Mitochondrial DNA-cGAS-STING-CISD1 axis mediates hypoxia-induced cognitive impairment by driving hippocampal neuronal ferroptosis

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Supplementary Information

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Supplementary Tables

Supplementary Table 1.Primary antibodies and secondary antibodies used in this study.

Target	Supplier	Catalog No.	Application
HIF-1α	ABMART	T55824F	WB
GPX4	ABMART	T56959F	WB
xCT	Immunoway	YM8243	WB
ACSL4	ABMART	T510198F	WB
TOM20	Proteintech	11802-1-AP	WB, IF
FTH1	Immunoway	YM8481	WB
cGAS	ABMART	PQA3430F	WB
STING	ABMART	TD12090F	WB, Co-IP
p-STING	ABMART	TA7416F	WB, IF
TBK1	Immunoway	YM8674	WB
p-TBK1	ABMART	T58364T	WB
IRF3	Proteintech	80519-1-RR	WB
p-IRF3	ABMART	TA2436F	WB
CISD1	Immunoway	YN5755	WB, IF, Co-IP
MAP2	ABclonal	AB11267	IF
NeuN	ABclonal	A26951	IF
IgG	Proteintech	30000-0-AP	Co-IP
dsDNA	Santa Cruz Biotechnology	HYB331-01	IF
β-actin	ABMART	P30002M	WB
Goat anti-rabbit IgG (H+L)	Abcam	ab150084	WB, IF

Supplementary Table 2.Primer sequences used for RT-qPCR.

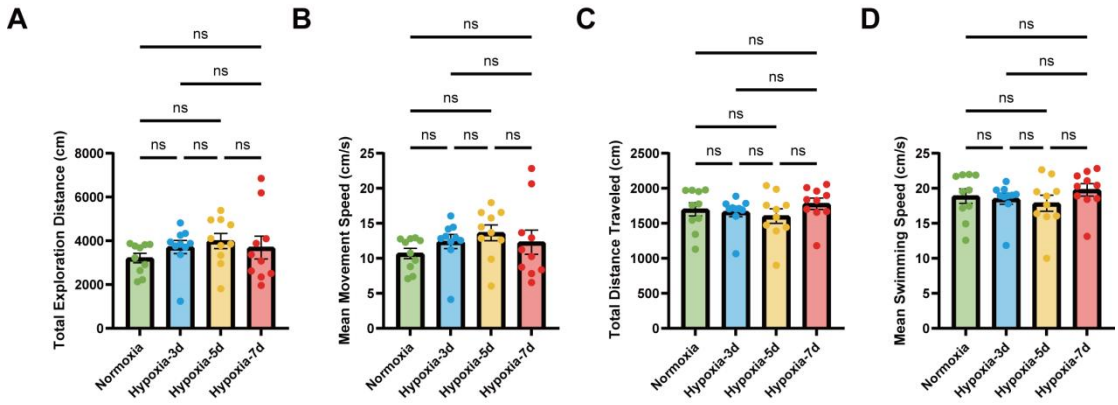
Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
Mus-Hif-1a	ACCTTCATCGGAAACTCCAAAG	CTGTTAGGCTGGGAAAAGTTAGG
Mus-Gpx4	TGTGCATCCCGCGATGATT	CCCTGTACTTATCCAGGCAGA
Mus-Slc7a11	GGCACCGTCATCGGATCAG	CTCCACAGGCAGACCAGAAAA
Mus-Acsl4	CTCACCATTATATTGCTGCCTGT	TCTCTTTGCCATAGCGTTTTTCT
Mus-Fth1	CAAGTGCGCCAGAACTACCA	ACAGATAGACGTAGGAGGCATAC
Mus-Cgas	TGGCTTTCAGCAAAAGTTAGG	AAGGATAGCCGCCATGTTTC
Mus-Irf3	GAGAGCCGAACGAGGTTCAG	CTTCCAGGTTGACACGTCCG
Mus-Ifnb1	AGCTCCAAGAAAGGACGAACA	GCCCTGTAGGTGAGGTTGAT
Mus-Cxcl10	CCAAGTGCTGCCGTCATTTTC	TCCCTATGGCCCTCATTCTCA
Mus-Isg15	GTCACGGACACCAGGAAATC	AAGCAGCCAGAAGCAGACTC
Mus-β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

Supplementary Table 3.Detailed information of reagents used in this study.

Category	Reagent/Antibody	Application	Catalog No.	Manufacturer
Cell culture reagent	DMEM medium	Cell culture	C11995500BT	Gibco,USA
Cell culture reagent	0.25% Trypsin–EDTA	Cell dissociation	25200-072	Gibco,USA
Cell culture reagent	Fetal bovine serum (FBS)	Cell culture supplement	C04001-500	DASHI Biotech, Shanghai,China
Cell culture reagent	Penicillin–Streptomycinsolution	Antibiotic supplement	SV30010	HyClone,USA
Cell culture reagent	Neurobasal™-A medium	Primary microglia culture	10888022	Gibco,USA
Cell culture reagent	B27® supplement	Primary cell culture	17404-044	Gibco,USA
Cell adhesion reagent	Poly-D-lysine hydrobromide	Cell coating	P0899-50MG	Sigma-Aldrich,USA
Fixation reagent	4% Paraformaldehyde	IF fixation	MM1506	Maokang Biotechnology,China
Permeabilization reagent	Triton™ X-100	IF staining	S15022	Maokang Biotechnology,China
Mounting reagent	DAPI-Fluoromount-G™	Fluorescence mounting	0100-20	SouthernBiotech, USA
Reverse transcription reagent	StarScript III RT Premix with gDNA removal	RT-qPCR	A230	GenStar, China
RNA extraction reagent	TRIGene™ reagent	RNA extraction	P118-05	GenStar, China
qPCR reagent	2× RealStar Fast SYBR qPCR Mix	RT-qPCR	A301-5	GenStar, China

Supplementary Figures

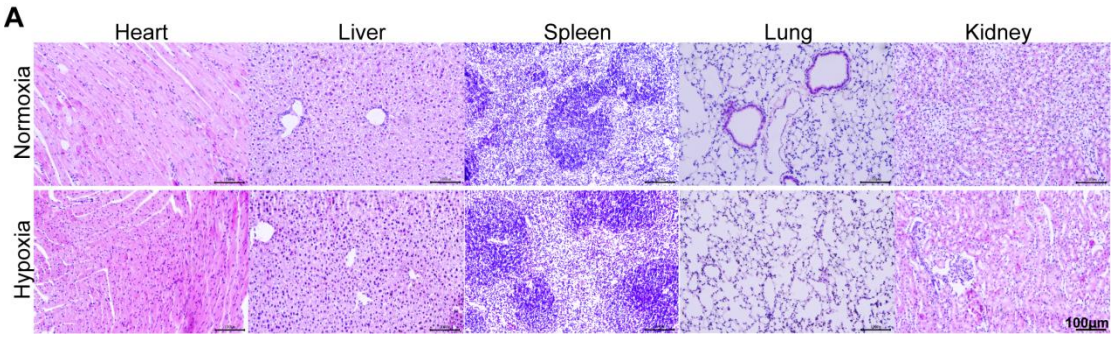
S1



Supplementary Fig.1 Hypoxia exposure does not significantly affect general locomotor activity or swimming performance in mice.

