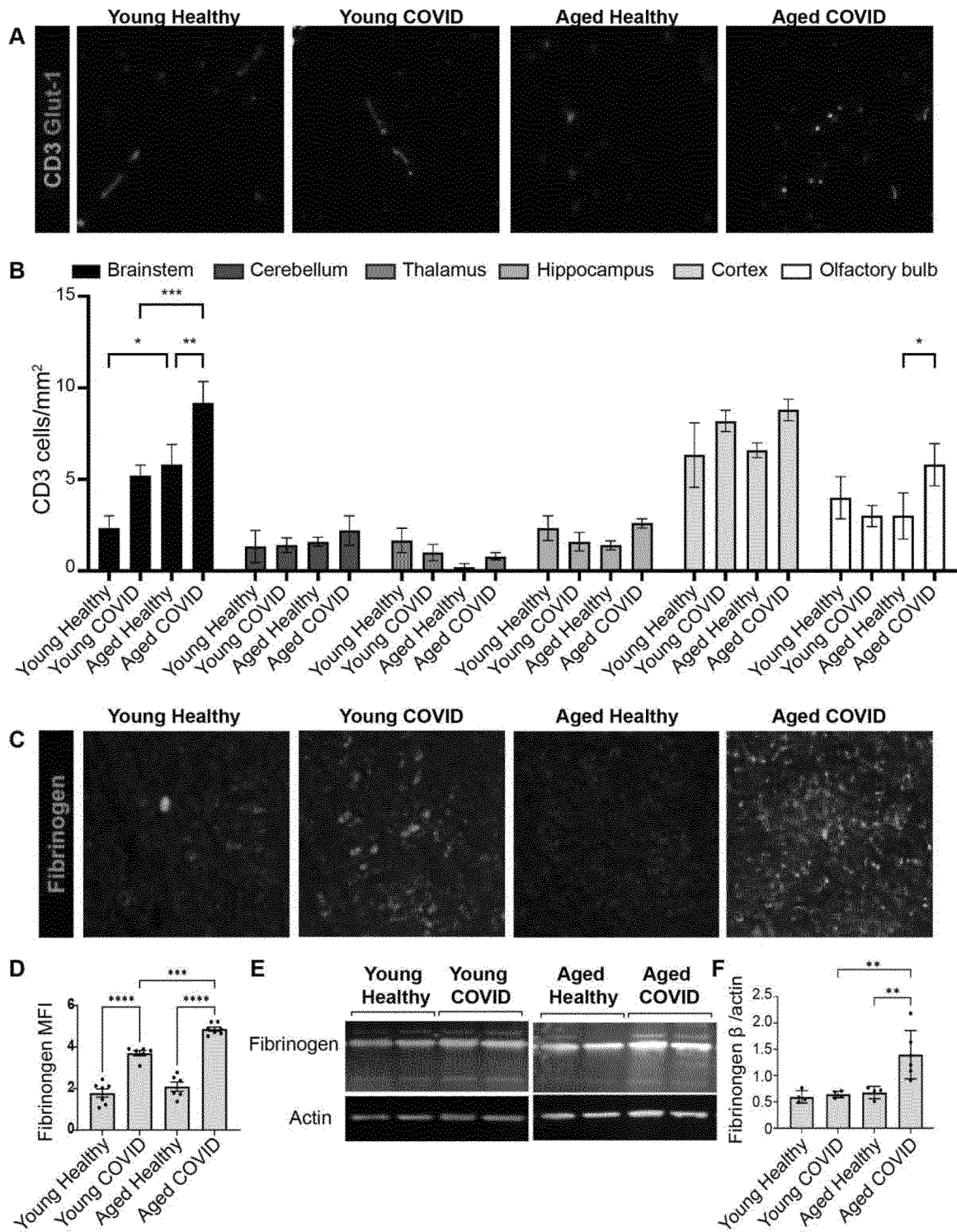


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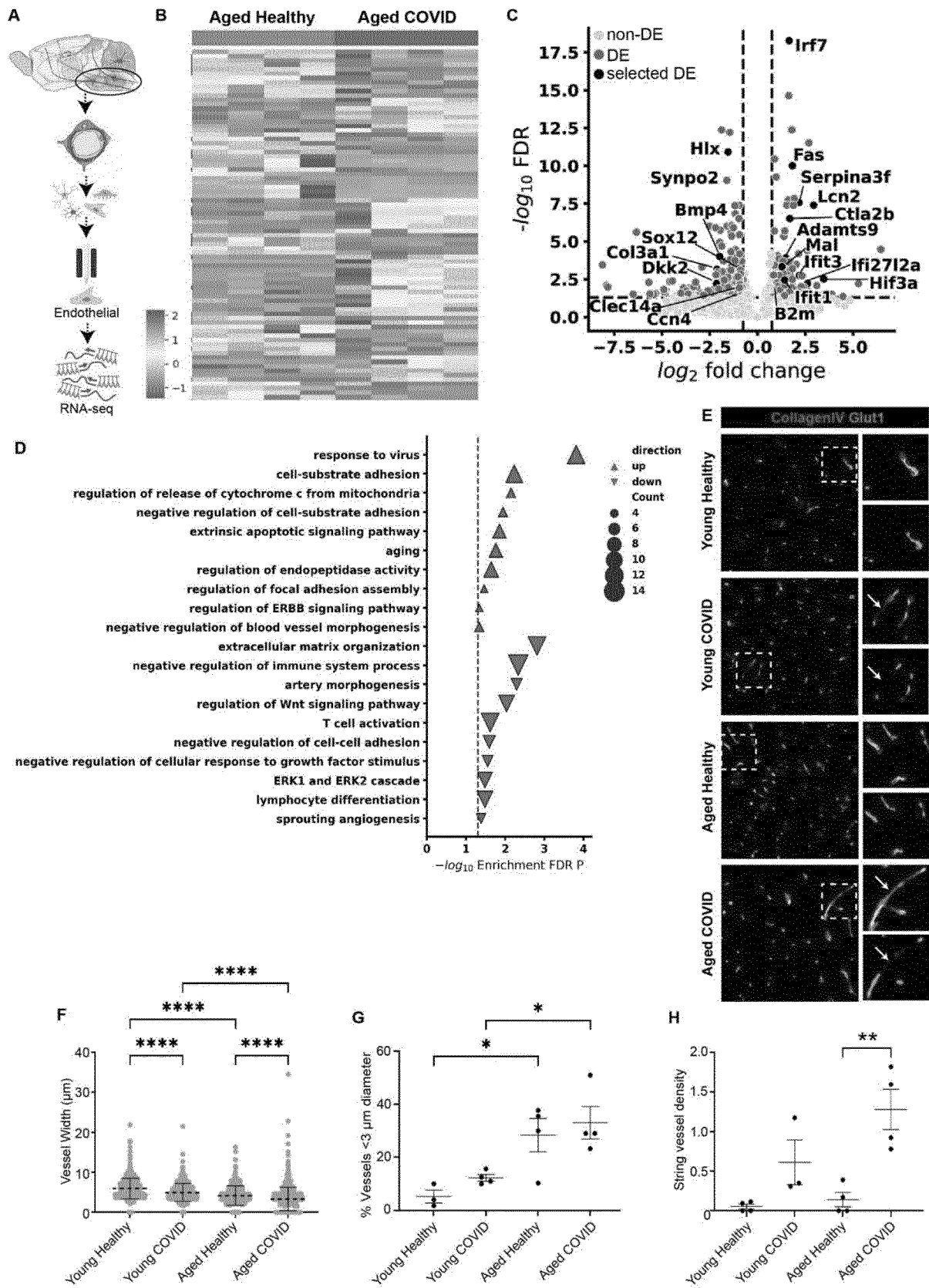


