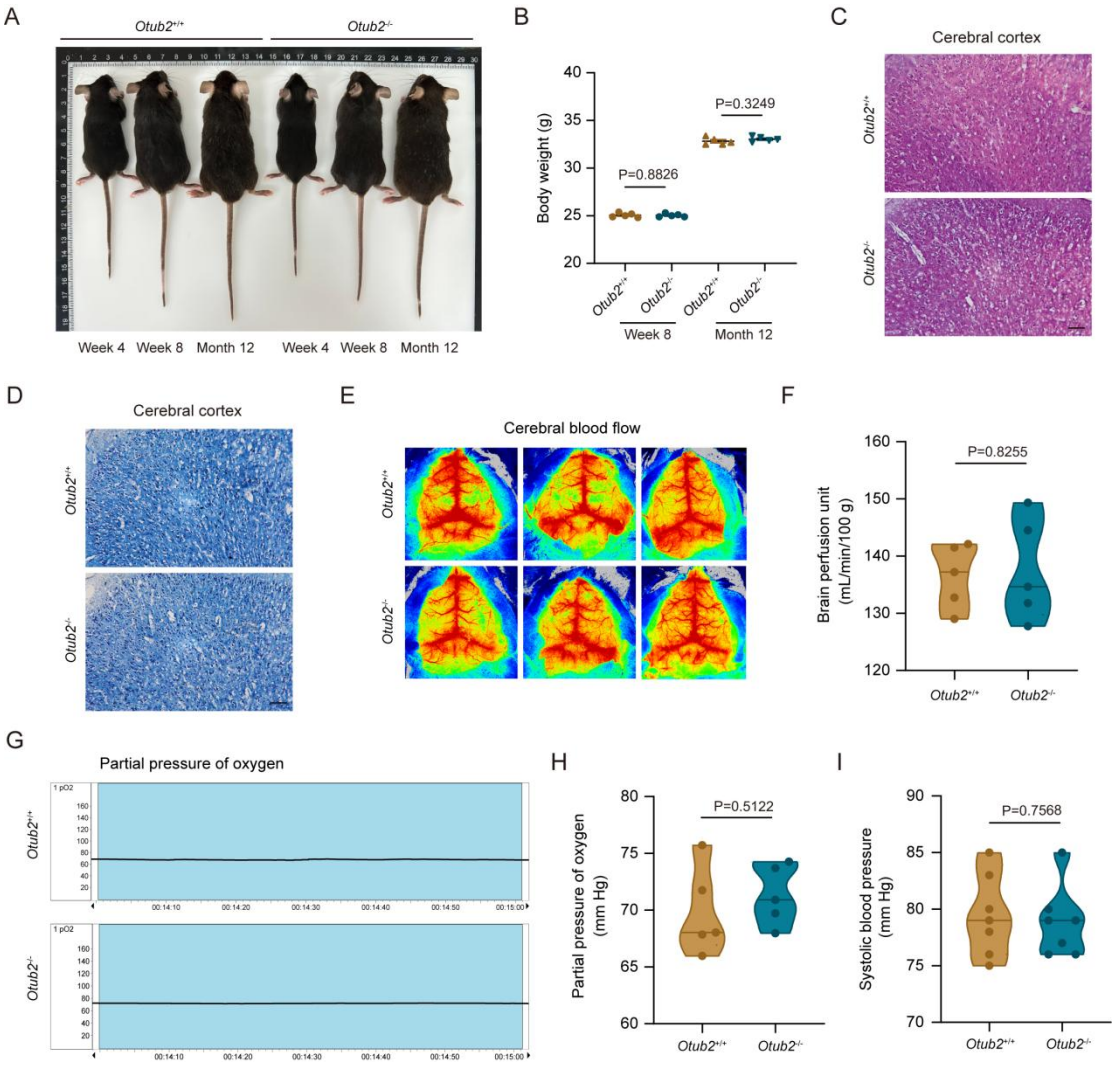
Appendix for

Deubiquitination of RIPK3 by OTUB2 potentiates neuronal necroptosis after ischemic stroke

Table of contents

Appendix Figure S1

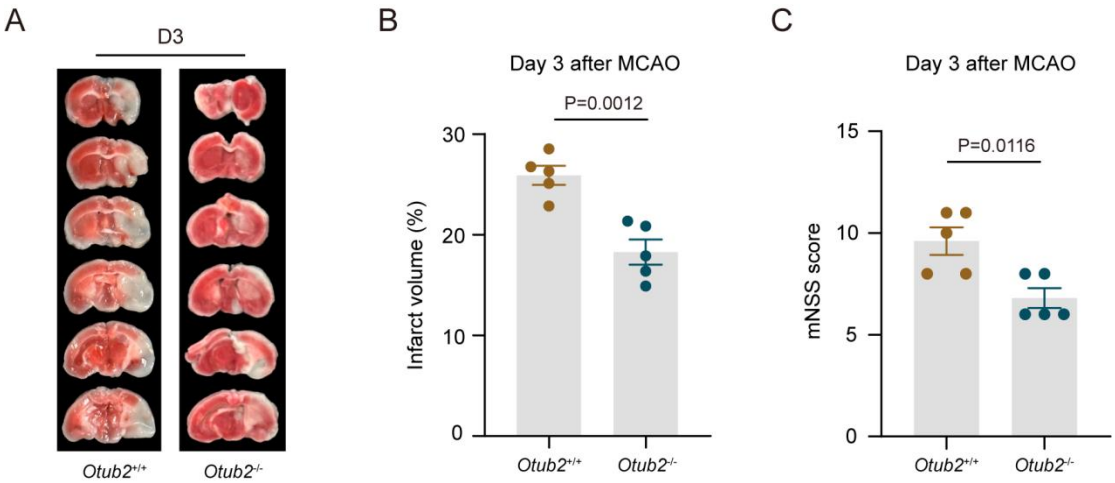


Appendix Figure S1. OTUB2 deficiency does not affect the growth and development of mice

(A) A representative image of *Otub2*$^{+/+}$ and *Otub2*$^{-/-}$ mice at indicated time after birth. (B) Body weight of male *Otub2*$^{+/+}$ and *Otub2*$^{-/-}$ mice at indicated time after birth. Multiple Student's t tests, n = 5 mice/group, biological replicates.(C-D) The cerebral cortex of 8-week-old male *Otub2*$^{+/+}$ and *Otub2*$^{-/-}$ mice was examined by H&E (C) and Nissl (D) staining. Scale bar, 100 μm.(E-F) Representative images (E) and statistics (F)

of the cerebral blood flow in 8-week-old male *Otub2*$^{+/+}$ and *Otub2*$^{-/-}$ mice. Unpaired Student's t test, n = 5 mice/group, biological replicates.(G-H) Representative images (G) and statistics (H) of the partial pressure of oxygen in 8-week-old male *Otub2*$^{+/+}$ and *Otub2*$^{-/-}$ mice. Unpaired Student's t test, n = 5 mice/group, biological replicates.(I) Systolic blood pressure of 8-week-old male *Otub2*$^{+/+}$ and *Otub2*$^{-/-}$ mice. Unpaired Student's t test, n = 5 mice/group, biological replicates. Data in (B) show the mean ± SEM. Source data are available online for this figure