A:Total exploration distance in the NOR test. B:Mean movement speed in the NOR test. C:Total swimming distance in the MWM test. D:Mean swimming speed in the MWM test. Comparisons among the four groups were performed using ordinary one-way ANOVA followed by Tukey's multiple-comparisons test. n = 10 mice per group. ns, not significant.

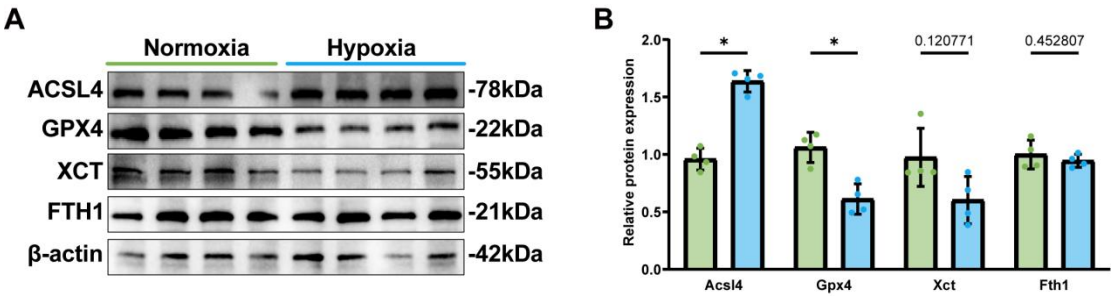
S2



Supplementary Fig. 2 Hypoxia exposure does not induce overt histopathological injury in major peripheral organs.

A:Representative H&E-stained sections of the heart, liver, spleen, lung, and kidney from normoxic and hypoxic mice. Scale bar, 100 μm.

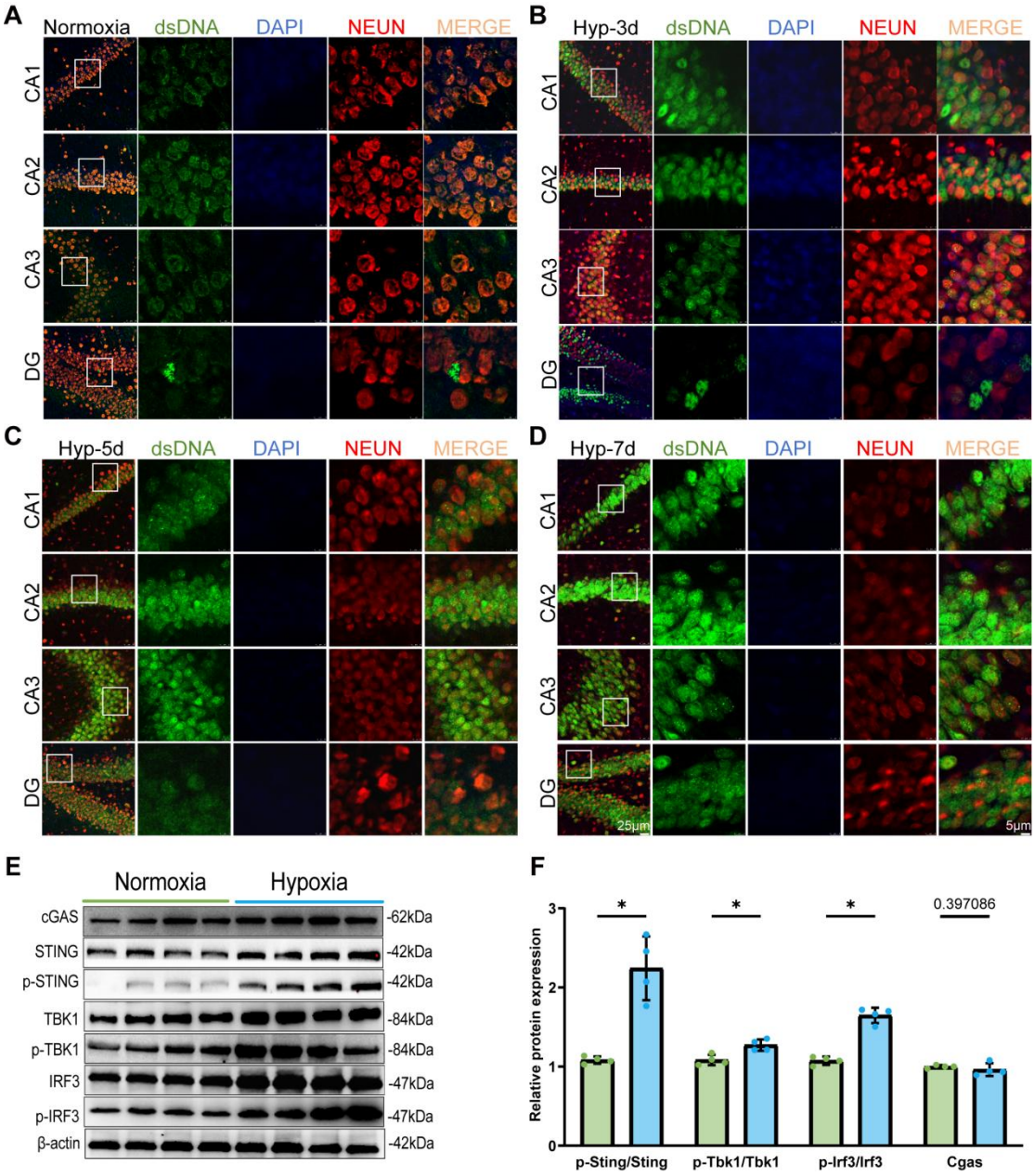
S3



Supplementary Fig. 3 Hypoxia induces ferroptosis-associated protein alterations in primary hippocampal neurons.

A:Representative Western blots of Acsl4, Gpx4, Xct, and Fth1 in primary hippocampal neurons cultured under normoxic or hypoxic conditions; β-actin served as the loading control. B:Densitometric quantification of the indicated proteins. n = 4 independent neuronal culture replicates per group. Exact P values are shown where indicated; *P < 0.05.

