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Supplemental information

SARS-CoV-2 Spike protein induces TLR4-mediated long-term cognitive dysfunction recapitulating post-COVID-19 syndrome in mice

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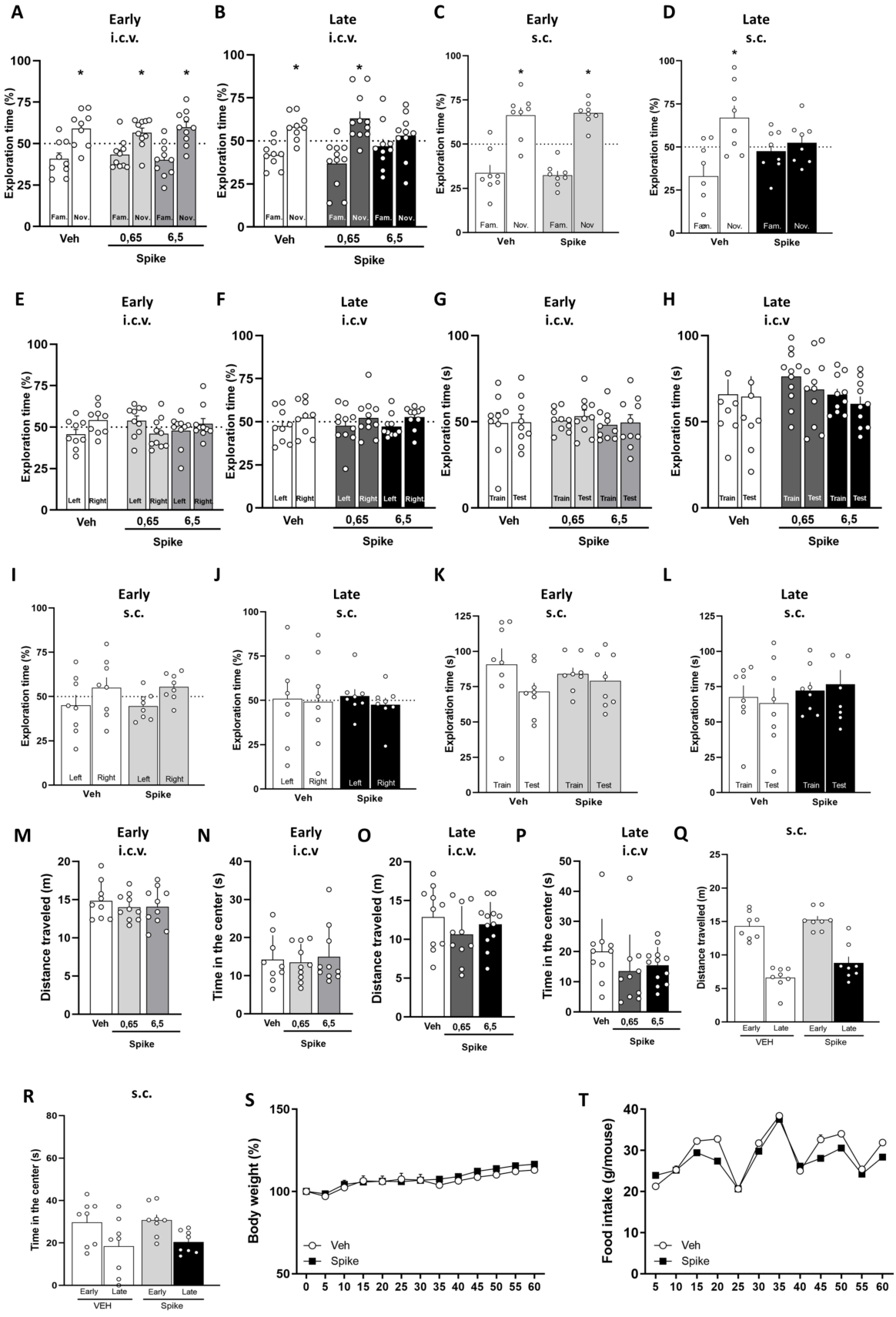
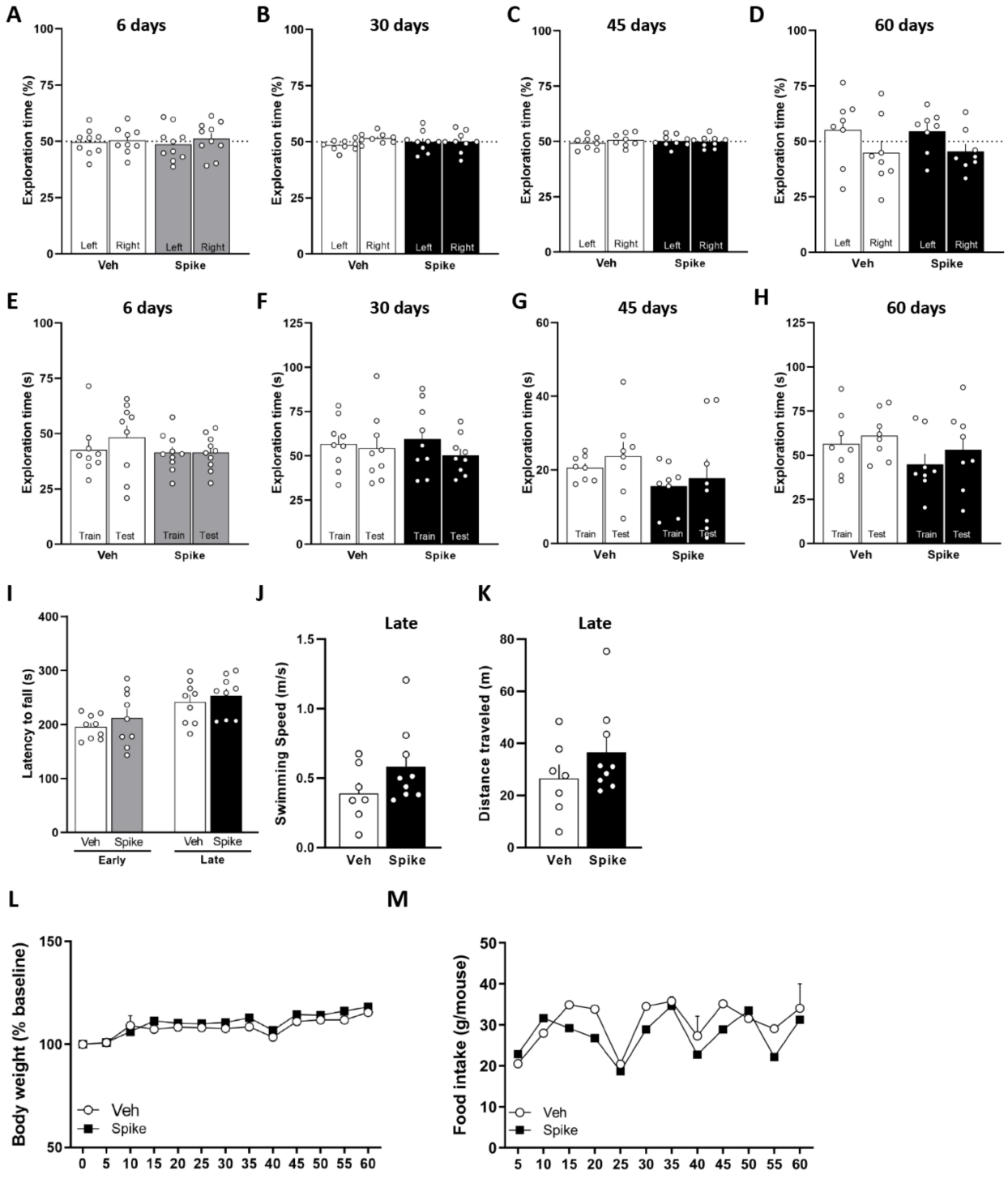
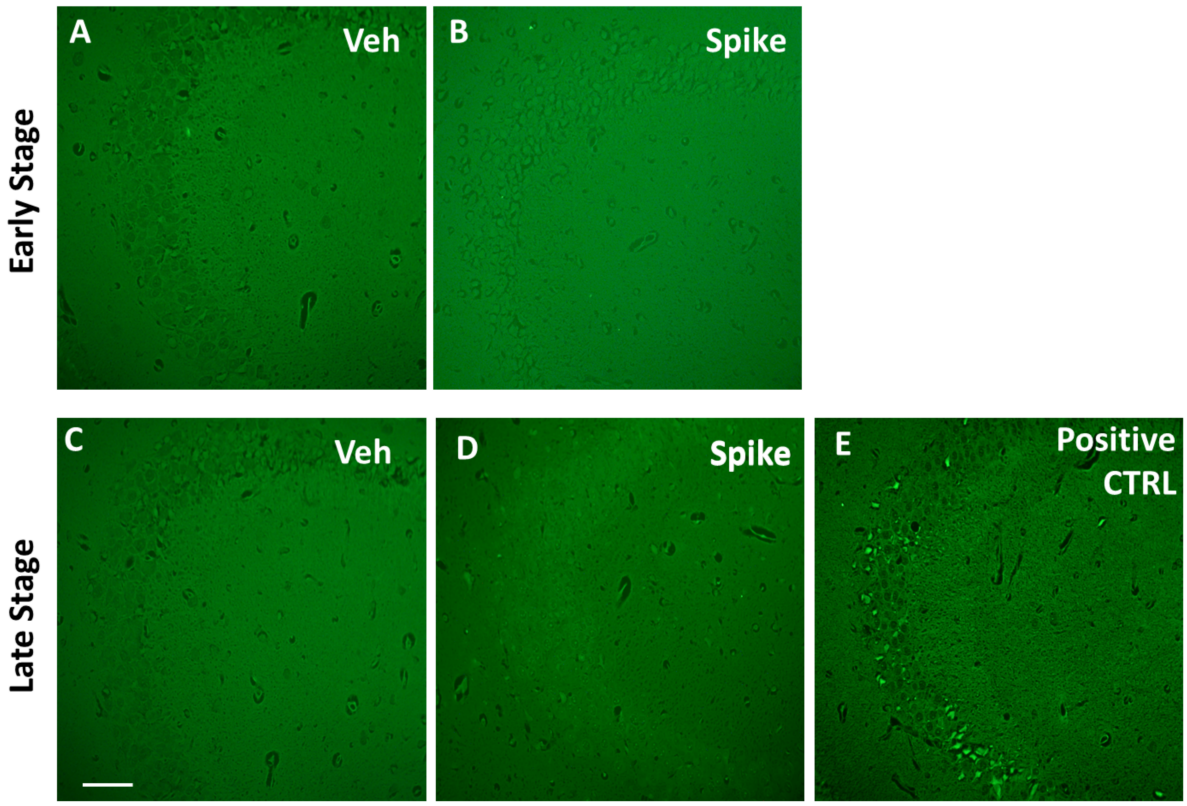