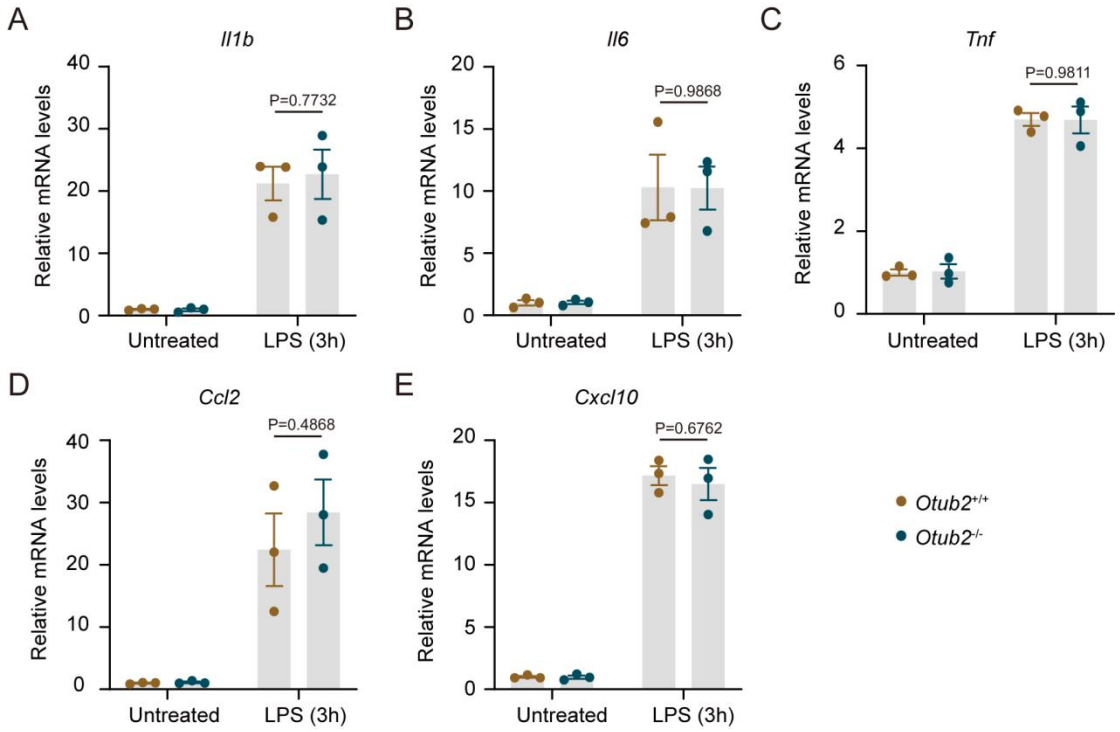
Appendix Figure S2



Appendix Figure S2. OTUB2 deficiency attenuates MCAO-induced cerebral injury in aged mice

(A) MCAO was induced in 12-month-old male *Otub2*$^{+/+}$ and *Otub2*$^{-/-}$ mice. On day 3 after MCAO, cerebral infarct size was analyzed by TTC staining. (B) Cerebral infarct volume was calculated based on TTC staining. Unpaired Student's t test, n = 5 mice/group, biological replicates. (C) Neurological function was evaluated by mNSS test. Mann-Whitney U test, n = 5 mice/group, biological replicates. Data in (B, C) show the mean ± SEM. Source data are available online for this figure.

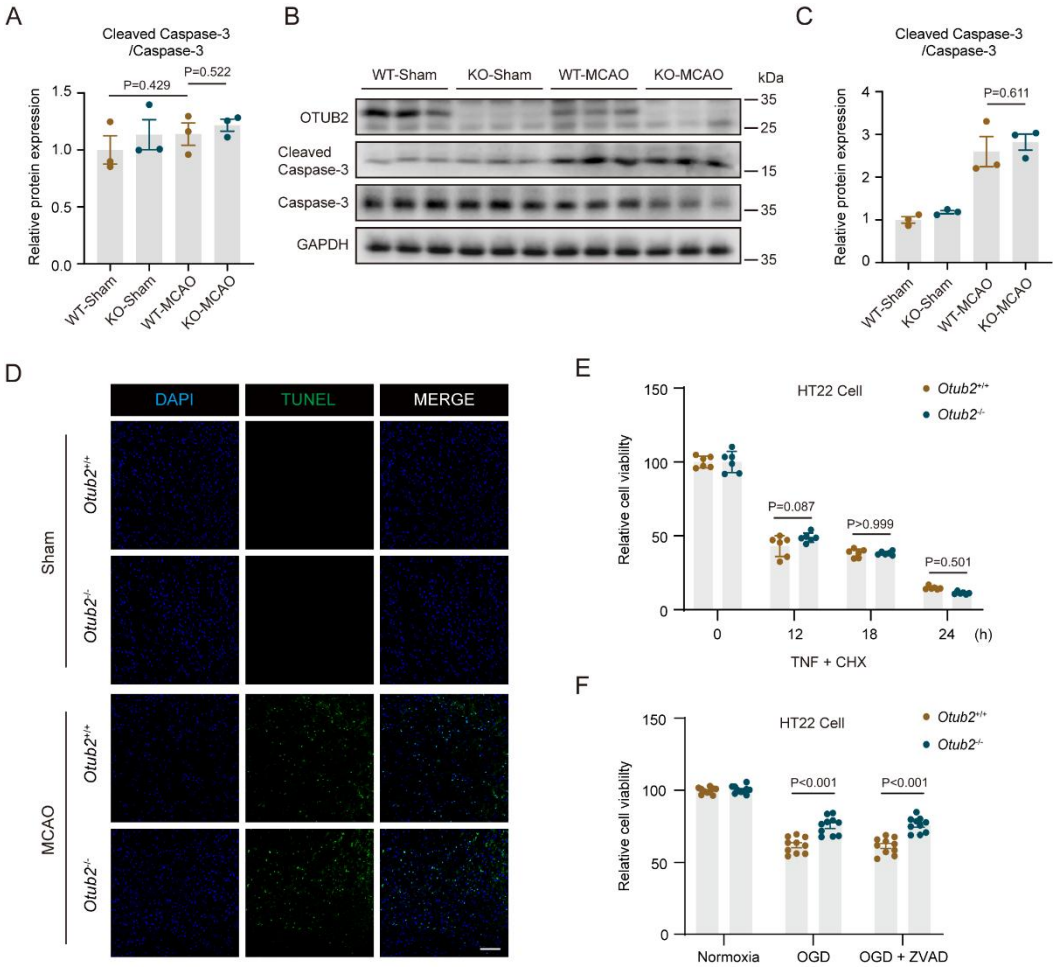
Appendix Figure S3



Appendix Figure S3. OTUB2 deficiency does not affect the production of pro-inflammatory cytokines in microglia

(A-E) Primary microglia from *Otub2*$^{+/+}$ and *Otub2*$^{-/-}$ mice were stimulated with LPS (500 ng/ml) for 3 h or left untreated. The transcription of *Il1b* (A), *Il6* (B), *Tnf* (C), *Ccl2* (D), and *Cxcl10* (E) was measured by qRT-PCR. Mann-Whitney U test (A) and unpaired Student's t test (B-E), n = 3/group, biological replicates. Data in (A-E) show the mean ± SEM. Source data are available online for this figure.