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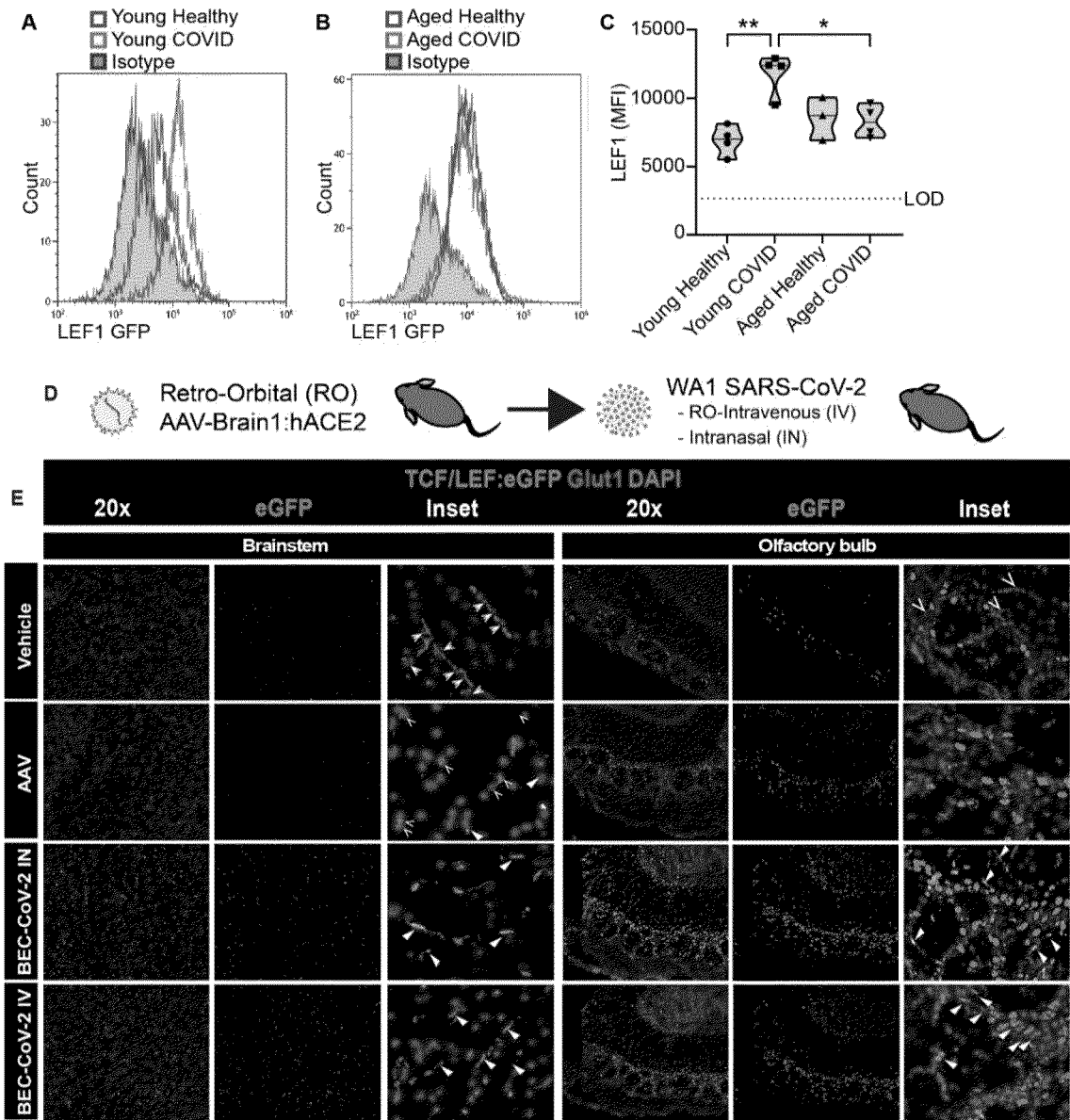


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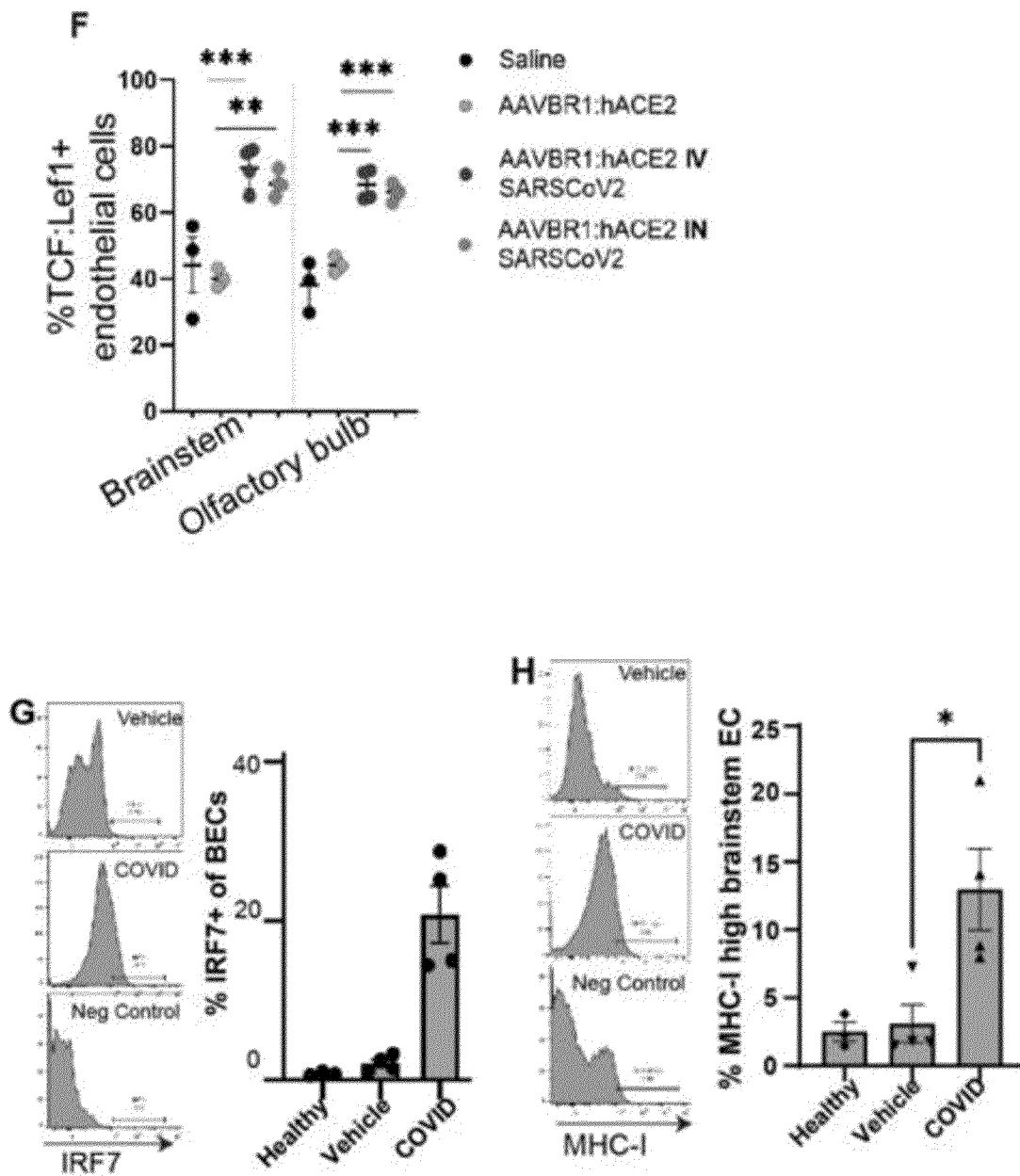


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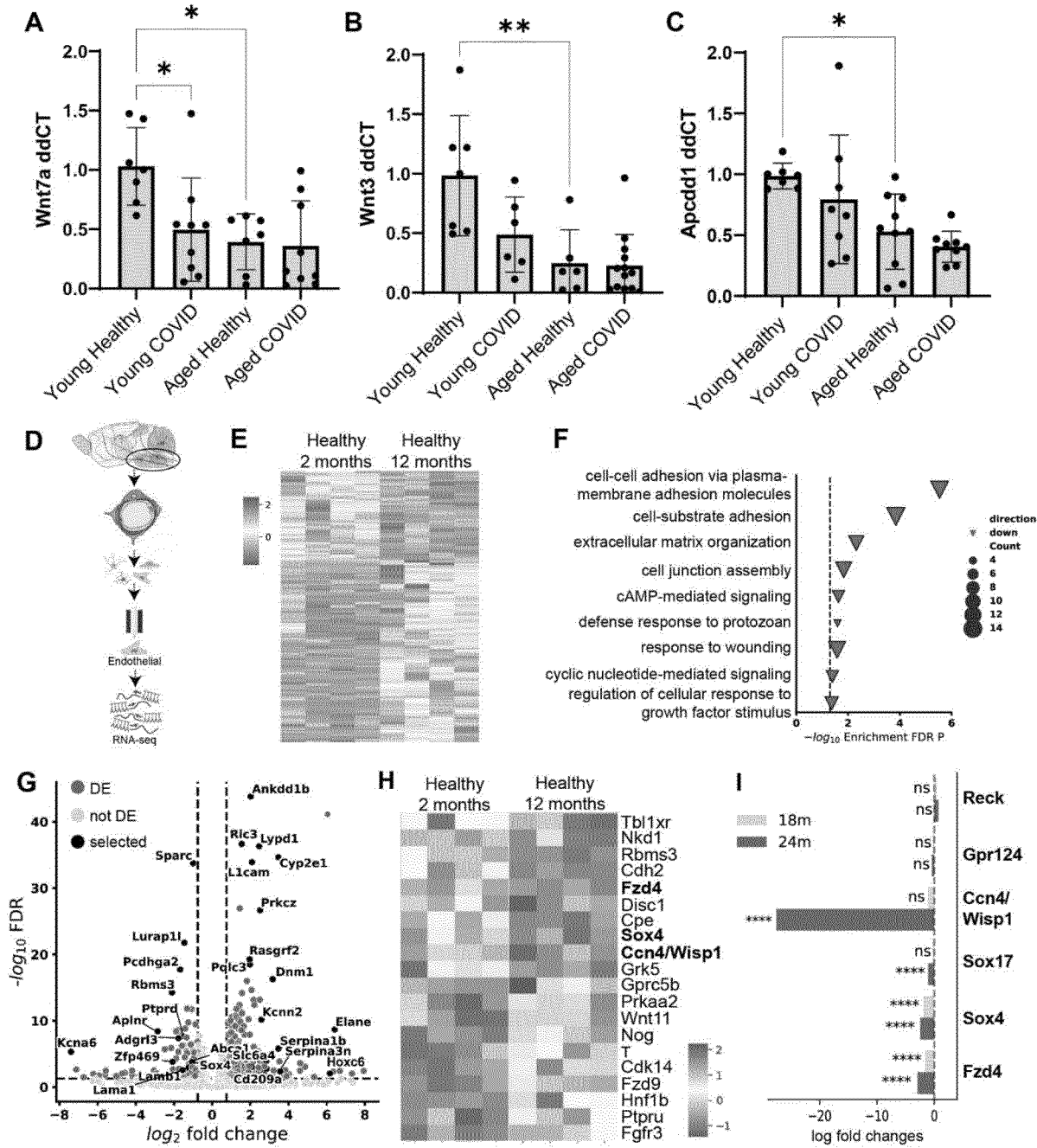


