

**Necroptosis and Mitophagy in the Pathogenesis
of Hypoxic-Ischaemic Encephalopathy**

A thesis submitted to Imperial College London for the
degree of Doctor of Philosophy

Lingzhi Wu

CID (00689134)

February 2023

Division of Anaesthetics, Pain Medicine and Intensive Care

Department of Surgery and Cancer

Faculty of Medicine

Chelsea and Westminster Hospital Campus

STATEMENT OF ORIGINALITY

I, Lingzhi Wu, hereby declare that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

The two MRes students that I had supervised during my PhD research, Miss Jiaqi Li and Dr Ge Huang, had contributed to experiments in Chapter Four and Chapter Five of this thesis. Western blot data from Fig. 3-13 and Fig. 3-14 were acquired and analysed with the help of Miss Jiaqi Li, and parts of Fig. 3-13 and Fig. 3-14 had been presented as Fig.5A and Fig.5B in her MRes project report titled “The Involvement of Necroptosis and Mitophagy in Hypoxic-ischaemic Encephalopathy”, dated March 2021. Western blot data from Fig. 3-11 and Fig. 3-12 and flow cytometry data from Supplementary Fig.1 and Fig.3-17 were obtained and analysed together with Dr Ge Huang. Parts of Supplementary Fig.1 and Fig.3-17 had been presented as Fig.1A, Fig.1B, Fig.2A and Fig.2B in Dr Huang’s MRes project report titled “Investigating the Regulatory Relationship between Necroptosis and Mitophagy in Hypoxic-Ischaemic Encephalopathy”, dated March 2022.

COPYRIGHT DECLARATION

The copyright of this thesis rests with the author. Unless otherwise indicated, its contents are licensed under a Creative Commons Attribution-Non Commercial-No Derivatives 4.0 International Licence (CC BY-NC-ND).

Under this licence, you may copy and redistribute the material in any medium or format on the condition that; you credit the author, do not use it for commercial purposes and do not distribute modified versions of the work.

When reusing or sharing this work, ensure you make the licence terms clear to others by naming the licence and linking to the licence text.

Please seek permission from the copyright holder for uses of this work that are not included in this licence or permitted under UK Copyright Law.

ABSTRACT

Hypoxic-ischaemic encephalopathy (HIE) is a devastating brain injury in newborns resulting from perinatal hypoxia event. A better understanding of the HIE pathophysiological mechanisms can help to identify targetable pathways for therapeutic development to improve HIE outcomes. In the present study, we hypothesise that necroptosis acts upstream to regulate mitophagy during HIE and ischaemia-reperfusion (I/R) brain injury.

Oxygen-glucose deprivation and reperfusion (OGD/Rep) was used to model I/R brain injury *in vitro* with mouse primary neurons or mouse neuroblastoma Neuro-2a cells. For murine model of HIE, right common carotid artery (CCA) coagulation plus hypoxia (8%O₂) was performed on postnatal day7 mice.

OGD/Rep induced necroptosis *in vitro* with increased phosphorylation of MLKL* and translocation of necrosome into the mitochondrial fraction. OGD/Rep also upregulated mitophagy mediators Parkin and BNIP3 and increased mitochondrial localisation of autophagosome marker LC3A/B-II to reflect enhanced mitophagy. Blocking necroptosis with RIPK1 inhibitor Nec-1s or RIPK3 inhibitor GSK'872 attenuated OGD/Rep-induced mitophagy, whereas siRNA-mediated MLKL knockdown further amplified mitophagy. Notwithstanding, either RIPK1/RIPK3 inhibition or MLKL knockdown restored mitochondrial function and morphology. In postnatal day7 mice, CCA plus hypoxia stimulated necroptosis and mitophagy at 24hrs of reperfusion, in particular in CA1 and CA3 of hippocampus. Intracerebrovascular administration of Nec-1s attenuated Parkin and BNIP3 in the hippocampus and decreased mitochondrial localisation of LC3A/B-II to indicate suppressed

* MLKL, mixed lineage kinase-domain like; BNIP3, Bcl2-interacting protein 3; LC3A/B-II, microtubule-associated light chain 3A/B-II; RIPK1, receptor-interacting protein kinase 1; RIPK3, receptor-interacting protein kinase 3; siRNA, small-interfering RNA; CA1, cornu ammonis 1; CA3, cornu ammonis 3.

mitophagy. Moreover, RIPK1 inhibition by Nec-1s was associated with long-term improvement in motor functions in HIE-injured mice.

In summary, the current study suggests RIPK1 and RIPK3 as likely positive regulators of mitophagy whereas MLKL likely suppresses mitophagy during HIE pathopathogenesis. Mitochondrial protection was evident from RIPK1/RIP3 inhibition or MLKL silencing, and RIPK1 inhibition may improve long-term motor function in HIE. Our findings provide fundamental rationale for developing therapeutic strategy that targets necroptosis and associated mitophagy pathway in treating HIE or other forms of I/R brain injury *per se*.

ACKNOWLEDGEMENT

I would like to express my most sincere gratitude to my PhD supervisor Professor Daqing Ma, Head of Anaesthetics Research at Division of Anaesthetics, Pain Medicine and Intensive Care, Imperial College London and Macintosh Professor at Royal College of Anaesthetists, who had provided me with tremendous support, insightful guidance and ongoing motivation throughout my PhD study. I am also extremely grateful to the generous support from National Institute of Academic Anaesthesia, from whom I received BJA/RCoA PhD studentship to fund my PhD study.

I wish to thank our research associate Dr Hailin Zhao for his technical inputs and valuable guidance on all the experiments. I also would like to extend my gratitude to my fellow PhD students Dr Enqiang Chang, Dr Qizhe Sun, Dr Cong Hu and Dr Qian Chen for sharing their knowledge and expertise that were of great help to my experiments, and also for their company during my study. I am also thankful to Miss Jiaqi Li and Dr Ge Huang, MRes students I had supervised on a daily basis, for their hard work and contributions to this project.

I wish to show my deep appreciation for Dr Tianyun Zhao, Miss Jingwen Qin and Miss Yaying Liang from Guangdong-Hongkong-Macau Institute of CNS Regeneration at Jinan University, Guangzhou, China, for their valuable assistance and guidance with the animal experiments.

Finally, I would like to thank my family and loved ones for their unwavering support, understanding and faith during my PhD study.

TABLE OF CONTENTS

STATEMENT OF ORIGINALITY	2
COPYRIGHT DECLARATION	3
ABSTRACT	4
ACKNOWLEDGEMENT	6
ABBREVIATIONS	14
CHAPTER ONE	19
Introduction	19
A. Hypoxic-Ischaemic Encephalopathy	20
A.1 Epidemiology	20
A.2 Clinical Definitions and Risk Factors	20
A.3 Clinical Presentation and Diagnostic Criteria	21
B. Management of HIE and Therapeutic Development	24
B.1 Systemic Support	24
B.2 Therapeutic Hypothermia	24
B.3 Experimental and Emerging Therapies	26
B.3.1 Xenon	26
B.3.2 Erythropoietin	27
B.3.3 Melatonin	27
B.3.4 Stem Cell Therapy	28
C. Animal Models for Studying Hypoxic-Ischaemic Encephalopathy	30
C.1 Rodents	30
C.1.1 Rats	30
C.1.2 Mice	31
C.1.3 Functional and Behavioural Studies in Rodent Models of HIE	32
C.2 Larger Mammals	34
D. The Pathophysiology of Hypoxic-Ischaemic Encephalopathy	35
D.1 Primary Phase and Latent Phase	36
D.2 Secondary Phase	37
D.2.1 Excitotoxicity	37
D.2.2 Calcium Imbalance, Mitochondrial Respiratory Chain Dysfunction and Oxidative Stress	38

D.2.3 Mitochondrial Permeabilisation and Apoptosis	40
D.2.4 The Intrinsic Apoptosis Pathway	41
D.2.5 PARP-1, AIF and Parthanatos	42
D.2.5.1 Sex Dimorphic Regulation on Apoptosis-Related Pathways	44
D.2.6 Inflammation and Immune Responses in HIE	46
D.2.6.1 Priming Effect of Inflammation	46
D2.6.2 Immune Responses Triggered by Hypoxia-ischaemia and Reperfusion	47
D.3 Tertiary Phase	51
D.3.1 Chronic Inflammation	51
D.3.2 Epigenetic Mechanisms	53
D.3.3 Sex Hormones	54
D.4 Susceptible Cell Types from Hypoxia-Ischaemia and Reperfusion Injury	56
E. Necroptosis	58
E.1 Molecular Regulation of Extrinsic Apoptosis and Necroptosis	58
E.1.1 Post-translational Modification of RIPK1	58
E.1.2 Extrinsic Apoptosis – The Formation of Complex IIa	61
E.1.3 Necroptosis – The Formation of Necrosome and Alternative Complexes	64
E.2 Necroptosis in CNS Disorders	67
F. Mitochondrial Function and Adaption During HIE	69
F.1 Mitochondrial Fission and Fusion	69
F.2 Mitochondrial Biogenesis	70
F.3 Mitophagy	71
F.3.1 Receptor-mediated Mitophagy	73
F.3.2 PINK/Parkin-mediated Ubiquitination Pathway	74
F.3.3 Measuring Mitophagy	78
F.3.4 Mitophagy in Hypoxic-Ischaemic Brain injury and HIE	78
F.4 Caseinolytic protease CLpP and Mitochondrial Quality Control	80
G. Mitochondrion as the Interface Between Necroptosis and Mitophagy?	82
G.1 Open Questions and Novelties of the current study	83
G.2 Aims, Hypothesis and Objectives	84

CHAPTER TWO	86
Materials and Methods	86
2.1 Mouse Primary Neuronal Cell Culture	87
2.2 Neuro-2a Cell Culture and Differentiation	88
2.3 Oxygen-Glucose Deprivation (OGD)	89
2.3.1 OGD in Primary Neuronal Cell Culture	89
2.3.2 OGD in Differentiated Neuro-2a Cells	90
2.4 Treatment with Pharmacological Inhibitors	91
2.4.1 Necroptosis Inhibition	91
2.4.2 Lysosome/autolysosome inhibition	91
2.5 Small interfering RNA (siRNA)-mediated knockdown	92
2.6 Immunofluorescence Staining	93
2.6.1 Immunofluorescence Staining for Cell Sample	94
2.6.2 Immunofluorescence Staining for Tissue Sample	94
2.7 Cresyl-Violet Staining	97
2.8 Live-cell Imaging of Neuro-2a Cells	98
2.9 Western Blot	99
2.9.1 Whole-cell Protein Lysis	100
2.9.2 Mitochondrial Protein Isolation	101
2.9.3 Gel Electrophoresis, Membrane Transfer and Detection	101
2.9.4 Co-immunoprecipitation	102
2.10 Flow Cytometry	104
2.10.1 JC-10 Detection of Mitochondrial Membrane Potential	105
2.10.2 TMRE Detection of Mitochondrial Membrane Potential	106
2.10.3 Propidium Iodide and Annexin V Staining	107
2.11 Cell Viability by Cell Counting Kit-8 (CCK8)	108
2.12 Murine Model of Hypoxic-Ischaemic Encephalopathy (HIE)	109
2.12.1 Animals	109
2.12.2 Animal Surgery and Hypoxia Exposure	109
2.12.3 Treatment with RIPK1 Inhibitor 7-ClO-O-Nec-1/Nec-1s	110
2.12.4 Motor and Behavioural Assessments	110

2.13 Statistical Analysis	112
CHAPTER THREE	113
Oxygen-Glucose Deprivation and Reperfusion (OGD/Rep) Upregulated Necroptosis and Mitophagy in Mouse Primary Neurons and Neuro-2a Cells <i>in vitro</i>	113
3.1 Introduction	114
3.2 Results	119
3.2.1 Oxygen-glucose deprivation plus reperfusion induced necroptosis and mitochondrial localisation of LC3A/B in mouse primary neurons	119
3.2.2 Oxygen-glucose deprivation plus reperfusion induced necroptosis and upregulated mitophagy mediators in differentiated Neuro-2a cells	124
3.2.3 RIPK1 inhibitor necrostatin-1 suppressed necrosome translocation and mitophagy	130
3.2.4 RIPK3 inhibitor GSK'872 suppressed necrosome translocation and mitophagy	135
3.2.5 MLKL knockdown prevented necrosome translocation and enhanced mitophagy	139
3.2.6 Interaction between MLKL and mitophagy mediators	144
3.2.7 RIPK1 inhibition, RIPK3 inhibition or MLKL knockdown restored mitochondrial membrane potential	146
3.2.8 RIPK1 inhibition or MLKL knockdown improved mitochondrial morphology	149
3.2.9 Cell Viability	149
3.3 Discussion	154
3.3.1 Concurrent activation of necroptosis and mitophagy in primary neurons and Neuro-2a cells from OGD and reperfusion	154
3.3.2 Necrosome components exert opposing effects on OGD/Rep-induced mitophagy	155
3.3.3 Necroptosis inhibition restores mitochondrial function decline from OGD/Rep challenge	158
CHAPTER FOUR	161
Activation of Necroptosis and Mitophagy in a Murine Model of Hypoxic-Ischaemic Encephalopathy	161
4.1 Introduction	162
4.2 Results	165
4.2.1 The development of brain lesion and motor dysfunction following HIE	165
4.2.2 Upregulated expression of necroptosis markers following HIE	168
4.2.3 Upregulated expression of mitophagy markers following HIE	174
4.2.4 Necrostatin-1s prevents MLKL and RIPK3 phosphorylation in the hippocampus post-HIE	181
4.2.5 Necrostatin-1s attenuates mitophagy in the hippocampus post-HIE	181

4.2.6 The effect of RIPK1 inhibition on long-term motor and behavioural outcomes	193
4.3 Discussion	195
4.3.1 Activation of regulated cell death during hypoxic-ischemic encephalopathy	195
4.3.2 Enhanced mitophagy during hypoxic-ischemic encephalopathy	196
4.3.3 The effect of RIPK1 inhibition on necroptosis and mitophagy markers	198
4.3.4 The effect of RIPK1 inhibition on long-term motor and behavioural outcomes	199
CHAPTER FIVE	203
Final Discussion	203
5.1 Rationale of the Study	204
5.2 Summary of the Findings	206
5.3 Scientific and Translational Implications	209
5.3.1 Mitochondrial Translocation of Necrosome	209
5.3.2 Necroptosis and Mitochondrial Dysfunction	210
5.3.3 Mitophagy as a Neuroprotective Mitochondrial Quality Control Mechanism	213
5.3.4 Targeting Necroptosis and Mitophagy for Treating I/R Brain Injury	216
5.4 Limitations and Future Works	217
5.4.1 Main Limitations	217
5.4.2 In vitro experiments	218
5.4.3 In vivo experiments	221
5.5 Conclusions	224
REFERENCES	225
APPENDICES	254

ABBREVIATIONS

3-MA	3-methyladenine
4HBD	four α -helix bundle
7-ClO-O-Nec-1	necrostatin-1s
ADC	apparent diffusion coefficients
AIF	apoptosis-inducing factor
ALS	amyotrophic lateral sclerosis
AML	acute myeloid leukemia
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Apaf-1	apoptotic peptidase-activating factor 1
ATF2	activating transcription factor 2
ATG	autophagy-related genes
AD	Alzheimer's disease
BAF	bocasparyl(OMe)-fluoromethylketone
Baf _{A1}	bafilomycin A1
BBB	blood brain barrier
BCL-2	B-cell lymphoma 2
Bcl-2-L-13	Bcl2-like protein 13
BCL-XL	B-cell lymphoma extra large
BDNF	brain-derived neurotrophic factors
BGT	basal ganglia and thalamus
BHA	butylated hydroxyanisole
BLA	basolateral amygdala
BNIP3	Bcl-2 interacting protein 3
BNIP3L/NIX	Bcl-2 interacting protein 3-like
BNST	bed nucleus of the stria terminalis
BSA	bovine serum albumin
CA	cornu ammonis
CAD	caspase-activated deoxyribonuclease
CALCOCO2/NDP52	calcium binding and coiled-coil domain 2
CBF	cerebral blood flow
CBP	CREB-binding protein
CCA	common carotid artery
CCCP	carbonyl cyanide chlorophenylhydrazone
CCI	controlled cortical impact
CCK-8	cell counting kit-8
CeA	central amygdala
c-FLIP	cellular FLICE-inhibitory peptide
CGU	cerebral glucose utilisation
CHMP	charged multivesicular body protein
CHX	cycloheximide
c-IAP	cellular inhibitor of apoptotic protein
CK2	casein kinase 2

CLpP	caseinolytic mitochondrial matrix protease proteolytic subunit
CLpX	caseinolytic mitochondrial matrix protease chaperone subunit x
CNTF	ciliary neutrophilic factors
CNS	central nervous system
COXI/IV	cytochrome c oxidase subunits 1 to 4
COX2	cyclooxygenase 2
CXCR4	C-X-C chemokine receptor 4
CP	cerebral palsy
CRE	CREB-responsive element
CREB	cyclic-AMP response element binding protein
CS	conditioned stimulus
CSF	cerebrospinal fluid
CYLD	cylindromatosis
DAI	DNA-dependent activator of interferon regulatory factors
DAMPs	danger-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DAPK1	death associated protein kinase 1
db-cAMP	dibutyryl-cyclic AMP
DG	dentate gyrus
DIABLO	direct IAP Binding Protein with Low pI
DISC	death-inducing signalling complex
DHT	dihydrotestosterone
DMEM	Dulbeccos' Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DRP-1	dynammin-related protein 1
EAA	excitatory amino acids
EEG	electroencephalography
eNOS	endothelial nitric oxide synthase
EPO	erythropoietin
ER	endoplasmic reticulum
ERR- α /g	oestrogen-related receptors- α and - γ
ESCRT-III	endosomal sorting complex required for transport III
ETC	electron transport chain
FasL	Fas ligand
FA	fractional anisotropy
FADD	Fas-associated death domain
FCCP	carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone
FiO ₂	fraction of inspired oxygen
FITC	fluorescein isothiocyanate
FLICE	FADD-like IL-1 β -converting enzyme
FOXO	forkhead box protein O
FSC	forward-scattered light
FUNDC1	FUN14 domain-containing protein 1
GABA	γ -aminobutyric acid

GABARAP	GABA receptor-associated protein
GFAP	glial fibrillary acidic protein
GLUD1	glutamate dehydrogenase 1
GLUL	glutamate-ammonia ligase
HAT	histone acetyltransferase
HBSS	Hank's balanced salt solution
HDAC	histone deacetylase
H/I	hypoxia-ischaemia
HIE	hypoxic-ischaemic encephalopathy
HIF	hypoxia-inducible factor
¹ H-MRS	proton magnetic resonance spectroscopy
HNF-4a	hepatic nuclear factor-4 alpha
HRP	horseradish peroxidase
HSP70	heat shock protein 70
IAP	inhibitor of apoptotic proteins
ICH	intracerebral haemorrhage
i.c.v	intracerebrovascular route of administration
IDO	indoleamine-2,3-dioxygenase
IFN- β	interferon-beta
IFN- γ	interferon-gamma
IL-1 β	interleukin 1 beta
IL-1R	Interleukin-1 receptor
IL-6	interleukin 6
IL-8	interleukin 8
IL-18	interleukin 18
I κ K	inhibitor of κ B kinase
I/R	ischaemia-reperfusion
IRF3	interferon regulatory factor 3
JC-1	5,5,6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide
JNK	c-Jun N-terminal kinases
JNK3	c-Jun N-terminal kinase 3
LAMP2	lysosomal membrane protein 2
LC3	microtubule-associated protein light chain 3
LC3A/B	microtubule-associated protein light chain 3 isoforms A and B
LIF	leukocyte inhibitory factor
LIR	LC3-interacting region
LPS	liposaccharides
LUBAC	linear ubiquitin chain assembly complex
MAP	mean arterial blood pressure
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MCAO	middle cerebral artery occlusion
MCAD	medium chain acyl-CoA dehydrogenase
MCP-1	monocyte chemotactic protein 1

mdivi-1	mitochondrial division inhibitor 1
MEM	eagle's minimum essential medium
MHC-II	major histocompatibility complex-II
MIP-1a	macrophage inflammatory protein 1-alpha
MLKL	mixed-lineage kinase-domain like
MMP	matrix metalloproteinase
MOM	mitochondrial outer membrane
MOMP	mitochondrial outer membrane permeabilization
mPFC	medial prefrontal cortex
MPO	myeloperoxidase
MPP	mitochondrial processing peptidase
mPTP	mitochondrial permeability transition pores
MRI	magnetic resonance imaging
MS	multiple sclerosis
MTF1/2	mitofusin 1/2
mTORC1	mammalian target of rapamycin complex 1
MTS	mitochondrial-targeting sequence
MWZ	Morris water maze
MyD88	myeloid differentiation primary response gene 88
NAA	N-acetyl aspartate
NBQX	6-nitro-7-sulfamoylbenzo(f)quinoxaline-2,3-dione
NDS	normal donkey serum
NDUFB8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8
NE	neonatal encephalopathy
Nec-1(s)	necrostatin-1(s)
NEMO	NF-kB essential modulator
pNF-H	phosphorylated axonal neurofilament heavy chain
NF-κB	nuclear factor-kappa light chain enhancer of activated B cell
NGF	nerve growth factor
NIRS	near-infrared spectroscopy
NLRP3	NLR family pyrin domain containing 3
NMDA	glutamate N-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NOR	novel object recognition
NOX4	NADPH oxidase 4
NPC	neural progenitor/precursor cells
NRF1/2	nuclear respiratory factors 1 and 2
NSA	necrosulfonamide
NSC	neural stem cell
NSE	neuron-specific enolase
NT	neurotrophin
NTP	nucleotide triphosphate
OFT	open field test
OGD	oxygen glucose deprivation

OGD/Rep	oxygen glucose deprivation and reperfusion
OLT	object location task
OPA1	optic atrophy 1
OPC	oligodendrocyte precursor cells
OPT	optineurin
OTULIN	OTU deubiquitinase with linear linkage specificity
PaCO ₂	arterial partial pressure of CO ₂
PAMPs	pathogen-associated molecular patterns
PaO ₂	arterial partial pressure of oxygen
PAR	poly ADP-ribose (PAR)
PARK2	parkin
PARL	presenilin-associated rhomboid-like protease
PARP	poly ADP-ribose polymerase
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PBS-Tween20	phosphate-buffered saline with 0.3% Tween 20
PBS-Triton X	phosphate-buffered saline with 0.3% Triton X-100
PCr	phosphocreatine
PD	Parkinson's disease
PDC	pyruvate dehydrogenase complex
PE	phosphatidylethanolamine
PE	phytoerythrin
PEPCK	phosphoenolpyruvate carboxykinase
PET	positron emission tomography
PFA	paraformaldehyde
PGAM	phosphoglycerate mutase 5
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1α
PGE ₂	prostaglandin E ₂
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PIER	protease-induced epitope retrieval
PINK1	PTEN-induced putative kinase 1
PLIC	posterior limb of the internal capsule
PMNL	polymorphonuclear leukocytes
³² P-MRS	phosphate magnetic resonance spectroscopy
PND	postnatal day
Poly I:C	poly inosinic: poly cytidylic acid
PPARα	peroxisome proliferator-activated receptor-α
PRRs	pattern recognition receptors
PS	phosphatidylserine
PSD95	postsynaptic density protein 95
PtdIns3P	phosphatidylinositol 3-phosphate
PYGL	glycogen phosphorylase
Q-VD-Oph	quinoline-Val-Asp(Ome)-CH ₂ -O-phenoxy