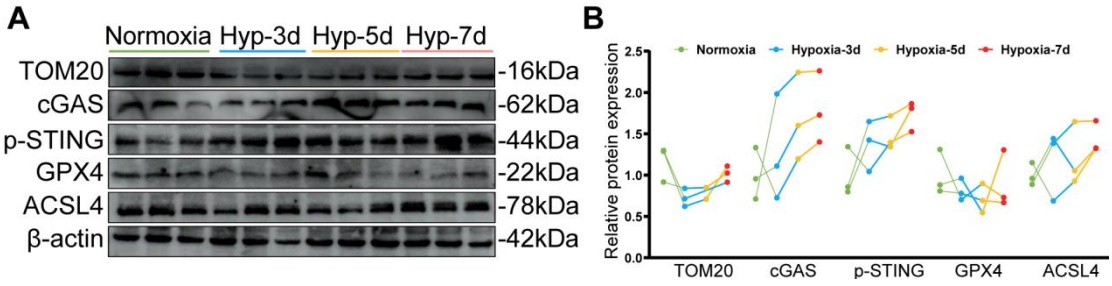
S4



Supplementary Fig. 4 Hypoxia promotes cytosolic dsDNA accumulation and activates cGAS–STING signaling in hippocampal neurons.

A–D:Representative immunofluorescence images of dsDNA (green), NeuN (red), and DAPI (blue) in the CA1, CA2, CA3, and DG regions under normoxia and after 3, 5, or 7 days of hypoxia. Boxed areas are shown at higher magnification. Scale bars, 25 μm for overview images and 5 μm for magnified images. E:Representative Western blots of cGAS, p-STING/STING, p-TBK1/TBK1, and p-IRF3/IRF3 in primary hippocampal neurons under normoxic and hypoxic conditions. F:Quantitative analyses of the indicated proteins. Comparisons were performed using two-tailed unpaired Student's t-tests. n = 4 independent biological replicates per group. Exact P values are shown where indicated; *P < 0.05.

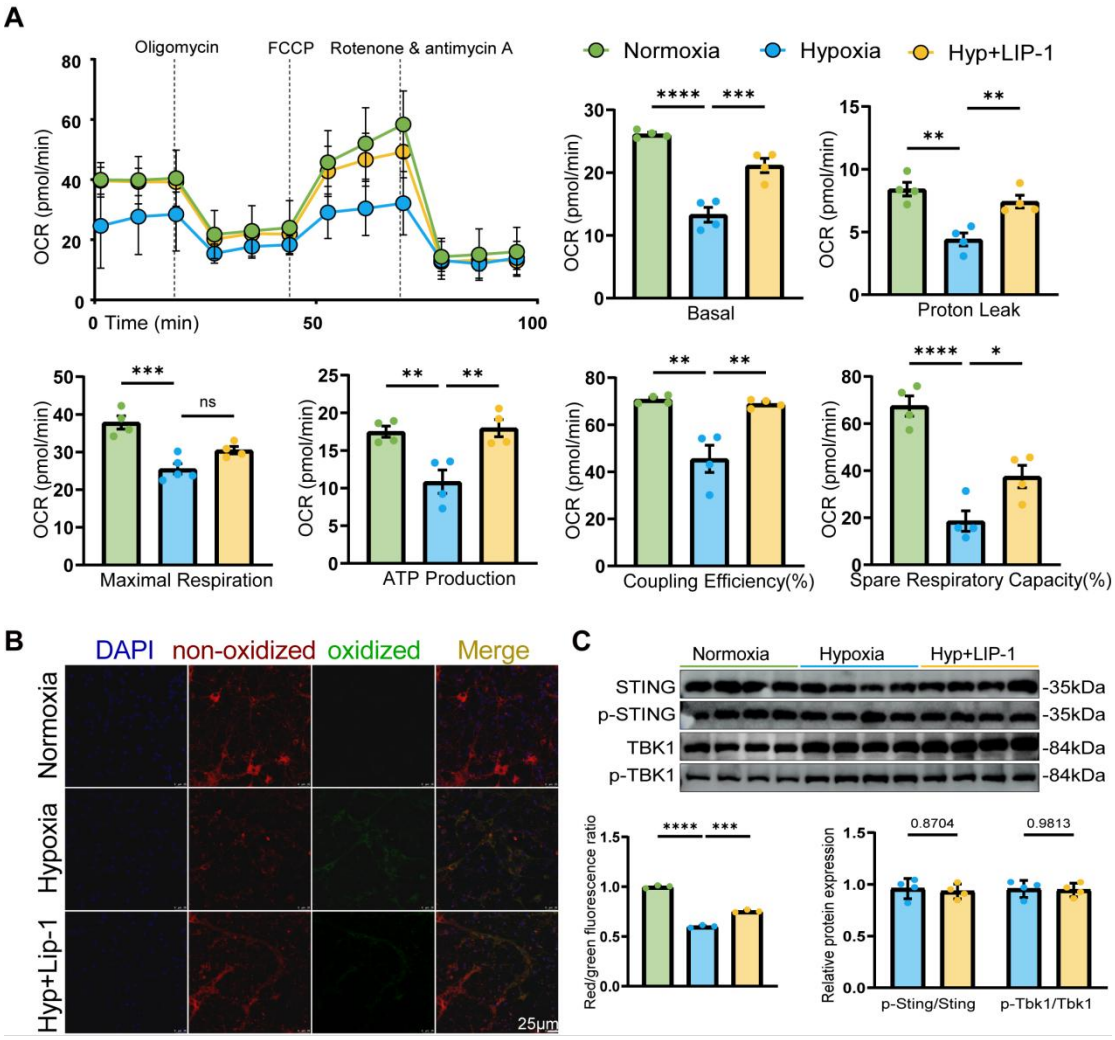
S5



Supplementary Fig. 5 Temporal changes in mitochondrial integrity, cGAS–STING signaling, and ferroptosis-associated proteins during hypoxia exposure.

A:Representative Western blots of Tom20, Cgas, p-Sting, Gpx4, and Acsl4 in hippocampal tissue from normoxic mice and mice exposed to hypoxia for 3, 5, or 7 days; β-actin served as the loading control. B:Relative expression profiles of the indicated proteins across the hypoxia time course. n = 3 independent biological samples per group.