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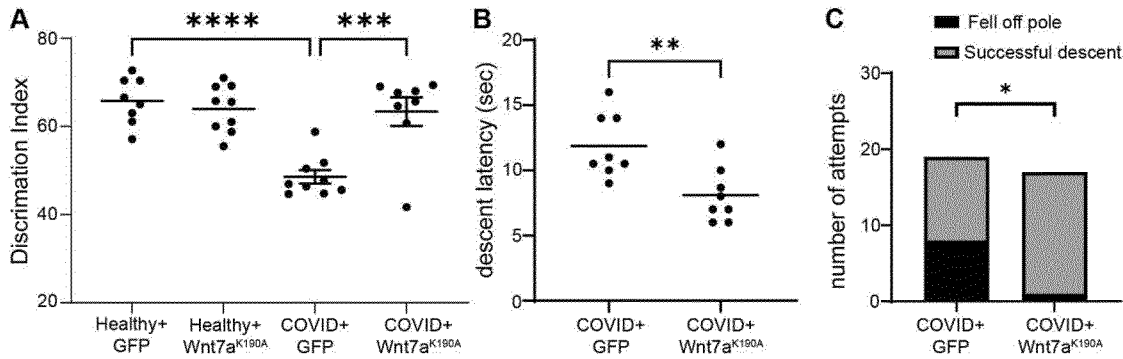
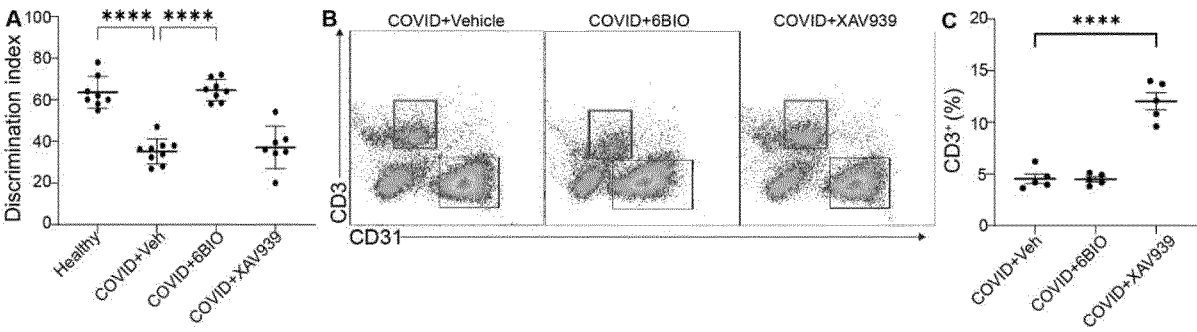


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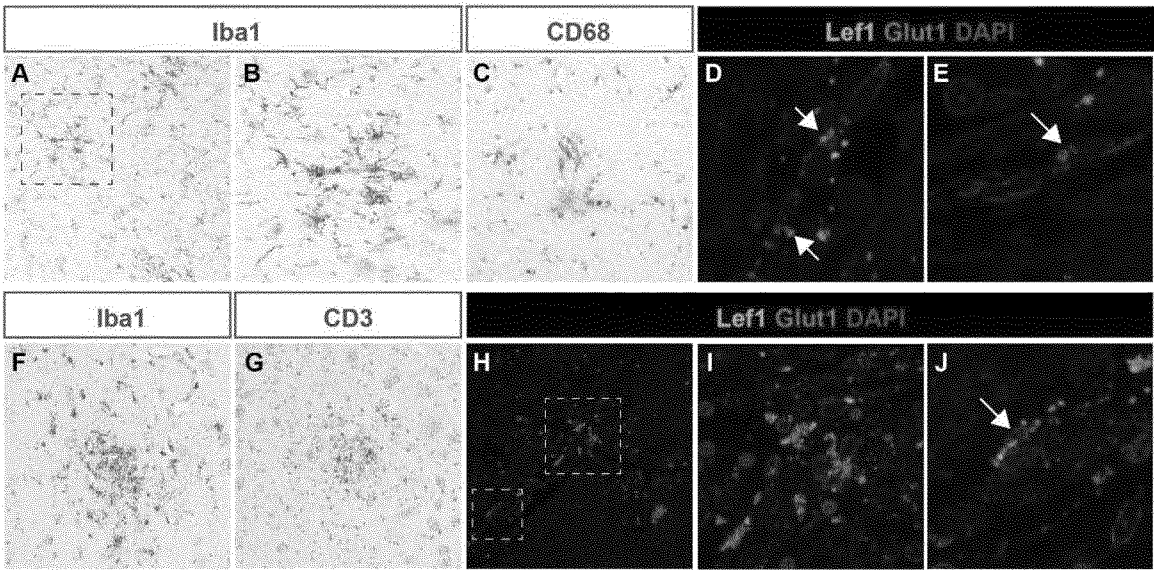


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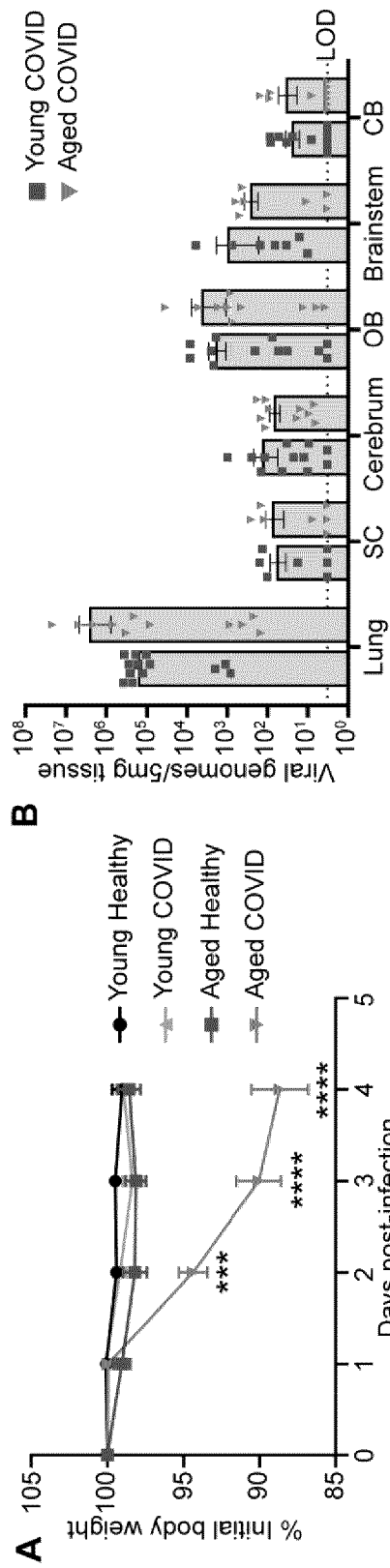