RCD	regulated cell death
RDA	RIPK1-dependent apoptosis
RIA	RIPK1-independent apoptosis
RING	really interesting new gene domain
RIPK1	receptor-interacting protein kinase 1
RIPK3	receptor-interacting protein kinase 3
RISC	RNA-induced silencing complex
RNS	reactive nitrogen species
ROS	reactive oxygen species
SAP	spontaneous alternation performance
SDF-1	stromal cell derived factor-1
SDS	sodium dodecyl sulphate
SDHA	succinate dehydrogenase subunit A
SGZ	subgranular zone
siRNA	small-interfering RNA
shRNA	short-hairpin RNA
SMAC	second mitochondria-derived activator of caspase
SOD	superoxide dismutase
SQSTM1/p62	sequestosome 1
SSC	side-scattered light
SVZ	subventricular zone
TAK1	transforming growth factor b-activated kinase 1
TAM	Tyro3, Axl, and Mer family of receptor kinases
TANK	TRAF-associated NF- κ B activator
TBI	traumatic brain injury
TBK1	TANK-binding protein 1
TBS-T	tris-buffered saline with 0.1% Tween-20
TFAM	mitochondrial transcription factor A
TGF-1 β	transforming growth factor 1-beta
TIM	translocase of inner membrane
TLR	toll-like receptor
TLR3	toll-like receptor 3
TLR4	toll-like receptor 4
TMRE	tetramethylrhodamine ethyl ester
TNF- α	tumour-necrosis factor alpha
TNFR1	tumour-necrosis factor receptor 1
TNFR2	tumour-necrosis factor receptor 2
TNFSF	tumour necrosis factors superfamily
TNFRSF	tumour necrosis factor receptors superfamily
TOM	translocase of outer membrane
TRADD	TNFR1-associated DD protein
TRAF2	TNFR-associated factor 2
TRAIL	TNF-related apoptosis-inducing ligand
TRIF	toll/IL-1R domain-containing adaptor-inducing interferon beta

Ubl	ubiquitin-like domain
UCB	umbilical cord blood
UCH-L1	ubiquitin c-terminal hydrolase-1
ULK	Unc-51 like autophagy activating kinase
UPR	unfolded protein response
US	unconditioned stimulus
VDAC1/3	voltage-dependent anion-selective channel protein 1 and 3
VEGF	vascular endothelial growth factor
vICA	viral-encoded inhibitor of caspases
WIPI	WD-repeat protein interacting with phosphoinositides
WMI	white matter injury
XIAP	X-linked inhibitor of apoptotic protein
ZBP-1	Z-DNA binding protein-1
Z-VAD-FMK	carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone
β-ME	β-mercaptoethanol

Introduction

A. Hypoxic-Ischaemic Encephalopathy – Epidemiology, Clinical Definition and Risk Factors

A.1 Epidemiology

Hypoxic-ischaemic encephalopathy (HIE) resulting from birth asphyxia contributes significantly to perinatal mortality and morbidity worldwide. The reported incidence of HIE ranges between 1-6 per 1,000 live births in developed nations¹ and can rise to 26/1,000 in developing countries². Among those, 10-15% affected infants would die during the neonatal period³, which is equal to approximately 1 million neonatal deaths (within 4 weeks of life) to account for a quarter of all neonatal mortality globally every year⁴. Similarly, World Health Organisation reported that birth asphyxia accounts for 9% of all under-five mortality worldwide (8.795 million) with an estimated death rate between 0.8-1 million annually⁵; 15-20% of those surviving from HIE continue to suffer from long-term cerebral palsy, learning disabilities, sensorimotor dysfunctions and behavioural disorders, even in those diagnosed with mild HIE^{3, 6}.

A.2 Clinical Definitions and Risk Factors

The terms of neonatal encephalopathy (NE) and hypoxic-ischaemic encephalopathy (HIE) have been used interchangeably to describe the collective neurological syndromes shortly after birth in termed or late preterm neonates. HIE specifically refers to a scenario of NE where hypoxia-ischaemia event during the perinatal period is evident^{3, 7, 8}. For decades intrapartum hypoxia-ischaemia insult was considered to be the primary cause of NE and thus HIE has been considered as the predominant form of NE clinically. Clinical studies reported that only a small proportion of NE directly results from birth asphyxia, with the principle aetiology being antepartum and encompasses diverse risk factors. Many have called for a revision to the use of HIE and recommended the more inclusive terminology NE as it was deemed difficult to ascertain the aetiological significance of hypoxia-ischaemia. In this regard, identifying the precise aetiologies of NE is of paramount importance, as this dictates the type and timing of treatment/intervention offered and is crucial to infant prognosis in short- and long-term.

In a series of population-based case-control studies on antepartum and intrapartum risk factors in termed newborns^{1, 9, 10}, Badawi and colleagues concluded that NE is primarily ascribed to antepartum factors (69%), followed by antepartum risk factors plus intrapartum hypoxia (25%), with intrapartum hypoxia alone only accounting for 4% of NE cases. In this Western Australian cohort, pre-partum risk factors most strongly associated with NE include maternal thyroid disease, family history of seizures/neurological disease, intrauterine growth restriction adjusted for gestational age, severe pre-eclampsia, infection and metabolic disorders. The Neonatal Encephalopathy Registry of Vermont Oxford Study examined the clinical antecedents in termed NE infants with seizures (n>4000), and reported that asphyxia sentinel event was present in 15% of NE cases and increases to 35% if foetal bradycardia was also included as an asphyxia indicator¹¹. Inflammation, small-for-gestational-age (growth restriction) and maternal metabolic disorders

have been also identified as predating risk factors. The notion that majority of NE has heterogeneous antepartum aetiologies, which could also predispose or sensitise newborns to intrapartum sentinel events, challenged the conventional assumption that perinatal asphyxia is the primary cause for NE^{7, 12, 13}. Understandably, the relative contributions from antepartum and intrapartum causes are also dependent on the level of medical care across different countries. A Nepali case-control study reported that intrapartum hypoxia along with other intrapartum risk factors was evident in 60% of NE, in stark contrast with 30% intrapartum events reported by the Western Australian cohort¹⁴. Both the Western Australian and Nepali studies identified infection and maternal hypothyroidism as significant antepartum risk factors for NE.

Magnetic resonance imaging (MRI) studies on NE brain injury pattern in termed newborns have offered convincing evidences to support intrapartum hypoxia-ischaemia as the primary aetiology to NE. In these studies, the MRI brain lesions observed in NE newborns were compared to the lesion pattern and topography documented in human neuropathological studies where hypoxia-ischaemia injury is evident, and to injury patterns to animal models of hypoxia-ischaemia brain injury. In a large-scale MRI study by Cowan et al.^{15, 16}, termed infants were divided into a NE group (either with or without seizures), where infants displayed foetal distress characteristic to NE (delayed respiration, umbilical cord acidosis, depressed Agpar score), and a seizures-only group. MRI revealed that 80% of NE infants with seizures exhibited mostly bilateral injury to the thalami, basal ganglia, cortex and/or white matter, and to a lesser extent the hippocampus, midbrain and brainstem were also implicated. These lesions conform to “acutely evolving lesions” and demonstrate marked similarity to the injury pattern resulting from hypoxia-ischaemia insult (e.g. brain swelling, cortical highlighting, loss of grey-white matter differentiation etc.). MRI scans from >1000 NE infants reported recurring abnormalities in the deep nucleus structures, the cerebral cortex and underlying white matter, the brainstem and/or the central white matter, with the different injury site and combinations thereof dependent on the severity and chronicity of the HI insult¹⁷⁻²⁰.

A.3 Clinical presentation and diagnostic criteria

Hypoxic-ischaemic encephalopathy is diagnosed based on the collective neurological syndromes of neonatal encephalopathy, in conjunction with evidences of perinatal/intrapartum asphyxia. Symptoms of neonatal encephalopathy evolve over the course of hours to days after birth, and the precise clinical features are dependent on the severity and duration of perinatal insults, thus continuous assessment and rigorous monitoring of infants with a combined multi-modal approach are required.

In the early hours after birth, encephalopathic infants exhibit depressed level of consciousness and alertness, together with depressed respiration or period breathing with apnoea. Early onset of seizures can be observed in infants sustaining most severe injury. Depending on the severity of insult, cranial nerve function, pupillary response and spontaneous eye movement may also be impaired^{2, 3, 21}. Weak muscle tone/posture (hypotonia)

with reduced movement are commonly accompanied with compromises to sucking and Moro reflexes. An APGAR score (appearance, pulse, grimace, activity and respiration) ≤ 5 with no improvement within 10 minutes after birth has been used to diagnose moderate to severe neonatal encephalopathy. To accurately diagnose HIE, metabolic acidosis of the cord blood (arterial and venous blood pH < 7) and the neonatal blood gas level, along with documented hypoxic-ischaemic events during the intrapartum period, should accompany the above neurological features. In addition, signs of systemic derangement of metabolites and asphyxia-associated multiorgan injury to the heart (elevated troponin T and creatinine kinase-MB levels), kidneys (elevated creatine level), liver (abnormal liver

Clinical feature	Stage 1 (mild)	Stage 2 (moderate)	Stage 3 (severe)
Level of consciousness	Hyperalert	Lethargic or obtund	Stuporous
Neuromuscular control			
Muscle tone	Normal	Mild hypotonia	Flaccid
Posture	Mild distal flexion	Strong distal flexion	Intermittent decerebration
Stretch reflexes	Overactive	Overactive	Decreased or absent
Segmental myoclonus	Present	Present	Absent
Complex reflexes			
Suck	Weak	Weak or absent	Absent
Moro	Strong, low threshold	Weak, incomplete, high threshold	Absent
Oculo vestibular	Normal	Overactive	Weak or absent
Tonic neck	Slight	Strong	Absent
Autonomic function			
Pupils	Mydriasis	Miosis	Variable, often unequal, poor light reflex
Heart rate	Tachycardia	Bradycardia	Variable
Bronchial and salivary secretions	Sparse	Profuse	Variable
Gastrointestinal motility	Normal or decreased	Increased, diarrhoea	Variable
Other			
Seizures	None	Common, focal or multifocal	Uncommon (excluding decerebration)
EEG findings	Normal (awake)	Early: low-voltage continuous delta and theta Later: periodic pattern (awake) Seizures: focal 1-to 1½-Hz spike-and-wave	Early: periodic pattern with isopotential phases Later: totally isopotential
Duration	Less than 24 hours	2–14 days	Hours to weeks

Sarnat and Sarnat staging of HIE, table acquired from Appendix D of Queensland Clinical Guidelines: Hypoxic-ischaemic encephalopathy (HIE)³³. Copyright © State of Queensland (Queensland Health) 2022, licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives V4.0 International licence, refer to Appendix A.

function test) and gastrointestinal tract (necrotising enterocolitis; abnormal bowel movement) would further corroborate the diagnosis of HIE. Sarnat and Sarnat staging system (right) is used to grade suspected HIE cases into stage I (mild), stage II (moderate) or stage III (severe), by assessing the level of consciousness, neuromuscular and reflex responses, autonomic functions and presence/absence of seizures²¹.

The presence of specific neurobiochemical markers within the serum and the cerebrospinal fluid (CSF) could also be used to monitor brain injury development in NE infants, and to identify those most at risk of long-

term neurodevelopmental deficiency, for example neuron-specific enolase (NSE)^{22, 23}. Conventional EEG and amplitude-integrated EEG have also been routinely used for monitoring cerebral activity and detecting seizure episodes and have been shown to effectively predict outcomes in HIE/NE neonates²⁴. Moreover, cerebral haemodynamics and oxygenation can be monitored in HIE subjects by near-infrared spectroscopy (NIRS), and increased cerebral oxygenation level seen with NIRS within 48hrs of life was strongly correlated with abnormal MRI findings and adverse outcomes in HIE infants²⁵⁻²⁸.

B. Management of HIE and Therapeutic Development

B.1 Systemic Support

Clinical management of HIE remains primarily supportive and focuses on maintaining the respiratory, cardiac and nutritional requirements of the infants, with the ultimate goal of achieving sufficient, sustained cerebral blood flow and avoiding impairment to other organs following HI insult^{2,3}. HIE infants are at an increased risk of hypocapnia and hyperoxia that may result from intense resuscitation and/or ventilation after birth; HI-induced metabolic disturbances also lead to decreased CO₂ production to worsen hypocapnia. Hypocapnia is detrimental to infants as it reduces cerebral perfusion and impairs oxygen release from haemoglobin, whereas hyperoxia increases oxidative stress and free radical production. Thus, vigilant monitoring of O₂ saturation (SpO₂), arterial partial pressure of oxygen (PaO₂) and arterial partial pressure of CO₂ (PaCO₂) are needed in HIE infants to prevent secondary brain injury from resuscitation and reperfusion.

Maintaining an optimal mean arterial blood pressure (MAP), which has been suggested to be within 40mmHg to 60mmHg², is also a critical component to managing HIE infants to avoid cerebral hypoperfusion and additional ischaemic injury. Treatment of hypotension in HIE subjects with normal versus impaired cardiac function is different. Fluid replacement is usually recommended for HIE neonates with hypotension and normal cardiac function whilst being cautious with the risk of cerebral oedema²⁹. In hypotensive HIE neonates with cardiac dysfunction, dopamine, dobutamine, noradrenaline and adrenaline have been suggested to increase vascular resistance and cardiac index/output hence augmenting MAP³⁰.

Moreover, HIE infants are at an increased risk of hypoglycaemia, as increased systemic anaerobic respiration during HI insult would deplete the hepatic glycogen store such that the diminishing hepatic glucose production may not fulfil the cerebral metabolic requirement. The newborn brain is particularly susceptible to hypoglycaemia, as neonatal cerebral glucose consumption accounts for approximately 70% of total glucose consumption, while the alternative substrates (lactate, ketone) may not sufficiently compensate for energy gap from glucose shortage². Clinical studies reported a significant, positive correlation between low serum glucose and higher (worse) Sarnat stage, and identified early hypoglycaemia after birth ($\leq 40\text{mg/dL}$) as a predictor of adverse neurologic outcome in HIE neonates^{31,32}.

B.2 Therapeutic hypothermia

Moderate hypothermia has become the standard of care recommended for termed infants (≥ 36 weeks gestation) born with hypoxic-ischaemic encephalopathy in developed countries^{2,33}. Therapeutic hypothermia can be performed as either systemic whole-body cooling or selective head cooling, with controlled reduction of core body temperature to 33-35°C for 72hr followed by gradual rewarming (0.5°C per hour) to within the normal body temperature range (36.5–37.5°C). For systemic hypothermia, infants were placed on a cooling

blanket that circulated cooled fluid³⁴, and selective heading cooling involved placing a thermostatically controlled cooling unit (cap) around the head^{35, 36}.

Meta-analysis of 7 independent trials enrolling a total of 1,214 HIE neonates demonstrated that therapeutic hypothermia initiated within 6hr of age reduced death and major disability (e.g. cerebral palsy, neurodevelopmental delay, blindness) in moderate (risk ratio 0.67; 95% CI, 0.56-0.81) and severe (risk ratio 0.83; 0.74-0.92) cases of HIE at 18 months of age³⁷, regardless of the mode of hypothermia (whole-body or selective head cooling). A systematic review of 11 randomised trials similarly reported that therapeutic hypothermia led to statistically significant reduction in mortality or major neurodevelopmental disability at 18 months of age (risk ratio 0.75; 95% CI, 0.68-0.83)³⁸. The therapeutic benefit of moderate hypothermia can be directly seen on MRI scans, which showed reduced lesions in the deep grey matter, white matter and posterior limb of the internal capsule, when compared to scans of non-cooled HIE infants. It was also concluded that therapeutic hypothermia did not interfere with the predictive value of MRI, and that improved scans correlated with decreased neuromotor impairments in cooled infants³⁹. Follow-up studies indicated that the therapeutic benefit of hypothermia persisted into early childhood, whereby mortality at 6-7 years of age was significantly lower in the cooled group (with a trend of improved IQ score)⁴⁰, and that favourable neurodevelopmental outcomes at 18-months from hypothermia predicted improved functional outcomes (self-care, mobility and cognition) at 7-8 years of age⁴¹. As recommended by the American Heart Association and National Institute for Health and Clinical Excellence (NICE), therapeutic cooling has become the standard resuscitation care for HIE infants who meet the treatment criteria in developed nations and high-resource settings.

More recent trials have examined if therapeutic cooling commenced at a later time points (6 to 24hrs after birth), for longer duration (120 hrs), at lower temperature (32°C) could confer similar survival benefit and neuroprotection. In view of the small size of the studies, it was not possible to demonstrate the therapeutic efficacy of delayed cooling⁴² or a modified combination of duration/temperature⁴³. The lack of evidences also justifies a continuation of the current hypothermia regimen (< 6hrs of life, for 72hrs and at 33.5-34.5°C) for moderate-to-severe cases of HIE.

B.3 Experimental and emerging therapies

Although the clinical benefit of therapeutic hypothermia is evident, there is still a significant proportion (~46%) of moderate to severe HIE cases that had major adverse outcomes (mortality and/or neurodevelopmental disability) at 18 months³⁴. There is great incentive to explore alternative, and ideally synergistic, interventions that can be administered together with therapeutic hypothermia after the HI insult to offer enhanced neuroprotection for HIE infants.

B.3.1 Xenon

Given that excitotoxicity is a central pathological event to ischaemia-reperfusion (I/R) brain injury, agents that either block excessive glutamate release or inhibit excessive postsynaptic actions would represent attractive future therapies for HIE. The anaesthetic noble gas xenon is a competitive NMDA antagonist, and has many desirable properties that would make it an ideal, safe agent for HIE-affected infants, such as blood-brain-barrier permeability, rapid onset/offset of action, limited cardiovascular depression, and myocardial protection⁴⁴.

The neuroprotective benefits of xenon have been demonstrated in rodent and piglet models of HIE, whereby 50% xenon inhalation alone after hypoxia-ischaemia (H/I) insult significantly reduced global neuropathological lesions in the cortex/white matter, hippocampus and deep grey matter⁴⁵. Additive neuroprotection from xenon (20-70%) and hypothermia (32-35°C) was reported, with the combined regime produced the greater amelioration to histopathology lesion, neurological score and long-term neuromotor function than hypothermia alone⁴⁶⁻⁴⁸. Importantly, xenon exposure for as brief as 1hr⁴⁹ was shown to provide additional neuroprotection, so was delayed xenon administration that commenced up to 6hr post-HI^{48, 50}, making xenon a pragmatic choice for HIE treatment at sub-anaesthetic concentration and with wide therapeutic timeframe. Xenon/hypothermia combination prevented the magnetic resonance spectroscopy abnormalities (e.g. lactate/NAA) associated with H/I injury, as well as reducing the number of TUNEL-positive nuclei/caspase-3 positive cells within the cortex, hippocampus and putamen to indicated attenuated apoptosis^{48, 51}.

The TOBY-Xe study is the first randomised controlled trial comparing the clinical benefits of xenon-augmented hypothermia to standard hypothermia in termed HIE infants, whereby 30% xenon was delivered for 24hr during the 72hr hypothermia. This small randomised control trial (n=92) reported that incorporating xenon into hypothermia is safe and feasible, however the addition of xenon did not further improve MRI injury (posterior limb of internal capsule MRI signal) and MRS biomarker (lactate/NAA ratio) compared to hypothermia alone, and the death rate was comparable between the two groups⁵².

Erythropoietin (EPO) is a naturally occurring glycoprotein hormone that acts as a pleiotropic growth factor, and is the best known for its ability to promote red blood cell synthesis (erythropoiesis) and angiogenesis. EPO is produced in the central nervous system (CNS) aside from its primary site of production in the kidneys, with the EPO receptors also being widely expressed across neurons, astrocytes and microglia^{53, 54}. In infants born with perinatal asphyxia, the elevated level of EPO was found in the cord blood, and disruption to the blood-brain barrier from HI insult can further increase the CNS permeability of EPO. The neuroprotective effect of EPO has been extensively studied in animal models of H/I injury and I/R injury in adult and neonatal animals. Systemic administration of EPO post-HI or post-I/R was shown to significantly decrease cerebral infarct volume, prevent neuronal apoptosis⁵⁵ and reduce pro-inflammatory cytokine production (IL-1 β)⁵⁶. Animal stroke and HIE studies also found the neurogenesis⁵⁷ and angiogenesis-promoting⁵⁸ effects of EPO, which increased the cerebral concentration of vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF), as well as increasing cerebral vasculature density around the ischaemic lesion and the proliferation of neural progenitor cells in subventricular zone (SVZ) and dentate gyrus (DG)^{58, 59}. Such findings may explain the improved sensorimotor and cognitive functions from EPO treatment, which was observed 3 months after the initial insult^{59, 60}.

In a double-blind, placebo-controlled phase II study, the additional benefit of high dose of erythropoietin to therapeutic hypothermia was investigated, where HIE infants were randomised to receive EPO (n=24) or placebo (n=26) plus hypothermia. EPO was administered in multiple doses within the first week of life, with the mean age of first dose given at 16.5hr after birth. Although EPO plus hypothermia did not lead to a lower death rate, the MRI brain injury score and frequency were significantly reduced in EPO cohort, whom also exhibited improved motor function at 1-year of age compared to the placebo plus hypothermia cohort⁶¹. In view such favourable outcomes, a larger-scale phase III randomised clinical trial is underway to study the neuroprotective efficacy of erythropoietin in combination with hypothermia in moderate-to-severe HIE infants⁶².

B.3.3 Melatonin

Melatonin (N-acetyl-5-methoxytryptamine) is a naturally-occurring hormone produced by the pineal gland and is the best-known for its role in regulating the circadian rhythm, which is mediated through G-protein coupled melatonin receptor subtype 1 and 2 (MT1 and MT2)⁶³. Melatonin is also a potent free radical scavenger towards a wide range of reactive oxygen (ROS) and nitrogen species (RNS), including hydroxyl radical ($\cdot$ OH), hydrogen peroxide (H₂O₂), nitric oxide (NO $\cdot$) and peroxynitrite anion (ONOO $^-$)⁶⁴. Melatonin readily crosses the blood-brain barrier, and its neuroprotective efficacy of melatonin has been demonstrated in H/I, I/R and excitotoxicity-induced brain injury models.