Appendix Figure S4

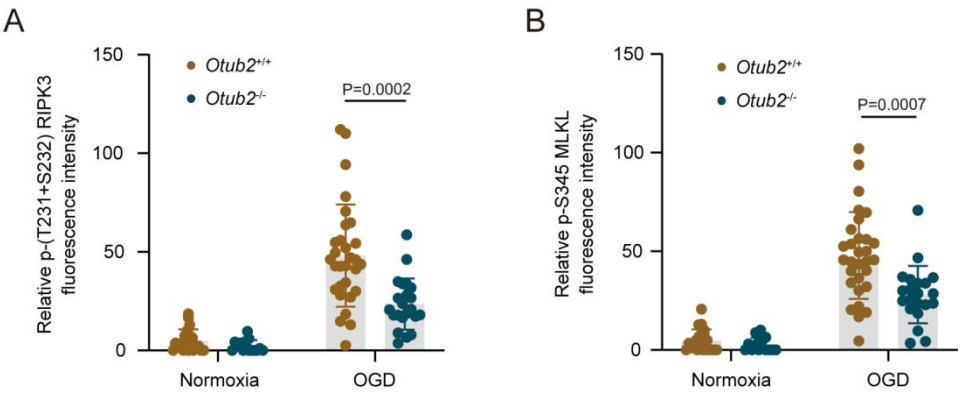


Appendix Figure S4. OTUB2 deficiency does not affect neuronal apoptosis

(A) Four hours after MCAO, the relative ratio of cleaved Caspase 3/Caspase 3 in the ischemic cerebral hemisphere was calculated based on Western blot results. Multiple Student's t tests, n = 3/group, biological replicates. (B-C) Twenty-four hours after MCAO, the ischemic cerebral hemisphere was analyzed by Western blot with indicated antibodies. Representative immunoblots (B) and the relative ratio of cleaved Caspase 3/Caspase 3 (C) are shown. Unpaired Student's t test, n = 3/group, biological replicates. (D) Twenty-four hours after MCAO, apoptotic cells in the ischemic

cerebral hemisphere were detected by TUNEL staining. Scale bar, 100 μm. (E) *Otub2*$^{+/+}$ and *Otub2*$^{-/-}$ HT22 cells were treated with TNF-α (20 ng/ml) and CHX (50 μM) for the indicated time. Cell viability was measured by CCK-8 test. Two-way ANOVA, n = 6/group, biological replicates. (F) *Otub2*$^{+/+}$ and *Otub2*$^{-/-}$ HT22 cells were subjected to OGD treatment in the presence or absence of Z-VAD-FMK (20 μM). Cell viability was measured by CCK-8 test. Two-way ANOVA, n = 10/group, biological replicates. Data in (A, C, E, F) show the mean ± SEM. Source data are available online for this figure.

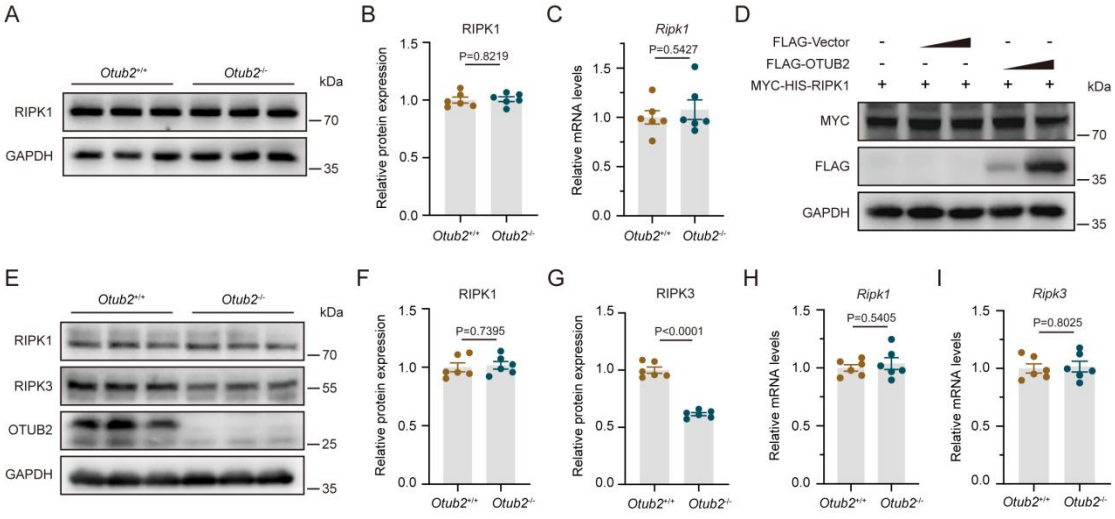
Appendix Figure S5



Appendix Figure S5. OTUB2 deficiency reduces the activation of RIPK3 and MLKL after OGD

(A-B) After OGD treatment for 6 h followed by reoxygenation for 12 h, *Otub2*$^{+/+}$ and *Otub2*$^{-/-}$ HT22 cells were analyzed by immunofluorescence with p-(T231+S232) RIPK3 and p-S345 MLKL antibodies. Data show the fluorescence intensity of p-RIPK3 (A) and p-MLKL (B). Unpaired Student's t test, n = 15-30/group, biological replicates. Data in (A, B) show the mean ± SEM. Source data are available online for this figure.

Appendix Figure S6

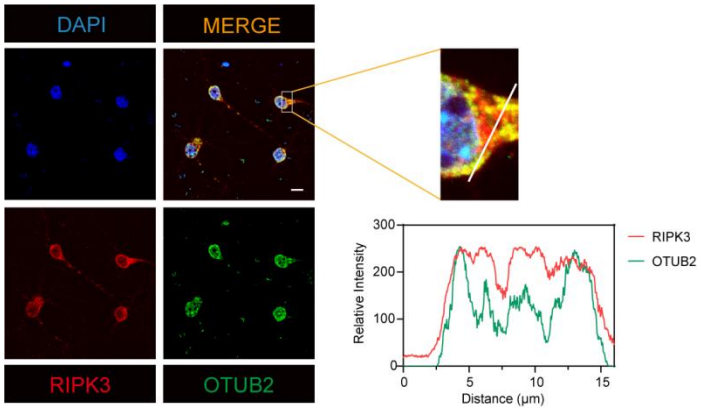


Appendix Figure S6. OTUB2 deficiency has no impact on RIPK1 abundance

(A-B) Whole-cell lysates of *Otub2*$^{+/+}$ and *Otub2*$^{-/-}$ HT22 cells were analyzed by Western blot with indicated antibodies. Representative immunoblots (A) and relative RIPK1 quantification (B) are shown. Unpaired Student's t test, n = 6/group, biological replicates. (C) Relative *Ripk1* mRNA levels in *Otub2*$^{+/+}$ and *Otub2*$^{-/-}$ HT22 cells were determined by qRT-PCR. Mann-Whitney U test, n = 6/group, biological replicates. (D) MYC-HIS-RIPK1 plasmids were co-transfected into NIH/3T3 cells with increasing amount of FLAG-Vector or FLAG-OTUB2 plasmids for 24 h. Whole-cell lysates were analyzed by Western blot. (E-G) Brains of untreated *Otub2*$^{+/+}$ and *Otub2*$^{-/-}$ mice were lysed and analyzed by Western blot with indicated antibodies. Representative immunoblots (E) as well as relative quantification of RIPK1 (F) and RIPK3 (G) are shown. Mann-Whitney U test (F) and unpaired Student's t test (G), n = 6/group, biological replicates. (H-I) Relative *Ripk1* (H) and *Ripk3* (I) mRNA levels in brains of

untreated *Otub2*$^{+/+}$ and *Otub2*$^{-/-}$ mice were determined by qRT-PCR. Unpaired Student's t test, n = 6/group, biological replicates. Data in (B, C, F-I) show the mean ± SEM. Source data are available online for this figure.