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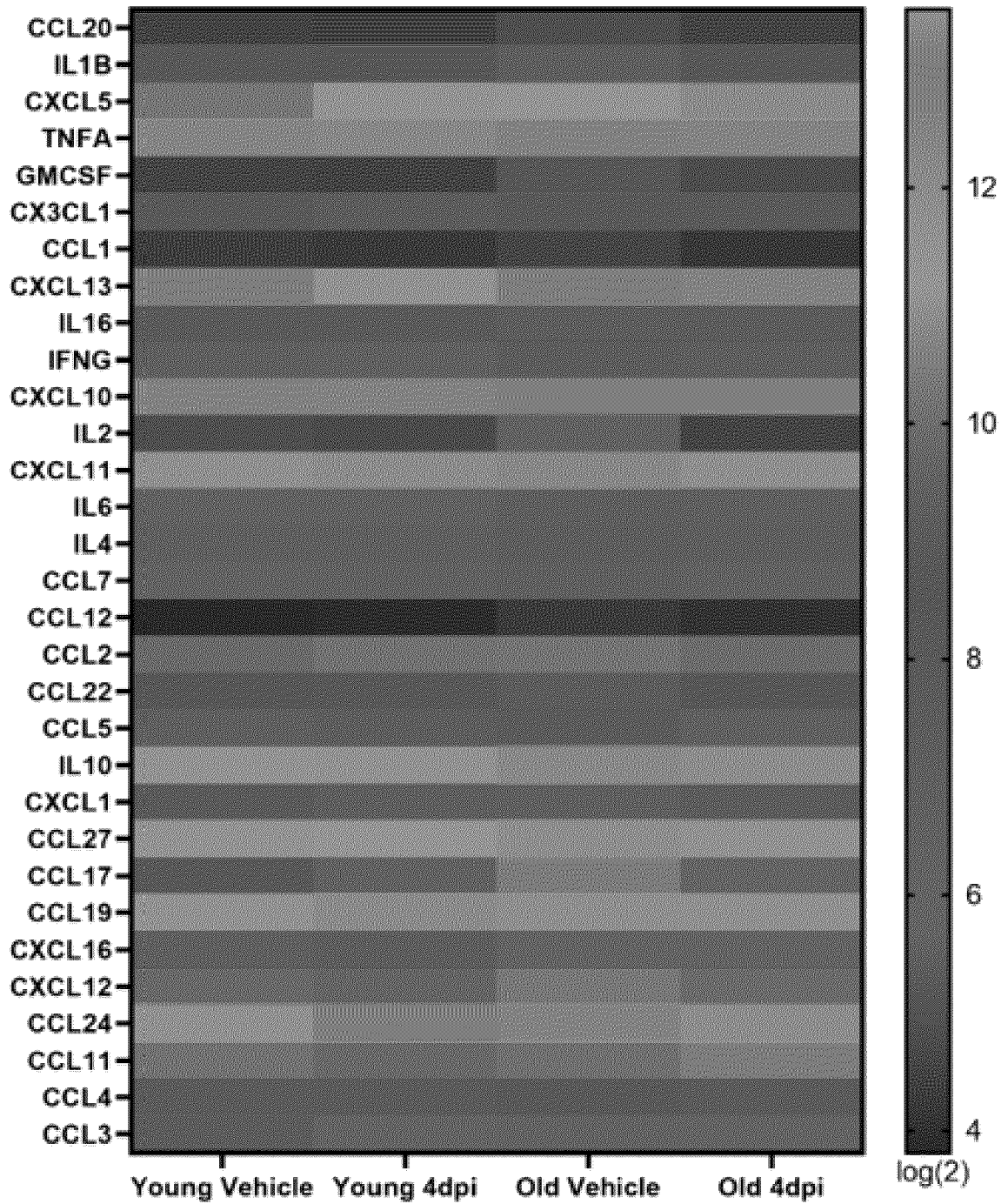
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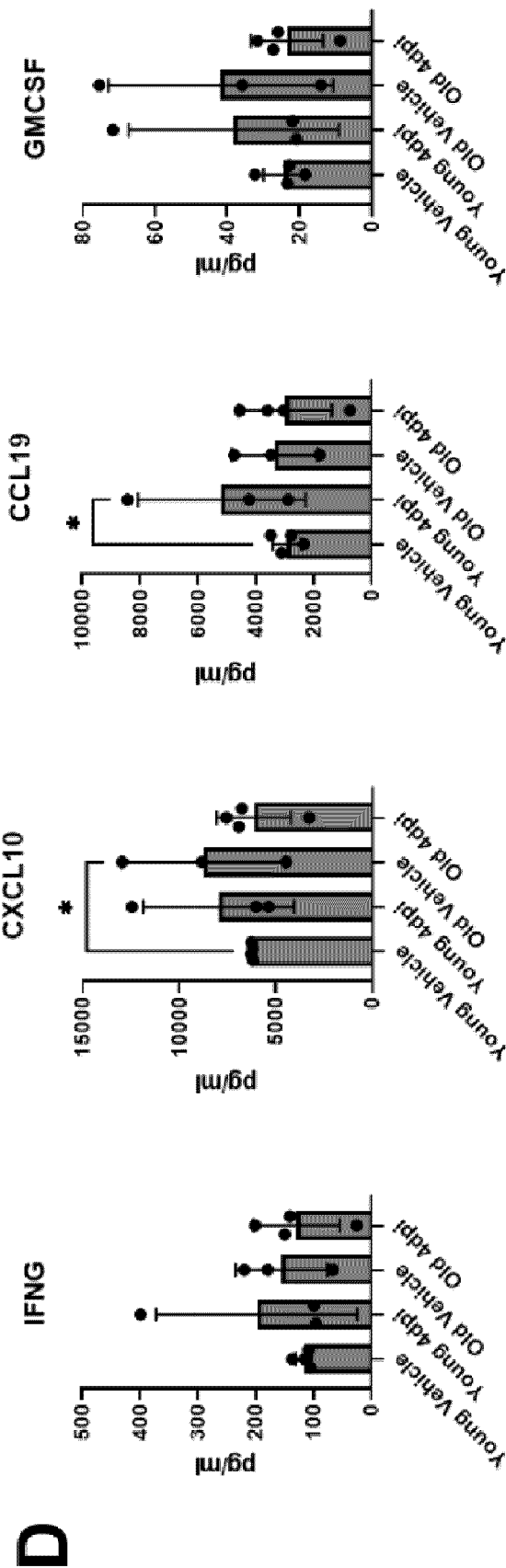


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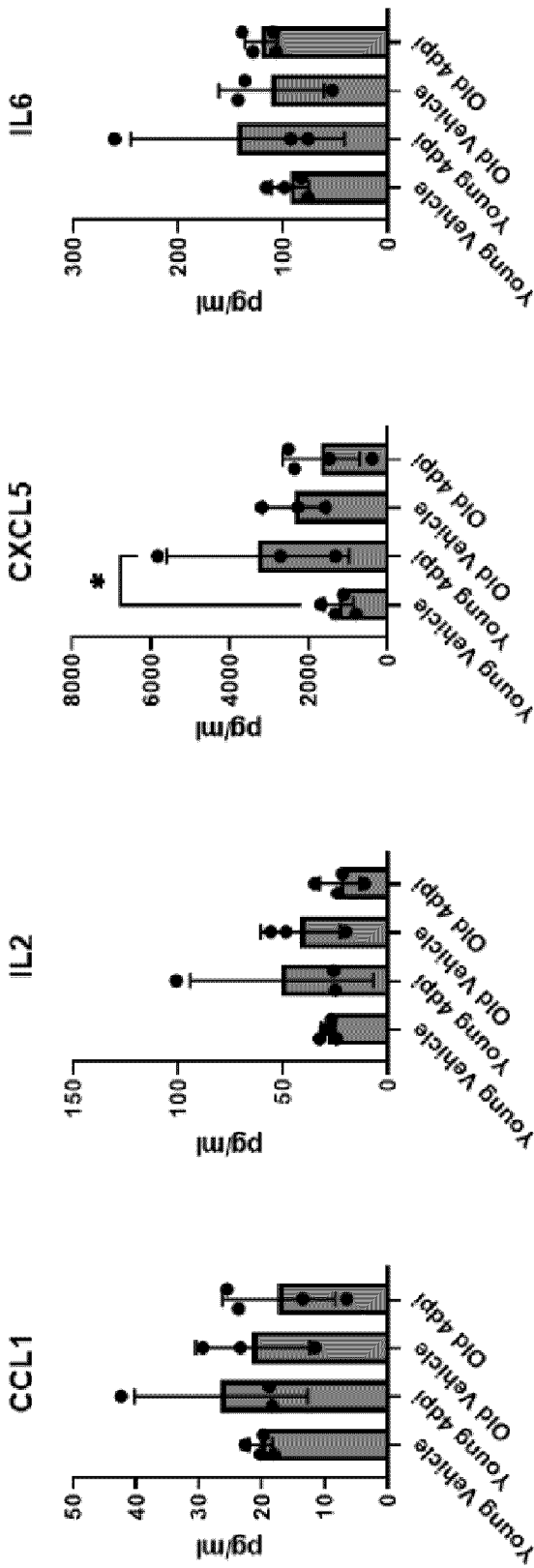