In postnatal day 7 rats, melatonin administration, either before or after the H/I insult, effectively prevented whole brain volume loss and hippocampal neuron loss and improved long-term learning assessed by circular water maze⁶⁵. In a foetal sheep model of white matter injury model (umbilical cord occlusion), melatonin administration post-insult reduced the number of TUNEL-positive apoptotic cells and the number/density of microglial cells in the white matter⁶⁶. The combination of melatonin and cooling was also demonstrated to provide superior neuroprotection to hypothermia in a piglet model of HIE. Melatonin-augmented hypothermia further ameliorated abnormal MRS signals (lactate/NAA and lactate/creatine ratios), elevated cerebral ATP content (nucleotide triphosphate/exchangeable phosphate pool ratio), suppressed microglial activation (CD68 expression), and reduced TUNEL positive and caspase-3 positive cells in deep grey matter, internal capsule, periventricular white matter and/or hippocampus⁶⁷. In a follow-up study comparing melatonin and erythropoietin as adjunct therapy to hypothermia, melatonin plus cooling was more effectively at lowering the lactate/NAA ratio and the number of TUNEL-positive nuclei in the sensorimotor cortex, whereas erythropoietin-augmented cooling preferentially enhanced oligodendrocyte survival⁶⁸.

B.3.4 Stem cell therapy

Stem cells-based treatment has attracted significant research attention as potential HIE therapy, as the multipotent/pluripotent, proliferative stem cells can differentiate and subsequently replace cells that are irreversibly damaged by I/R insult and unlikely to regenerate (neurons). The therapeutic potential of different types of stem cell have been studied in HIE, including neural stem cell (NSC), haematopoietic stem cells and mesenchymal stem cells.

In adult stroke^{69, 70} and neonatal H/I animal studies⁷¹⁻⁷³, neural stem cells were demonstrated to preferentially migrate to and integrate into the ischaemic site when transplanted after brain injury, even when NSCs were implanted into contralateral side of the lesion. Importantly, the transplanted NSCs were capable to re-enter proliferative cycle, differentiate into neurons and oligodendrocytes, replenish the host resident progenitor cells and prevent astrocyte scarring in neonatal mice subjected to brain H/I, with the most robust responses observed at day 3-7 following H/I injury^{71, 72}. Similar enrichment of endogenous, host progenitor cells at subventricular zone was observed when NCSs were transplanted after focal H/I in adult animals⁷⁰. The migratory behaviour of NSCs is protracted and lasted up to 58 weeks after H/I without adverse effects and remained within the ischaemic lesion at a quiescent state⁷³. The cellular responses from NSC transplantation also translated into motor function recovery and preservation of brain volume⁷⁰. In these studies, the neural stem cells were either harvested from another animal or induced from a progenitor stem cell lineage (e.g. embryonic stem cells), which would hinder the development of neural stem cell-based therapy in clinical practice as such approach is ethically and logistically challenging.

Multipotent mesenchymal stem cells are capable of differentiating into cells other than those from the mesodermal lineage, such as neurons. Compared to neural stem cells, autologous transplantation of mesenchymal stem cells derived from umbilical cord blood or bone marrow represent a safer, more pragmatic option as future HIE therapy. However, it must be emphasised that the observed therapeutic benefits from bone marrow (BM) cell or umbilical cord blood (UCB) cell transplantation cannot be attributed to solely to the MSCs, which only constitute a small proportion of the BM and UCB cell population^{74, 75}. The haematopoietic stem cells, endothelial progenitor cells, lymphocytes and monocytes produce various growth factors (brain-derived neurotrophic factor – BDNF, vascular-endothelial growth factor – VEGF, nerve growth factor – NGF, neurotrophin 3/5 – NT3/NT5) and anti-inflammatory cytokines (IL-10) to achieve neuroprotection and immune-modulation⁷⁵. In rodent models of HIE, transplantation of human UCBCs after H/I injury ameliorated sensorimotor dysfunctions, with neuroprotection achieved when UCBCs were transplanted within 3hr post-injury, seen as reduced numbers of caspase-3 positive neurons and activated microglia^{76, 77}. Human studies also demonstrated the feasibility of autologous transplantation to subjects with traumatic brain injury or cerebral palsy following procurement of MSCs from bone marrow or umbilical cord blood⁷⁸⁻⁸¹. Preliminary studies on HIE infants did not report significant improvement in motor and cognitive functions after autologous MSC transplantation, and sufficiently-powered, randomised trials are needed in the future^{80, 81}.

C. Animal models for studying hypoxic-ischaemic encephalopathy

Accurately correlating the neurodevelopmental stages of animals to those of human is the foundation for developing appropriate animal models to study HIE/perinatal hypoxic-ischaemic brain injury. Cross-species comparison of neurodevelopment was traditionally based on brain growth rate (size/weight of whole brain)^{82, 83}, to more recent multivariate analysis that compared >100 neural events on histological and functional maturity from various brain regions between different mammalian species^{84, 85}. Such multivariate analyses have been instrumental for equating key prenatal and postnatal developmental events between species and have provided justifications for the mammalian HIE models currently in use.

C.1 Rodents

C.1.1 Rats

Rodents are most commonly used mammals for HIE research to account for approximately 26% of animal studies⁸⁶. Rodents, including rats and mice, are born altricial like human with significant brain development taking place after birth, with different brain regions maturing at different pace. Cross-species comparison on neuroanatomy, neurogenesis, neurotransmission, synaptogenesis, myelination, immune maturation and other biochemical signatures between human and rodents offered compelling evidences to correlate postnatal day 7 to day 10 (PND7-10) in rodents to 36- to 40-week gestation in human⁸²⁻⁸⁴. PND 7-10 rodents are deemed appropriate for studying HIE in near-term or termed infants, whereas more immature rodents of PND 1-5 are needed to model pre-term HI injury that predominantly manifests as white matter injury⁸⁷.

Since its inception in 1981, the Rice-Vannucci H/I model on PND7 rats and its later adaptation on PND7-10 mice have provided extensive insights into the pathophysiological mechanisms of HIE in termed newborns and facilitated testing of various potential therapeutic interventions. The original Rice-Vannucci model described unilateral common carotid artery (CCA) ligation (i.e. ischaemia) in PND7 rats followed by hypoxia exposure to 8%O₂/92%N₂ (fraction of inspired oxygen, FiO₂=0.08) in a humidified chamber at 37°C for 3.5 hrs⁸⁸. The hypoxia component is necessary to produce apparent brain injury, as rats have a complete and robust Circle of Willis such that the collateral blood flow will compensate for the ligated carotid artery. It is only during systemic hypoxia that the metabolic demands from both hemispheres will reduce blood flow into the hemisphere ipsilateral to carotid ligation to inflict ipsilateral brain injury⁸⁹. As reported in the initial study by Rice and Vannucci⁸⁸, ischaemic neuronal damage was observed in the ipsilateral cerebral cortex, hippocampus, striatum and thalamus; however, considerable animal-to-animal variation in injury severity was noted. Neuronal loss was most accentuated in cortical laminar layers 3/5/6, hippocampal cornu ammonis (CA1) and the lateral thalamus. Importantly, injuries are confined to the ipsilateral brain with the contralateral hemisphere seldomly affected, and brain lesions were never observed in rats subjected to hypoxia only.

The neuropathological changes correlated well with the changes to cerebral blood flow (CBF) and cerebral energy metabolisms during and after H/I injury in PND7 rats. At 2hrs, regional CBF in the ipsilateral hemisphere was reduced to 15-40% of baseline rCBF with neocortex and subcortical white matters showing greatest reductions, whereas rCBF remained largely unchanged in the contralateral hemisphere⁹⁰. Cerebral glucose utilisation (CGU) assay revealed increases in glucose usage in both hemispheres during and following H/I^{89, 91}. The level of high-energy phosphates including ATP and phosphocreatine decreased to 10% of the baseline in the ipsilateral hemisphere during H/I, compared to the contralateral hemisphere that only displayed a reduction of 20-30%. Lactate as the main glycolytic product was increased in both hemispheres, however the lactate-to-pyruvate (glycolytic versus aerobic) ratio of the ipsilateral side was three times that of the contralateral. Upon reperfusion, lactate level fell gradually overtime; high-energy phosphate reserves were restored transiently but fell again at 12-18hrs (secondary energy failure, more details in D2.2) in the ipsilateral hemisphere, and the level of ATP and adenine nucleotide remained suppressed compared to that of the baseline. The metabolic perturbations indicate that 1) the ipsilateral hemisphere was restricted to anaerobic respiration and experienced energy failure from lack of high-energy phosphates during H/I, and 2) onset of secondary energy failure after H/I with protracted depletion of high-energy phosphate reserves.

C.1.2 Mice

The Rice-Vannucci model was adapted for neonatal mice in view of the increasing availability of different transgenic mice strains, which enabled researchers to investigate the effect from gain- or loss-of-function of a protein/pathway in relation to the pathogenesis of HIE. Unlike rats, newborn mice are less resilient to H/I and careful titration of the duration/severity of hypoxia exposure is required. Most research groups found that 40-70mins of hypoxia ($\text{FiO}_2 = 0.08$)⁹²⁻⁹⁵ can be tolerated by mice without excessive mortality, and that is significantly shorter than the 3.5hrs hypoxia used for neonatal rats. Significant variations in susceptibility and injury severity between different mouse strains were reported by Sheldon and colleagues. When comparing CD1, C57BL/6 and 129Sv PND7 mice and cross-strain breeds, 129Sv background led to significant mortality (up to 50%) with 30mins hypoxia when no death was reported from the other strains, whereas CD1 background produced the most severe neuropathology and lesion in the ipsilateral hemisphere⁹⁶. The inter-strain discrepancy maybe ascribed to the different cerebrovascular anatomy, including differences in the Circle of Willis, as the successful induction of ipsilateral ischaemia from ligation *plus* hypoxia is dependent on the robustness and integrity of the Circle of Willis⁹⁷. An incomplete Circle of Willis would render the ipsilateral hemisphere susceptible from solely carotid artery ligation, and the ensuing brain lesion will be further aggravated by subsequent hypoxia.

C.1.3 Functional and behavioural studies in rodent models of HIE

An increasing number of rodent HIE studies have incorporated long-term functional and behavioural tests to systematically examine locomotion, cognition/learning, emotion and sensory functions. These functional tests allow researchers to 1) determine whether the long-term functional impairments in rodent HIE models resemble those of termed human (validity of the rodent models), 2) assess whether potential therapeutic intervention is equally effective at improving functional outcomes as biochemical and pathological outcomes, and 3) correlate biochemical and neuropathological changes with functional disturbances for more in-depth, comprehensive understanding of HIE pathophysiology⁸². However, the immature human and rodent brains are fundamentally different; the human brain has more compartmentalised structures, complex neural circuits and prolonged neurogenesis, in particular in the cerebral cortex that is particularly susceptible to H/I insult⁹⁸. The functional and behavioural consequences from rodent HIE models have limited clinical relevance to human, nonetheless are still indispensable for correlating pathology with symptoms, and behavioural/functional study has become compulsory to the rigorous testing of candidate treatment for hypoxic-ischaemic brain injury.

Open field test (OFT) examines the general locomotion activity and exploratory behaviour in rodents aged 4 weeks or older. Exploration level and pattern in OFT are also sensitive to the emotional state of the animals, hence OFT has also been used to identify anxiety-like behaviours. Studies on rats and mice found that H/I injury do not alter the overall locomotion and activity level, and changes to relative time spent in centre versus peripheral areas have been reported and are gender-dependent⁹⁹⁻¹⁰¹. Rodents naturally prefer periphery and corners over the unprotected centre in the open field, and further increase in periphery-covered time is believed to reflect increased anxiety-like behaviours subsequent to H/I injury.

Cognition, learning and memory in rodents can be studied using a versatile range of tests. The Morris water maze (MWM) is a sensitive test for spatial learning and memory that dependent on hippocampal integrity, and therefore MWM performance strongly correlates with hippocampal synaptic connectivity and NMDA neurotransmission¹⁰². Moreover, it was reported that MWM learning is independent from overall locomotion, as reduced locomotor activity does not affect swimming ability¹⁰². As demonstrated by many, H/I brain injury consistently impaired MWM performance during both learning phase and memory recall in juvenile rodents (4 weeks and older)^{100, 103, 104}, seen as increased escape latency and increased distance travelled to locate the platform.

Fear conditioning is a form of associative learning task that examines the ability of an animal to anticipate events when exposed to specific context and/or cue. The principle of FC is to pair a neutral conditioned stimulus (CS; environment, tone, odour) with an aversive unconditioned stimulus (US; typically foot shock in mice), and the mice will demonstrate freezing behaviour when exposed to context/cue^{105, 106}. The main brain

regions involved in contextual/cued fear conditioning include the amygdala, hippocampus, frontal cortex and the cingulate cortex. The basolateral amygdala has been suggested to be crucial for the acquisition and expression of conditional fear¹⁰⁷, and the hippocampus is necessary for context learning and becomes increasingly engaged with longer trace interval for the animal to associate CS and US^{105, 108}. In this regard, H/I-injured juvenile mice displayed significantly fewer freezing behaviour to the contextual and/or auditory cues compared to naïve control¹⁰⁹⁻¹¹¹.

Accelerating rotarod is well-validated test of motor learning, coordination and balance in juvenile rodents aged 4 weeks and older with high sensitivity and relative ease. Animals placed on the rotating cylinder will need walk continuously and avoid from falling off, and the amount of time the animals remain on the rod until falling (latency to fall) or time until the first complete revolution (movement stops but grasps the rod) is recorded as a quantitative endpoint measure of motor function. H/I-injured mice were repeatedly shown to have shorter latency to fall from the rotarod than control animals^{100, 112-114}. Motor learning is an important component to rotarod as performance (latency to fall) should improve with increased training, however in HIE mice learning was impaired¹¹⁵. Moreover, poor rotarod performance was shown to be positively correlated with volume loss of the hippocampus and corpus callosum in HIE mice¹¹⁴.

The appearance of various sensorimotor reflexes in early postnatal days is valuable for studying age-appropriate neurological development in rodents, and a battery test of different sensorimotor reflexes can be applied to evaluate the severity of H/I brain injury in neonatal rodents from 24hrs to 1 week after the insult^{116, 117}. Importantly, diminished/impaired short-term sensorimotor reflexes also correlated with poor cognitive function and learning (Morris water maze) at 8 weeks of age¹¹⁶. Negative geotaxis is an innate reflex that develops in postnatal day 7 pups, and can be used to evaluate motor coordination and vestibular processing of postural orientation¹¹⁶⁻¹¹⁸. The rodent is placed head down on an inclined slope of 45°, and due to vestibular cues of gravity, the rodent will turn to face up the slope and climb up. In pups with extensive motor deficit or are unable to recognise the downward orientation on a slope, they may fail to display turning (taxis) and slide down the slope. Cliff aversion reflex assesses the ability of pups to turn away from a cliff edge when their head and forepaws are positioned over the edge^{116, 117}. Because their eyes are still closed at the time of testing (postnatal day 7-14), the avoidance response is not triggered by visual cues but vestibular cues and also requires motor strength. The experimenter will place the pup with snout and the forepaws overhanging the cliff, and the time taken for the snout and paws to move away from the cliff edge is recorded.

Sheep, pigs and rabbits represent some of the most commonly used larger mammals for the study of HIE, in part because these animals have a similar grey/white matter ratio to that of human.

Sheep are precocial such that foetal sheep (total gestation ~150days) between gestational day90-100 and day120-135 had been used to model HIE to correlate to pre-term and near-term/term human infants, respectively. Hypoxemia of the pregnant ewes, umbilical cord occlusion or bilateral carotid artery occlusion have been used to produce cerebral ischaemia in foetal sheep, which displayed reduced cerebral oxygen content, increased lactate level¹¹⁹, ongoing seizure activities¹²⁰ and substantial neuronal loss in the cortex, hippocampus, thalamus and striatum¹²¹.

The newborn piglet HIE model is highly relevant to HIE in termed human, as the brain growth spurt for piglets is around birth time, and the brain weight for newborn piglets and human infants is 35-40% of that of the adults¹²². Piglets born within 24hr were subjected to either global hypoxia (4-6% O₂)^{123, 124} or underwent bilateral carotid artery ligation plus hypoxia (~10%)¹²⁵ lasting for 30-45 minutes. MRI studies highlighted lesion in the basal ganglia of injured piglets along with increased tissue lactate level¹²⁶. Phosphate ³²P-MR spectroscopy further demonstrated cerebral energy failure in H/I piglets, seen as decreased [nucleotide triphosphate (NTP)/exchangeable phosphate pool (EPP)] and [phosphocreatine (PCr)/inorganic phosphoate (Pi)] ratios¹²⁵. Histology examination identified lesions in the deep grey matters, hippocampus, parasagittal cortex, subcortical white matter and even in the cerebellum, from discrete neuronal necrosis to region of confluent infarct^{123, 124}. Moreover, piglet HIE model has been routinely used to screen for potential neuroprotective agent/treatment and understand their neuroprotective mechanisms, for example hypothermia alone¹²⁵ or in combination with xenon (50%Xe for 18hr)⁴⁷, melatonin (20 mg/kg)⁶⁷ or erythropoietin (3000U/kg)⁶⁸.

Rabbit model of HIE was established to facilitate study on cerebral palsy and other motor deficits resulting from perinatal H/I brain injury, as rabbits also undergo motor function development during the perinatal period like human. To model pre-term and term HIE in human, rabbit foetus of embryonic day22-25 (70-80% gestation) and day29 (90% gestation) were subjected to global hypoxia from uterine ischaemia for 40 mins, respectively. In the pre-term study, PND1 kits after sustained global hypoxia displayed marked hypertonia with increased forelimb tone, decreased locomotor control in head and limbs, and impaired righting, sucking and swallow reflexes¹²⁷. Findings from near-term study produced similar motor deficits to those from pre-term hypoxia, and both models provoked neuronal death in the basal ganglia and thalamus, and to a lesser extent also in the cortex and hippocampus¹²⁷, with increased caspase-3 expression indicate elevated apoptosis activity¹²⁸. MRI studies further revealed white matter lesion in the internal capsule¹²⁹, as well as lesions of the deep grey matter (basal ganglia, thalamus) and the brainstem are predictive of hypertonia¹³⁰.

The advent of reliable and versatile animal models as described above have propelled research into the molecular mechanisms underlying the pathogenesis of HIE. Although still nowhere near elucidating the full picture, the current consensus is that H/I brain injury in the newborns unfolds in distinct stages and can be divided into three main phases^{33, 131-133} (**Fig.1-1**).

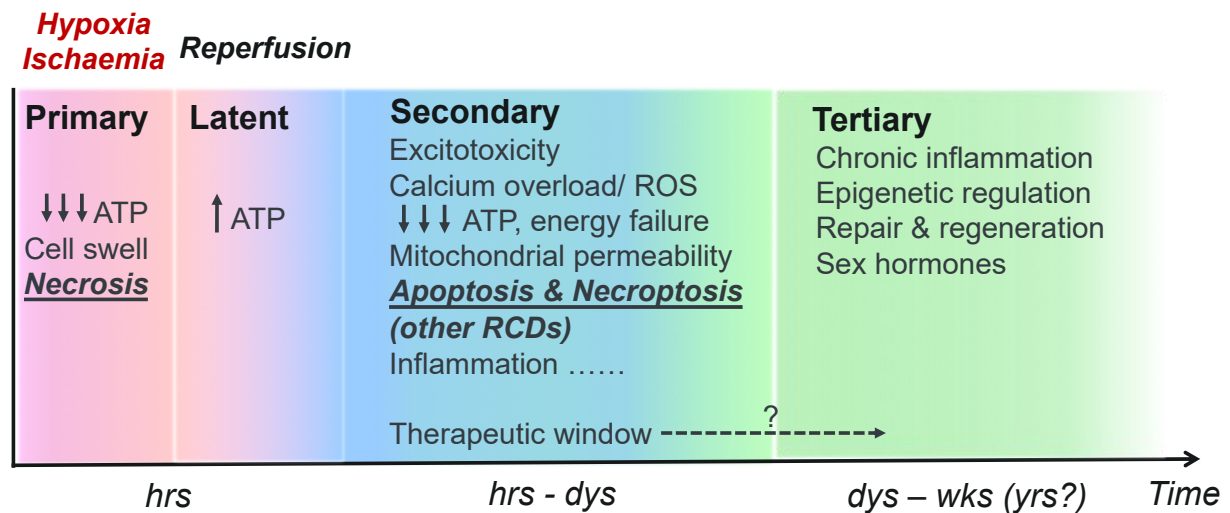


Figure 1-1. The distinct phases of hypoxic-ischaemic encephalopathy (HIE) in termed infants.

Brain hypoxia-ischaemia (I/R) triggers an immediate, early primary injury phase characterised by rapid ATP decline, cell swelling and necrotic cell death, and is followed a latent phase of temporary ATP recovery from reperfusion. The secondary injury phase encompasses a chain of pathological events highlighted by mitochondrial dysfunction contributing to severe energy failure and initiation of intrinsic apoptosis pathway amongst other parallel regulated cell death mechanisms. I/R brain injury also provokes a potent pro-inflammatory response that sustains and propagates into chronic inflammation to alter brain development and neural plasticity up to years. During tertiary injury phase, in addition to inflammatory/anti-inflammatory modulation, epigenetic regulation on neurogenesis, differentiation and myelination and the influence of sex hormones are also involved in adolescence to underscore the complex interplay between different factors in the pathogenesis of HIE in the long-term.

D.1 Primary Phase and Latent Phase

In the primary injury phase, cerebral hypoxia-ischaemia of sufficient severity, with abrupt or gradual onset, will cease oxidative phosphorylation reaction and deplete high-energy phosphates (ATP, phosphocreatine) store³³. The imminent consequence to the initial energy failure is disruption to ATP-dependent cell membrane ion transporter (Na^+ , K^+ ATPase) to cause influx of Na^+ ions and water, thus leading to cell swelling and membrane collapse. It is thought that necrosis, or accidental cell death, prevails during this acute injury phase, with necrosis being primarily responsible for neuronal loss at the ischaemic core where hypoxia-ischaemia is most severe. Necrosis is deemed as a passive form of cell demise, and it unlikely encompasses specific molecular pathways that can be modulated to prevent primary neuronal loss¹³³. Upon reperfusion and reoxygenation, a near-complete recovery of oxidative phosphorylation, cerebral metabolism and high-energy phosphate will take place (latent phase); however, such latent phase is quickly succeeded by a secondary energy failure phase with prominent neuronal death, and the secondary phase can persist up to days after initial H/I insult.

The secondary injury phase by far has received the most research attention, as studies have incrementally revealed that the pathophysiological events defining the secondary injury phase are “regulated” in nature, and therefore can be modulated and targeted to limit the progression of brain injury in animal models of HIE. Importantly, current evidence also supports the therapeutic benefit of hypothermia to be confined to the secondary phase “window” (6-24 hrs after birth). Key mechanisms of the secondary injury phase encompass excitotoxicity, calcium overload, oxidative and nitrosative stress, and mitochondrial dysfunction. The chain of pathophysiological events has been shown to culminate to apoptosis, the most-well studied form of regulated cell death in HIE. Regulated cell death (RCD) is believed to predominate in the secondary injury phase, as opposed to accidental cell death, and this notion offered great hope and incentive for selective targeting of RCD as potential therapeutic interventions for HIE.