S6

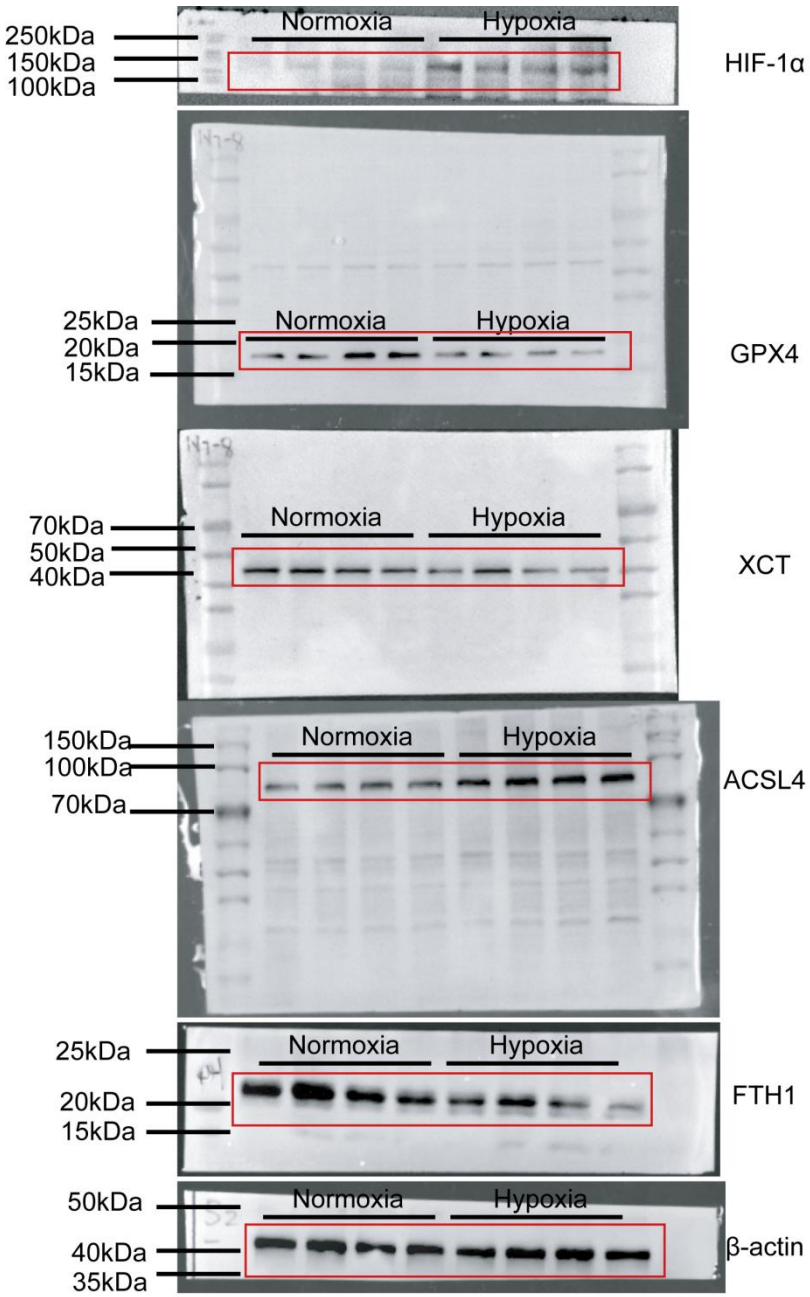


Supplementary Fig. 6 Liproxstatin-1 partially restores mitochondrial bioenergetics and lipid redox homeostasis without suppressing Sting signaling.

A:Seahorse mitochondrial stress-test OCR profiles in the normoxia, hypoxia, and hypoxia + LIP-1 groups, with quantification of basal respiration, proton leak, maximal respiration, ATP production, coupling efficiency, and spare respiratory capacity. Oligomycin, FCCP, and rotenone/antimycin A were sequentially added as indicated. B:Representative C11-BODIPY 581/591 fluorescence images showing non-oxidized lipid signals (red) and oxidized lipid signals (green). Scale bar, 25 μm. C:Representative Western blots and quantitative analyses of STING signaling-related proteins in the normoxia, hypoxia, and hypoxia + LIP-1 groups. Seahorse assays included n = 4 independent biological replicates per group; panels B–C included n = 4 per group. Three-group comparisons were performed using ordinary one-way ANOVA followed by Tukey's multiple-comparisons test. ns, not significant; *P<0.05,**P<0.01,****P<0.0001.

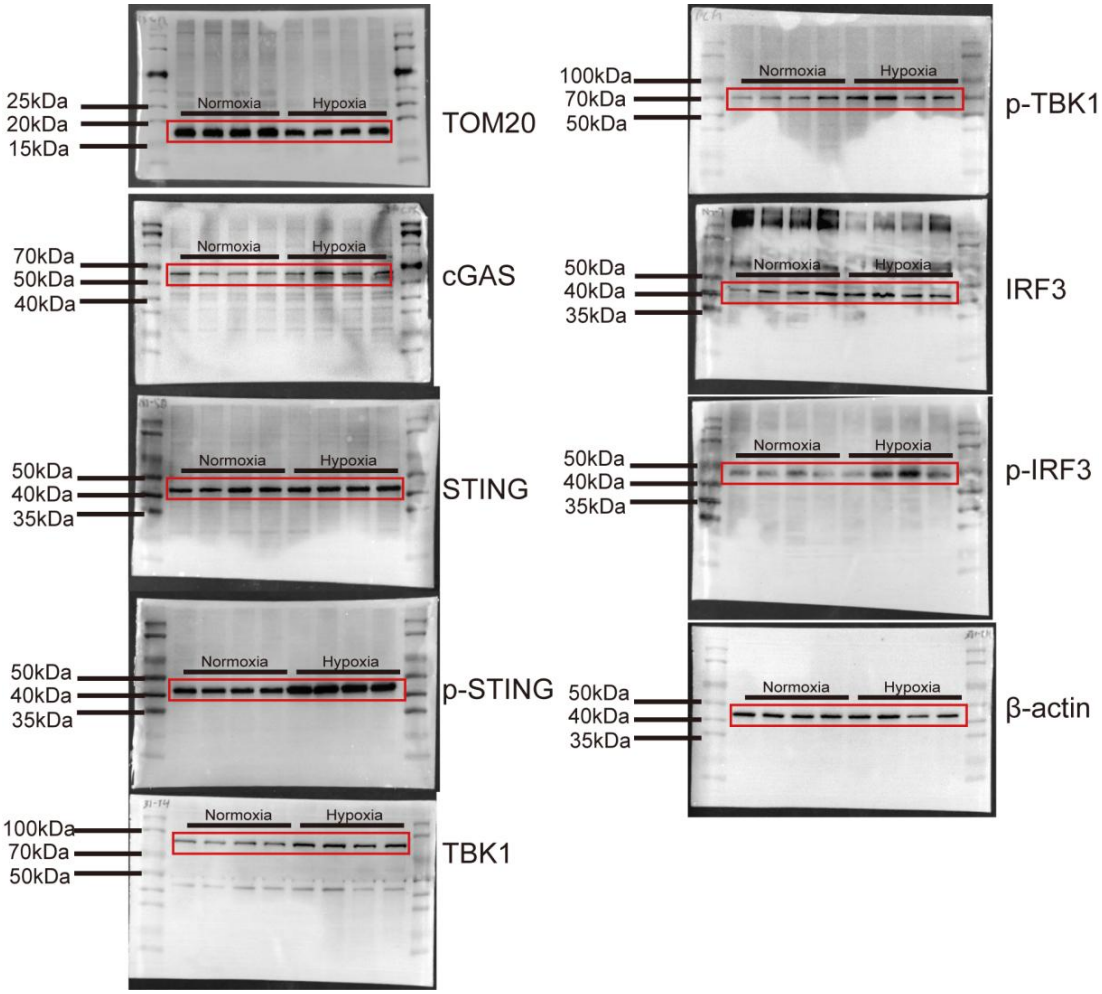
Original Western Blot Data and Full Blot Images