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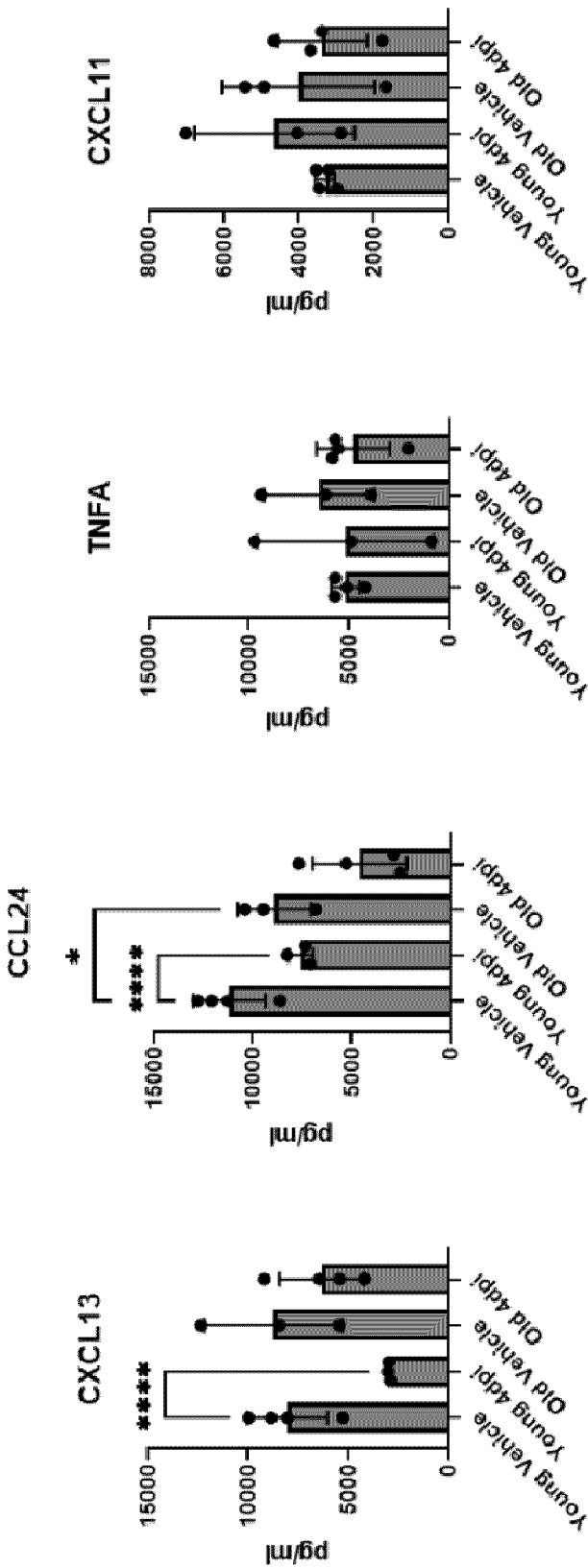


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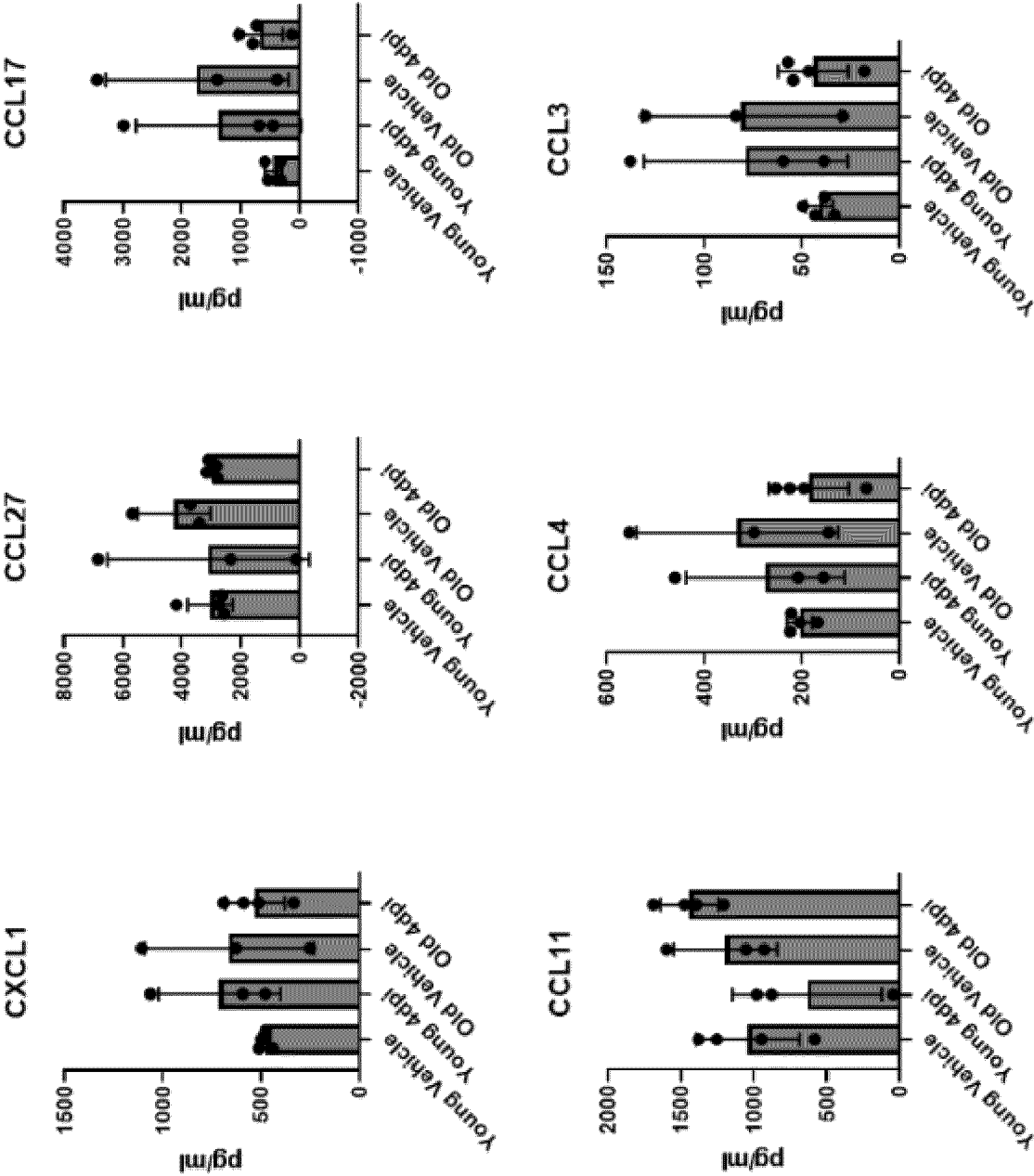