A
Early
i.c.v.
Exploration time (%)
Fam.
Nov.
Veh
0,65
6,5
Spike
B
Late
i.c.v.
C
Early
s.c.
D
Late
s.c.
E
Early
i.c.v.
Left
Right
F
Late
i.c.v
G
Early
i.c.v.
Exploration time (s)
Train
Test
H
Late
i.c.v
I
Early
s.c.
J
Late
s.c.
K
Early
s.c.
L
Late
s.c.
M
Early
i.c.v.
Distance traveled (m)
N
Early
i.c.v
Time in the center (s)
O
Late
i.c.v.
P
Late
i.c.v
Q
s.c.
Distance travelled (m)
Early
Late
VEH
R
s.c.
S
Body weight (%)
Veh
Spike
T
Food intake (g/mouse)

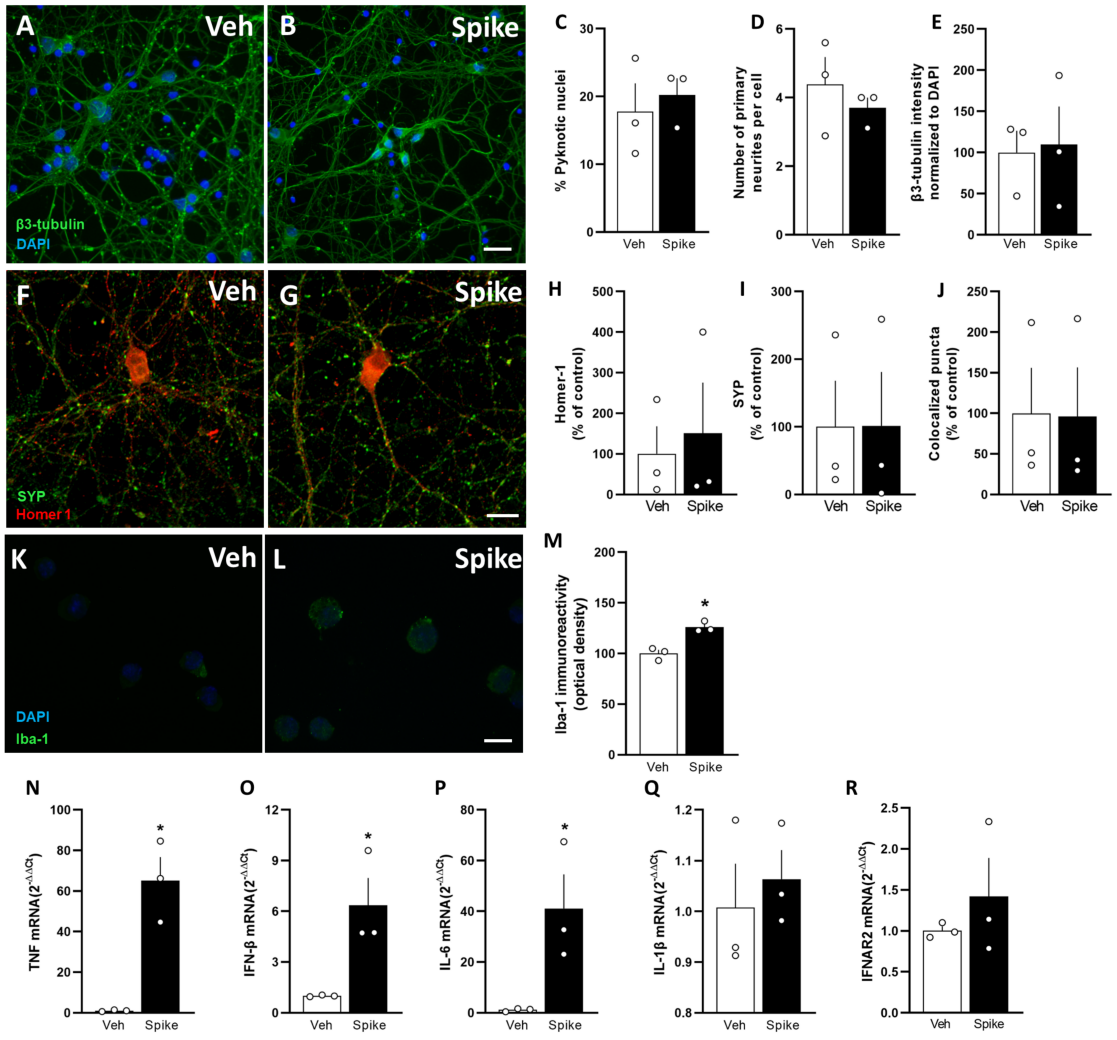
Supplementary Fig. 1 Behavioral analysis of mice infused with SARS-CoV-2 Spike protein by intracerebroventricular (i.c.v.) or subcutaneous (s.c.) route. Related to Figure 1. Mice were infused with vehicle (Veh) or Spike protein by i.c.v. (0,65 or 6,5 μg/site) or s.c. (10 μg) route and were evaluated at early (6 days) and late (45 days) time points. (**A and B**) Mice were tested in the novel object recognition (NOR) test at early (**A**; $t = 2.578$, $^*p = 0.0327$ for Veh, $t = 2.400$ $^*p = 0.0399$, for 0,65 μg Spike, $t = 3.052$ $^*p = 0.0138$, for 6,5 μg Spike) or late (**B**; $t = 3.307$, $^*p = 0.0107$ for Veh, $t = 3.214$ $^*p = 0.0093$, for 0,65 μg Spike, $t = 0.7246$ $p = 0.4871$, for 6,5 μg Spike) time points after i.c.v. infusion. (**C and D**) Mice were tested in the novel object recognition (NOR) test at early (**C**: $t = 3.647$, $^*p = 0.0082$ for Veh; and $t = 7.466$, $^*p = 0.0001$, for Spike) or late (**D**) $t = 2.416$, $^*p = 0.0463$ for Veh and $t = 0.5562$, $p = 0.5954$, for Spike) time points after s.c. infusion. One-sample Student's t-test compared to the chance level of 50%; N = 8-11 mice per group). (**E-L**) Neither i.c.v. nor s.c. Spike protein infusion affected innate object preferences during the training session (**E and F, I and J**), or exploratory activity (**G and H, K and L**) during the test session of NOR at early and late time points after protein infusion. (**E**) Early stage ($t = 1.477$, $p = 0.1789$ for Veh, $t = 1.357$, $p = 0.2079$, for 0,65 μg Spike, $t = 0.6648$ $p = 0.5228$, for 6,5 μg Spike), and (**F**) late stage ($t = 0.7313$, $p = 0.4855$ for Veh, $t = 0.7105$ $p = 