D.2.1 Excitotoxicity

Excitotoxicity is defined as extracellular accumulation of excitatory amino acids (EAAs), including glutamate and aspartate, to cause over-activation of glutamate N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors, leading to excessive Ca^{2+} and Na^{+} ion influx into vulnerable cell types, in particular neurons and oligodendrocyte-precursor cells (OPCs). During hypoxia-ischaemia and subsequent reperfusion, there is significant glutamate release from excitatory nerve terminals, and impaired energy-dependent glutamate re-uptake into astrocytes further exacerbates extracellular glutamate overflow. Blocking the NMDA and AMPA receptors before or immediately after H/I insult was shown to significantly attenuate brain injury in animal models of HIE. For example, NMDA receptor antagonist MK-801^{134, 135} or AMPA receptor antagonist 6-nitro-7-sulfamoylbenzo(f)quinoxaline-2,3-dione (NBQX)^{136, 137} effectively suppressed epileptiform activity, reduced cerebral infarct, preserved cortical and hippocampal volume, prevented death of neurons and OPCs and improved myelination in neonatal rats and foetal sheep. The anti-convulsant topiramate was also shown to antagonise AMPA/kainate receptor and blocked AMPA/kainate-invoked inward currents and calcium influx in cultured neurons and OPCs^{138, 139}. Topiramate effectively prevented neuronal loss, white matter injury and motor deficits in animal models of HIE¹³⁸⁻¹⁴⁰. Topiramate also extended the therapeutic window of hypothermia, such that delayed hypothermia (3hr post-H/I) was also neuroprotective against HIE, which was not feasible with delayed hypothermia alone¹⁴¹.

It is important to appreciate that the overflow of EAAs stimulates both the intrasynaptic and the extrasynaptic NMDA receptors, and the excitotoxic effect of glutamate is believed to ascribe to over-activation of the extra-synaptic NMDA receptors^{33, 132}. Studies have shown stimulation of extra-synaptic versus synaptic NMDA receptors elicited opposing effects on the pro-survival cyclic-AMP response element

binding protein (CREB)-dependent and pro-apoptosis forkhead box protein O (FOXO)-dependent gene transcriptions, by differentially regulate the phosphorylation status of transcriptional factors¹⁴². Synaptic NMDA signalling promotes the phosphor-activation of CREB and its co-activator CBP (CREB binding protein) to facilitate their recruitment to CRE (CREB response element) to activate transcription, whereas extrasynaptic NMDA signalling impairs CREB activity through reduced phosphorylation and increased dephosphorylation. Synaptic NMDA signalling phosphorylates FOXO to prevent its nuclear translocation, whereas extrasynaptic NMDA receptor stimulation dephosphorylates FOXO to favour its nuclear import and transcriptional activity. Excitotoxic downstream events to extra-synaptic NMDA signalling could also involve inactivation of the pro-survival Ras-ERK1/2 pathway¹⁴², disinhibition of the neurotoxic p38 mitogen-activated protein (MAP) kinase pathway (via inhibition of calpain-mediated cleavage of p38 MAPK)¹⁴², activation of c-Jun N-terminal kinases (JNK)^{143, 144} and induction of neuronal nitric oxide synthase (nNOS) activity⁹². Selective inhibition of the extra-synaptic NMDA signalling represents a more favourable strategy, as inhibition to intra-synaptic NMDA activity was shown to offset the endogenous pro-survival pathways to trigger apoptosis in immature brain. In this regard, the efficacy of inhibitors selectively targeting extra-synaptic NMDA receptor associated proteins, e.g. postsynaptic density protein 95 (PSD95)¹⁴⁵, death associated protein kinase 1 (DAPK1) or NMDA receptor subunit NR2B¹⁴⁶, needs to be evaluated in appropriate animal models of HIE.

D.2.2 Calcium Imbalance, Mitochondrial Respiratory Chain Dysfunction and Oxidative Stress

Disruption to intracellular calcium homeostasis is characteristic to H/I brain injury of the immature brain and significantly contributes to disease pathogenesis. H/I is associated with increased intracellular Ca^{2+} level with further, incremental increases during the reperfusion phase¹⁴⁷, from over-activation of extrasynaptic NMDA receptor and opening of voltage-gated Ca^{2+} channels. Cytosolic Ca^{2+} increase is closely correlated with mitochondrial uptake and sequestration of Ca^{2+} , and the mitochondrial Ca^{2+} response from intracellular Ca^{2+} rise is faster during NMDA receptor stimulation than during activation of non-NMDA receptor (AMPA/kainate) or voltage-dependent calcium channels¹⁴⁸. In neonatal rat, mitochondrial Ca^{2+} accumulation is associated mitochondrial swelling/dilation as early as 3hrs into reperfusion¹⁴⁹, when mitochondrial respiratory chain and oxidative phosphorylation have transiently recovered from H/I (transition between latent and secondary injury phase). Intramitochondrial Ca^{2+} overload initiates a series of injurious processes to the mitochondria, including production of reactive oxygen species, respiratory chain inhibition, mitochondrial permeability and release of pro-apoptotic factors.

It is theorised that the respiratory chain complexes/components (e.g. complex IV, cytochrome c) are phosphorylated at specific residues, and the overall phosphorylation status is essential for regulating the electron transfer rate, allosteric feedback inhibition by ATP and respiratory chain efficiency¹⁵⁰. In this regard, mitochondrial Ca^{2+} overload could activate calcium-dependent mitochondrial dephosphatases to

dephosphorylate electron transport chain (ETC) components¹⁵¹ (notably cytochrome C and cytochrome C oxidase), to promote maximal activity of ETC^{152, 153}. The lack of oxygen during H/I phase suspends ETC to deplete ATP and depolarise the mitochondrial inner membrane (the H^+ proton gradient dissipates). Nonetheless, the dephosphorylated status is permissive for a hyperactive ETC during the reperfusion stage. When oxygen is replenished, the dephosphorylated ETC will hyperpolarise¹⁵⁴, and loss of ATP allosteric feedback further aggravates the hyperpolarised state to exceed the mitochondrial membrane potential ($\Delta\Psi_m$) above 140mV.

The phosphorylation/desphosphorylation “switch” of ETC has physiological importance, as it would enable rapid restoration of mitochondrial membrane potential and replenishment of ATP upon reperfusion and reflow. However sustained ETC hyperpolarisation during reperfusion is pathological, and the transient recovery is preceded by extended period of mitochondrial dysfunction. Studies have shown that in hyperpolarised mitochondria ($\Delta\Psi_m$ surpasses 140mV), a mild increase in membrane potential evokes exponential production of reactive oxygen species (ROS)^{150, 155}. In the post-ischaemic brain/neurons, the primary site of ROS production is at complex I and complex III of ETC¹⁵⁵⁻¹⁵⁷. ROS is generated when electrons (e^-) escape from complexes I and III and react with O_2 to form superoxide free radical ($^{\bullet}O_2^-$). $^{\bullet}O_2^-$ is the precursor molecule and reacts with H_2O or H^+ to form other more reactive ROS such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$). Superoxide radical could also react with nitric oxide ($NO^{\bullet}$) to form the highly unstable nitrosative species peroxynitrite ($^{\bullet}ONOO^-$). During H/I brain injury, $NO^{\bullet}$ is produced by neuronal and endothelial nitric oxide synthase (nNOS and eNOS), and selective deficiency of *nNOS* gene was shown to protect neonatal mice from cerebral H/I insult and preserved cortical and hippocampal integrity⁹².

Excessive ROS synthesis from mitochondria overwhelms the endogenous anti-oxidant reservoir and could directly damage ETC components¹⁵⁸ (complex IV, cytochrome c oxidase) and membrane lipids crucial to ETC function, to suppress mitochondrial respiration and collapse mitochondrial membrane potential¹⁵⁰. In I/R brain injury, respiration inhibition occurs to complex IV first (early reperfusion) and gradually extends to complex I (24hr after reperfusion) to persists through the secondary injury phase¹⁵⁰. ROS oxidation of mitochondrial structural proteins and lipids has been suggested to promote apoptosis. ROS oxidises the mitochondrial phospholipid cardiolipin for it to translocate from inner to outer mitochondrial membrane, which destabilises cytochrome c and is conducive for cytochrome c release into the cytosol¹⁵⁹. Mitochondrial oxidative stress was also shown to impair the oligomerisation of optic atrophy 1 (OPA1) to disrupt mitochondrial cristae junctions, again facilitating the displacement/diffusion of cytochrome c from mitochondria¹⁶⁰. The significance of cytochrome c release will be discussed in more details in the upcoming paragraphs.

D.2.3 Mitochondrial Permeabilisation and Apoptosis

Electron microscope revealed mitochondrial matrix swelling and mitochondrial membrane rupture during later reperfusion in immature brain¹⁴⁹. Such morphological changes are regarded as mitochondrial permeabilization, and the molecular events culminating to mitochondrial permeabilization in I/R injured brain are incompletely understood. Two main routes to mitochondrial permeabilization have been proposed to contribute ischaemic brain injury. The mitochondrial permeability transition pores (mPTP) is a voltage- and calcium-dependent conductance channel positioned in the mitochondrial inner membrane, and opening of mPTP is believed to be triggered by mitochondrial calcium overload and bioenergetic failure (from NMDA overactivation)^{161, 162}. Suppression of mPTP opening by genetic knockdown of cyclophilin D (*Ppid* gene deficiency), a central component to mPTP, effectively ablated mitochondrial depolarisation and ameliorated primary neuron death induced by glutamate¹⁶³. Similarly, treating oxygen glucose deprivation (OGD) challenged neurons with pharmacological inhibitor of mPTP opening cyclosporin A also attenuated cell death *in vitro*¹⁶⁴.

Studies on adult and immature brains reported dichotomised findings on mPTP opening in ischaemic brain injury. *Ppid* deficiency markedly reduced infarct volume following focal cerebral ischemia in adult mice¹⁶⁵, whereas *Ppid*-KO neonatal mice had significantly worse tissue loss from H/I than WT¹⁶⁶. Interestingly, *Ppid*-deficient neonatal mice displayed greater caspase activation and cytochrome C release than WT neonates following H/I insult, and the opposite is observed for adult *Ppid*-KO and WT mice. These findings suggest that in the injured immature brain, cyclophilin D encoded by *Ppid* gene negatively regulates mitochondrial permeabilization, which is primarily achieved through BAX-BAK pore formation.

BAX and BAK are pore-forming pro-apoptotic proteins of the B-cell lymphoma 2 (BCL-2) protein family, which further comprises of anti-apoptotic proteins (e.g. BCL-2, BCL-X_L) and BH3-only pro-apoptotic proteins (e.g. BAD, BID, PUMA)¹⁶⁷. The pro- and anti-apoptotic BCL-2 proteins interact with each other through BH3 domain, and partnering between different members carefully modulates the oligomerisation of BAX/BAD on mitochondrial outer membrane (MOM), which ultimately leads to MOM permeabilization (MOMP)¹⁶⁷ and release of cytochrome c and SMAC/DIABLO. Studies demonstrated that BAX knockdown or BAX-inhibitory-peptide attenuated mitochondrial permeabilization, cytochrome c release and caspase-3 activation, and conferred significant neuroprotection in neonatal mice subjected to H/I injury^{109, 168}. Other manipulations that impaired BAX/BAK-dependent pore formation and MOMP also attenuated apoptosis and injury development in neonatal mice subjected to HIE, for example genetic deficiency of BAD or BIM (but not BID)¹⁶⁹, forced overexpression of anti-apoptotic protein BCL-X_L¹⁷⁰, or TAT-delivery of exogenous BCL-X_L protein¹⁷¹.

BAX/BAK-mediated MOMP is also pliable to modulation by different upstream signalling events. In neonatal rats, H/I insult led to increased level of the tumour suppressor p53 within nucleus and mitochondria¹⁷².

Nuclear p53 was shown to upregulate transcription of pro-apoptotic proteins (BAX, PUMA)¹³³. In the mitochondrial fraction, p53 interacts with anti-apoptotic BCL-X_L and is later displaced by increased level of PUMA (p53-dependent upregulation); the available p53 then functions as an “activator” of BAX to trigger MOMP¹⁷³. Pharmacological inhibitor of p53 pifithrin- μ suppressed H/I-induced mitochondrial p53 accumulation, prevented cytochrome c release and caspase 3 activation, and ultimately improved histological and behavioural scores in injured neonatal rats¹⁷². Cerebral H/I also enhanced phosphorylation of c-Jun N-terminal kinases (JNK), which are members of the stress-activated group of MAP kinases, and JNK3 is the predominant JNK isoform in the brain. Phosphorylated JNK (p-JNK) subsequently phosphor-activates the transcriptional factors c-Jun and ATF-2 to induce cellular stress response and/or death. Selective knockdown of JNK3¹⁴³ or treatment with JNK small peptide inhibitor D-JNKi¹⁴⁴ decreased pro-apoptotic protein expression (PUMA, BIM), cytochrome c release and caspase 3 cleavage and increased expression of anti-apoptotic proteins (BCL-2, BCL-X_L), which translated to reduced neuronal loss in the cortex, hippocampus, striatum and thalamus in neonatal mice following cerebral H/I.

D.2.4. The Intrinsic Apoptosis Pathway

Mitochondrial permeabilization/MOMP and subsequent release of pro-apoptotic factors into the cytosol are regarded as the triggering events of “intrinsic” apoptosis pathway (**Fig.1-2**), which contrasts with “extrinsic” apoptosis pathway triggered by the activation of cell surface death receptor from cognate ligand binding, as will be discussed in more depth in E.1.2. The most well-studied mitochondrial-originated pro-apoptotic signals include cytochrome c, SMAC¹⁷⁴/DIABLO¹⁷⁵ (Second Mitochondria-derived Activator of Caspase/ Direct IAP Binding Protein with Low pI) and apoptosis-inducing factor (AIF). Once released into the cytosol, cytochrome c oligomerises with apoptotic peptidase-activating factor 1 (Apaf-1) at a ratio of 2:1 in the presence of ATP or deoxy-ATP¹⁷⁶. The cytochrome c/Apaf-1 apoptosome then recruits pro-caspase 9 as the initiator caspase to promote its auto-activation through proteolytic cleavage¹⁷⁶. Activated caspase-9 subsequently activates and cleaves downstream executor caspases such as caspase-3 and caspase-7. Of all known caspases, caspase-3 has the most substantive range of substrates such as actin, fodrin, lamin, poly ADP-ribose polymerase (PARP), caspase-activated deoxyribonuclease (CAD), and it also cleaves/processes other pro-caspases (-2, -6, -7 and -9) in a feed-forward fashion. Caspase-3 has been shown to be indispensable for the hallmarks of apoptotic cell death, including DNA fragmentation, chromatin condensation and blebbing; however, caspase-3 deficiency does not eliminate cell death from known apoptosis-inducers¹⁷⁶. In H/I brain injury studies (including HIE), caspase-3 activation is routinely regarded as the apoptotic endpoint where different pathophysiological signalling pathways converge, and reduced caspase-3 activation has become a convincing surrogate to evaluate the therapeutic efficacy of neuroprotective interventions. Studies demonstrated that pan-caspase inhibitors bocaspartyI(OMe)-

fluoromethylketone (BAF) or quinoline-Val-Asp(Ome)-CH₂-O-phenoxy (Q-VD-Oph) provided significant neuroprotection to neonatal rodents subjected to HIE even when administered after H/I insult¹⁷⁷⁻¹⁷⁹.

Concurrent with cytochrome c, SMAC/DIABLO is also released into the cytosol during MOMP. SMAC/DIABLO functions as an endogenous inhibitor/neutraliser of a class of anti-apoptotic proteins known as inhibitor-of-apoptotic-proteins (IAPs; e.g. X-linked IAP, XIAP; cellular IAP, c-IAP; survivin), thus providing an additional layer of control over apoptosis^{174, 175}. IAPs could bind to pro-caspase 9 to prevent its activation by cytochrome/Apaf-1 (apoptosome) or associate with activated caspases (caspase-3, caspase-7) to inhibit their cleavage activity. Thus, unlike the BCL-2 anti-apoptotic proteins that only block mitochondrial-originated intrinsic apoptosis, IAPs are effective inhibitors of both intrinsic and extrinsic (death receptor-mediated) apoptosis pathways¹⁸⁰. The presence of SMAC/DIABLO in cytosol would remove the inhibitory brake of IAPs on caspases and is conducive for caspase-dependent apoptosis, and the cellular availability of SMAC/DIABLO predicts the sensitivity of cells to apoptotic stimuli^{174, 175}. Cerebral H/I studies primarily focussed on XIAP as the most potent member of the IAP family, and reported increased SMAC/DIABLO release, SMAC/XIAP interaction and caspase-9/-3 activation after cerebral H/I insult¹⁸¹⁻¹⁸³. In neonatal brain, over-expression of *XIAP* gene significantly reduced cell death and tissue loss from I/R injury, whereby XIAP expression increases during reperfusion and XIAP nuclear translocation is regarded as the principle anti-apoptotic event^{182, 183} (to counteract caspase-3 mediated DNA fragmentation and chromatinolysis).

D.2.5 PARP-1, AIF and Parthanatos

Apoptosis-inducing factor (AIF) is a NADH oxidase located on mitochondrial inner membrane, where it serves as an essential component to mitochondrial respiratory chain to maintain complex I and complex III functionality¹⁸⁴. Mobilisation of AIF from mitochondrial compartment into cytosol and its subsequent nuclear translocation induces chromatin condensation and DNA loss, which is considered to be a parallel, substitute mechanism to caspase 3-mediated DNA degradation and cell death^{184, 185}. The cytosolic release of AIF requires 1) protease cleavage of AIF to liberate it from mitochondrial inner membrane and 2) MOMP. In this regard, mitochondrial calcium overload could activate Ca²⁺-dependent calpains and cathepsins (cysteine proteases) to cleave AIF^{184, 185}. Poly (ADP-ribose) polymerase-1 (PARP-1)-mediated AIF digest represents an alternative processing pathway. PARP-1 is a nuclear enzyme activated by DNA damage and oxidative stress during H/I insult, and activated PARP-1 catalyses the synthesis of poly ADP-ribose (PAR) polymers, which have been shown to relocate to mitochondria to trigger AIF release^{133, 185}. Cytosolic AIF then binds with cyclophilin A to facilitate its nuclear import, whereas association with heat shock protein 70 (HSP70) leads to AIF sequestration in the cytosol^{184, 185}. PARP-1/AIF mediated DNA degradation and cell lethality have been termed parthanatos and is distinct from caspase-dependent apoptosis.

In neonatal HIE studies, mitochondrial AIF release and AIF nuclear translocation are observed immediately after ischaemic insult¹⁸⁶. Importantly, the number of AIF-positive nuclei correlated with infarct volume, and treatment with pan-caspase inhibitor did not reduce the number AIF-positive nuclei^{179, 186}. Neuronal loss and infarct size were significantly reduced in an AIF-deficient background, which did not alter cytochrome c release and caspase 3 activation following cerebral H/I; the combination of pan-caspase inhibitor and AIF deficiency provided additive neuroprotection¹⁷⁹. Moreover, HSP70 overexpression conferred neuroprotection in neonatal mice, which displayed significantly greater AIF sequestration in the cytosol and lesser nuclear translocation after H/I challenge¹⁸⁷.

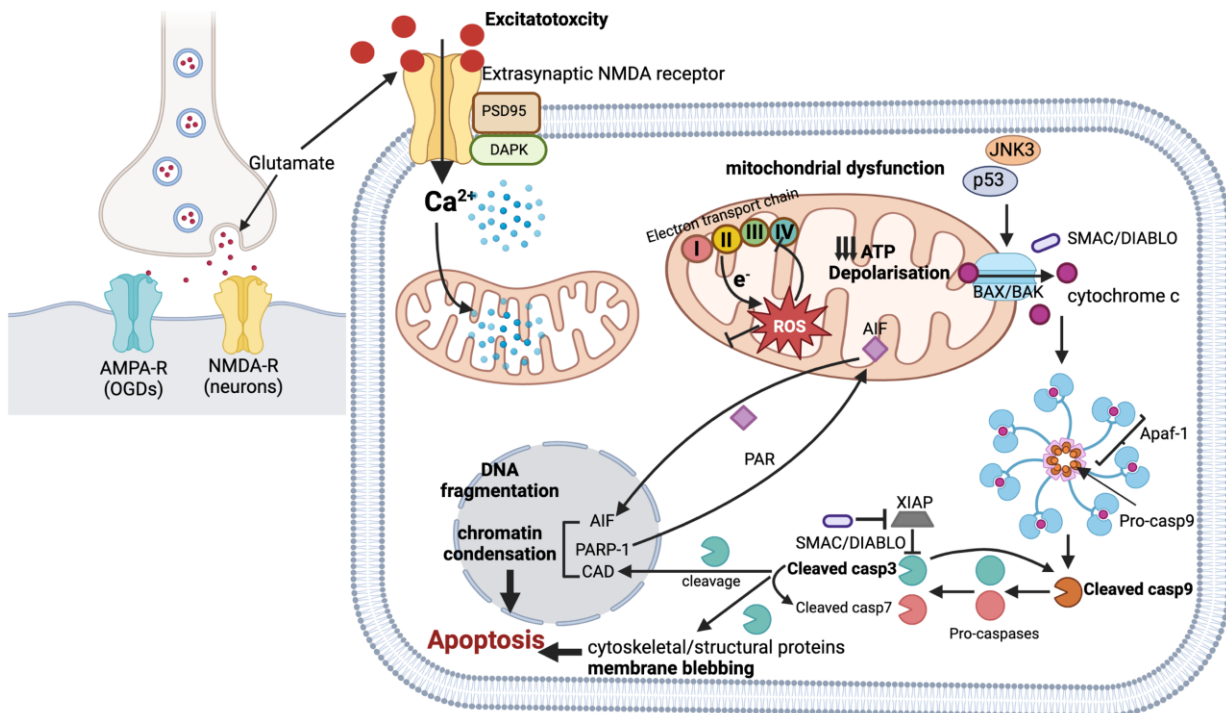


Figure 1-2. Secondary phase injury and mechanisms leading up to intrinsic apoptosis. During the hypoxia-ischaemia and reperfusion (I/R) stages of hypoxic-ischaemic encephalopathy (HIE), significant glutamate release from excitatory nerve terminals compounded by defective astrocytic reuptake of glutamate causes over-stimulation of the extra-synaptic N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and opening of voltage-gated Ca^{2+} channels. Excessive calcium influx predates cytosolic and mitochondrial Ca^{2+} overload, and intramitochondrial Ca^{2+} accumulation is associated with respiratory chain failure, ROS synthesis and mitochondrial swelling. In the immature brain, mitochondrial outer member permeabilisation (MOMP) is found with a 3-24 hrs delay after the onset of I/R insult and is dependent on the pore-forming pro-apoptotic Bcl-2 family proteins BAX and BAD, which oligomerise on mitochondrial outer membrane to trigger the release of pro-apoptotic cytochrome C and second mitochondria-derived activator of caspase/direct IAP binding protein with low pI (SMAC/DIABLO). The expression of pro-apoptotic and anti-apoptotic Bcl-2 family proteins is differentially regulated by p53 and c-Jun N-terminal kinase 3 (JNK3) signalling pathways. Upon release, cytochrome c assembles with apoptotic peptidase-activating factor 1 (Apaf-1) and pro-caspase 9 to form the apoptosome for the proteolytic cleavage of pro-caspase 9. Activated caspase 9 proceeds to cleave downstream caspases including pro-caspase 3 and pro-caspase 7. Activated caspase 3 cleaves a wide range of structural proteins as well as caspase-activated deoxyribonuclease (CAD) to membrane blebbing, DNA fragmentation and chromatin condensation, collectively contributing to apoptotic cell death. As the endogenous inhibitor for X-linked inhibitor of apoptotic proteins (XIAP), SMAC/DIABLO disinhibits the caspase blockade by XIAP to facilitate caspase-dependent apoptosis. Moreover, DNA damage and oxidative stress activates the nuclear enzyme poly (ADP-ribose) polymerase-1 (PARP-1) to generate the (ADP-ribose) polymers (PARs), which subsequently translocate into the mitochondrial compartment to prompt the cytosolic release of apoptosis-inducing factor (AIF) before its nuclear importation. As a parallel mechanism to caspase 3-dependent CAD activity, AIF also catalyses DNA loss and chromatin condensation.