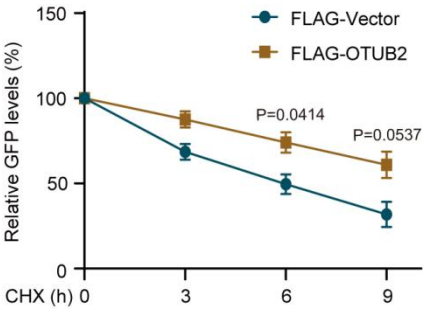
Appendix Figure S7



Appendix Figure S7. Co-localization of OTUB2 and RIPK3 in primary neurons

Subcellular distribution of OTUB2 (green) and RIPK3 (red) in primary neurons from C57BL/6 mice was analyzed by immunofluorescence. Scale bar, 10 μm. Source data are available online for this figure.

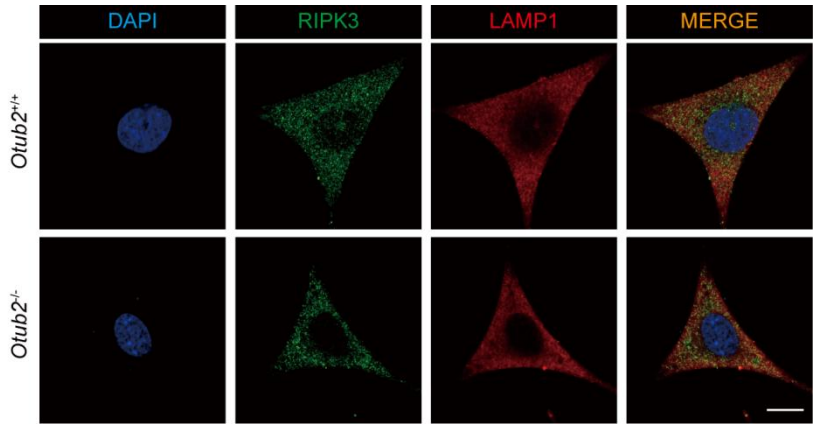
Appendix Figure S8



Appendix Figure S8. OTUB2 inhibits the degradation of RIPK3

GFP-RIPK3 plasmids were co-transfected into NIH/3T3 cells with FLAG-Vector or FLAG-OTUB2 plasmids for 24 h, followed by treatment with CHX (50 μM) for indicated time. Whole-cell lysates were analyzed by Western blot and relative levels of GFP-RIPK3 were quantified based on Western blot results. Multiple Student's t tests, n = 3/group, biological replicates. Data show the mean ± SEM. Source data are available online for this figure.

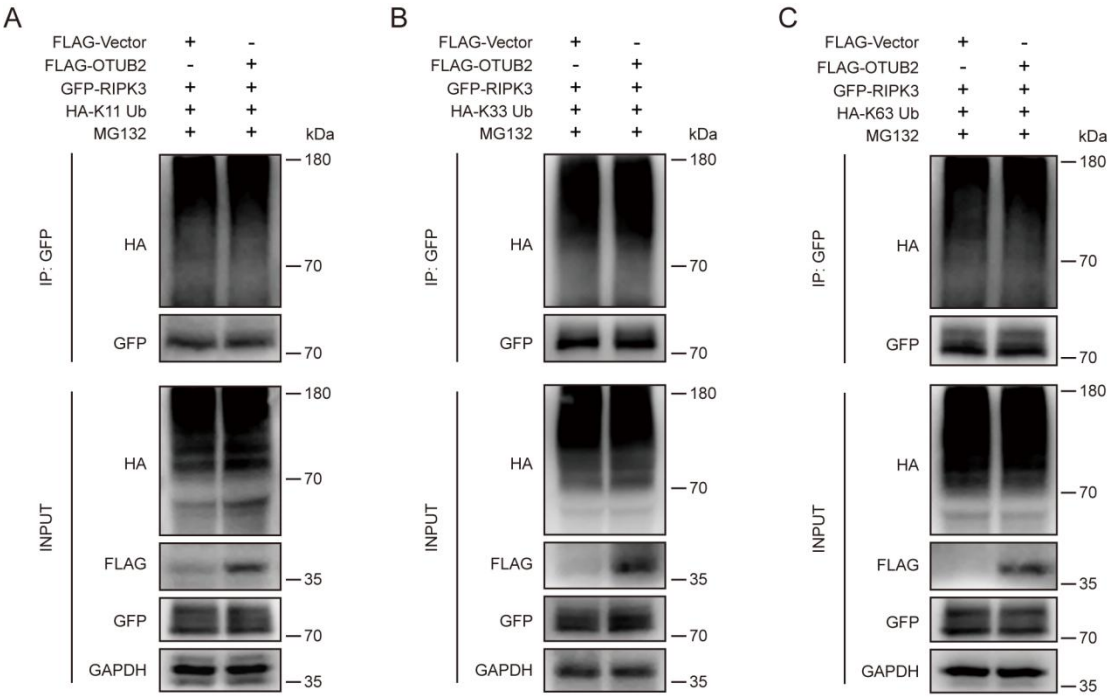
Appendix Figure S9



Appendix Figure S9. OTUB2 deficiency does not increase the co-localization of RIPK3 with LAMP1

Subcellular distribution of RIPK3 (green) and LAMP1 (red) in HT22 cells was determined by immunofluorescence. Scale bar, 10 μm. Source data are available online for this figure.