0.4937$, for 0,65 μg Spike, $t = 1.277$, $p = 0.2336$, for 6,5 μg Spike) after i.c.v. infusion. One-sample Student's t-test compared to the chance level of 50% (N = 9-11 mice per group). (**G**)Early stage ($F = 1.1411$, $p = 0.3345$ for Training and $F = 0.2435$, $p = 0.7857$ for Test), and (**H**) late stage ($F = 0.1117$, $p = 0.8947$ for Training and $F = 0.3122$, $p = 0.7344$ for Test) after i.c.v. infusion. One-way ANOVA test, followed by Tukey's test ($N = 9-11$ mice per group). (**I**) Early stage ($t = 0.8437$, $p = 0.4267$ for Veh; and $t = 2.008$, $p = 0.0846$, for Spike), and (**J**) late stage ($t = 0.9215$, $p = 0.9292$ for Veh and $t = 0.6250$, $p = 0.5518$, for Spike) after s.c. infusion. One-sample Student's t-test compared to the chance level of 50%; $N = 8$ mice per group. (**K**) Early stage ($t = 0.5526$, $p = 0.5893$ for Training and $t = 0.8203$ $p = 0.4258$, for Test), and (**L**) Late stage ($t = 0.4536$, $p = 0.6570$ for Training and $t = 0.9041$, $p = 0.3812$, for Test) after s.c. infusion; Student's t-test; $N = 8$ mice per group. (**M, O and Q**) Total distance traveled and (**N, P and R**) time spent at the center of the open field arena by or i.c.v.- (**M-P**), or s.c.-infused (**Q and R**) mice. (**M**) Early stage ($F = 0.4086$, $p = 0.6688$). (**O**) Late stage ($F = 1.231$, $p = 0.3074$). One-way ANOVA test, followed by Tukey's test; $N = 9-11$ mice per group. (**N**) Early stage ($F = 0.1360$, $p = 0.8734$, One-way ANOVA test, followed by Tukey's test). (**P**) Late stage ($p = 0.1103$, Kruskal-Wallis test). $N = 9-11$ mice per group. (**Q**) $t = 1.057$, $p = 0.3085$ for early, and $t = 1.967$, $p = 0.0693$ for late stage; (**R**) $t = 0.2321$, $p = 0.8191$ for early, and $t = 0.3775$, $p = 0.7115$ for late stage. Student's t-test; $N = 8$ mice per group. (**S**) Body weight ($F(12,182) = 0.3791$, $p = 0.9696$, and (**T**) food intake ($F(11,168 = 1.444$, $p = 0.1576$) measured for up to 60 days following Veh or Spike s.c. infusion. Two-way ANOVA test followed by Bonferroni ($N = 8$ mice per group). Bars or points represent means ±SEM. Symbols represent individual mice.