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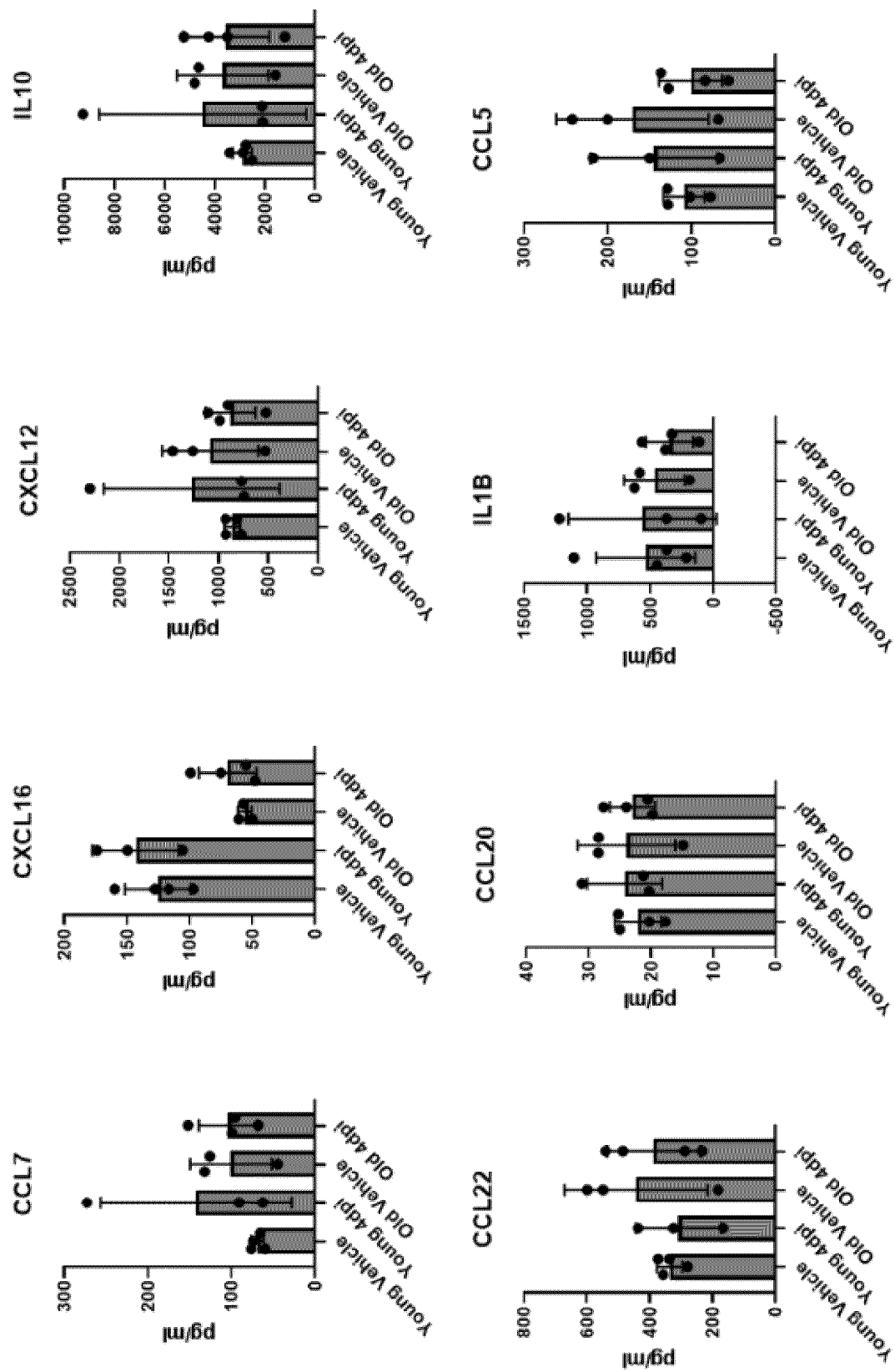
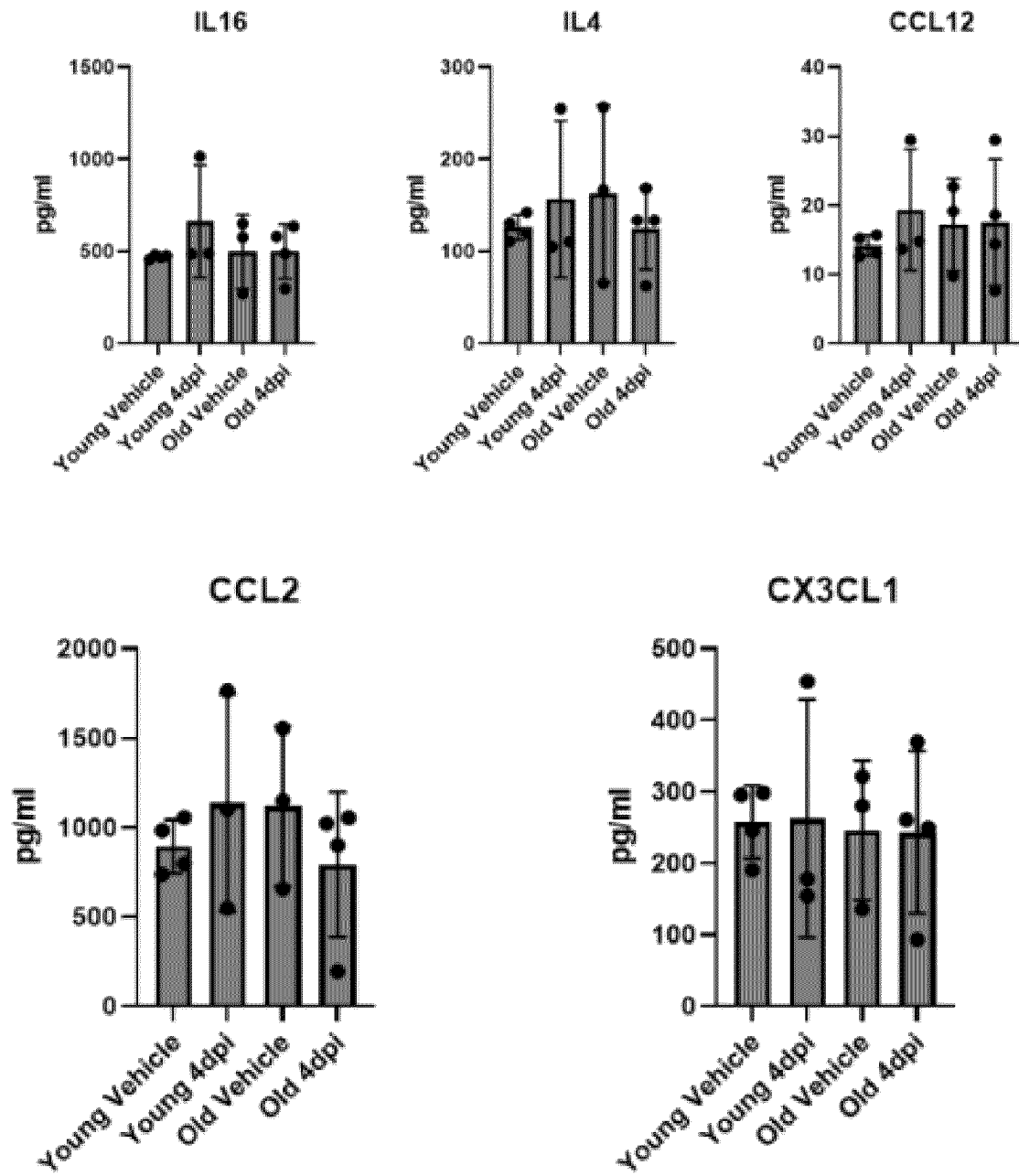


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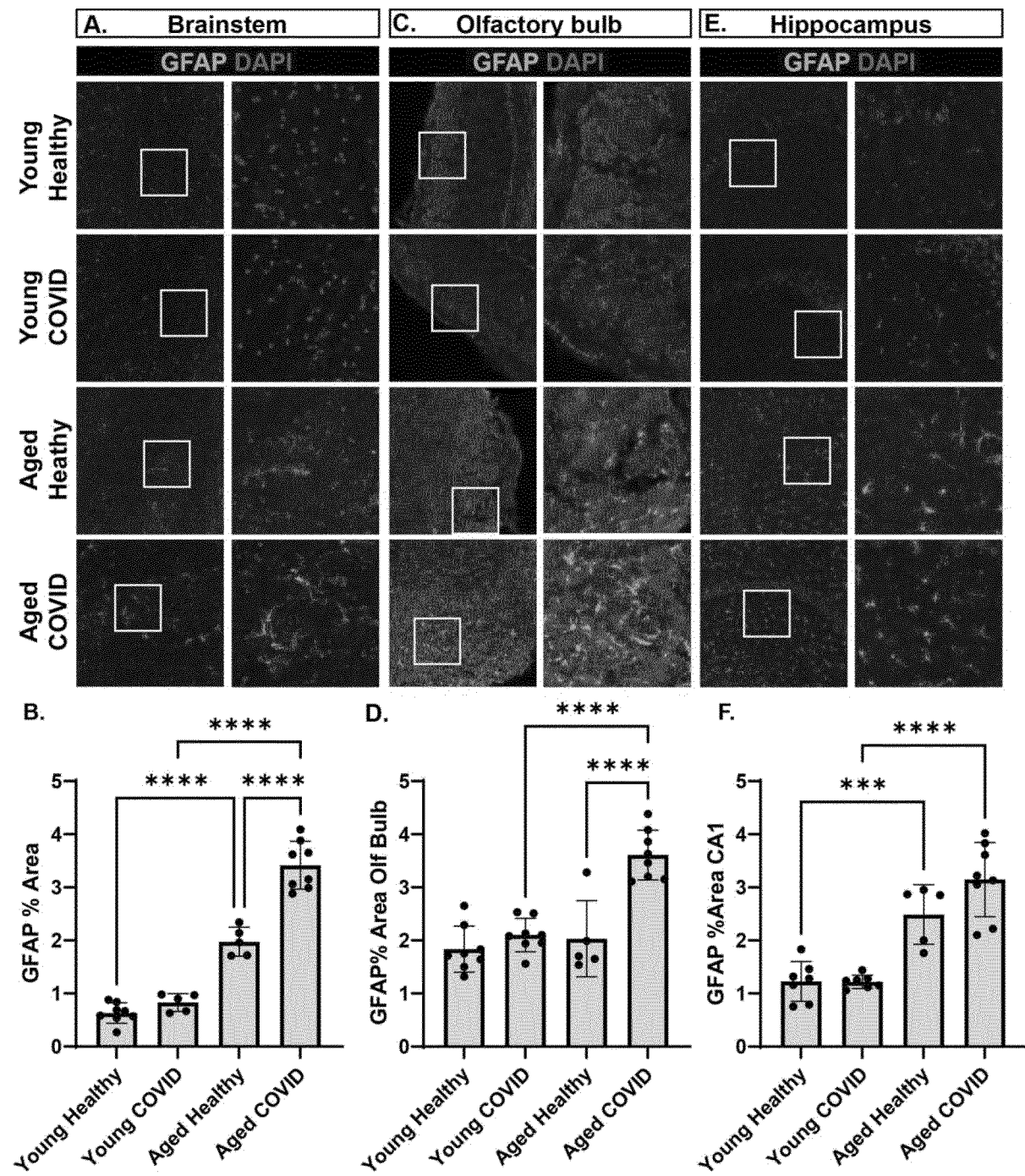