Figure modified from Figure 1 of Hagberg et al⁷².

D.2.5.1 Sex Dimorphic Regulation on Apoptosis-Related Pathways

Pre-clinical and clinical evidences consistently reported female advantage in ischaemic brain injury, which has been attributed to the neuroprotective property of circulating oestrogen and/or the differential regulation of cell death mechanisms. A study on isolated neurons observed that upon cytotoxic challenge, XY neurons preferentially activated AIF-dependent cell death pathway whereas cytochrome c/caspase 3-mediated apoptosis was more upregulated in XX neurons¹⁸⁸. Animal studies have revealed sex-dependent susceptibility to apoptosis and related pathways in H/I brain injury, with preferential activation of caspase-dependent apoptosis in females and alternative activation of caspase-independent parthanatos pathway in males.

Stroke studies reported a prominent sexual dimorphism in PARP/AIF-mediated parthanatos pathway in adult animals. Stroke-induced PAR polymer formation was more pronounced in the male brain but AIF nuclear translocation was equivalent between genders; PARP-1 knockout or AIF deficiency was effective at preventing PAR accumulation and AIF nuclear translocation in both sexes¹⁸⁹. Complete or partial deletion of PARP-1 reduced infarct volume in male mice, and in stark contrast, the absence of PARP-1 exacerbated volume loss in females; treatment with PARP inhibitor PJ-34 similarly ameliorated and aggravated injury in wildtype males and females, respectively^{189, 190}. PARP-1 deficiency also abrogated oestradiol-17 β conferred neuroprotection in ovariectomised females¹⁹⁰. Moreover, AIF deficiency was demonstrated to be neuroprotective in males but did not advance ischemic injury in females¹⁸⁹. In this regard, PARP1/AIF-dependent parthanatos likely represents a predominant contributor to ischaemic brain injury in males. On the other hand, the suppression of PARP-1/AIF pathway is uncoupled from a neuroprotective phenotype in females, to suggest that parthanatos deficiency triggers excessive compensatory upregulation of other cell death pathway (i.e. apoptosis) to worsen brain injury.

In neonatal mice, cerebral H/I on PND9 produced brain lesion of equivalent size in both sexes, with male pups showing more PAR accumulation and AIF nuclear translocation in the damaged hemisphere, whereas injured brain from females showed greater caspase 3 cleavage and activation¹⁹¹. Similar to the observations made in adult males, PARP-1 deletion significantly reduced H/I brain injury in the cortex, hippocampus, thalamus and striatum in male pups; unlike their adult counterparts, PARP-1 deficiency did not further alter brain lesions in female pups⁹⁴. On the other hand, equivalent neuroprotection was attained in both genders from AIF-deficiency after perinatal H/I brain injury¹⁷⁹. In a reciprocal study, pan-caspase inhibitor Q-VD-Oph conferred more significant neuroprotection in female than male pups following cerebral H/I insult¹⁷⁸. The collective findings support the notion that parthanatos and apoptosis are preferentially activated by male and female neonatal brains, respectively, upon H/I challenge. Moreover, it has been speculated that females exhibit a higher endogenous level of the anti-apoptotic regulator X-linked IAP than males¹⁹², and such feature

maybe associated with increased neuroprotection from caspase-dependent apoptosis, which females have been shown to be intrinsically more vulnerable to. During genetic balancing, random inactivation to genes on the second X chromosome occurs yet 10-15% of the genes escape inactivation, which maybe applicable to the *XIAP* gene¹⁹². However, XIAP knockout or overexpression studies did not identify sex difference in the severity of tissue damage amongst neonatal mice subjected to cerebral H/I^{182, 183}, and the lack of difference may be ascribed to compensatory changes to other IAPs (e.g. cIAP-1 and cIAP-2) such that overall inhibition of apoptosis is unaltered. Current understanding on the molecular mechanisms governing sex dimorphic susceptibility to HIE injury is still very limited, and more studies are needed to identify potential gender-specific regulations over cell death to be developed as future therapy.

D.2.6 Inflammation and Immune Responses in HIE

In the termed infants, prenatal inflammation and infection during mid- to late-gestation could “prime” the developing brain to hypoxic-ischaemic event during birth (sensitisation), and H/I injury *per se* mounts a potent immune response that is initiated by the innate immune system¹⁹³. In the case of preterm encephalopathy, the brain injury is believed to be a direct result of inflammation/infection during very early development stage to predominantly affect the oligodendrocyte precursor cells, which is associated with substantial myelination deficiency and permanent white matter injury¹⁹³.

D.2.6.1 Priming Effect of Inflammation

A number of clinical studies examined the association between placental infection (chorioamnionitis) and cerebral palsy¹⁹³, although study that specifically evaluates chorioamnionitis as a risk factor for hypoxic-ischemic encephalopathy (HIE) is lacking. This is in part due to the stringent inclusion criteria for HIE (very difficult to recruit sufficient eligible participants) and the difficulty to ascertain H/I event as the definitive cause in cases of neonatal encephalopathy, as explained in A.2 and A.2.1. In a large-scale case-control study on termed, singleton birth, chorioamnionitis was shown to be independently associated with four-fold increased risk of cerebral palsy, and chorioamnionitis had a population attributable fraction of 11% for cerebral palsy (CP) in termed infants (27% in CP with spastic quadriplegia)¹⁹⁴. Potential mechanisms linking chorioamnionitis/placental infection to cerebral palsy include 1) maternal infection directly leads to infection of foetal brain or meninges (very rare), or 2) increased pro-inflammatory cytokines from foetal circulation/brain in response to maternal intrauterine response could be perilous the immature brain¹⁹⁴. This study also identified a particular strong association between chorioamnionitis and CP subjects showing hypoxic-ischaemic brain injury pattern (i.e. watershed injury), whom constituted a small proportion of all CP studied, to suggest potential interaction between chorioamnionitis and HI in the pathogenesis of HIE. In this regard, placental inflammation from chorioamnionitis could constrict placental blood flow and gas exchange to subject foetus to hypoxia-ischaemia, and/or elevated maternal temperature may increase the susceptibility of foetal brain (i.e. increased metabolic rate) to cerebral H/I event¹⁹⁴. On the other hand, a prospective cohort study on encephalopathic infants showing signs of perinatal hypoxia-ischaemia (blood gas pH <7.1, blood gas base deficit >10) found that maternal chorioamnionitis was associated with lower incidences of abnormal MRI findings at 5days of age and adverse neurological outcomes at 30 months, while a diagnosis of neonatal sepsis (within 7days of birth) was associated with more severe watershed brain injury¹⁹⁵. The discrepant findings might be attributed to the timings of inflammation, the different immune responses mounted and/or the distinct vulnerability window of various brain cell types; it is even plausible that a less severe condition (exemplified by chorioamnionitis) could have “preconditioned” the foetal brain

for the more severe hypoxic-ischaemic insult, by upregulating hypoxia-inducible factor (HIF) signalling pathway, nitric oxide synthesis and anti-apoptotic cascades¹⁹⁵.

Animal studies highlighted that the innate immune system is instrumental to the sensitisation/priming effect of inflammation in the immature brain in response to hypoxia-ischaemia. Administration of bacterial endotoxin liposaccharides (LPS) prior to unilateral carotid artery ligation plus hypoxia exacerbated brain injury than H/I alone, and is associated with upregulation of its cognate receptor toll-like receptor 4 (TLR4)¹⁹⁶. LPS-induced sensitisation to H/I brain injury is dependent on TLR4 and its adaptor protein myeloid differentiation primary response gene 88 (MyD88), as MyD88 deficiency avoided the potentiation of tissue loss, white matter injury, nuclear factor-kappa light chain enhancer of activated B cell (NF- κ B) activation and pro-inflammatory cytokine and chemokine (e.g. IL-1 β , IL-6, TNF- α , and MCP-1 monocyte chemoattractant protein-1) production following H/I + LPS, such that MyD88-KO mice exhibited injury similar in severity to that of H/I WT mice¹⁹⁷. LPS sensitised H/I injury was also characterised by greater increases in TNF- α and JNK phosphor-activation, and treatment with TNF- α inhibitor (Etanercept, prevents TNF- α binding to TNF receptor 1, TNFR1) or JNK inhibitor (AS601245) significantly reduced cerebral infarct, microgliosis and astrogliosis, demyelination and caspase-3 activation following cerebral H/I; TNF receptor 1 (TNFR1) knockdown conferred similar neuroprotection¹⁹⁸. In addition to TLR4, involvement of toll-like receptor 3 (TLR3) in HIE sensitisation was confirmed. TLR3 is naturally activated by viral double stranded RNA and is also activated by synthetic ligand poly inosinic: poly cytidylic acid (Poly I:C). Neonatal mice pre-treated with poly I:C sustained greater infarct and white matter loss than WT following cerebral H/I and downregulated pro-survival Akt pathway¹⁹⁹. Deficiency of TLR3 adaptor protein Toll/IL-1R domain-containing adaptor-inducing interferon-beta (TRIF) effectively prevented the exacerbation of injury and the heightened pro-inflammatory response¹⁹⁹. Moreover, proteolytic processing of TLR3 by cysteine protease cathepsin H (CatH) is necessary for TLR3-mediated activation of interferon regulatory factor 3 (IRF3) and downstream production of interferon-beta (IFN- β)²⁰⁰. CatH knockout neonatal mice displayed significantly lesser hippocampal atrophy but greater astrocyte scar formation after cerebral H/I. Such observations were attributed to decreased microglia activation and increased microglia death due to attenuated TLR3/IRF3 signalling and reduced availability of IFN- β , whereby IFN- β could stimulate microglia in an autocrine fashion following H/I²⁰⁰.

D.2.6.2 Immune Responses Triggered by Hypoxia-ischaemia and Reperfusion

Perinatal hypoxia-ischaemia triggers an immediate pro-inflammatory state that lasts for days after the initial insult, and precedes an anti-inflammatory and reparative phase that continues for weeks. Increasing evidences also support a more protracted remodelling phase into early childhood, whereby inflammation either resolves or propagates as chronic inflammation^{193, 201}.

In rodent HIE studies, H/I challenge leads to rapid and profound activation of resident astrocytes and microglia in the injured hemisphere/region, and is followed by subsequent infiltration of neutrophils, myeloid cells, T-cells, natural killer cells, and mast cells during the reperfusion when the blood-brain barrier is compromised (6 hrs to 24 hrs post-H/I)¹⁹³. The resident and infiltrating immune cells collectively produce pro-inflammatory cytokines, chemokines, death receptor ligands (e.g. TNF- α , FasL), along with excitatory amino acids and reactive oxygen species, to cause further damage to neurons and oligodendrocytes/OPCs¹⁹³.

Activation of the innate immune system initiates the pro-inflammatory cascade, whereby H/I-injured neurons and oligodendrocytes/OPCs could release intracellular contents as danger-associated molecular patterns (DAMPs) and/or pathogen-associated molecular patterns (PAMPs) to stimulate the pattern recognition receptors (PRRs) expressed by intrinsic and infiltrating immune cells¹⁹³. The toll-like receptor (TLR) family as classical PRRs has been extensively studied in the context of HIE^{193, 201}. Of the nine toll-like receptors (TLR1-TLR9), the expressions of TLR1, TLR2 and TLR7 were most strongly upregulated by cerebral H/I and reperfusion, however only gene knockdown of *TLR2* conferred significantly neuroprotection in neonatal mice²⁰². Deficiency of TLR1 or adaptor proteins of TLR3 and TLR4 (TRIF-KO or MyD88-KO) failed to reduce infarct volume and dampen inflammatory response; nonetheless TRIF or MyD88 deficiency prevented TLR3 and TLR4-mediated sensitisation to H/I injury, respectively, as discussed above^{197, 199, 202}. In this regard, stimulation of TLR2 by the synthetic ligand Pam₃CSK₄ significantly increased the level of pro-inflammatory cytokines (IL-1 β , IL-6, MCP-1) in the immature brain and led to a transient reduction of grey matter volume²⁰³. The importance of pro-inflammatory cytokines IL-1 β and IL-18 has been highlighted in rodent HIE studies, as IL-1 receptor blockade (IL1R antagonist)^{204, 205}, impaired IL-1 β processing (caspase 1 deficiency)²⁰⁶ or genetic knockout of IL-18²⁰⁷ all significantly attenuated tissue loss in neonatal mice after H/I with or without LPS sensitisation. Antagonising IL1 β signalling also suppressed NF κ B nuclear translocation and NF κ B-dependent transcription of nitric oxide synthase (NOS) and cyclooxygenase 2 (COX2)²⁰⁸. H/I-induced upregulation of IL-1 β , IL-18 and caspase 1 also suggest activation of the purinergic receptor and NLRP3 inflammasome 3, which are predominantly expressed by activated microglia^{206, 207}.

The activities and responses of microglia need to be carefully orchestrated during the course of HIE. Appropriate and timely microglial activation is essential for phagocytic removal of cellular debris during the acute phase of H/I injury, as well as releasing anti-inflammatory factors and different trophic factors to promote repair and regeneration^{209, 210}. On the other hand, activated microglia could also release excessive cytokines, chemokines, ROS and prostaglandins to exacerbate neural and OPC damage^{209, 210}. Studies also demonstrated that microglia-astrocyte communication could aggravate excitotoxicity during neuroinflammation. Inflammation-activated microglia releases L-glutamate to increase extracellular EAA concentration, which subsequently downregulated L-Glu transporter expression on astrocyte to impair astrocytic L-glutamate re-uptake²¹¹. Moreover, TNF- α released from activated microglia could potentiate

astrocytic glutamate release triggered by stromal cell derived factor-1 (SDF-1)/C-X-C chemokine receptor 4 (CXCR4) chemokine signalling, whereby SDF-1/CXCR4 induced microglial TNF- α increases intracellular Ca²⁺ in astrocyte to culminate to excessive glutamate release²¹². In light of the revisited understanding of the highly fluid, dynamic nature of different microglial “states” under physiological and diseased conditions, it is likely that microglia activation in the neonatal brain following cerebral H/I also encompasses multiple subpopulations each with distinct transcriptomic, epigenomic, proteomic and metabolomic signatures to endow significant functional and biological diversity²¹³. In view of this very recent revelation, future studies are warranted to examine and profile the different microglial states following H/I challenge. Partial understanding, and therefore unselective targeting of macroglia have unsurprisingly yielded conflicting results in neonatal H/I brain injury. Pharmacological elimination of microglia (liposome-encapsulated clodronate) further increased lesion area and elevated brain pro-inflammatory cytokine level than H/I insult alone²¹⁴, whereas treatment with minocycline, a tetracycline that selectively inhibits microglia with a pro-inflammatory signature²¹⁵, alleviated tissue loss and improved neurobehavioural outcomes in neonatal mice after H/I injury²¹⁶. Similarly, astrocytic activation from cerebral H/I also should not be confined to the binary classifications of neuroprotective/good versus neurotoxic/bad, instead specific transcriptomic, proteomic, signaling and functional characteristics are preferred to accurately describe the reactive astrocyte state under pathological conditions²¹⁷. In this regard, improved understanding of microglia and astrocyte subtypes and selective targeting of cerebral H/I-relevant microglia and astrocyte populations would pave the way for novel HIE treatment.

Mast cells are potently activated by H/I challenge in the adult brain, with immediately degranulation of cytoplasmic contents (histamine, TNF- α , heparin, proteases) to cause blood brain barrier (BBB) disruption, extravasation and brain oedema²¹⁸. Prolonged activation of mast cells further leads to de-novo synthesis of leukotrienes, prostaglandins and additional cytokines/chemokines to facilitate the recruitment of polymorphonuclear leukocytes (PMNL) to site of injury²¹⁸. During development, mast cells continue to migrate into the brain through vasculatures to permanently reside at perivascular locations (meninges and BBB), and the density of mast cells is believed to be higher in the immature brain than in the adult brain²¹⁸. Studies on H/I injury in the developing brain also supports a detrimental effect from mast cell activation. H/I significantly increased the number of mast cells (in particular TNF- α positive mast cells) and histamine accumulation in the ipsilateral hemisphere, where mast cells are located at pia and brain parenchyma^{219, 220}. Mast cell stabiliser disodium cromoglycate effectively prevented mast cell migration, reduced infarct volume/neuronal loss and microglial activation in rodent HIE studies^{219, 221}. Moreover, excitotoxicity increased transforming growth factor-beta 1 (TGF- β 1) level in the brain, and mast cells and mast cell-derived histamine are required for the neurotoxic effect of TGF- β 1, whereby mast cell-deficient mice or treatment with anti-histamine or mast cell stabiliser reduced brain lesions from TGF- β 1 stimulation²²².

Increased blood brain barrier (BBB) permeability was detected from 2 hrs to 24 hrs after cerebral H/I in neonatal mice along with dysregulated expressions of tight junction proteins²²³. The disruption to BBB coupled with prominent chemokine/chemoattractant release from resident immune cells promote the recruitment and influx of peripheral immune cells into brain to propagate inflammation further. In neonatal mice, cerebral H/I prompt an initial rise in local CD11b⁺ (microglia) and CD11c⁺ (dendritic cells) populations with upregulated co-stimulatory molecules (major histocompatibility complex-II, MHC-II and CD86⁺) to enable antigen presentation²²⁴. The first wave of antigen-presentation response peaked at 24 hrs and extended to one week after H/I challenge, likely corresponding to a shift from local microglia to infiltrating macrophages as the main effector cell type; a second, delayed episode of antigen presentation occurred at 3 months²²⁴. Infiltration of Ly-6G positive granulocytes²²⁴ and myeloperoxidase (MPO)-positive neutrophils²²⁵ were also evident from 6 hrs to one week after hypoxia-ischaemia with or without LPS sensitisation. Prophylactic Ly-6G antibody treatment to neonatal mice prior to HI/LPS insult was shown to prevent brain infiltration of neutrophils, and reduced H/I-induced tissue loss and release of pro-inflammatory cytokines (IL-1 β , TNF- α), chemokines (monocyte chemoattractant protein 1, MCP-1; macrophage inflammatory protein 1- α , MIP1- α) and degradative enzyme (matrix metalloproteinase 9, MMP9)²²⁶. Sex dimorphic immune and inflammatory responses were reported at day3 post-H/I in neonatal mice. Compared to female pups, male pups had significantly higher serum concentration of IL-1 β and TNF- α and more MHC-II positive (antigen-presenting) microglia, neutrophils and monocytes in the ipsilateral hemisphere, which predicted greater infarct/atrophy at day30²²⁷.

The neonatal brain also displayed significant influxes of CD4⁺ T-helper and CD8⁺ T-cytotoxic cells from one week to up to 7 months after H/I insult²²⁴. T-lymphocyte trafficking post-H/I is believed to be primarily through the choroid plexus²²⁸. Although studies on immature brain have not specifically investigated the significance of lymphocytes in HIE/perinatal hypoxia-ischaemia injury, stroke studies in adult rodents demonstrated that SCID mice that are naturally deficient in T-lymphocytes and B-lymphocytes exhibited smaller infarct volume after H/I and lacked most of the pro-inflammatory factors responsive to H/I challenge²²⁹. The pathogenic processes of H/I brain injury were attributed to T-cells, as neuroprotection was still apparent when SCID mice were reconstituted with functional B cells²³⁰. Future studies are needed to better elucidate the pathogenic and immune-modulatory roles of adaptive immunity in HIE. The relative immaturity of the adaptive immune system at birth may have led studies to overlook the importance of adaptive immunity, which likely contributes to the chronic modulation of brain inflammation and/or repair after HIE (i.e. tertiary phase) as the immune system gradually matures.

In addition to the immediate cell death and metabolic disturbances that characterise the primary and secondary phases of injury, there is increasing appreciation of a possible tertiary injury phase that features prolonged inflammation, epigenetic changes and tissue remodelling from months to years after the initial hypoxic-ischaemic insult. It has been suggested that ongoing pathological processes continue to exacerbate existing brain injury, prevent endogenous regeneration/repair mechanisms, and/or sensitise the brain to subsequent injury. Persistent structural modifications to the developing brain after initial H/I injury may include hypomyelination, reduced neurogenesis, impaired axonal growth, aberrant synaptogenesis and increased glial scar formation.

D.3.1 Chronic Inflammation

To date, no animal or clinical studies on hypoxic-ischaemic encephalopathy have specifically and systemically investigated long-term neuroinflammation and resolution thereof into childhood and adulthood. In 7-year-old cerebral palsy children who were born preterm, peripheral blood level of TNF- α was found to be persistently elevated, and peripheral blood mononuclear cells (PBMCs) from these children produced higher concentration of TNF- α when challenged by LPS²³¹. Similarly, in a rat model of preterm H/I brain injury with LPS sensitisation, increased serum level of pro-inflammatory cytokines (IL-1 β , TNF- α , MCP-1) persisted into adulthood (postnatal day 60 and day 120), and PBMCs from injured, adult rats remained hyper-responsive to LPS²³². Persistent infiltration of immune cells was also evident in adulthood, whereby injured rats had significantly more T-helper cells, T-regulatory cells and macrophages in the brain at postnatal day 120 compared to controls²³². Findings from these studies support the notion that perinatal brain injury is associated with chronic, ongoing inflammatory response (CNS and peripheral).