A
6 days
B
30 days
C
45 days
D
60 days
Exploration time (%)
Left
Right
Veh
Spike
E
6 days
F
30 days
G
45 days
H
60 days
Exploration time (s)
Train
Test
I
Latency to fall (s)
Early
Late
J
Late
Swimming Speed (m/s)
K
Late
Distance traveled (m)
L
Body weight (% baseline)
Veh
Spike
M
Food intake (g/mouse)

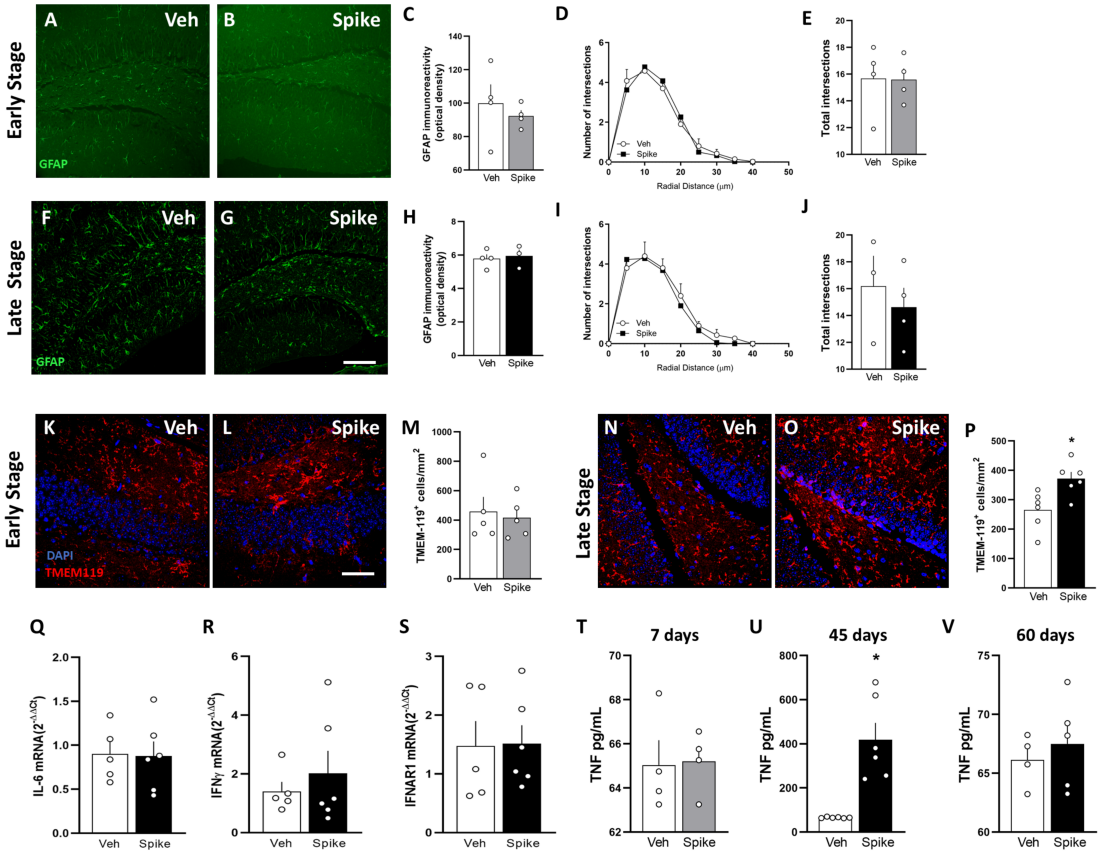
Supplementary Fig. 2 Controls for behavioral analysis of mice infused with SARS-CoV-2 Spike protein. Related to Figure 1. Mice were infused with vehicle (Veh) or Spike protein by i.c.v. (6,5 μg/site) route, and were evaluated at different time points after infusion. Intracerebroventricular (i.c.v.) infusion of Spike protein had no effect on innate preference for the objects during the training session (**A-D**), or exploratory activity (**E-H**) during the test session of novel object recognition (NOR) test at 6, 30, 45 and 60 days after protein infusion. (**A**) 6 days ($t = 0.1869$, $p = 0.8564$ for Veh; and $t = 0.5302$, $p = 0.6088$, for Spike), (**B**) 30 days ($t = 2.009$, $p = 0.0794$ for Veh; and $t = 0.03443$, $p = 0.9734$, for Spike), (**C**) 45 days ($t = 0.6465$, $p = 0.5386$ for Veh; and $t = 0.2022$, $p = 0.8448$, for Spike), and (**D**) 60 days ($t = 0.9527$, $p = 0.3725$ for Veh; and $t = 1.381$, $p = 0.2098$, for Spike). One-sample Student's t-test compared to the chance level of 50%; $N = 8-10$ mice per group. (**E**) 6 days ($t = 0.2549$, $p = 0.8019$ for Training and $t = 1.174$, $p = 0.2565$ for Test), (**F**) 30 days ($t = 0.3569$, $p = 0.7258$ for Training and $t = 0.8627$, $p = 0.4011$, for Test), (**G**) 45 days ($t = 1.921$, $p = 0.07553$ for Training and $t = 0.9256$, $p = 0.3793$, for Test), (**H**) 60 days $t = 1.346$, $p = 0.1998$ for Training and $t = 0.8578$, $p = 0.4055$, for Test). Student's t-test; $N = 8-10$ mice per group. (**I**) No difference between groups was found when mice were tested in the Rotarod task at early (6 days; $t = 0.9060$, $p = 0.3784$) and late (45 days; $t = 0.6381$, $p = 0.5325$) time points following Veh or Spike infusion. Student's t-test; $N = 9$ mice per group. Spike protein had no effect on swimming speed (**J** $p = 0.1416$) or total distance traveled (**K** $p = 0.2523$) in the Morris Water Maze at the late stage (45 days post infusion). Mann-Whitney U test; $N = 7-9$ mice per group. (**L**) Body weight ($F(12,182) = 0.2997$, $p = 0.9888$, and (**M**) food intake ($F(11,168) = 1.592$, $p = 0.1051$) measured for up to 60 days following Veh or Spike i.c.v. infusion. Two-way ANOVA test followed by Bonferroni ($N = 8$ mice per group). Bars or points represent means ±SEM. Symbols represent individual mice.