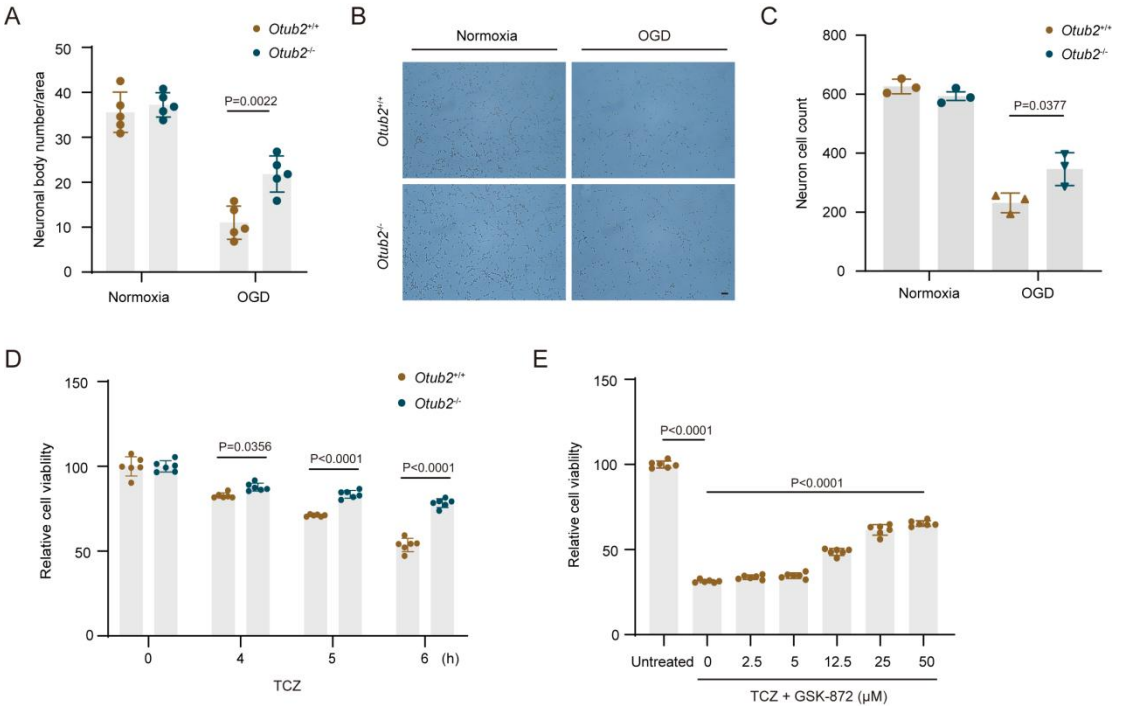
Appendix Figure S10



Appendix Figure S10. OTUB2 does not affect the K11, K33, and K63 ubiquitination of RIPK3

(A-C) NIH/3T3 cells were transfected with indicated plasmids for 24 h, followed by treatment with MG132 (5 μM) for 6 h. Immunocomplexes were harvested from whole-cell lysates by immunoprecipitation and analyzed by Western blot. Source data are available online for this figure.

Appendix Figure S11

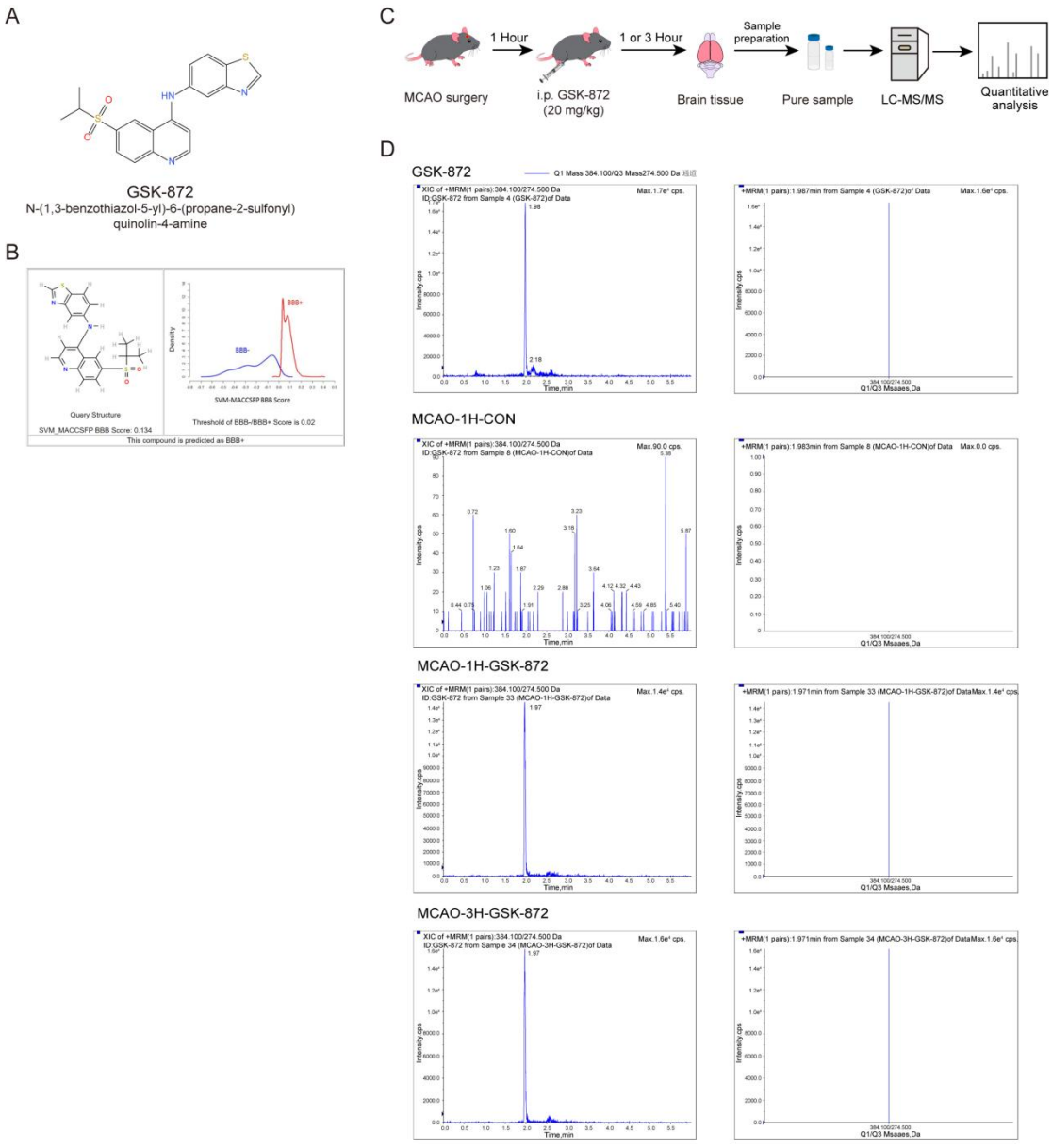


Appendix Figure S11. OTUB2 deficiency attenuates necroptosis

(A) Primary neurons from *Otub2*$^{+/+}$ and *Otub2*$^{-/-}$ mice were subjected to OGD for 6 h followed by reoxygenation for 12 h. The number of neurons was determined by immunofluorescence with anti-MAP2 antibody. Unpaired Student's t test, n = 5/group, biological replicates. (B-C) Primary neurons from *Otub2*$^{+/+}$ and *Otub2*$^{-/-}$ mice were subjected to OGD for 6 h followed by reoxygenation for 12 h. The number of neurons was counted with a brightfield microscope. Representative images (B) and cell number (C) are shown. Unpaired Student's t test, n = 3 /group, biological replicates. (D) *Otub2*$^{+/+}$ and *Otub2*$^{-/-}$ HT22 cells were stimulated with TNF-α (20 ng/ml) + CHX (50 μM) + Z-VAD-FMK (20 μM) for indicated time. Cell viability was determined by CCK-8 test. Two-way ANOVA, n = 6/group, biological replicates. (E) HT22 cells

were stimulated with TNF-α (20 ng/ml) + CHX (50 μM) + Z-VAD-FMK (20 μM) in the presence of indicated concentrations of GSK-872 for 6 h. Cell viability was determined by CCK-8 test. Multiple Student's t tests, n = 6/group, biological replicates. Data in (A, C, D, E) show the mean ± SEM. Source data are available online for this figure.