Positron emission tomography (PET) studies on traumatic brain injury (TBI) survivors revealed persistent microglia activation (microgliosis) lasting for more than 10 years, in particular in the deep grey matter and posterior limb of internal capsule (PLIC), and microglial activation in the thalamus is associated with worse cognitive performance²³³. It is plausible that long-term microgliosis also takes place in HIE survivors, which needs to be substantiated by future imaging study. Chronic activation of microglia could exert lasting effects on neural regeneration and repair in the developing brain. In neonatal rodents, cerebral H/I enhanced overall proliferation of neural progenitor/precursor cells (NPCs) in the subventricular zone (SVZ) concurrent with enlargement of SVZ^{234, 235}. Flow cytometric analysis of H/I-injured SVZ revealed expansion of two types of multipotent precursors and three clusters of glial-restricted precursors, which were at the expense of diminished neural stem cell (NSC) population²³⁶. Increased neurons and glia from SVZ were shown to migrate into the lesioned cortex, and such neurogenesis and migratory responses continued for months after H/I in neonatal mice²³⁷. Microglial cytokines are potent regulators of NPC expansion, self-renewal and pluripotency.

Microglial-derived IL-6 was demonstrated to stimulate NP proliferation and renewal in SVZ, which maybe dependent on IL-6 mediated cyclo-oxygenase 2 (COX2) activation and subsequent prostaglandin E2 (PGE2) synthesis, as COX inhibitor indomethacin prevented IL-6 induced NPC expansion and H/I-induced microglia accumulation in SVZ. On the contrary, IL-6 suppressed neural progenitor/precursor proliferation and neurogenesis from hippocampal subgranular zone (SGZ) during neuroinflammation²³⁸, and indomethacin treatment restored the proliferative ability of SGZ NPCs in neonatal rats after H/I²³⁵. Leukocyte inhibitory factor (LIF) and ciliary neurotrophic factors (CNTF) are also members of the IL-6 cytokine family and have been individually shown to stimulate self-renewal of SVZ NPCs *in vitro*²³⁹. Taken together, IL-6 produced by microglia may exert niche-specific effect on different NPC populations (SVZ vs. SGZ), and the different self-renewal capacity and pluripotency/"stemness" of NPCs between the immature and mature brains will also dictate the cellular response to IL-6. Oligodendrocytes and oligodendrocyte precursor cells (OPCs) are also susceptible to microglia-originated pro-inflammatory cytokines, such that persistent microglia activation will eventually lead to hypomyelination/dysmyelination. TNF- α and IL-1 β from LPS-activated microglia have been shown to be highly toxic to oligodendrocytes and immature oligodendrocytes^{240, 241}, nonetheless TNF- α signalling through TNF receptor 2 (TNFR2) may favour remyelination. Moreover, excess glutamate release from activated microglia (and injured neurons) could induce excitotoxicity to OPCs primarily through the AMPA/kainate receptor¹³⁷. Microglia also participate in phagocytosis of synaptic materials and are essential to synaptic pruning during brain development^{193, 209}.

Astrocyte activation during the early injury phase can be protective by several means, such as uptake of excitotoxic glutamate, release of antioxidants glutathione and superoxide dismutase (SOD), restore BBB integrity by forming glial scar, prevent vasogenic oedema and stabilise extracellular fluid and ion balance, thus facilitating regeneration and repair. Excessive glial scar formation is the most well-studied negative consequence of reactive astrogliosis²⁴². Persistent astrogliosis and scar formation cordon off areas of severe tissue damage and inflammation, to limit the propagation of neuroinflammation but also at the expense of axonal regeneration^{201, 242}. Astrocytes are immune-competent cells and respond to a plethora of cytokines/chemokines/lipids released from the injured site. For example, IL-17 released by T lymphocytes was shown to activate NF κ B signalling pathway within astrocytes to upregulate transcription of pro-inflammatory cytokines, whereas TGF- β 1 binding to astrocytic receptor suppressed NF κ B activation and nuclear translocation to ameliorate neuroinflammation²⁴³. in light of the renewed appreciation of the diverse and dynamic states of glial cells^{213, 217}, there is great impetus to systemically and comprehensively study the long-term effects of microglia and astrocyte activation on neurogenesis, synaptic plasticity and connectivity in HIE.

D.3.2 Epigenetic Mechanisms

Epigenetic modification is achieved through either regulation of gene expression by enzymatic control of permissive tags (methylation, acetylation, ubiquitination, sumoylation) on histones or DNA or through microRNA-dependent regulation over protein translation²⁰¹. Epigenetic changes to specific amino acids residues at the N-terminal of histones and to DNA enable precise control over the complex organisation of chromatin and the binding of transcription factors, to promote or depress gene transcription²⁴⁴. In general, a transcriptionally active chromatin is characterised by unmethylated CpGs (5' cytosine-phosphate-3 guanine) and increased acetylation of H3 histone residues, and such chromatin configuration requires the cooperative activities from DNA demethylase and histone acetyltransferase (HAT). Histone deacetylase (HDAC) antagonises the action of HAT by removing the acetyl tags from histone to reduce transcription activation²⁴⁴. The acetylation status of histone has been implicated in different CNS disorders including ischaemic brain injury, whereby the acetyl tags on H3 histone were significantly depleted during the acute ischaemic phase following middle cerebral artery occlusion (MCAO), and treatment with inhibitor of class I/II HDAC (HDACi) reversed H/I-induced H3 deacetylation, increased expression of neuroprotective and anti-apoptotic proteins (HSP70, BCL-2) and reduced brain volume loss²⁴⁵. Paradoxically, HDACi-induced histone acetylation could either increase gene expression by reducing chromatin condensation, or decrease expression by increasing the level of transcriptional repressors. In a rodent model of LPS-sensitised HIE injury, class I/II HDACi pre-treatment only promoted the accumulation of acetyl tags on histones H3 and H4 in female pups but not males after LPS+H/I, and only HDACi-treated females demonstrated reduced grey and white matter injury on day5. HDACi-treated females also displayed significant improvement in open-field (more time spend/distance travelled in the centre of the field) and fear conditioning (longer freezing time to cued stimuli) tests on PND34 compared to non-treated females²⁴⁶. Unlike in adults, in neonatal LPS+H/I injury paradigm, HDACi was not associated with altered expressions of pro-inflammatory cytokines and apoptosis-related proteins, to suggest that the neuroprotective effect of HDACi is channelled through alternative pathways.

Histone acetylation/deacetylation exerts complex regulation over neurogenesis and progenitor cell differentiation in the developing brain, and perinatal H/I brain injury could perturb epigenetic processes associated with neurogenesis/differentiation to produce long-lasting damages. In rats, progressive histone H3 deacetylation within oligodendrocyte precursor cells (OPCs) in early postnatal days was demonstrated to be crucial for the timely differentiation into myelin-producing oligodendrocytes, as exposure to HDACi prevented class I HDAC-mediated histone deacetylation, downregulated oligodendrocyte marker genes and delayed oligodendrocyte differentiation, leading to hypomyelination of axons in the corpus callosum²⁴⁷; it is believed that histone deacetylation is needed to downregulate OL differentiation inhibitors/repressors. In mice that are double-deficient in HDAC1 and HDAC2, both belonging to class I HDAC, neural progenitors showed increased cell death and impaired differentiation into mature neurons, which manifests into

defective cortical laminar organisation and severe abnormalities in the hippocampus and cerebellum²⁴⁸. On the other hand, administration of HDACi enhanced the expression of neuron-specific gene *NeuroD* to favour neuronal differentiation²⁴⁹. The discrepant outcomes maybe explained by the different level/magnitude of (re)acetylation accomplished by gene deletion or pharmacological inhibition, such that the effect of latter is less severe and also transient when compared to complete gene knockout; effect from HDAC manipulation could also be cell type-specific and promoter-specific. Future studies will need to address whether and how perinatal H/I insult may lead to chronic epigenetic changes to contribute to tertiary injury mechanisms, and to validate the therapeutic efficacy of HDAC inhibitors and the likes in HIE in the long-term.

D.3.3 Sex Hormones

Hypoxia-ischaemia encephalopathy studies have repeatedly demonstrated a strong gender effect on outcomes, where males are significantly more susceptible and sustain worse brain injury than females¹⁹². In addition to sex-dependent differential regulation of apoptosis and related pathways (section D.2.5.1), hormonal exposures during gestation/perinatal period (early life) and after puberty (later life) can also substantially influence brain morphology and neurodevelopment trajectory between genders after H/I injury. During human foetal development, testosterone level temporarily peaks in male foetuses between gestational weeks 10-20 and rises again on the day of birth. High testosterone level remains throughout the first year of life, and between 3-4 months of age testosterone concentration is equivalent to level of second stage of puberty¹⁹². Early life exposure to high level of testosterone is believed to be the main contributor to male vulnerability to perinatal H/I injury. In this regard, hippocampal slides from neonatal male rats were more sensitive to γ -aminobutyric acid A (GABAA)-induced excitotoxicity, whereby excessive activation of GABAA receptor by muscimol caused more cell death in male than female-derived slides; application of dihydrotestosterone (DHT) significantly potentiated muscimol-induced cell death from both sexes²⁵⁰. Moreover, pre-treating PND7 female rats with exogenous testosterone prior to HIE procedure significantly exacerbated tissue loss and cognitive and behavioural performance on PND30, when compared to non-treated female pups²⁵¹. Neonatal male mice also exhibited greater microglial activation (MHC-II antigen presentation) and neutrophil infiltration into the lesioned hemisphere than female counterparts after H/I²²⁷. In a perinatal microgyria study where rats received cortical freezing lesion on the day of birth, sex divergent outcomes persisted into adulthood (PND80), whereby male rats and androgenised female rats (testosterone-treated) had significantly smaller neuronal size than untreated females²⁵².

The neuroprotective property of oestrogen is well-established in animal models of global and focal ischaemic brain injury; however there lacks consensus on the beneficial effect of oestrogen is in human, as the successful neuroprotection from oestrogen replacement observed in animals do not always translate to human population¹⁹². Nonetheless, female advantage in the context of hypoxic-ischaemic encephalopathy is

unlikely to be attributable to gonadal hormones, as neonatal female animals and pre-pubertal girls have negligible level of circulating oestrogen (and progesterone) from the quiescent ovaries, although the possibility of central steroidogenesis is feasible. It should be noted that ovarian oestrogen maybe associated with favourable, long-term neurodevelopment and reorganisation/repair later on into puberty.

D.4 Susceptible Cell Types from Hypoxia-Ischaemia and Reperfusion Injury

Cellular and animal models of hypoxic-ischaemic brain injury have demonstrated that neurons are extremely sensitive to oxygen deprivation and the ensuing excitotoxicity, ROS stress and mitochondrial dysfunction. As reported by different animal models of HIE, neuronal loss is prominent across multiple brain regions including basal ganglia, thalamus, hippocampus and cortex, with neuronal loss starting as early as 1 hr after H/I in dentate gyrus of hippocampus before detectable injury in hippocampal CA regions, thalamus and cortex²⁵³. Little is known regarding the relative susceptibilities of different neuronal subtypes of the immature brain. Early study using glutamate binding capacity as a surrogate measure of glutamate receptor-expressing cells found that H/I led to progressive and significant reduction in glutamate binding in the hippocampus and striatum of neonatal rat, with the earliest and most profound decrease seen in the dentate gyrus²⁵⁴. In the lesioned striatum, reduced level of different neurotransmitters that correspond to cholinergic, dopaminergic and GABAergic neurons was detected 3 weeks after H/I; the biochemical perturbation persisted into week 7, by which point GABAergic markers had fallen to 50% of that of control to highlight greater susceptibility of GABAergic neurotransmission²⁵⁵. Such reductions reflect susceptibility of the presynaptic cholinergic, dopaminergic and GABAergic nerve terminals, cell bodies or both in H/I-injured neonatal brain. Cholinergic neuron loss was also found in the medial septal nucleus, which sends projections into the hippocampus, while striatal cholinergic neurons displayed dysmorphic dendrites in a rat model of HIE²⁵⁶. Moreover, the neurogenic niche could also be susceptible to perinatal H/I, whereby caspase 3-activated neural progenitor cells were identified in the dorsolateral but not medial subventricular zone (SVZ)²⁵⁷. Others reported an expansion of SVZ size following perinatal H/I, which was attributed to expansion of glia-restricted progenitors and multipotential progenitors at the expense of neural stem cells (NSC)²³⁶. Whether hippocampal subgranular zone (SGZ) as the other main neurogenic niche also harbours similar susceptibility and/or plasticity following H/I remains unknown, but the prominent neuronal loss in dentate gyrus is suggestive of neural stem/progenitor cell vulnerability.

Vulnerability of myelinating oligodendrocytes (OLs) and oligodendrocyte precursor cells (OPCs) to hypoxia is also well-documented in the adult²⁵⁸ and developing brains^{259, 260}. Hypomyelination resulting from the demise, dysfunction or altered differentiation of OLs and OPCs directly contribute to the development of white matter injury in HIE subjects. Depending on the timing of perinatal cerebral H/I challenge and the age of the newborn animals as distinct models for pre-term versus term HIE, white matter injury may manifest as the predominant pathology over grey matter lesion²⁵⁹, as in the case of preterm periventricular leukomalacia (PVL). The susceptibility of OLs and OPCs to hypoxia has been ascribed to AMPA/kainate receptor-mediated excitotoxicity²⁶¹, a heightened sensitivity towards oxidative stress²⁶² and/or glia-driven pro-inflammatory cytokine release²⁴¹ during H/I insult. Maturation-dependent vulnerability in the oligodendrocyte lineage has

also been described, whereby late OL progenitor population suffered greater loss when compared to early OL progenitors and mature OLs from H/I in the neonatal brain²⁶³.

In addition to neuron- and oligodendrocyte-lineage cells, endothelial cells of the brain microvasculature are also susceptible during H/I brain injury to compromise blood brain barrier integrity. Cultured endothelial cells were shown to be more sensitive to oxygen-glucose deprivation (OGD) than other cellular types (pericytes, astrocytes) of the perivascular unit²⁶⁴, exhibiting increased DNA fragmentation and elevated level of reactive nitrogen species²⁶⁵. In a mouse model of transient middle cerebral artery occlusion (MCAO), early death of endothelial cells was observed at 1-2hrs after stroke in the form of necroptosis²⁶⁶, as will be discussed in more detail in the following chapters. Finally, evidence of astrocytic or microglial susceptibility during H/I brain injury is scarce, as the glial cells are considered to be more resistant to oxygen deprivation and rather become activated to release pro-inflammatory factors and coordinate immune responses (as discussed in Introduction, section D.2.6.2); one study found significant astrocyte loss in the H/I-injured striatum of newborn animal at 24-48hrs and is followed by rapid proliferation to replenish the astrocyte population²⁶⁷.

An overwhelming body of studies from the last decade have confirmed the involvement of alternative regulated cell death (RCD) pathways in the pathogenesis of ischaemic, traumatic and inflammatory CNS disorders, and these RCD mechanisms are distinct from apoptosis and utilise unique molecular machineries to execute cell death. In hypoxia-ischaemia injured immature brain exists a “continuum”/spectrum of cell death phenotypes where classical apoptosis and classical necrosis are at the two ends of extreme, and in between falls different hybrid forms of cell death²⁶⁸. Under electron microscope, apoptosis is characterised by compaction and marginalisation of chromatin, nuclear fragmentation, intact plasma membrane, condensed cytosol and intact organelles until late phase of apoptosis. In stark contrast, necrotic cells typically present with disrupted plasma membrane, organelle swelling and random dissolution of chromatin without condensation^{268, 269}. Regulated necrosis, termed necroptosis, as one of the most extensively studied RCD pathways more closely resembles the appearance of classical necrosis, with early plasma membrane permeabilisation, swollen organelles (endoplasmic reticulum, mitochondria), translucent cytoplasm and random nuclear dissolution^{133, 269}. Necroptosis is orchestrated by receptor-interacting protein kinases 1 and 3 (RIPK1/3) and their downstream pseudokinase mixed-lineage kinase-domain like (MLKL) through a chain of phosphor-activation^{270, 271}. Common triggers of necroptosis include stimulation by cell death receptor ligands (e.g. TNF- α , FasL, TNF-related apoptosis-inducing ligand – TRAIL), viral infection (activation of toll-like receptors 3 and 4) and ischaemic injury *in vivo*, and the successful induction of necroptosis is reliant on specific cellular contexts and sophisticated, interactive molecular regulations, as will be discussed in the following sections²⁶⁹⁻²⁷¹.

E.1 Molecular Regulation of Extrinsic Apoptosis and Necroptosis

During ischaemic brain injury, damaged cells and activated immune cells (microglia, astrocytes and infiltrated macrophages and leukocytes) will release a variety of pro-inflammatory cytokines and tumour necrosis factors (TNFs). The tumour necrosis factors superfamily (TNFSF) consists of 19 ligands to correspond to 28 receptors of the tumour necrosis factor receptor superfamily (TNFRSF); because the similarity in structure between different TNFs and between TNF receptors, multiple ligands may bind to the same receptor, and vice versa one ligand could stimulate different receptors²⁷². A few ligand/receptor pairs have been shown to participate in the induction of extrinsic apoptosis and necroptosis pathways, for example TNF- α / TNF receptor 1 (TNFR1), Fas ligand (FasL, CD95L)/ Fas (CD95), tumour necrosis factor-related apoptosis inducing ligand (TRAIL)/ TRAIL receptor 1 and 2 [TRAIL-R1 (death receptor 5, DR5), TRAIL-R2 (death receptor 6, DR6)].

E.1.1 Post-translational modification of RIPK1

The necroptosis pathway is tightly regulated by a constellation of kinases, E3 ubiquitin ligases and deubiquitinases to finetune the phosphorylation and ubiquitination statuses of necroptosis mediators (**Fig.1-3**). Classical necroptosis signalling requires the activation of RIPK1 that is triggered by stimulation of cell death receptors from their cognate ligands, as exemplified by TNF- α binding to TNF receptor 1 (TNFR1), before the death domain (DD) of the cell death receptors recruits DD-containing TNFR1-associated DD protein (TRADD) and RIPK1 via homotypic binding to form a membrane-bound *complex I*^{273, 274}. Within complex I, RIPK1 undergoes complex post-translational modifications to its ubiquitination and phosphorylation states, which dictate the precise downstream pathway to be pursued, to include pro-survival, pro-inflammatory NF κ B pathway or the induction of apoptosis or necroptosis.

Under conditions that the cell do not die, TRADD further recruits the E3 ubiquitin ligases cellular inhibitor of apoptosis 1 or 2 (cIAP1 or cIAP2) via the adaptor protein TNFR-associated factor 2 (TRAF2), to promote K63 ubiquitination of RIPK1 at amino acid residue Lys377. The K63 ubiquitin chain on RIPK1 then recruits and activates transforming growth factor β -activated kinase 1 (TAK1), which was shown to induce pro-survival MAPK signalling pathway as well as I κ B (inhibitor of κ B) kinases (I κ K) to promote NF κ B-dependent gene transcription. Activation of NF κ B signalling subsequently upregulates the expression of antiapoptotic proteins cIAP1/2 and c-FLIP (cellular FLICE (FADD-like IL-1 β -converting enzyme)-inhibitory peptide) along with pro-inflammatory cytokines, to suppress cell death and sustain inflammation. The presence of cIAP1/2 and K63 polyubiquitination is conducive for the recruitment of the linear ubiquitin chain assembly complex (LUBAC) to complex I to permit M1-linear ubiquitination of RIPK1. The M1 linear ubiquitin chain was shown to further engage NF κ B essential modulator (NEMO), optineurin (OPTN) and TANK-binding protein 1 (TBK1), to promote I κ K/NF κ B signalling, regulate K48 ubiquitination and to phosphorylate RIPK1 at T189 residue, respectively; binding of these proteins to M1 ubiquitin chain was shown to suppress RIPK1 activation. The ubiquitin chains on RIPK1 can be removed endogenous deubiquitinases, whereby K63 ubiquitination is antagonised by cylindromatosis (CYLD) and A20 and M1 linear chain is deubiquitinated by OTU deubiquitinase with linear linkage specificity (OTULIN). Therefore, it is conceivable that polyubiquitinated, inactive RIPK1 acts as an “anchor” to transduce pro-inflammatory and pro-survival signals to increase cellular resistance to cell death signals. The loss of K63 and M1-linear ubiquitins by CYLD and A20, in conjunction with cIAP1/2 degradation via autoubiquitination or by SMAC mimetic (synthetic compound that mimic the action of endogenous SMAC/DIABLO)²⁷⁵, will release RIPK1 from complex I and marks the irreversible commitment by RIPK1 to regulated cell death pathway.