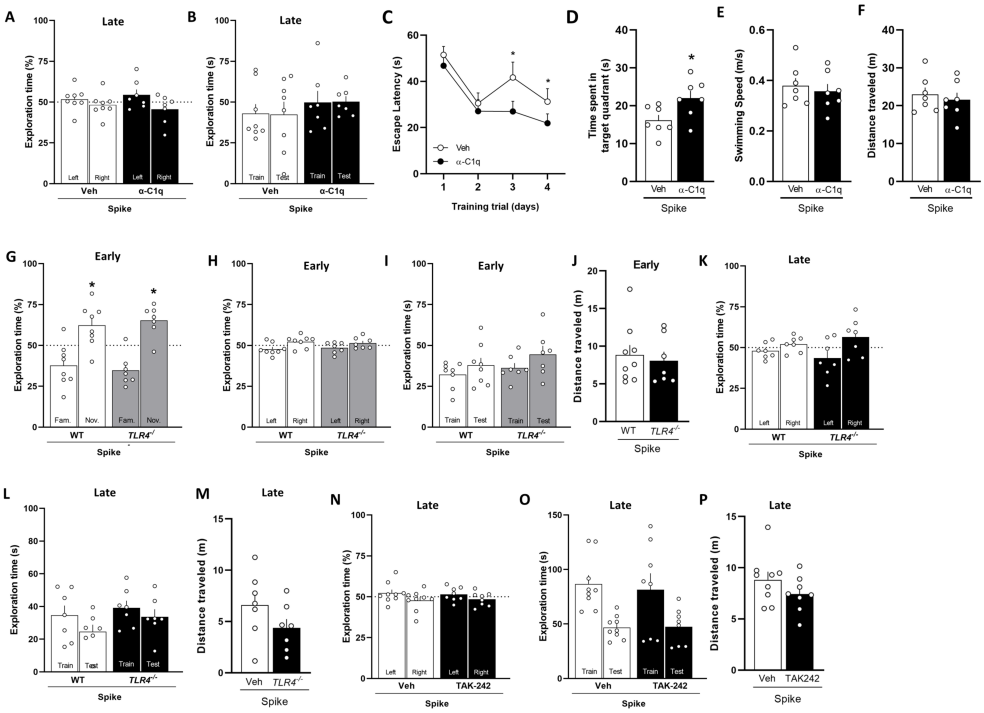
Supplementary Fig. 3 Analysis of neuronal cell death in the hippocampus of SARS-CoV-2 Spike protein-infused mice. Related to Figure 1. Mice received an i.c.v. infusion of 6,5 μg SARS-CoV-2 spike protein (Spike) or vehicle (Veh), and brains were processed for Fluoro-Jade B staining. Representative staining of the hippocampal DG region at early (7 days; **A and B**) and late (45 days; **C and D**) time points after infusion. $N = 4$ mice per group. (**E**) Fluoro-Jade B staining positive control consisted of brain sections of a mouse infused i.c.v. with the neurotoxin quinolinic acid. Scale bar $= 50\mu m$.