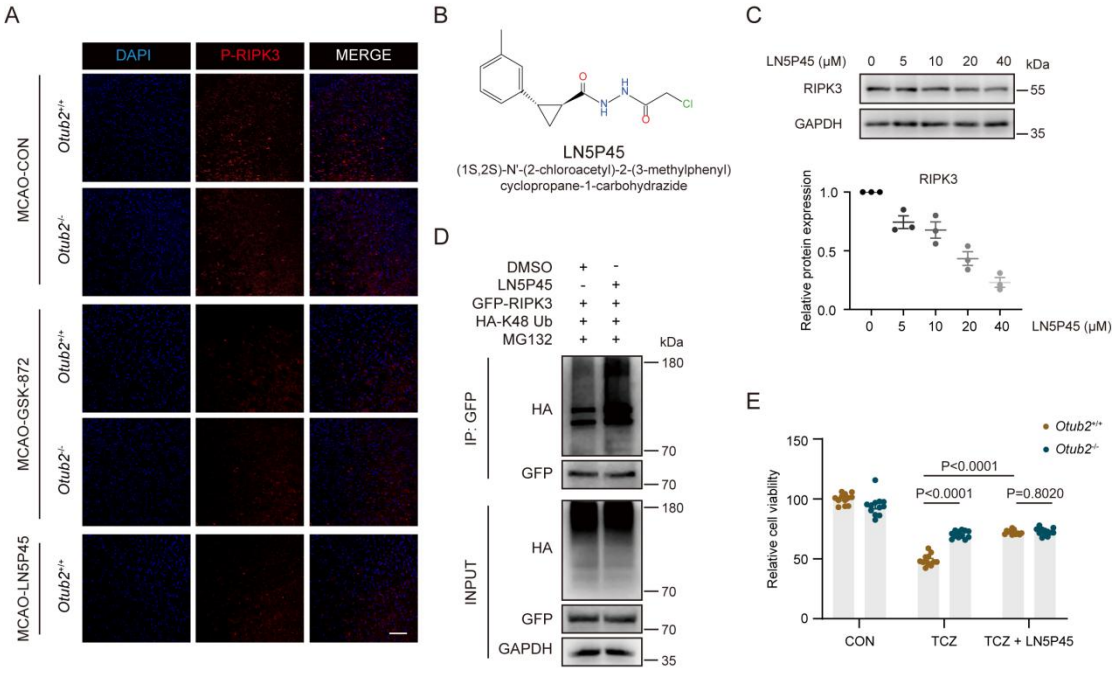
Appendix Figure S12



Appendix Figure S12. GSK-872 can pass the BBB

(A) Chemical structure of GSK-872. (B) GSK-872 was predicted to pass the BBB by the online tool (GraphBBB). (C) Experimental flowchart for the analysis of GSK-872 in brain cells. (D) Results of the LC-MS/MS analysis. Source data are available online for this figure.

Appendix Figure S13.

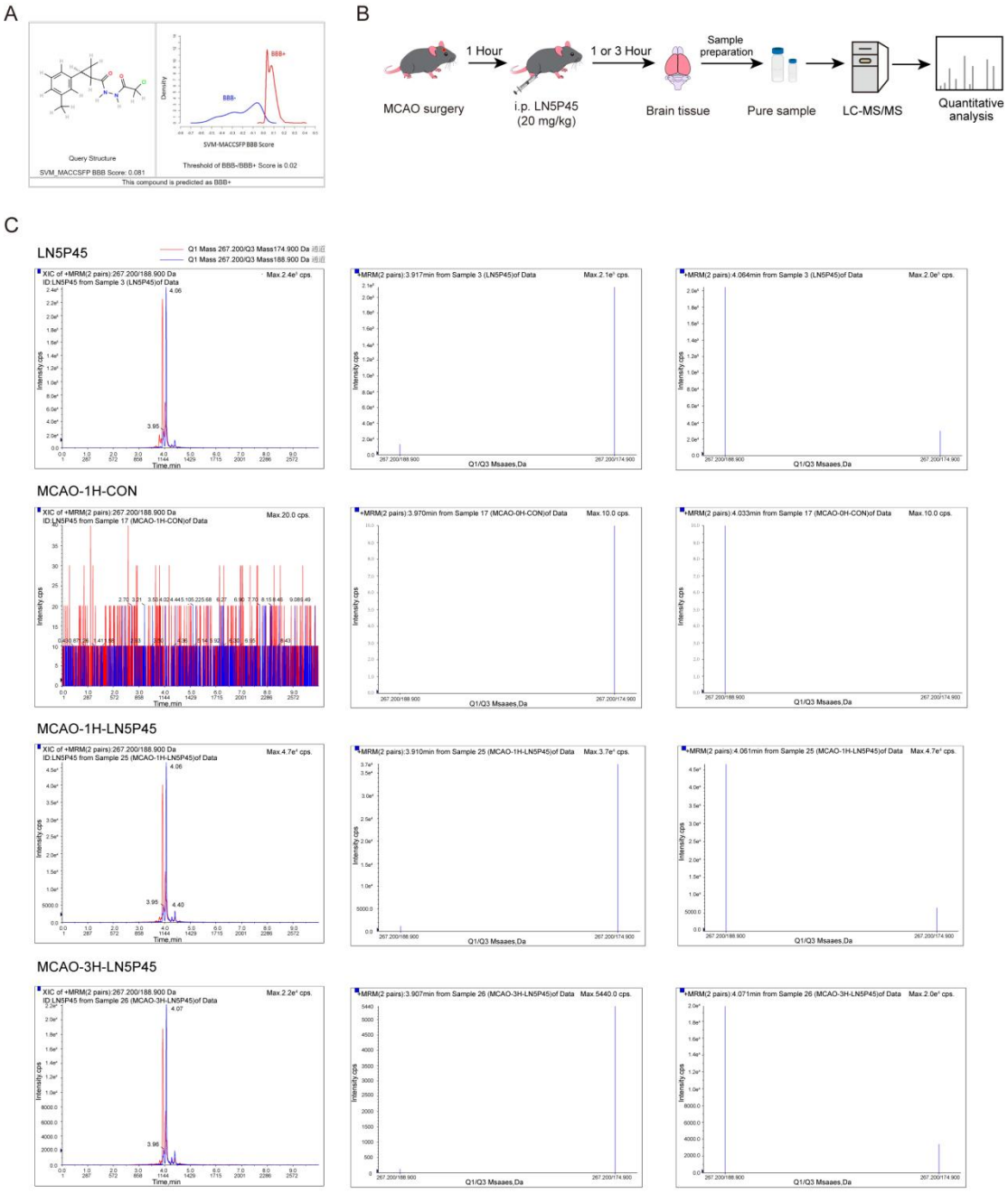


Appendix Figure S13. Pharmacological inhibition of OTUB2 reduces RIPK3-mediated necroptosis

(A) Mice were intraperitoneally injected with LN5P45 (10 mg/kg) prior to MCAO surgery. The ischemic penumbra was analyzed by immunofluorescence at 6 h after MCAO. Scale bar, 100 μm. (B) Chemical structure of LN5P45. (C) HT22 cells were treated with indicated concentrations of LN5P45 for 6 h before lysis. Whole-cell lysates were analyzed by Western blot. Representative immunoblots (upper panel) and relative RIPK3 protein levels (lower panel) are shown. (D) NIH/3T3 cells were transfected with indicated plasmids for 24 h. After transfection, cells were treated with MG132 (5 μM) in the presence or absence of LN5P45 (20 μM) for 6 h. Immunocomplexes and whole-cell lysates were analyzed by Western blot. (E) After treatment with LN5P45 (20 μM) for 6 h, HT22 cells were stimulated with TNF-α (20

193 ng/ml) + CHX (50 μM) + Z-VAD-FMK (20 μM) for another 6 h. Cell viability was
194 determined by CCK-8 test. Mann-Whitney U test, n = 12/group, biological replicates.
195 Data in (C, E) show the mean ± SEM. Source data are available online for this figure.