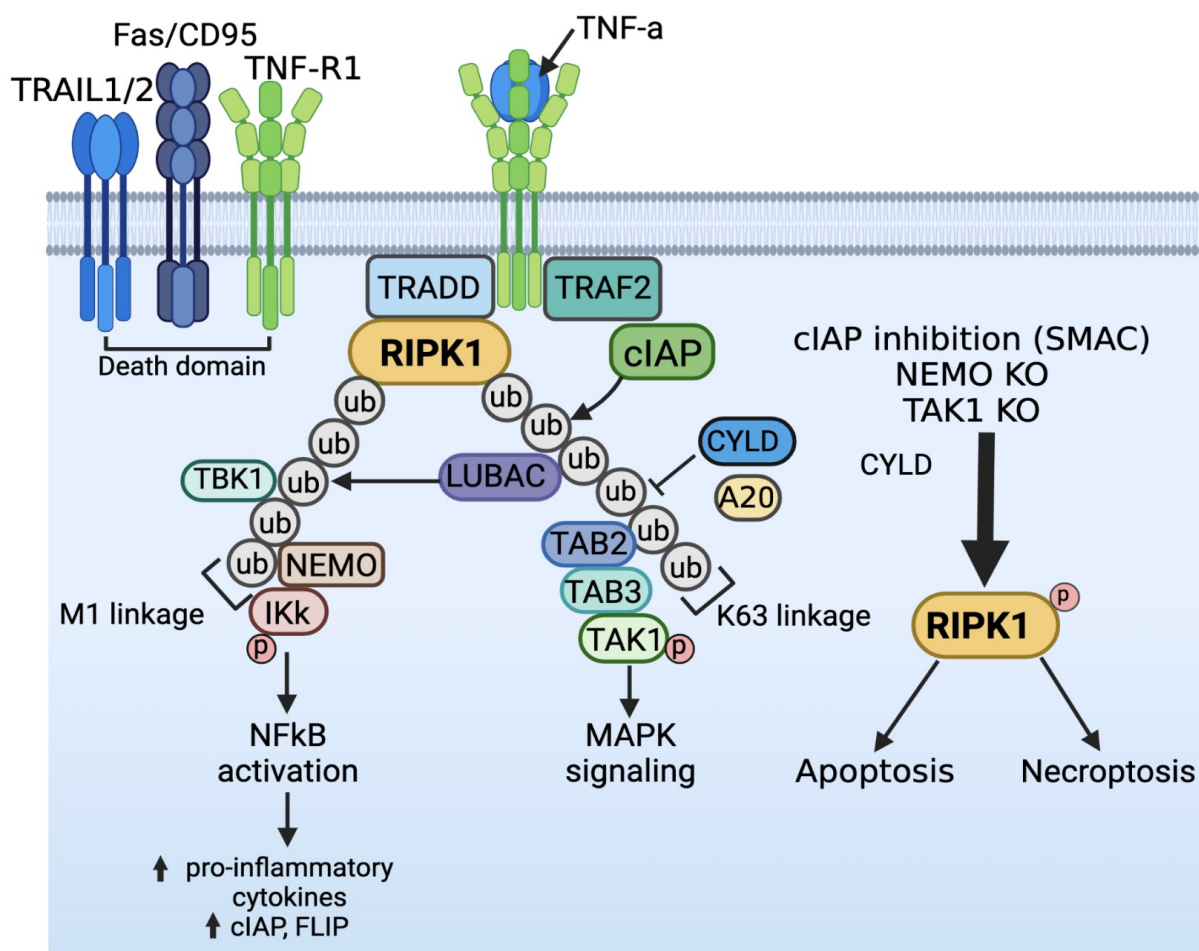


Figure 1-3. Post-translational modification of RIPK1 and formation of complex I. Binding of the tumour necrosis factor superfamily (TNFSF) to the tumour necrosis factor receptor superfamily (TNFRSF) leads to TRADD (TNFR1-associated DD protein)-dependent recruitment of receptor-interacting protein kinase (RIPK1) through via homotypic interaction. The E3 ubiquitin ligase cellular inhibitor of apoptosis 1 or 2 (cIAP1/2) is subsequently recruited by TNFR-associated factor 2 (TRAF2) to promote K63 ubiquitination of RIPK1 to form the K63 polyubiquitin linkage. The K63 linkage facilitates the recruitment and activation of transforming growth factor β -activated kinase 1 (TAK1) via (TAK-associated binding protein 2 and 3) TAB2/3 to induce the pro-survival MAPK signalling pathway. The presence of K63 linkage also promotes the recruitment of linear ubiquitination assembly complex (LUBAC) to lead to M1 (linear) polyubiquitination of RIPK1. The M1 ubiquitin linkage can be bound by NF κ B essential modulator (NEMO) and inhibitor of κ B kinases (IkK) to stimulate the NF κ B signalling pathway and upregulate the transcription of pro-inflammatory cytokines; transcription of anti-apoptotic proteins such as cIAP and c-FLIP (cellular FLICE (FADD-like IL-1 β -converting enzyme)-inhibitory peptide) is also activated by NF κ B. Endogenous deubiquitinases such as CYLD and A20 are also recruited to the polyubiquitin chains to disassemble M1 and K63 linkages, respectively. Moreover, RIPK1 can be phosphorylated by TAK1 or by TANK-binding kinase 1 (TBK1) recruited to the M1 linkage, and such inhibitory phosphorylation further prevents RIPK1 activity. The aggregation and stabilisation of RIPK1 with the handful of post-translational editing enzymes is termed as *complex I*. Under conditions of cIAP inhibition by SMAC/DIABLO or SMAC synthetics, NEMO knockout or TAK1 knockout, RIPK1 is liberated from the membrane-bound complex I to activate downstream apoptosis or necroptosis by forming the respective signalling complexes.

Figure adapted from **Figure 1** from Yuan et al³⁵³.

E.1.2 Extrinsic Apoptosis – The Formation of Complex IIa

As explained above, activation of transmembrane cell death receptors by cognate death ligands (e.g. TNF α /TNFR1, FasL/Fas, TRAIL/TRAIL-R1 and TRAIL-R2), and the subsequent liberation of RIPK1 from polyubiquitination, are shared upstream events to extrinsic apoptosis and necroptosis pathways. The death-promoting TNFRSF members function in trimers and all contain an intracellular death domain (DD), and upon receptor stimulation, the receptor DD proceeds to recruit DD-containing adaptor proteins (e.g. TRADD, FADD) via homotypic interaction^{273, 274}. Depending on the specific extracellular cell death trigger and availability/activity of intracellular anti-apoptotic factors (cIAP1/2, c-FLIP), RIPK1-dependent or RIPK1-independent pathway may take place to culminate in caspase 8 activation and extrinsic apoptosis (**Fig.1-4**).

In RIPK1-dependent apoptosis (RDA), upon its dissociation from complex I, RIPK1 engages with FADD (Fas-associated death domain) to facilitate FADD and pro-caspase 8 interaction thus forming *complex IIa*, or death-inducing signalling complex (DISC)^{269-271, 275}, to promote the auto-proteolysis and activation of pro-caspase 8 homodimers. The kinase activity of RIPK1 was shown to be essential for the successful assembly of *complex IIa* (FADD, pro-caspase 8 and RIPK1), where RIPK1 directly interacts with both caspase-8 and FADD²⁷⁵. RIPK1-independent apoptosis (RIA) was observed when cells were treated with TNF- α plus protein synthesis inhibitor cycloheximide (CHX), whereby DD of activated cell death receptors recruit TRADD, FADD and caspase-8 to form DISC without RIPK1 (*complex IIb*). In this instance, CHX hinders the translation and expression of c-FLIP to sensitise cells to apoptosis from uninterrupted caspase 8 processing (see below)^{275, 276}. RIA was believed to be an “artificial” cell death programme as protein synthesis inhibitor is not present *in vivo*²⁷⁶, unlike RDA whereby the ubiquitinases/deubiquitinases of RIPK1 function in homeostasis and can be antagonised by endogenous molecules (e.g. cIAP1/2 and SMAC/DIABLO). More recent study revealed that TAK1-mediated phosphorylation of the intermediate domain of RIPK1 serves as a molecular switch to selectively activate RDA, RIA or necroptosis following TNF- α stimulation²⁷⁷. Increased RIA was reported with transient phosphorylation of RIPK1 S321 serine residue by TAK1, while S321A phosphorylation-resistant RIPK1 mutant was sensitised towards the cytotoxicity of TNF- α and preferentially induced RDA, which can be inhibited by RIPK1 inhibitor necrostatin-1. Hyperphosphorylation of RIPK1 intermediate domain in quadruple S321A/332/334/336E mutant favoured activation of RIPK3 and MLKL to trigger necroptosis²⁷⁷.

Regardless of RDA or RIA, when sufficient amount of mature caspase 8 is produced by DISC, downstream caspases (pro-caspase 3 and 7) can be efficiently cleaved to proceed with apoptosis²⁷⁸. Alternatively, when only a small quantity of activated caspase 8 is generated from death receptor signalling, caspase 8 could then cleave the pro-apoptotic Bcl-2 family member BID to generate truncated BID (tBID)²⁷⁹, which subsequently participates in MOMP to integrate the extrinsic and intrinsic apoptosis pathways. Importantly, activated caspase 8 was also shown to directly cleave RIPK1 to separate its death domain from intermediate and kinase

domains, hence abolishing the kinase activity of RIPK1²⁸⁰. Similar studies also demonstrated RIPK3²⁸¹ and the deubiquitinase CYLD²⁸² as proteolytic substrates of caspase 8, representing plausible mechanisms by which apoptosis could inhibit necroptosis.

Complex IIa/IIb additionally may incorporate c-FLIP, an inactive homolog of caspase 8, for c-FLIP to heterodimerise with caspase 8 via its N-terminal death domain^{270, 271, 283}. c-FLIP exists as short (c-FLIP_s) and long (c-FLIP_L) isoforms and is transcriptionally upregulated by NFκB, and depending on which isoform caspase 8 complexes with, the caspase 8/c-FLIP heterodimers could differentially regulate apoptosis and necroptosis. c-FLIP_s competes with pro-caspase 8 for binding with FADD and pro-caspase 8 cleavage is completely blocked in c-FLIP_s/caspase-8 heterodimer²⁸⁴ to inhibit apoptosis. C-FLIP_L/pro-caspase 8 heterodimer impairs the efficiency of caspase-8 processing (and downstream apoptosis) yet the heterodimer still retains sufficient proteolytic activity towards RIPK1²⁸⁵ and suppresses RIPK3-dependent cell death²⁸⁶, thus eliciting negatively regulation over necroptosis. Genetic studies demonstrated that embryonic lethality resulting from complete ablation of caspase 8 can be rescued by a concomitant knockdown of RIPK3^{286, 287}. Specifically, RIPK3-dependent cell death triggered by TNF-α can be inhibited by catalytically active caspase 8/c-FLIP_L heterodimer²⁸⁶, and genetic depletion of caspase 8 and/or c-FLIP resulted in increased association between RIPK1, RIPK3 and FADD²⁸⁶ to facilitate the assembly of the *necrosome*. Similarly, tissue-specific knockout (small intestine, epidermis) of caspase 8 (Casp8^{-/-}) or FADD (FADD^{-/-}) culminated in overt RIPK3-dependent necroptosis, and the ensuing tissue lesion and inflammation could be reversed by simultaneous depletion of RIPK3^{288, 289}. Thus, it could be inferred that caspase 8 acts as an intrinsic inhibitor of RIPK3-dependent, apoptosis-independent cell death, and lethality from caspase 8 deficiency is consequent to aberrant, excessive activation of RIPK3-dependent necroptosis. In view of the above, loss of caspase-8 expression, compromise of caspase 8 activity from c-FLIP complexation (specifically c-FLIP_s), increased endogenous level of IAPs, or blockade of caspase 8 activity by virally-expressed or synthetic caspase inhibitor, represent some of the conducive, precipitating conditions that could drive the transition from complex IIa/IIb-directed apoptosis to necrosome-induced necroptosis.

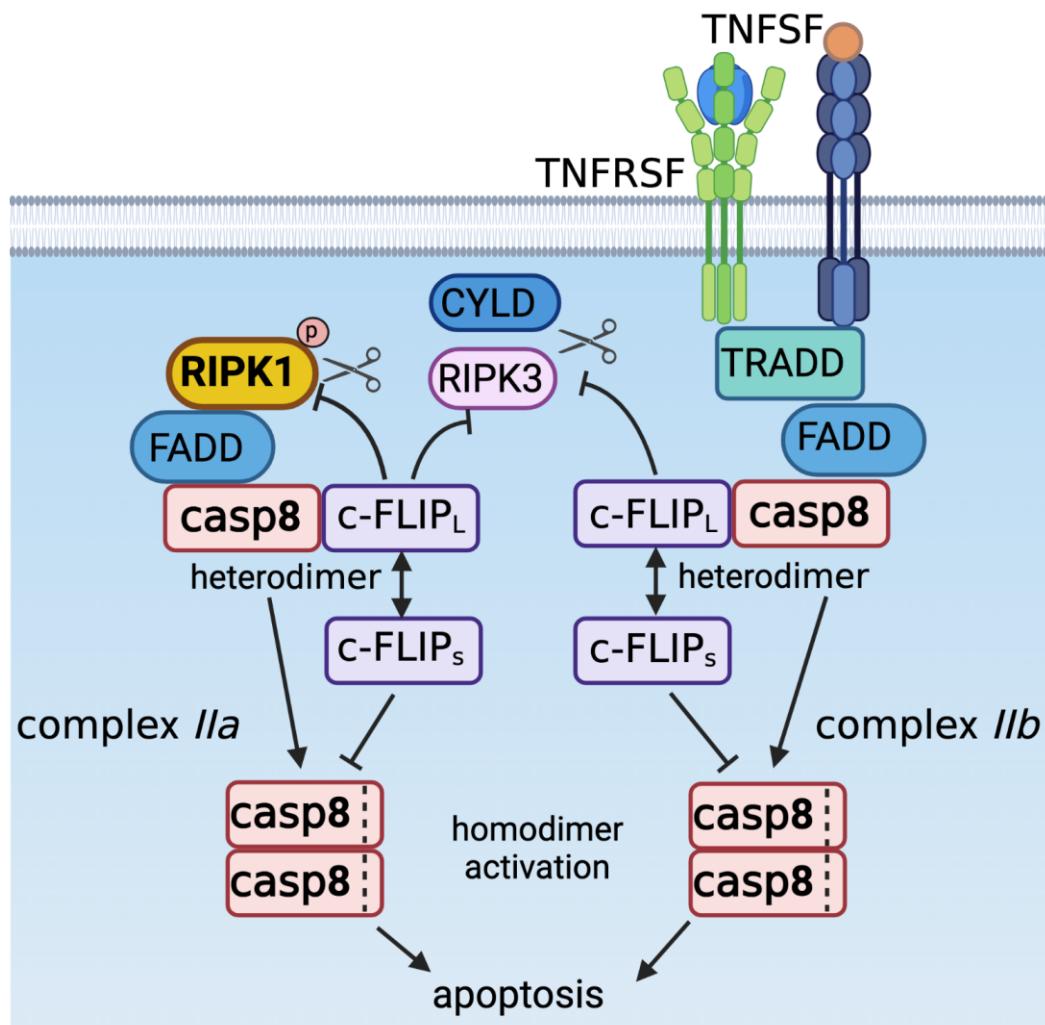


Figure 1-4. Extrinsic apoptosis and the formation of complex IIa and IIb. Subsequent to disassembly of polyubiquitin chains, RIPK1 is dissociated from complex I and associates with Fas-associated death domain (FADD) for the recruitment of pro-caspase 8 and its inactive homolog c-FLIP, to form the cytosolic death inducing complex (DISC) or complex IIa. Alternatively, activation of the TNFRSF promotes the assembly of FADD/pro-caspase 8/c-FLIP DISC via TRADD as a membrane-bound DISC, or complex IIb. Within DISC, pro-caspase 8 forms heterodimer with the short or the long isoform of c-FLIP, whereby pro-caspase 8/c-FLIP_S completely prevents caspase 8 proteolysis and activation, and the pro-caspase 8/c-FLIP_L dimer retains basal caspase 8 processing while cleaves RIPK1, RIPK3 and CYLD as potential substrates to inhibit necroptosis. Formation of the pro-caspase 8 homodimer is required for efficient auto-proteolysis and generation of sufficient cleaved caspase 8, which then cleaves downstream caspases (pro-caspase 7 and 3) to promote apoptosis.

E.1.3 Necroptosis – The Formation of Necrosome and Alternative Complexes

When caspase 8 becomes inactivated or unavailable, RIPK1 proceeds to recruit RIPK3²⁹⁰ through their RIP-homotypic interaction motif (RHIM) to form *necrosome*²⁹¹ (**Fig.1-5**). Within necrosome, RIPK1 and RIPK3 undergo autophosphorylation and trans-phosphorylation²⁹², and the extensive phosphorylation stabilises the RIPK1/RIPK3 complex to eventually lead to the formation of necrosome with a filamentous, amyloid-like structure²⁹³. Within the necrosome scaffold, further recruitment of RIPK3 takes place and RIPK3-RIPK3 dimer/oligomer was shown to augment the autophosphorylation of RIPK3²⁹⁴. Phosphorylation of RIPK1 at Ser161 residue was shown to confer its kinase activity, and phosphorylation of Ser199 (Ser204 in mouse) and Ser227 (Ser232 in mouse) residues of RIPK3 is crucial for the pro-necroptotic activity of RIPK3^{295, 296}.

Aside from the classical modality of RIPK1/RIPK3 activation, RIPK3 could also interact with the other two RHIM-containing adaptor proteins TRIF (TIR-domain-containing adapter-inducing IFN β) and DAI (DNA-dependent activator of interferon regulatory factors)^{270, 271, 297}. Activation of RIPK3-mediated necroptosis downstream to TRIF and DAI signalling represents an essential pathogen sensing and host defence mechanism. The ability to eliminate infected cells through necroptosis is a critical evolutionary defence strategy, as viruses are known to express a variety of anti-apoptotic factors to circumvent apoptosis upon infection. For example, viral-encoded inhibitor of caspases (vICA) blocks caspase 8 activity, and viral-encoded FLIP (vFLIP) is analogous to FLIP_s to prevent caspase 8 homo-dimerisation²⁹⁷. In addition, necroptosis is regarded as a pro-inflammatory cell death pathway, and a potentiated immune response could accelerate the clearance of pathogen and pathogen-infected cells. Activation of necroptosis signalling could directly trigger the assembly of NLR family pyrin domain containing 3 (NLRP3) inflammasome for the processing and release of pro-inflammatory IL-1 β and IL-18^{298, 299}; alternatively, intracellular contents liberated from necroptotic cells may function as danger-associated molecular patterns (DAMPs) to stimulate pro-inflammatory transcription and cytokine processing³⁰⁰. In this regard, RIPK3-mediated necroptosis was shown to be essential to protect mice against vaccinia virus infection, which induced RIPK1/RIPK3 complexation in infected liver tissue and a potent inflammatory response in WT; RIPK3 deficient mice failed to mount an effective inflammatory response, displayed higher viral titres and succumbed to the infection²⁹².

As endosomal PRR sensor, TLR3 is naturally activated by double-stranded RNAs and requires TRIF as the sole signal transduction adaptor protein. *In vitro* stimulation with synthetic TLR3 ligand poly (I:C), alone or plus caspase inhibitor, induced TRIF- and RIPK3-dependent necroptosis, concurrent with increased TRIF/RIPK3 oligomerisation^{301, 302}. Depending on cell types (macrophages vs. fibroblasts), TLR3/TRIF-mediated necroptosis may also require the presence of RIPK1³⁰². Unlike TLR3, TLR4 signals through both TRIF and MyD88, and in the latter case, MyD88-mediated TNF- α transcription could boost classical death receptor-induced necroptosis secondary to TLR4 activation³⁰¹; the other TLRs coupled to MyD88 could similarly

enhance necroptosis by upregulating TNF- α . DAI (also known as Z-DNA binding protein-1, ZBP-1) is a cytosolic DNA sensor that could be activated by DNAs from different sources (e.g. infection, incomplete clearance of cellular damage), to induce potent type I interferon response and innate immunity by activating interferon regulatory factor 3 (IRF3)-associated pathways³⁰³. DAI was shown to directly interact with RIPK1 and RIPK3 via the homotypic RIHMs, and stable complexation of DAI/RIPK1/RIPK3 is needed to induce NF κ B signalling and IFN β expression upon stimulatory double-stranded DNA treatment³⁰⁴. RIPK3/DAI aggregation and signalling are implicated in virus-induced necroptosis to confer resistance against viral infection^{305, 306}. Knockdown of DAI or RIPK3 impaired necroptosis in susceptible, murine cytomegalovirus (MCMV)-infected cells, which exhibited higher viral titre than infected WT cells. DAI^{-/-} or RIPK3^{-/-} mice also displayed worse foot swelling with greater viral dissemination (spleen, liver and salivary glands) than WT upon foot inoculation of MCMV^{305, 306}.

RIPK3 is the only identified kinase to date that could phosphorylate the pseudokinase MLKL. Regardless of its RIHM binding partner (RIPK1, TRIF, DAI), RIPK3 activation and oligomerisation are necessary for the recruitment and phosphor-activation of MLKL as the indispensable step preceding necroptosis. Within the activated necrosome, the kinase domain of RIPK3 associates with the C-terminal kinase-like domain of MLKL, before phosphorylating MLKL at threonine T357 and serine S358 residues²⁹⁶ located within the activation loop; in murine cells, S345, S347, T349 and S352 residues on MLKL could be phosphorylated by RIPK3³⁰⁷. Phosphorylation of MLKL at certain residues (T357 and S358 in human cells, and S345 in murine cells) is necessary for the execution of necroptosis, as mutation to these residues significantly attenuated cell death to necroptosis-inducing stimulus (TNF- α + caspase inhibitor)^{296, 308}. Phosphorylation of the C-terminal pseudokinase domain of MLKL was suggested to alleviate the suppression of pseudokinase domain on the N-terminal four α -helix bundle (4HBD) domain, which is required for the formation of high-molecular weight MLKL oligomers and insertion of MLKL oligomers into the plasma membrane^{309, 310}. Different studies have reported various conformations of MLKL oligomers comprising three, four or even six monomers. The 4HBD domain of MLKL contains stretches of positive charged residues that directly interact with the negatively charged phospholipids of the plasma membrane (specifically, phosphatidylinositol phosphates, PIP) to form pores and cause membrane permeabilisation^{311, 312}. An intrinsic membrane shedding mechanism was identified to antagonise phosphor-MLKL mediated plasma membrane rupture. When MLKL phosphor-activation/ necroptosis stimulus is limited, membrane-bound MLKL recruits the endosomal sorting complex required for transport III (ESCRT-III) complex containing charged multivesicular body protein 2A and 4B (CHMP2A and CHMP4B), to enclose the damaged membrane fractions as “bubbles” for extrusion, thus maintaining plasma membrane integrity to favour cell survival³¹³. ESCRT-III organised plasma membrane bubbles from necroptotic cells also contained chemokines that could participate in cross-priming of CD8⁺ T cells in vivo³¹³. The signalling events downstream to MLKL phosphorylation and membrane localisation

remain poorly understood, and studies are ongoing to elucidate downstream pathways that either promote or antagonise cellular demise in necroptotic cells.