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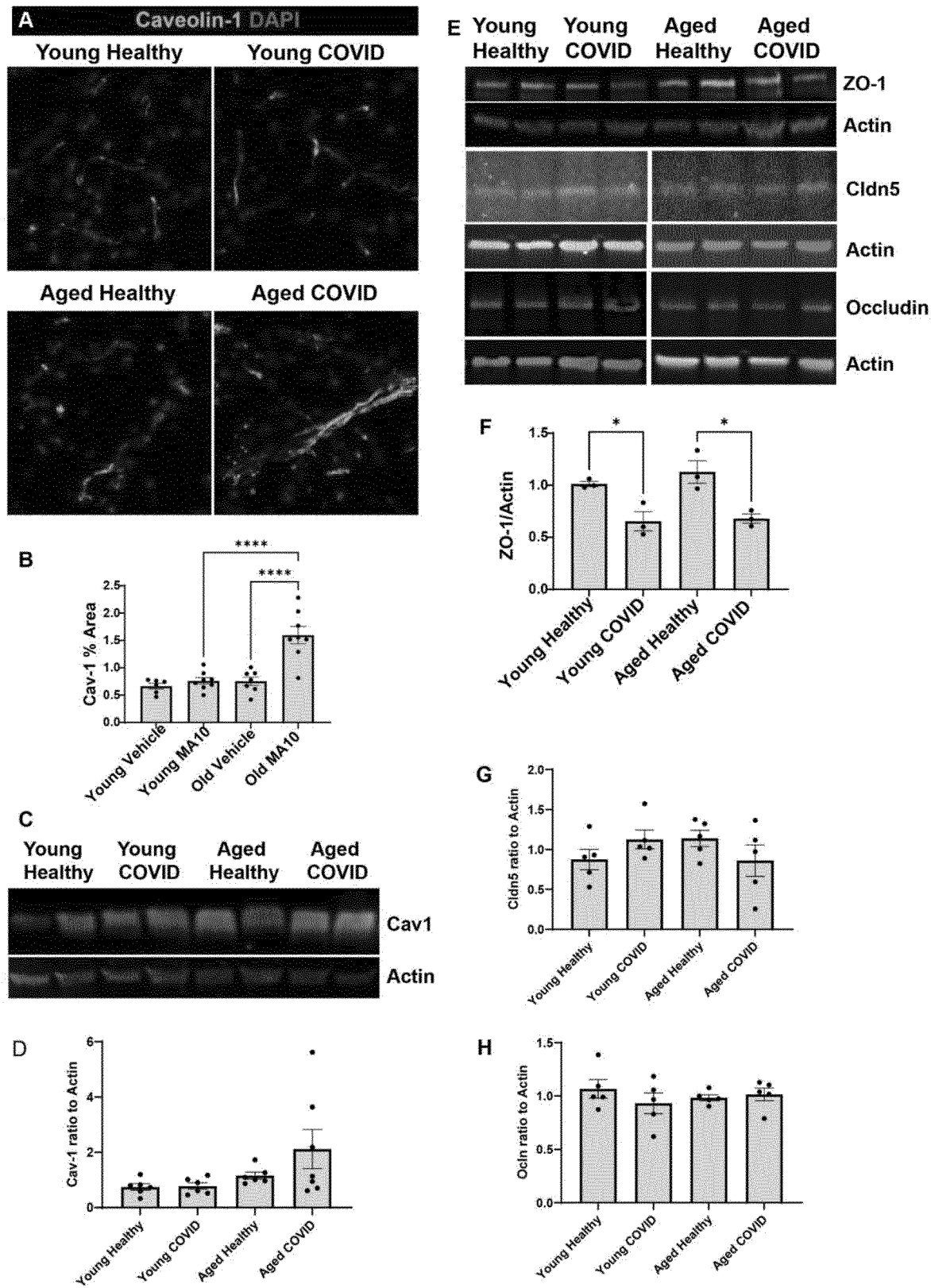
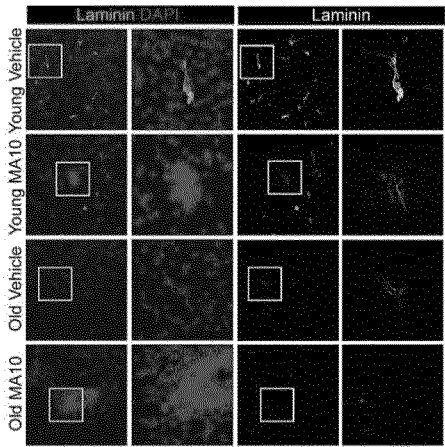


Figure 12:

A



B

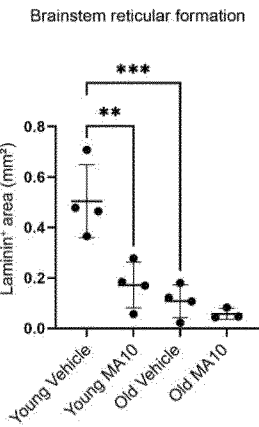


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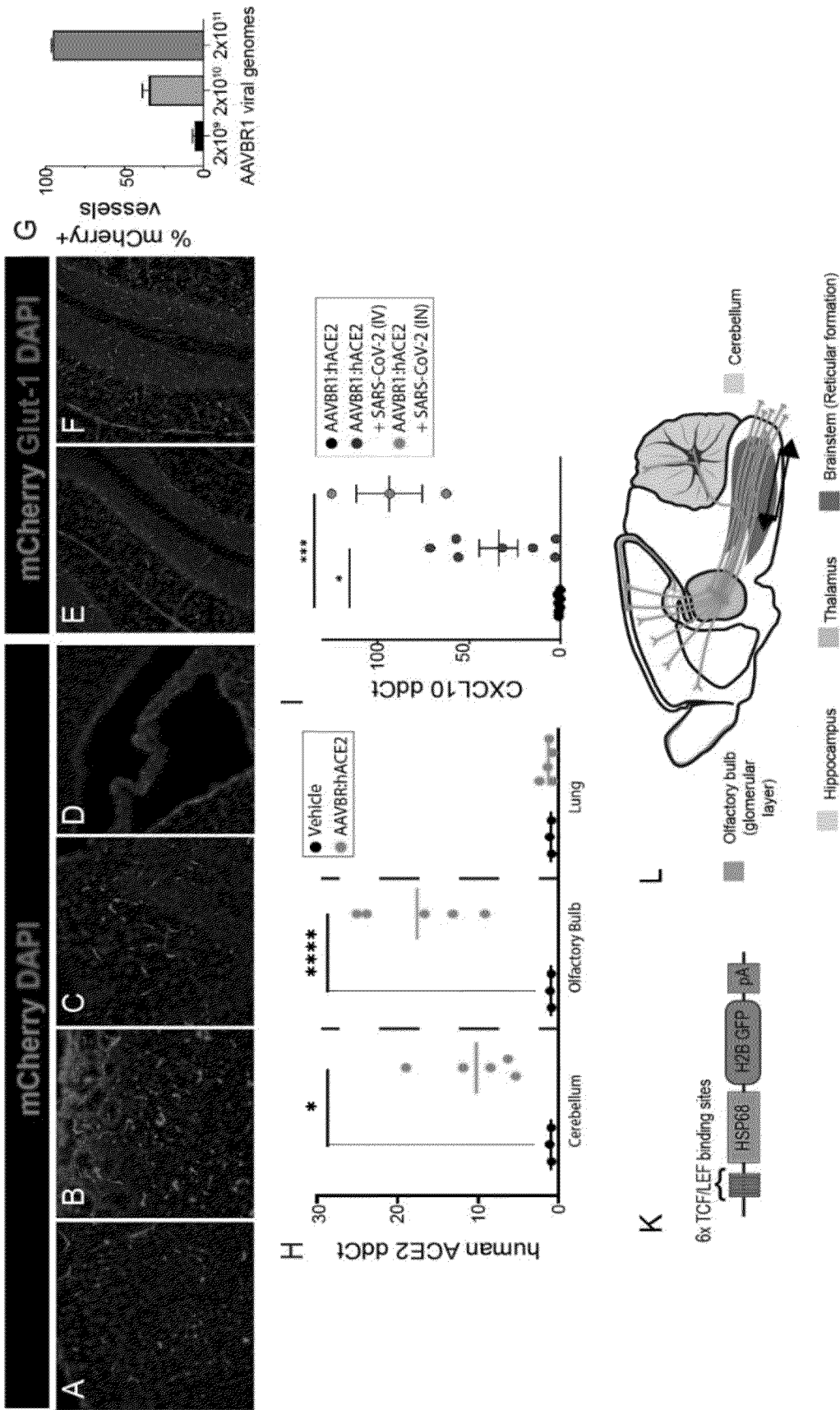