Fig. 2H



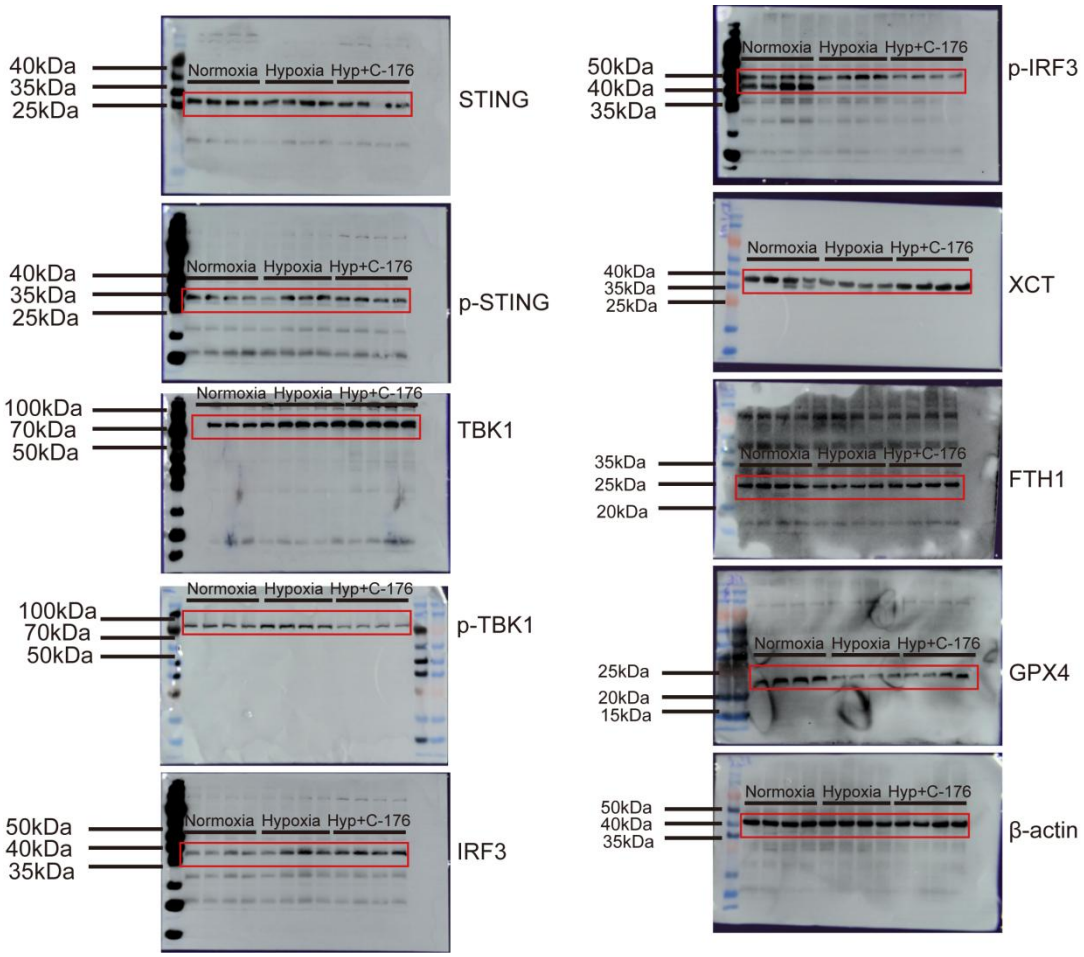
Original Western blot images showing HIF-1α, GPX4, XCT, ACSL4, FTH1, and β-actin in hippocampal tissue from normoxic and hypoxic mice. Molecular-weight markers (kDa) are indicated on the left, and the corresponding target proteins are indicated on the right. Red boxes indicate the target band regions used for representative presentation and densitometric analysis in Fig. 2H. β-actin served as the loading control for normalization of protein loading.

Fig.4F



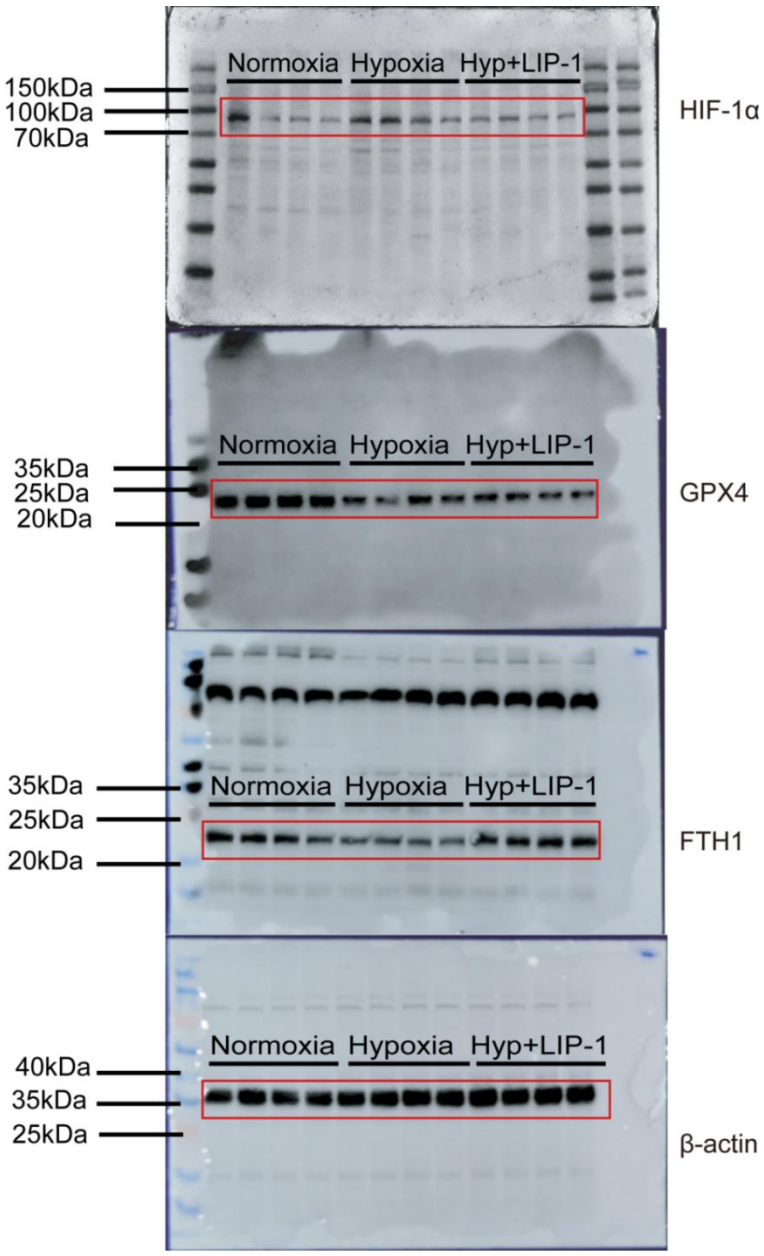
The original blots corresponding to Fig. 4F, including TOM20, cGAS, STING, p-STING, TBK1, p-TBK1, IRF3, p-IRF3, and β-actin in hippocampal tissue from normoxic and hypoxic mice. Phosphorylated proteins were normalized to their corresponding total proteins, whereas non-phosphorylated proteins were normalized to β-actin. Red boxes indicate the target band regions used for presentation and densitometric analysis in the corresponding figures.