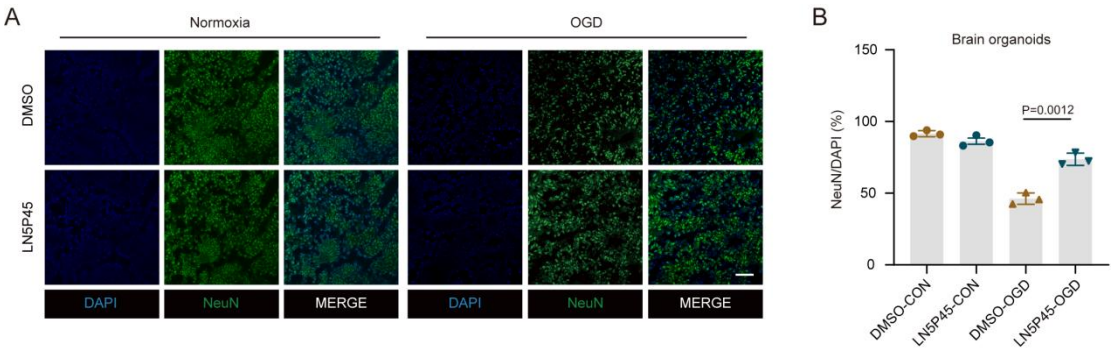
Appendix Figure S14



Appendix Figure S14. LN5P45 can pass the BBB

(A) LN5P45 was predicted to pass the BBB by the online tool (GraphBBB). (B) Experimental flowchart for the detection of LN5P45 in brain cells. (C) Results of the LC-MS/MS analysis. Source data are available online for this figure.

Appendix Figure S15



Appendix Figure S15. Pharmacological inhibition of OTUB2 reduces OGD-induced neuronal loss in human brain organoids

(A-B) On day 30 after culture, brain organoids were subjected to OGD for 12 h, followed by 12 h of reoxygenation. The brain organoids were then analyzed by immunofluorescence with indicated antibodies. Representative images (A) and quantification (B) are shown. Scale bar, 50 μm. Unpaired Student's t test, n = 3/group, biological replicates. Data in (B) show the mean ± SEM. Source data are available online for this figure.

Appendix Table S1 Primers for quantitative Real-Time PCR (qRT-PCR)

Reagent/Resource	Reference or source	Identifier or Catalog Number
Ccl2 reverse primer (M. musculus)	5'-GCATTAGCTTCAGATTTACGGGT-3'	N/A
Ccl2 forward primer (M. musculus)	5'-TAAAAACCTGGATCGGAACCAAA-3'	N/A
Cxcl10 reverse primer (M. musculus)	5'-CCTATGGCCCTCATTCTCAC-3'	N/A
Cxcl10 forward primer (M. musculus)	5'-CTCATCCTGCTGGGTCTGAG-3'	N/A
Il1b reverse primer (M. musculus)	5'-TGAGGCCCAAGGCCACAGGT-3'	N/A
Il1b forward primer (M. musculus)	5'-AAGCCTCGTGCTGTCGGACC-3'	N/A
Il6 reverse primer (M. musculus)	5'-AAGTGCATCATCGTTGTTCATACA-3'	N/A
Il6 forward primer (M. musculus)	5'-ACACATGTTCTCTGGGAAATCGT-3'	N/A
Otub2 reverse primer (M. musculus)	5'-GTAAGACAAGACGGAGAACAGAAGG-3'	N/A
Otub2 forward primer (M. musculus)	5'-ACCTCATTCCTCGTTCCATCTG-3'	N/A
Ripk1 reverse primer (M. musculus)	5'-TGCCCAAAGGACAATGCCAAAG-3'	N/A
Ripk1 forward primer (M. musculus)	5'-AGCAGCACCACTAAGAAGAACAATG-3'	N/A

Reagent/Resource	Reference or source	Identifier or Catalog Number
Ripk3 reverse primer (M. musculus)	5'-TCAGAACAGTTGTT GAAGACGAGAG-3'	N/A
Ripk3 forward primer (M. musculus)	5'-ACACGGCACTCCTT GGTATCC-3'	N/A
Tnf reverse primer (M. musculus)	5'-ATAGCAAATCGGCT GACGGT-3'	N/A
Tnf forward primer (M. musculus)	5'-ACGTCGTAGCAAA CCACCAA-3'	N/A
β-actin reverse primer (M. musculus)	5'-CACAGCTTCTCTTT GATGTCAC-3'	N/A
β-actin forward primer (M. musculus)	5'-CTACCTCATGAAGA TCCTGACC-3'	N/A

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Statistics Tables

Appendix Table S2

Figure	Analysis of normality	Statistical method	Power	p value
Fig 1 B	Yes	two-tailed unpaired Student's t test	1.000	<0.0001
Fig 1 F(day3/day7)	Yes	two-way ANOVA followed by the Sidak post-test	0.945/0.644	0.0004/0.0310
Fig 1 G (day1/day3/day7)	Yes	two-way ANOVA followed by the Sidak post-test	0.925/0.717/0.903	0.0052/0.0393/0.0052
Fig 1 H (day1/day3/day7)	Yes	two-way ANOVA followed by the Sidak post-test	0.855/0.953/0.807	0.0019/0.0015/0.0070
Fig 1 I (day1/day3/day7)	Yes	two-way ANOVA followed by the Sidak post-test	0.963/0.292/0.566	0.0289/0.2859/0.1794
Fig 1 J (day1/day3/day7)	Yes	two-way ANOVA followed by the Sidak post-test	0.949/0.822/0.836	0.0004/0.0080/0.1074
Fig 1 K (day1/day3/day7)	Yes	two-way ANOVA followed by the Sidak post-test	0.755/0.310/0.212	0.0410/0.2718/0.5269