Supplementary Fig. 4 Effect of SARS-CoV-2 Spike protein incubation in microglial and neuronal cultures. Related to Figure 2. **(A-J)** Cultured primary cortical neurons were incubated with Spike protein (1μg/mL) or vehicle (Veh) for 24h, and analyzed by immunocytochemistry. (**A and B**) Representative images of β3-tubulin and DAPI immunoreactivity. Scale bar = 50μm. (**A-E**) Spike protein causes no changes in neither number of pyknotic nuclei (**C**; $p > 0.9999$, Mann-Whitney U test) and primary neurites (**D**; $t = 0.8031$, $p = 0.4669$, Student's t-test), nor β3-tubulin intensity (**E**; $t = 0.1824$, $p = 0.8642$, Student's t-test). (**F and G**) Representative images of Homer-1 and synaptophysin (SYP) immunoreactivity. Scale bar = 10μm. (**F-J**) Spike protein also induces no difference in the number of synapses in cortical neurons, as demonstrated by double immunostaining for Homer-1 (**H**; $p > 0.9999$, Mann-Whitney U test), SYP (**I**; $t = 0.01403$, $p = 0.9895$, Student's t-test), and colocalized Homer-1/SYP puncta (**J**; $t = 0.04320$, $p = 0.9676$, Student's t-test). $N = 3$ experiments with independent neuron cultures. (**K and L**) Representative images of IBA-1 immunoreactivity in BV-2 cells incubated for 24 h with vehicle (Veh; **K**) or Spike protein (**L**; 1 μg/mL). Scale bar = 50μm. (**M**) Iba-1 and DAPI immunoreactivity ($t = 5.567$, $*p = 0.0051$). (**N-R**) BV2 cells incubated with Spike or Veh were analyzed by qPCR for mRNA levels of TNF (**N**; $t = 5.557$, $*p = 0.0051$), IFN-β (**O**; $t = 3.307$, $*p = 0.0297$), IL-6 (**P**; $t = 2.968$, $*p = 0.0412$), IL-1β (**Q**; $t = 0.5398$, $p = 0.6180$), and IFNAR2 (**R**; $t = 0.8884$, $p = 0.4245$). Student's t-test; $N = 3$. Bars represent means ±SEM.



Supplementary Fig 5 Analysis of glial cell activation and cytokine expression in the hippocampus of SARS-CoV-2 Spike protein-infused mice. Related to Figure 2. Mice received an i.c.v. infusion of 6,5 μg SARS-CoV-2 spike protein (Spike) or vehicle (Veh), and brains were processed for analysis at early (7 days) and late (45 and 60 days) time points. (**A-J**) Spike protein had no effect on GFAP immunoreactivity or GFAP-positive cell morphology in the DG region of the hippocampus. Representative images of GFAP immunoreactivity at early (**A and B**) and late (**F and G**; 45 days) time points. Scale bar $= 20\mu m$. GFAP immunoreactivity (**C** $t = 0.6543$, $p = 0.5372$), and Sholl analysis (**D and E**; $F(8,54) = 0.5484$, $p = 0.8147$, and $t = 0.05462$, $p = 0.9582$, respectively) at the early stage of the model. GFAP immunoreactivity (**H**; $t = 0.3638$, $p = 0.7309$), and Sholl analysis (**I and J**;$F(8,45) = 0.3151$, $p = 0.9563$, and $t = 0.6199$, $p = 0.5625$, respectively) at the late stage of the model (45 days). Two-way ANOVA test followed by Bonferroni (**D and I**), and Student's t-test (**E and J**). $N = 3-4$ mice per group. Representative images of TMEM-119 immunoreactivity at early (**K and L**) and late (**N and O**; 45 days) time points in hippocampal DG region. Scale bar $= 20\mu m$. TMEM-119-positive cells in the hippocampi of Veh- or Spike-infused mice in the early (**M**; $t = 0.3669$; $p = 0.7232$) and late (**P**; $t = 3.036$; $*p = 0.0125$; 45 days) stages of the model. Student's t-test, $N = 5$ mice per group). (**Q-S**) qPCR analysis of indicated mRNA isolated from the hippocampus in the late stage of the model (45 days). Spike protein infusion had no effect on mRNA levels of IL-6 (**Q**; $t = 0.0979$; $p = 0.9241$), IFNγ (**R**; $t = 0.9586$; $p = 0.3304$) and IFNAR1 (**S**; $t = 0.3336$; $p = 0.7456$). $N = 5-6$ mice per group. (**T-V**) ELISA analysis of time-dependent serum levels of TNF in Veh- or Spike-infused mice at 7 days (**T**; $t = 0.128$; $p = 0.9021$), 45 days (**U**; $t = 4.636$; $*p = 0.009$), and 60 days post-infusion (**V**; $t = 0.6137$, $p = 0.5588$). Student's t-test; $N = 4-6$ mice per group. Bars or points represent means ±SEM. Symbols represent individual mice.