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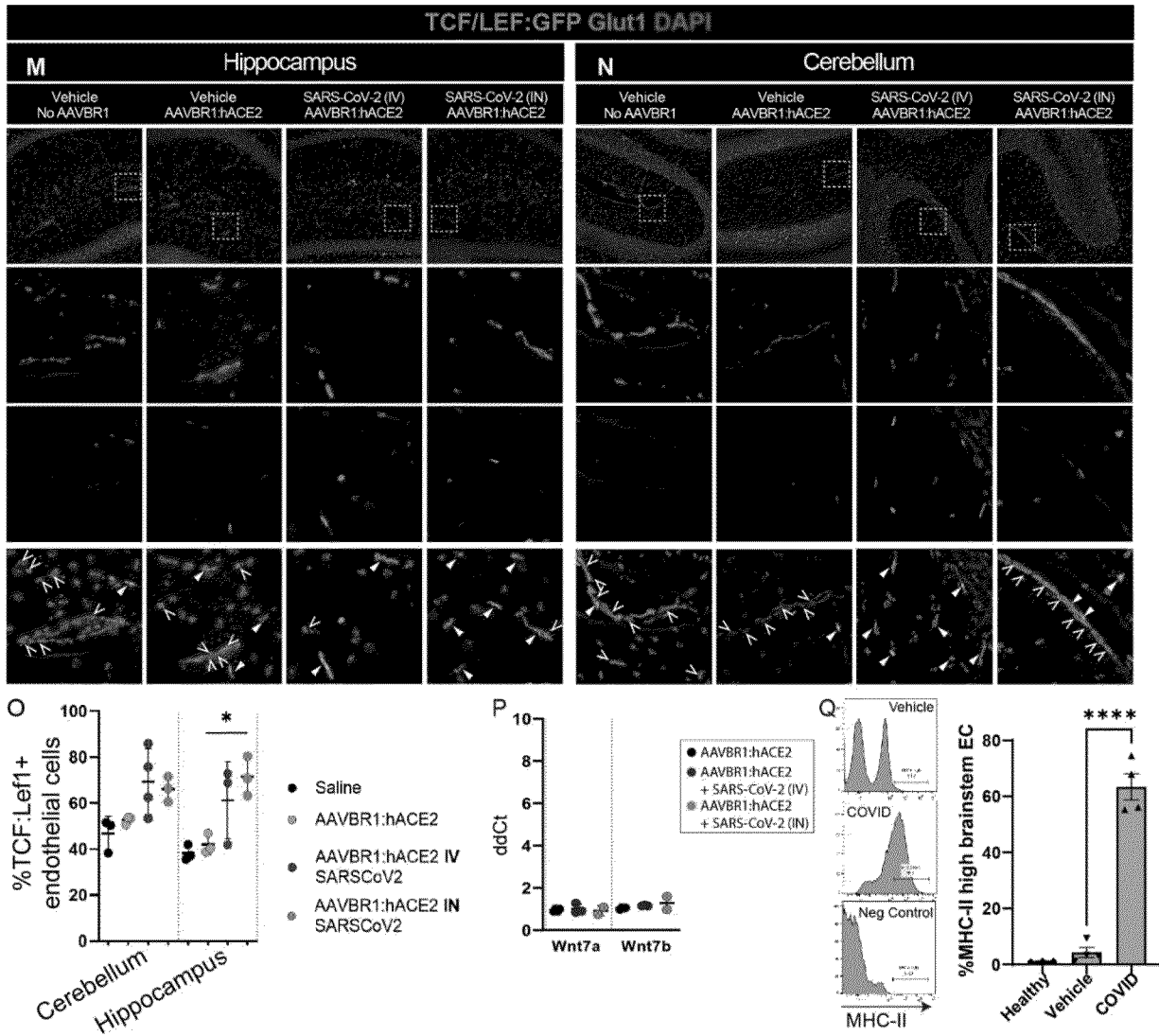
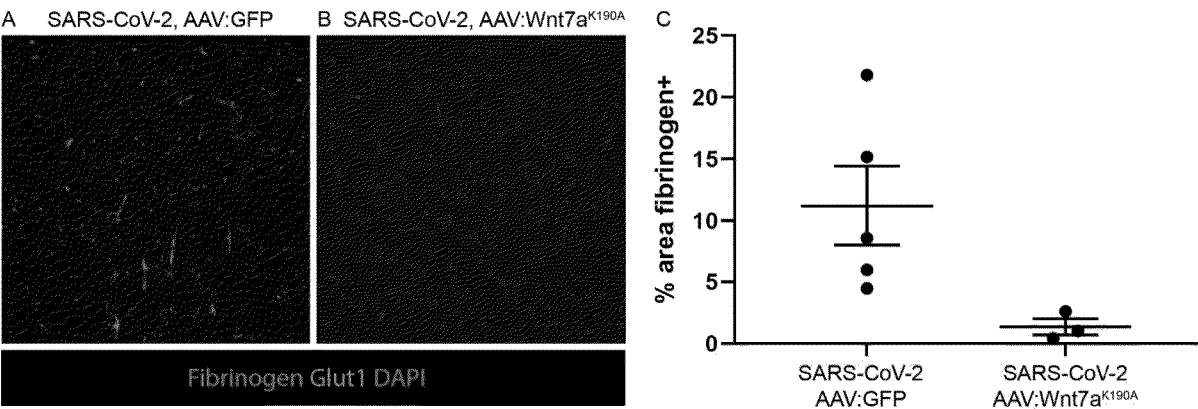


Figure 14



INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2024/057952

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed.
 - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).
☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/057952

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K38/17 A61K9/00 A61P25/00 A61P25/28 A61P31/14
C12N15/01 A61K31/70

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K C12N A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HANNAH E. DAVIS: "Long COVID: major findings, mechanisms and recommendations", NATURE REVIEWS MICROBIOLOGY, vol. 21, no. 3, 13 January 2023 (2023-01-13), pages 133-146, XP093166873, GB ISSN: 1740-1526, DOI: 10.1038/s41579-022-00846-2 Retrieved from the Internet: URL:https://www.nature.com/articles/s41579-022-00846-2> table 1	1-15
X	WO 2008/134632 A1 (HARVARD COLLEGE [US]; MCMAHON ANDREW [US]; STENMAN JAN [US]) 6 November 2008 (2008-11-06) claims 1-36	1-15



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search

28 May 2024

Date of mailing of the international search report

07/06/2024

Name and mailing address of the ISA/

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Authorized officer

Rodrigo-Simón, Ana

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/057952

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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X	WO 2021/077105 A1 (MCLEAN HOSPITAL CORP [US]) 22 April 2021 (2021-04-22) claims 1-28 -----	1-15
X	WO 2019/180204 A1 (UNIV BRUXELLES [BE]) 26 September 2019 (2019-09-26) claims 1-25 -----	1-15
X	WO 2010/052203 A1 (NOVARTIS FORSCHUNGSSTIFTUNG [CH]; CARONI PICO [CH]) 14 May 2010 (2010-05-14) claims 1-14; example 1 -----	1-15
A	Benoit Vanhollebeke ET AL: "Tip cell-specific requirement for an atypical Gpr124- and Reck-dependent Wnt/[beta]-catenin pathway during brain angiogenesis", eLife, 1 January 2015 (2015-01-01), pages 1-25, XP055511303, DOI: 10.7554/eLife.06489 Retrieved from the Internet: URL:https://elifesciences.org/articles/06489 [retrieved on 2018-10-01] the whole document -----	1-15
X,P	TROY N TREVINO: "Engineered Wnt7a ligands rescue blood-brain barrier and cognitive deficits in a COVID-19 mouse model", BRAIN, vol. 147, no. 5, 2 February 2024 (2024-02-02), pages 1636-1643, XP093166585, GB ISSN: 0006-8950, DOI: 10.1093/brain/awae031 Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC11068107/pdf/awae031.pdf> the whole document ----- -/-	1-15

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/057952

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2024/057952

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