Fig. 5I



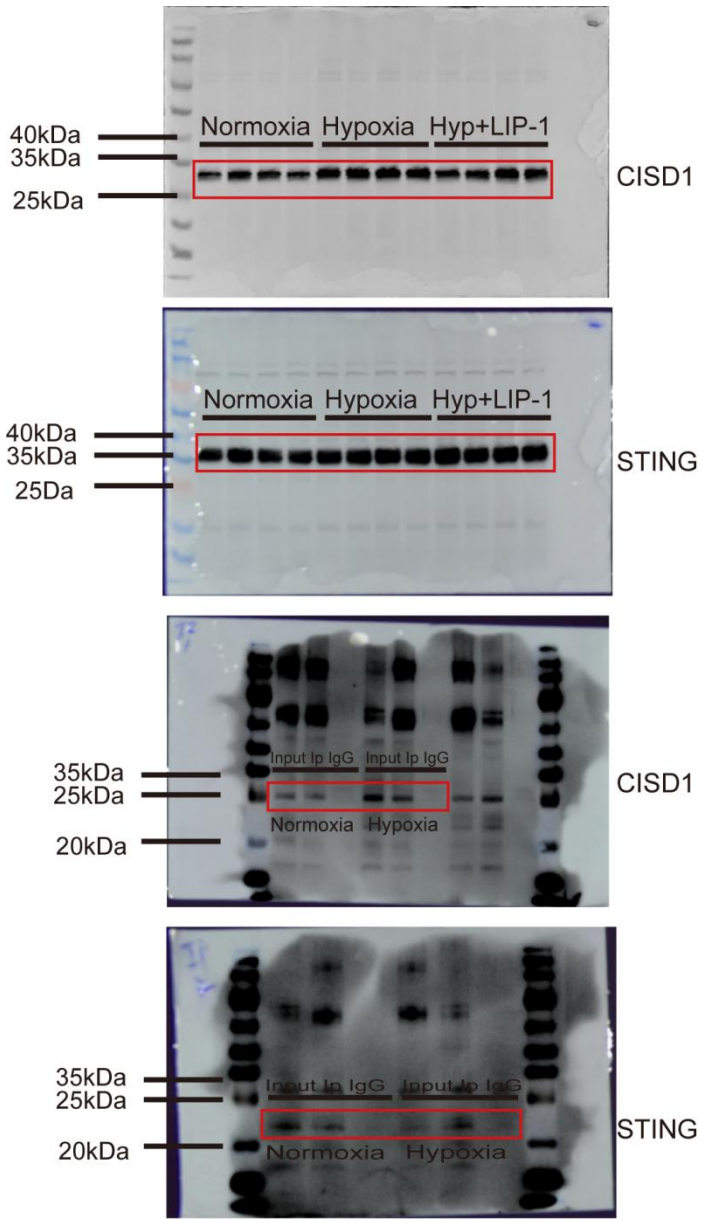
The original blots corresponding to Fig. 5I, including p-STING, STING, p-TBK1, TBK1, p-IRF3, IRF3, XCT, FTH1, GPX4, and β-actin in hippocampal tissue from the normoxia, hypoxia, and hypoxia + C-176 groups. Phosphorylated STING, TBK1, and IRF3 were analyzed relative to their corresponding total proteins, whereas β-actin served as the loading control for non-phosphorylated proteins.

Fig. 6H.



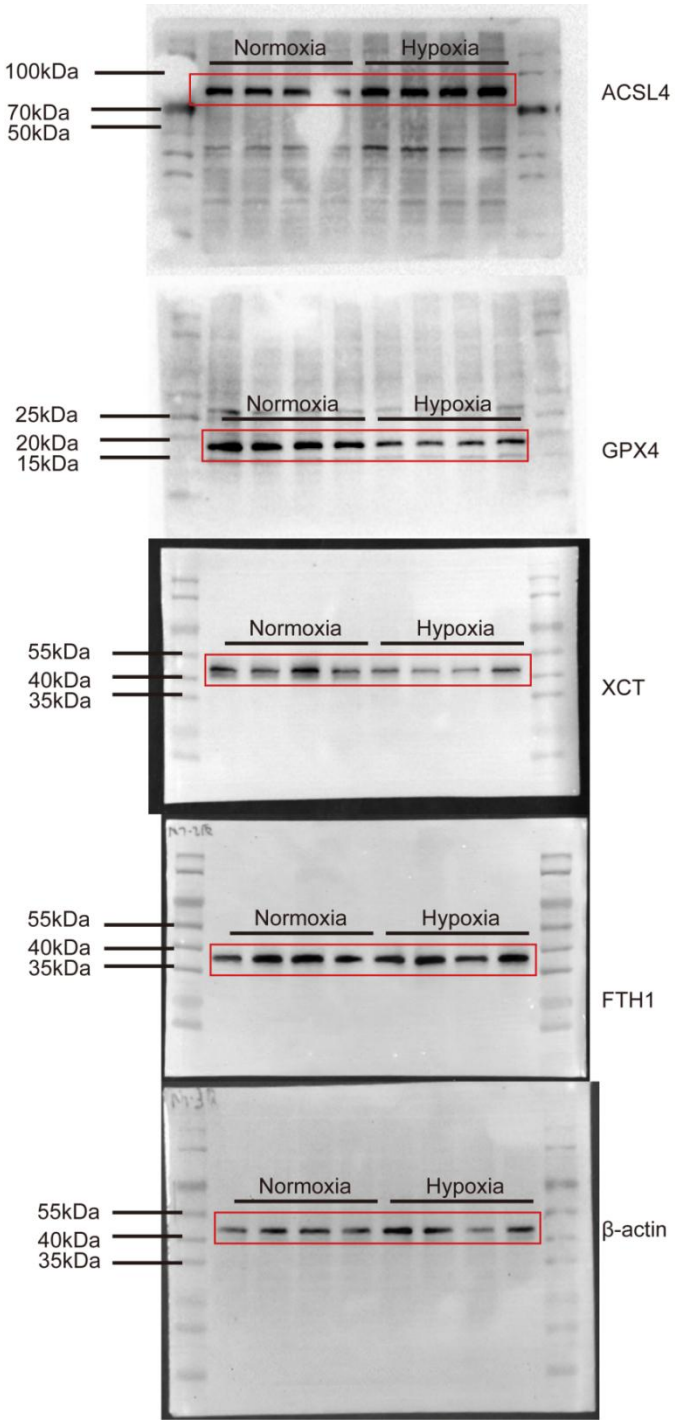
Original Western blot images showing HIF-1α, GPX4, FTH1, and β-actin in hippocampal tissue from the normoxia, hypoxia, and hypoxia + Liproxstatin-1 (Hyp + LIP-1) groups. Molecular-weight markers (kDa) are indicated on the left and the corresponding target proteins on the right. Red boxes indicate the target band regions used for representative presentation and densitometric analysis in Fig. 6H. β-actin served as the loading control.