Appendix Table S3

Figure	Analysis of normality	Statistical Method	Power	p value
Fig 2 C	Yes	two-tailed unpaired Student's t test	0.568	0.0438
Fig 2 D	Yes	two-tailed unpaired Student's t test	0.102	0.4637
Fig 2 F	Yes	two-tailed unpaired Student's t test	0.999	0.0004
Fig 2 I	No	Mann-Whitney U test	0.758	0.0164
Fig 2 J	Yes	two-tailed unpaired Student's t test	0.708	0.0435
Fig 2 K	Yes	two-tailed unpaired Student's t test	0.945	0.0022
Fig 2 L	Yes	two-tailed unpaired Student's t test	0.520	0.0492
Fig 2 M	Yes	two-tailed unpaired Student's t test	0.364	0.1056

Appendix Table S4

Figure	Analysis of normality	Statistical method	Power	p value
Fig 3 B	Yes	two-way ANOVA followed by the Sidak post-test	1.000/0.221	0.0003/0.3863
Fig 3 C	Yes	two-way ANOVA followed by the Sidak post-test	1.000/0.604	0.0011/0.0329
Fig 3 D	Yes	two-way ANOVA followed by the Sidak post-test	1.000/0.585	0.0011/0.0477
Fig 3 F	Yes	two-way ANOVA followed by the Sidak post-test	0.848/0.970	0.0202/0.0461
Fig 3 I	Yes	two-tailed unpaired Student's t test	1.000	0.0008
Fig 3 J	Yes	two-tailed unpaired Student's t test	0.610	0.0341
Fig 3 K	Yes	two-tailed unpaired Student's t test	0.999	0.0004
Fig 3 L	Yes	two-tailed unpaired Student's t test	0.586	0.0382
Fig 3 N	Yes	two-tailed unpaired Student's t test	0.956	0.0030
Fig 3 O	Yes	two-way ANOVA followed by the Sidak post-test	0.866/0.070	0.0005/0.9566

220 **Appendix Table S5**

Figure	Analysis of normality	Statistical method	Power	p value
Fig 4 B	Yes	two-tailed unpaired Student's t test	1.000	<0.0001
Fig 4 C	Yes	two-tailed unpaired Student's t test	0.104	0.4711

221 **Appendix Table S6**

Figure	Analysis of normality	Statistical method	Power	p value
Fig 6 A	Yes	two-tailed unpaired Student's t test	1.000	<0.0001
Fig 6 E	No	Mann-Whitney U test	0.996	<0.0001
Fig 6 F	Yes	two-tailed unpaired Student's t test	0.953	<0.0001
Fig 6 G	Yes	two-way ANOVA followed by the Sidak post-test	1.000/0.606/0.311	<0.0001/0.0603/0.6878
Fig 6 H	Yes	two-way ANOVA followed by the Sidak post-test	0.995/0.112	0.0005/0.7816

222 **Appendix Table S7**

Figure	Analysis of normality	Statistical method	Power	p value
Fig 7 C	Yes	Multiple Student's t tests	0.997/0.904/0.984	0.0006/0.0057/0.0015
Fig 7 D	Yes	Multiple Student's t tests	0.878/0.675/0.963	0.0072/0.0249/0.0026
Fig 7 H	Yes	Multiple Student's t tests	0.992/0.864/0.971	0.0010/0.0081/0.0022
Fig 7 I	No	Mann-Whitney U test	0.891/0.759/0.979	0.0063/0.0193/0.0064
Fig 7 N	Yes	two-tailed unpaired Student's t test	0.986	0.0045

223

224 **Appendix Table S8.**

Figure	Analysis of normality	Statistical method	Power	p value
Appendix Fig S1 B	Yes	Multiple Student's t tests	0.052/0.153	0.8826/0.3249
Appendix Fig S1 F	Yes	two-tailed unpaired Student's t test	0.055	0.8255
Appendix Fig S1 H	Yes	two-tailed unpaired Student's t test	0.093	0.5122
Appendix Fig S1 I	Yes	two-tailed unpaired Student's t test	0.060	0.7568

225 **Appendix Table S9**

Figure	Analysis of normality	Statistical method	Power	p value
Appendix Fig S2 B	Yes	two-tailed unpaired Student's t test	0.989	0.0012
Appendix Fig S2 C	No	Mann-Whitney U test	0.810	0.0116

226 **Appendix Table S10**

Figure	Analysis of normality	Statistical method	Power	p value
Appendix Fig S3 A	No	Mann-Whitney U test	0.056	0.7732
Appendix Fig S3 B	Yes	two-tailed unpaired Student's t test	0.050	0.9868
Appendix Fig S3 C	Yes	two-tailed unpaired Student's t test	0.050	0.9811
Appendix Fig S3 D	Yes	two-tailed unpaired Student's t test	0.092	0.4868
Appendix Fig S3 E	Yes	two-tailed unpaired Student's t test	0.064	0.6762

227

228 **Appendix Table S11**

Figure	Analysis of normality	Statistical method	Power	p value
Appendix Fig S4 A	Yes	Multiple Student's t tests	0.106/0.085	0.4289/0.5225
Appendix Fig S4 C	Yes	two-tailed unpaired Student's t test	0.072	0.6115
Appendix Fig S4 E	Yes	two-way ANOVA followed by the Sidak post-test	0.372/0.050/0.999	0.0869/>0.9999/0.5012
Appendix Fig S4 F	Yes	two-way ANOVA followed by the Sidak post-test	0.998/1.000	<0.0001/<0.0001

229 **Appendix Table S12**

Figure	Analysis of normality	Statistical method	Power	p value
Appendix Fig S5 A	Yes	two-tailed unpaired Student's t test	0.992	0.0002
Appendix Fig S5 B	Yes	two-tailed unpaired Student's t test	0.967	0.0007

230 **Appendix Table S13**

Figure	Analysis of normality	Statistical method	Power	p value
Appendix Fig S6 B	Yes	two-tailed unpaired Student's t test	0.055	0.8219
Appendix Fig S6 C	No	Mann-Whitney U test	0.086	0.5427
Appendix Fig S6 F	No	Mann-Whitney U test	0.060	0.7395
Appendix Fig S6 G	Yes	two-tailed unpaired Student's t test	1.000	<0.0001
Appendix Fig S6 H	Yes	two-tailed unpaired Student's t test	0.089	0.5405
Appendix Fig S6 I	Yes	two-tailed unpaired Student's t test	0.056	0.8205

231

Appendix Table S14

Figure	Analysis of normality	Statistical method	Power	p value
Appendix Fig S8	Yes	Multiple Student's t tests	0.609/0.537	0.0414/0.0537

Appendix Table S15

Figure	Analysis of normality	Statistical method	Power	p value
Appendix Fig S11 A	Yes	two-tailed unpaired Student's t test	0.971	0.0022
Appendix Fig S11 C	Yes	two-tailed unpaired Student's t test	0.635	0.0377
Appendix Fig S11 D	Yes	two-way ANOVA followed by the Sidak post-test	0.975/1.000/1.000	0.0356/<0.0001/<0.0001
Appendix Fig S11 E	Yes	Multiple Student's t tests	1.000/1.000	<0.0001/<0.0001

Appendix Table S16

Figure	Analysis of normality	Statistical method	Power	p value
Appendix Fig S13 E	No	Mann-Whitney U test	1.000/1.000/0.056	<0.0001/<0.0001/0.8020

Appendix Table S17

Figure	Analysis of normality	Statistical method	Power	p value
Appendix Fig S15 B	Yes	two-tailed unpaired Student's t test	1.000	0.0012