Supplementary Fig 6 Controls for behavioral analysis of SARS-CoV-2 Spike protein-infused mice with TLR4 or C1q blockade. Related to Figure 3 and Figure 4. Mice were infused with Spike protein (6,5 μg/site, i.c.v.), and were treated with vehicle (Veh) or an anti-C1q antibody (α-C1q; 0.3 μg twice a week for 30 days) or the TLR4 inhibitor TAK-242 (2mg/kg i.p., daily for one week). In some experiments, TLR4-/- mice on the C57BL/6 background were used. Mice were evaluated in behavioral tests at early (6 days) and/or late (45 days) time points. Spike infusion had no effect on innate preferences for the objects during the training session (**A, H, K and N**) or the exploratory activity during the test session (**B, I, L and O**) of the NOR test ($N = 7-9$ mice per group). (**A**) t=0.7062, $p = 0.5029$ for Veh; and $t = 1.323$, $p = 0.2340$, for α-C1q. One-sample Student's t-test compared to the chance level of 50%. (**B**) $t = 0.7542$, $p = 0.4642$ for Training and $t = 0.8826$, $p = 0.3835$ for Test. Student's t-test. (**C**) Escape latencies across 4 consecutive training trials $F(3,36) = 0.6463$, $p = 0.5904$, repeated measures ANOVA followed by Tukey's test), and (**D**) time spent in the target quadrant ($t = 2.439$, $*p = 0.0312$), (**E**) swimming speed ($t = 0.5104$, $p = 0.6190$), and (**F**) total distance traveled ($t = 0.5370$, $p = 0.6011$) during the probe trial of the MWM test performed at the late stage. Student's t-test; $N = 7-9$ mice per group). (**G**) Spike protein does not impair object recognition memory in WT and TLR4-/- mice, early after protein infusion ($t = 2.66$ $*p = 0.0323$ for WT and $t = 4.18$; $*p = 0.0058$ for TLR4-/-); one-sample Student's t-test compared to the chance level of 50%($N = 7-8$ mice per group). (**H**) $t = 1.756$, $p = 0.1225$ for WT; and $t = 1.132$, $p = 0.3007$, for TLR4-/-. One-sample Student's t-test compared to the chance level of 50%. (**I**) $t = 1.005$, $p = 0.3334$ for Training and $t = 0.9718$, $p = 0.3489$, for Test.. Student's t-test. (**K**) $t = 1.128$, $p = 0.3025$ for WT; and $t = 1.495$, $p = 0.1854$, for TLR4-/-. One-sample Student's t-test compared to the chance level of 50%. (**L**) $t = 1.433$, $p = 0.1775$ for Training and $t = 1.433$, $p = 0.1775$ for Test. Student's t-test. (**N**) $t = 1.081$, $p = 0.3114$ for Veh; and $t = 0.9918$, $p = 0.3543$ for TAK-242. One-sample Student's t-test compared to the chance level of 50%. (**O**) $t = 0.3194$, $p = 0.7539$ for Training and $t = 0.08751$, $p = 0.9314$ for Test. Student's t-test. Genetic (**J and M**) or pharmacological (**P**) inhibition of TLR4 signaling does not affect total distance traveled in the open field arena. (**J**) $t = 0.4239$, $p = 0.6781$. (**M**) $t = 1.498$, $p = 0.1600$. (**P**) $t = 1.349$, $p = 0.1974$. Student's t-test, $N = 7-9$ mice per group. Bars or points represent means ±SEM. Symbols represent individual mice.

Supplementary Table 1. Participant demographics of the study sample. Related to Figure 4.

Sample demographics	Number of individuals (%) (total N = 86)
Sex	
Female	70 (81.4%)
Male	16 (18.6%)
Age (years)[a]	45.6 (19-71)
Time between onset of clinical symptoms and cognitive assessment (months)	5.89 (1-15)
Education[a] (years)	17.02 (5-28)
Comorbidities	
1. None	40 (45.5%)
2. Obesity	19 (22.1%)
3. Hypertension	17 (19.7%)
4. Diabetes	10 (11.6%)

a = mean (range)

Supplementary Table 2. List of primers used in qPCR analyses for mouse and human samples. Related to Figure 2, Figure 3 and Figure 4.

Target gene	Forward primer	Reverse primer
Mouse		
β-Actin	GCCCTGAGGCTCTTTTCCAG	TGCCACAGGATTCCATACCC
TNF	CCCTCACACTCAGATCATCTTCT	GCTACGACGACGTGGGCTACAG
IFNβ	CACAGCCCTCTCCATCAACTA	CATTTCCGAATGTTCGTCCT
Il6	GCTACCAAACTGGATATAATCAGGA	CCAGGTAGCTATGGTACTCCAGAA
IL1-β	GTAATGAAAGACGGCACACC-	ATTAGAAACAGTCCAGCCCA-
IFNAR1	CTGGTCTGTGAGCTGTACTT	TCCCCGCAGTATTGATGAGT
IFNAR2	CTATCGTAATGCTGAAACGG	CGTAATTCCACAGTCTCTTCT
IFNγ	AGCAACAGCAAGGCGAAAA	CTGGACCTGTGGGTTGTTGA
C1q	CTCAGGGATGGCTGGTGGCC	CCTTTGAGACCCGGCCTCCCC
TLR4	GTCAGTGTGATTGTGGTATCC	ACCCAGTCCTCATTCTGACTC
Human		
β-Actin	ACCAACTGGGACGACATGGA	CCAGAGGCGTACAGGGATAG
TLR4	AAGCCGAAAGGTGATTGTTG	CTGAGCAGGGTCTTCTCCAC