Fig. 7F–G



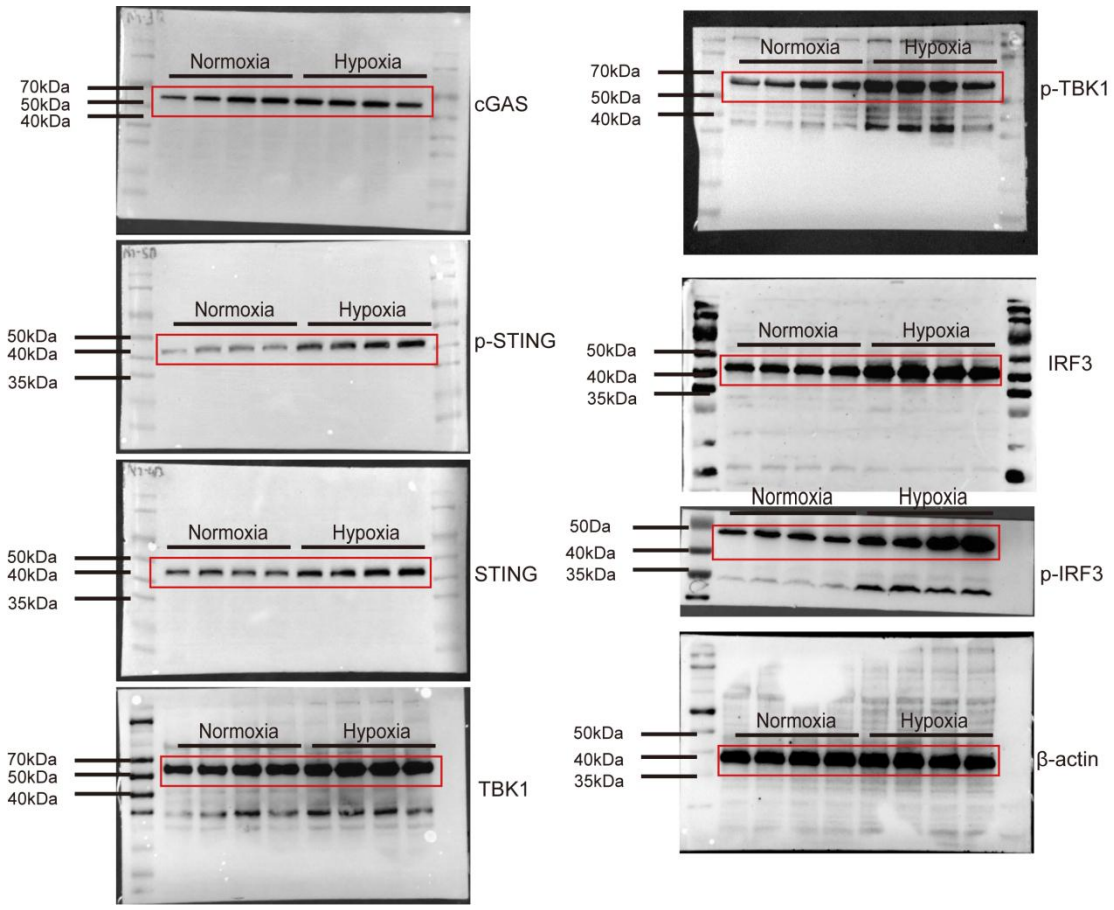
The upper panels show original Western blot signals for CISD1 and STING under normoxic, hypoxic, and hypoxia + Liproxstatin-1 (Hyp + LIP-1) conditions. The CISD1 blot provides source data supporting the CISD1 protein-expression analysis presented in Fig. 7F. The lower panels show original co-immunoprecipitation (Co-IP) blots assessing the STING–CISD1 interaction. Input, IP, and IgG control lanes are indicated. The normoxia and hypoxia Co-IP data correspond to Fig. 7G. The CISD1 blot additionally contains Co-IP data obtained under the hypoxia + C-176 condition; because this C-176 experiment is not currently described as a separate component of Fig. 7, it is designated here as additional validation data.

Fig. S3A



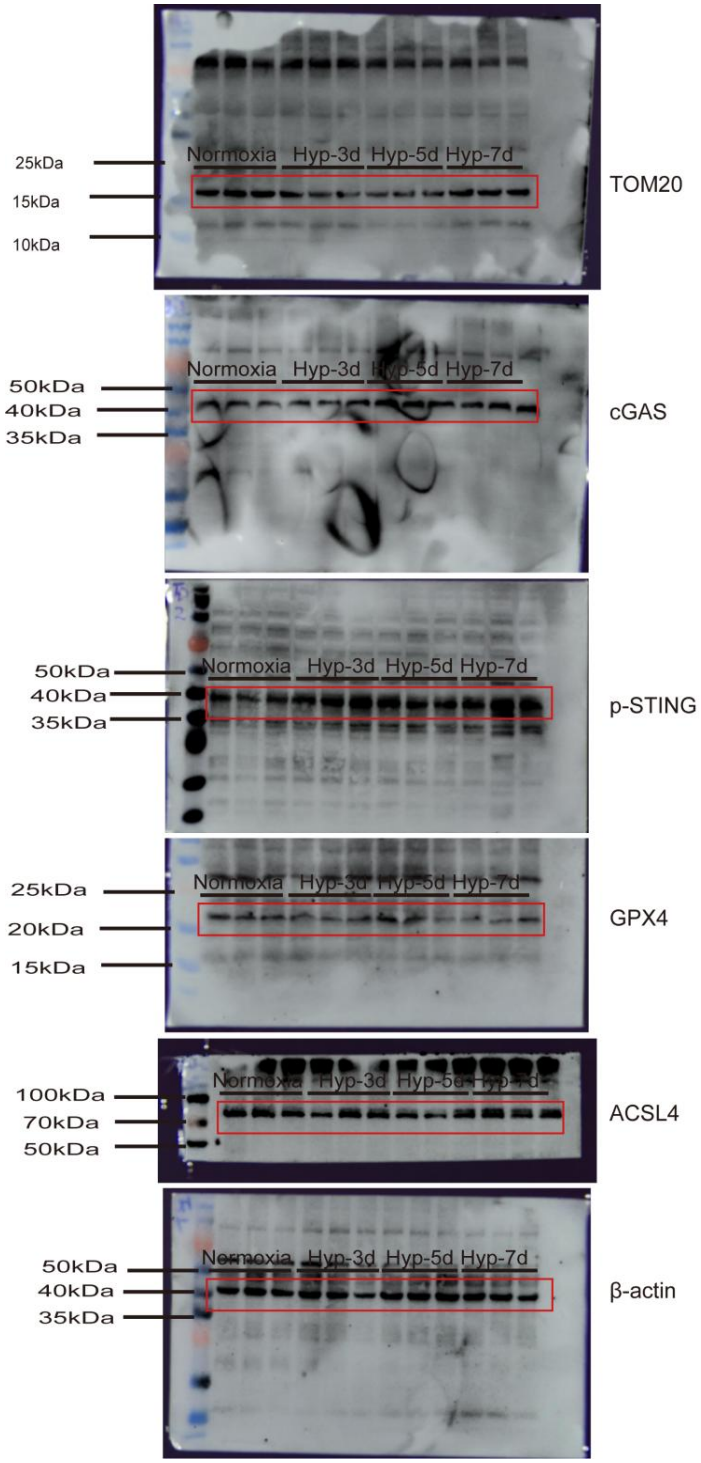
Original Western blot images showing ACSL4, GPX4, XCT, FTH1, and β-actin in primary hippocampal neurons cultured under normoxic or hypoxic conditions. Red boxes indicate the target band regions used for representative presentation and densitometric analysis in Supplementary Fig. S3A. β-actin served as the loading control. Four independent neuronal culture replicates were included per group.

Fig. S4E



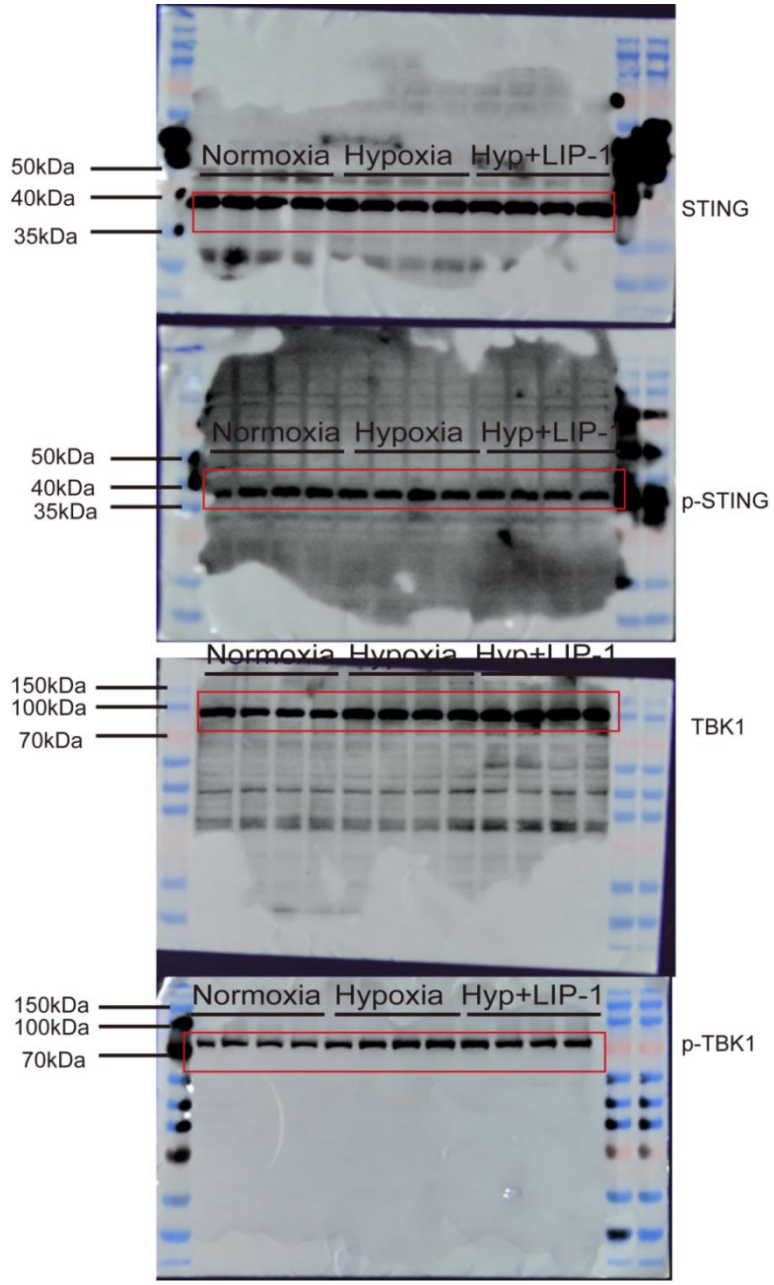
Original Western blot images showing cGAS, p-STING, STING, p-TBK1, TBK1, p-IRF3, IRF3, and β-actin in primary hippocampal neurons under normoxic and hypoxic conditions. Red boxes indicate the target band regions used for representative presentation and quantitative analysis in Supplementary Fig. S4E–F. Phosphorylated proteins were analyzed relative to their corresponding total proteins, whereas β-actin served as the loading control for non-phosphorylated proteins. Four independent biological replicates were included per group.

Fig. S5A



Original Western blot images showing TOM20, cGAS, p-STING, GPX4, ACSL4, and β-actin in hippocampal tissue from normoxic mice and mice exposed to hypoxia for 3, 5, or 7 days. These blots were used to assess temporal changes in mitochondrial integrity, cGAS–STING signaling, and ferroptosis-associated proteins during hypoxia exposure. Red boxes indicate the target band regions used for presentation and quantitative analysis in Supplementary Fig. S5A–B. β-actin served as the loading control. Four independent biological samples were included per group.

Fig. S6C



Original Western blot images showing STING, p-STING, TBK1, and p-TBK1 in the normoxia, hypoxia, and hypoxia + Liproxstatin-1 (Hyp + LIP-1) groups. Red boxes indicate the target band regions used for representative presentation and quantitative analysis in Supplementary Fig. S6C. p-STING and p-TBK1 were analyzed relative to their corresponding total STING and TBK1 proteins, respectively, to determine whether Liproxstatin-1 treatment affected activation of the STING signaling pathway. Four independent biological replicates were included per group.