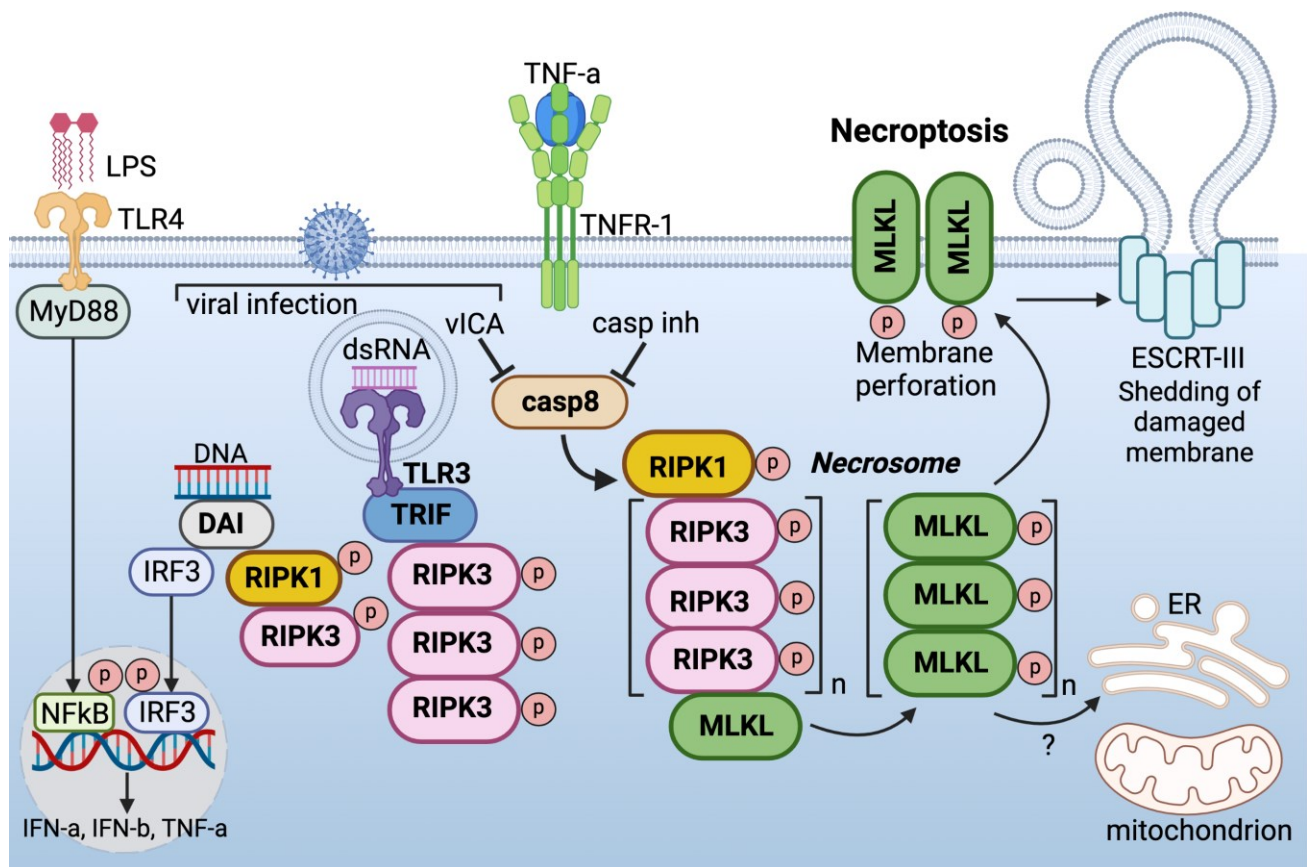


Figure 1-5. Necroptosis and the formation of necrosome and alternative complexes. When caspase-8 is deficient or inhibited, for example, by endogenous XIAP or virus-encoded inhibitor of caspases (vICA), RIPK1 proceeds to recruit RIPK3 to facilitate RIPK3 oligomerisation and trans-phosphorylation between RIPK1 and RIPK3. During viral infection, RIPK3 was also shown to complex with TIR-domain-containing adapter-inducing IFN β (TRIF) downstream to toll-like receptor 3 (TLR3) activation by double-stranded RNA, and the requirement of RIPK1 in this complex is cell-dependent. The presence of cytosolic DNA (from infection or damage cellular DNA) can be recognised by Z-DNA binding protein-1 (DAI) to similarly lead to RIPK3 recruitment and activation. Moreover, toll-like receptor 4 (TLR4) signalling through myeloid differentiation primary response gene 88 (MyD88) and DAI induces NF κ B-dependent and interferon regulatory factor 3 (IRF3)-dependent transcription, respectively, to upregulate the expression of pro-inflammatory cytokines and interferons. The stabilised and phosphor-activated RIPK3 oligomer is necessary for the subsequent recruitment and activation of the pseudokinase mixed lineage kinase-domain like (MLKL) to collectively form the necrosome. RIPK3-mediated phosphorylation of MLKL leads to conformational change that is conducive for MLKL oligomerisation, whereby the MLKL oligomer can be of different configurations. Oligomerised MLKL was shown to translocate to plasma membrane to perforate the lipid bilayer, the putative mechanism by which MLKL execute necroptosis. Membrane-localised MLKL may also interact with the endosomal sorting complex required for transport III (ESCRT-III) complex, to promote shedding of the damaged plasma membrane to preserve membrane integrity and delay necroptotic death. MLKL-containing necrosome may also translocate to the lipid membrane of endoplasmic reticulum and mitochondrion.

The pathogenic role of necroptosis has been consistently demonstrated in ischaemia-reperfusion injury of solid organs, graft injury from transplantation, drug-related organ failure and chronic inflammatory conditions (including autoimmune disorders). In the CNS, increased necroptosis activity and elevated necrosome expression have been linked to oligodendrocyte vulnerability and axonal degeneration in chronic neurodegenerative conditions, including multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD) and Parkinson's disease (PD)^{276, 314}. Acute neuronal injury from hypoxia-ischaemia or traumatic insult also promoted the formation of active necrosome (RIPK1/RIPK3/MLKL) and necroptotic neuronal death. Moreover, increased RIPK1/NF κ B signalling within microglia was shown to upregulate classical pro-inflammatory cytokines (TNF- α , IL-1 β , IFN- γ) and genes involved in innate immunity^{276, 315}. For example, expression of *Cst7*, which encodes the cathepsin inhibitor cystatin F to regulate natural killer cell (NK) cytotoxicity, is elevated in RIPK1-activated microglia in mice AD model³¹⁶. In an integral necroptosis-driven neurodegeneration cascade, neurons and oligodendrocytes undergoing necroptosis (from environmental stress or genetic predisposition) could liberate DAMPs or immunogenic factors (as detailed in D.2.6.2) to trigger microglial RIPK1-dependent neuroinflammatory transcription, thus generating a progressive inflammatory milieu to propagate CNS injury^{276, 314}. In this regard, treatment with RIPK1-specific inhibitor necrostatin-1 (Nec-1) or genetic deficiency of RIPK3 effectively prevented neuronal and oligodendrocyte necroptosis in these neuropathologies, while Nec-1 conferred additional anti-inflammatory benefits, including reduced cytokine release and immune cell recruitment, as well as a shift of microglial phenotype to become more phagocytic (evidenced as increased degradation of amyloid-beta in AD mice).

The very first evidence supporting that selective targeting of RIPK1 and necroptosis with necrostatin-1 was therapeutically effective came from adult ischaemic brain injury study^{317, 318}. In mice subjected to middle cerebral artery occlusion (MCAO, stroke), intracerebrovascular administration of Nec-1 reduced infarct size in a dose-dependent manner and the neuroprotection was evident even when given 6hrs after the injury³¹⁷. Serine 161 residue of RIPK1 was shown to be crucial for the inhibitory effect of Nec-1 on RIPK1 and necroptosis, and as an allosteric kinase inhibitor Nec-1 preferentially binds to the inactive conformation of RIPK1 to prevent RIPK1 activation^{276, 318}. Nec-1 was also found to alleviate neurovascular injury from intracerebral haemorrhage (ICH) by decreasing haematoma volume, BBB opening and brain oedema³¹⁹. Subsequent study on traumatic brain injury (from controlled cortical impact, CCI) confirmed the neuroprotective efficacy of Nec-1, even with delayed administration, with reduced neuronal death in the cortex and hippocampus, suppressed microglial activation and improved motor function and spatial learning³²⁰.

The pathogenic contribution of necroptosis to hypoxic-ischaemic injury of the immature brain has also been studied. Necroptosis was found to predominate in the peri-infarct zone of the H/I lesion, whereas apoptotic death was more prevalent in the core following HIE insult (when RIPK3 and caspase-3 were used as markers for necroptosis and apoptosis, respectively)³²¹. In primary culture of hippocampal neurons obtained from foetal rats (gestational day 18), 2 hrs oxygen-glucose deprivation followed by reperfusion significantly elevated RIPK3 expression along with neuronal death; forced upregulation of RIPK1 or RIPK3 potentiated OGD-induced neuron injury whereas Nec-1 treatment reduced neuron death³²². In PND7 mice, Nec-1 administration (i.c.v) after cerebral H/I significantly decreased cerebral, thalamic and hippocampal infarcts, and prevented RIPK1/RIPK3 interaction in the injured hemisphere³²³. Nec-1 treatment was also elicited anti-inflammatory benefits, seen as reduced NF κ B activation and cytokine synthesis (TNF- α , IL-1 β , IL-6) to indicate inhibition of the pro-inflammatory RIPK1/NF κ B axis³²³. Moreover, Nec-1 treated immature brains also displayed less protein oxidation and nitration and improved mitochondrial function, such as preserved complex I activity and ATP production against secondary energy failure³²⁴, when compared to untreated, H/I challenged controls. Mitochondrial protection by Nec-1 maybe ascribed to reduced oxidative stress or through inhibition of specific molecular mechanisms that remain elusive. The collective findings support therapeutic targeting of necroptosis as novel treatment option for HIE. From a therapeutic perspective, inhibition of the master regulator kinase RIPK1 represents an attractive strategy to enable concomitant suppression of inflammation, apoptosis and necroptosis in the H/I-injured brain. For mechanistic studies, on the other hand, blockade or deletion of RIPK3 and MLKL as necroptosis-exclusive mediators will be necessary to ascertain the contribution of necroptosis in HIE. Studies are ongoing to unravel necroptosis-related signalling events to enrich current understanding of HIE pathogenesis and to identify novel molecular candidates for therapy development.

F. Mitochondrial Function and Adaption during HIE

As discussed in D.2.2 to D.2.3, mitochondrial dysfunction during hypoxic-ischaemic brain injury is characterised by respiratory chain failure, reactive oxygen/nitrogen species generation and mitochondrial membrane permeabilisation leading to intrinsic apoptosis. Hypoxia-ischaemia events also strongly influence mitochondrial dynamics including biogenesis, mitophagy and fission/fusion cycle to promote mitochondrial adaptation to meet the energy requirement as well as to eliminate defective mitochondria as sources of ROS and pro-apoptotic factors. Compared to the adult brain, mitochondrial networks in the immature brain are more robust and dynamic during early postnatal life to fulfil the metabolic needs from accelerated neurodevelopment. In the neonatal brain, intrinsic mitochondrial rescue mechanisms could be activated to prevent irreversible mitochondrial permeabilisation from H/I or I/R challenge. Thus, selective manipulation to the mito-protective pathways during the secondary injury phase may be adopted to achieve neuroprotection.

F.1 Mitochondrial Fission and Fusion

Mitochondria are highly plastic and malleable organelles and are trafficked to different cytosolic sites where there is increased ATP demand, for example at synaptic junctions and dendritic spines of neurons. Mitochondria continuously undergo the fission-fusion cycle and utilise cytoskeletal tracks for intracellular trafficking, such that appropriate distribution of mitochondrial number, size, mass and energy production capacity can be achieved depending on cellular needs. Mitochondrial fission is mediated by dynamin-related protein 1 (DRP1), which is recruited to mitochondrial outer membrane to form oligomers that encircle the fission site and cause constrict to segregate mitochondria. Mitochondrial fusion enables exchange of proteins and DNA between mitochondrial units and repairing of damaged mitochondria. Mitochondrial fusion requires the sequential fusion of outer and then inner mitochondrial membranes, whereby mitofusin 1 and 2 (MFN1/2) mediate outer membrane fusion and optic atrophy 1 (OPA1) promotes inner membrane fusion and formation of cristae junction.

In the immature brain, the mitochondrial networks become concentrated at the perinuclear region following H/I insult, together with a change in mitochondrial morphology from elongated, fibrous to granular and truncated^{325, 326}. The fragmented appearance of mitochondria has been linked to a greater propensity for apoptosis, and studies have attempted to decipher the molecular interactions between apoptosis and mitochondrial fission-fusion. Bcl-2 family proteins BAX and BAK were shown to mediate mitofusin 2 (MTF2) localisation to the mitochondrial inner membrane to promote mitochondrial fusion and elongation³²⁷. Another study reported that BAX activation and mitochondrial translocation impeded mitochondrial fusion in apoptotic cells undergoing membrane permeabilisation³²⁸, to suggest BAX promotes mitochondrial fission and fragmentation. The discrepancy may arise from the different assay systems used and relative time

courses studied. It is also plausible that BAX/BAK adopts different conformations at different phases of apoptosis; BAX/BAK is required to recruit MTF2 and DRP1 to form aggregates/foci on the OMM, and later on during mitochondrial fragmentation BAX/BAK inactivates MTF2-mediated fusion while favours DRP1-induced fission. Studies further demonstrated that overexpression of MTF2³²⁹ or inhibition/downregulation of DRP1³³⁰⁻³³², strategies that enhance mitochondrial fusion and inhibits division, effectively prevented BAX-mediated cytochrome c release, mitochondrial membrane depolarisation and apoptotic death; silencing of MTF2 increased mitochondrial fragmentation and cellular sensitivity to apoptosis³³⁰.

F.2 Mitochondrial Biogenesis

Mitochondrial turnover and total mitochondrial mass are carefully controlled by the complementary processes of biogenesis and mitophagy. In conditions of H/I and I/R, timely and appropriate mitophagy is critical for removal of injured mitochondria which need to be replenished through biogenesis¹³². Mitochondrial biogenesis is highly coordinated process involving transcription of both nuclear and mitochondrial DNA and mitochondrial import of approximately 1000 proteins and lipids^{132, 333}.

Peroxisome proliferator-activated receptor gamma coactivator 1a (PGC-1 α) is considered to be the central regulator to mitochondrial biogenesis and is highly expressed in neurons to fulfil the high energy demand of neurons in particular during neurodevelopment^{132, 333}. PGC-1 α expression could be induced by physiological stresses such as cold, exercise and starvation³³⁴. PGC-1 α was demonstrated to potently upregulate the expression of nuclear respiratory factors 1 and 2 (NRF1/2) and PGC-1 α complexation with NRF1 enhanced the transcriptional activity of NRF1³³⁵. Activated NRF1 subsequently promotes the nuclear transcription of mitochondrial transcription factor A (mtTFA/TFAM)³³⁵, which is indispensable for transcription of mtDNA-encoded genes, mtDNA replication, and mtDNA packaging. NRF1/TFAM signalling could then upregulate the expression of mtDNA-encoded oxidative phosphorylation enzymes, such as cytochrome c oxidase subunit II (COX-II) and cytochrome c; NRF1 also enhances transcription of nuclear DNA-encoded cytochrome c oxidase subunit IV (COX-IV) and ATP synthase- β ^{335, 336}. PGC-1 α could also coactivate peroxisome proliferator-activated receptor-alpha (PPAR α)³³⁷, oestrogen-related receptors-alpha and -gamma (ERR- α / γ)³³⁸ and hepatic nuclear factor-4 alpha (HNF-4 α)³³⁹ to induce the nuclear transcription of mitochondrial enzymes involved in fatty acid beta-oxidation (medium chain acyl-CoA dehydrogenase, MCAD) and gluconeogenesis from pyruvate (phosphoenolpyruvate carboxykinase, PEPCK). Significant mitochondrial biogenesis was evident in PND7 rats following hypoxic-ischaemic brain injury, whereby mitochondrial DNA and mitochondrial number were increased in the cortical infarct area 6-24 hrs after the insult, along with upregulated expression of TFAM and NRF1 but not PGC-1 α ³⁴⁰.

Mitophagy refers to the selective removal of mitochondria via autophagy machineries, whereby mitophagy and macroautophagy share the same molecular mechanisms for autophagosome biogenesis (initiation) and lysosomal-mediated degradation (execution)³⁴¹. In addition to serving as a critical mitochondrial quality control mechanism by eliminating defective or excess mitochondria, mitophagy also operates during erythrocyte maturation to remove mitochondria and during maternal inheritance of mitochondrial DNA by degrading sperm-originated mitochondria³⁴². In the simplest concept, mitophagy and autophagy require the formation of a double-membrane vesicular autophagosome, which derives from the progressive expansion of a small, flattened isolation membrane within the cytoplasm, and the subsequent engulfment of cellular components/organelles (e.g. mitochondria) by the autophagosome. In mammalian cells, the enclosed autophagosome is transported to lysosomes and the inner autophagosomal membrane fuses with the lysosomal membrane, enabling the formation of autolysosome and the degradation of inner autophagosomal membrane and sequestered materials by various hydrolases³⁴¹⁻³⁴³.

Autophagosome biogenesis is an intricately regulated processes mediated by autophagy-related (ATG) genes and the ATG proteins encoded, which are highly conserved across eukaryotes and nearly 20 core ATGs have been identified to participate in different steps of autophagosome synthesis^{344, 345} (**Fig.1-6**). Autophagosome biogenesis can be triggered by a variety of intracellular and extracellular stimuli, which are relayed to the ULK/ATG13 initiation complex comprising mammalian target of rapamycin complex 1 (mTORC1), Unc-51 like autophagy activating kinase (ULK), and ATG13. mTORC1 exerts basal inhibition on ULK1 and ATG13, and attenuation of mTORC1 activity during stress/starvation, for example by AMP-activated kinase (AMPK) under low glucose condition, facilitates the assembly of ULK/ATG13 as a pre-autophagosome platform to recruit downstream ATGs. In mammals, the autophagosome formation site is located at endoplasmic reticulum (ER) subdomains, where active ULK/ATG13 complex recruits ATG9-containing vesicles derived from trans-Golgi network and plasma membrane as autophagosome precursor or isolation membrane. ATG9 vesicles/isolation membranes are modified by phosphatidylinositol 3-kinase (PI3K) complex I, which generates phosphatidylinositol 3-phosphate (PtdIns3P) to be inserted into ATG9 vesicles to enable subsequent recruitment of PtdIns3P-binding proteins. In mammals, PtdIns3P is recognised by WD-repeat protein interacting with phosphoinositides (WIPIs, WIPI1-4), and the binding of ATG2/WIPI complex via PtdIns3P was shown to tether ATG9 vesicles to ER to promote lipid transfer from ER, resulting in expansion of the initiation membranes. PtdIns3P also recruits WIPI2b (isoform of WIPI2) as a linkage protein to localise the ATG12/ATG5/ATG16L1 complex to autophagosome precursor, for ATG12/ATG5/ATG16L1 complex to catalyse the lipidation of ATG8-family proteins. In mammals, ATG8-family proteins consist of microtubule-associated protein light chain 3 (LC3) isoforms and GABA receptor-associated proteins (GABARAPs), and these proteins require conjugation with the lipid phosphatidylethanolamine (PE) for insertion into the

autophagosome membrane. ATG8-family proteins are firstly cleaved by ATG4 to expose their c-terminals, before ATG3, ATG7 and the ATG12/ATG5/ATG16L1 complex acting sequentially as E1, E2 and E3 enzymes to conjugate PE to ATG8-family proteins. For example, pro-LC3 is initially cleaved to generate cytoplasmic LC3-I, before conjugation with PE to form LC3-II to be inserted into the autophagosome membrane. Once anchored to the autophagosome precursor, ATG8-family proteins could then stimulate further membrane expansion and elongation, recruit autophagy receptors to direct selective autophagy (e.g. mitophagy, explained in more details below), as well as facilitating fusion of mature autophagosome with lysosome³⁴⁴. In this regard, conjugated ATG8-family proteins (e.g. LC3-II) have been routinely used as a surrogate marker to quantify the level, or the “flux”, of autophagy and selective autophagy of subcellular cargoes.

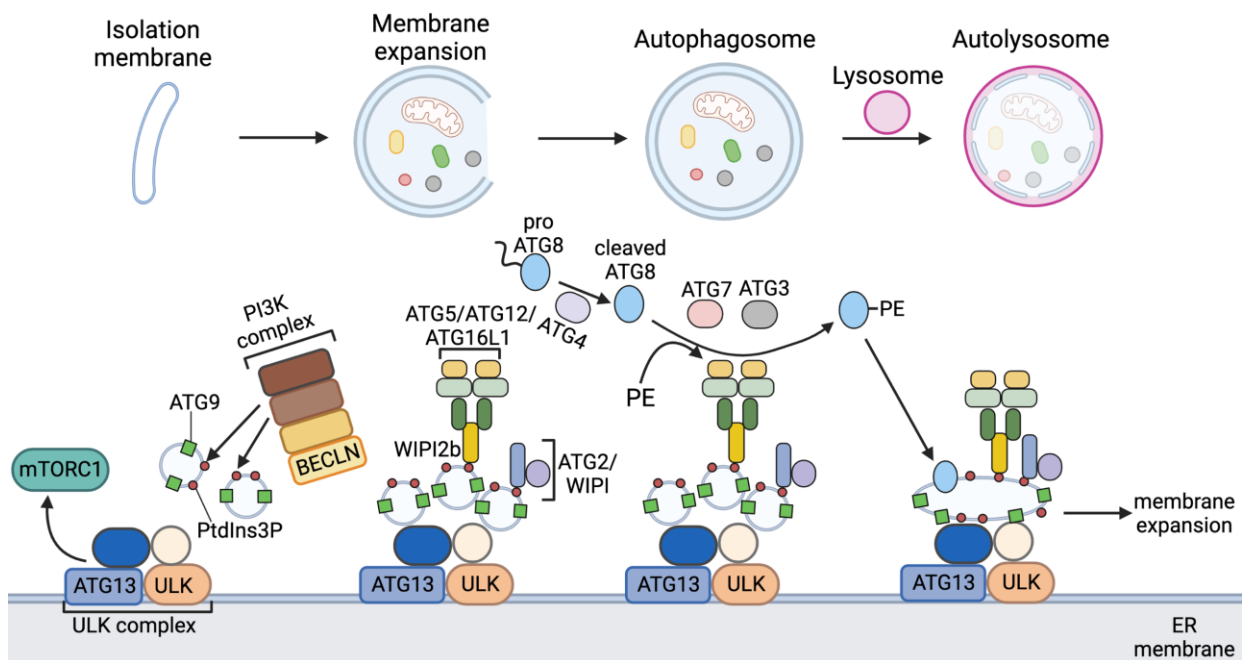


Figure 1-6. Autophagosome biogenesis. In mammalian cells, autophagosome biogenesis commences at the endoplasmic reticulum subdomains with the formation of an isolation membrane. The isolation membrane continues to expand and bend into a spherical shape, while various intracellular components are encapsulated into the expanding membrane, before the isolation membrane completely closes to form the mature autophagosome. The outer autophagosomal membrane fuses with lysosome to deliver the sequestered contents for degradation in the acidic environment of autolysosome. Autophagosome biogenesis is intricately coordinated by numerous autophagy-related gene (ATG) proteins, and can be triggered during cellular starvation, hypoxia, and endoplasmic reticulum stress. The dissociation of mTORC1 from the ULK/ATG13 complex initiates isolation membrane formation at the ER subdomain. ATG9-containing vesicles are recruited to the ULK/ATG13 complex, and the PI3K complex generates phosphatidylinositol 3-phosphate (PtdIns3P) to be inserted into the ATG9-vesicles for recruitment of downstream ATGs. ATG2/WD-repeat protein interacting with phosphoinositides (WIPI) complex is recruited to the ATG9 vesicles via PtdIns3P to facilitate membrane expansion. The ATG12/ATG5/ATG16L1 complex can be localised to the initiation membrane to catalyse the lipidation of ATG8-family proteins (LC3, GABARAP), through the linkage WIPI2b that recognises PtdIns3P. In this regard, conjugation with the lipid phosphatidylethanolamine (PE) is required for insertion of the ATG8-family proteins into the expanding isolation membrane. ATG8-family protein is first cleaved by ATG4, before the lipidation reaction is sequentially catalysed by ATG7, ATG3 and ATG12/ATG5/ATG16L1 complex. Once anchored to the precursor autophagosome membrane, ATG8-family protein could trigger membrane tethering and expansion, recruit autophagy receptors on autophagic cargoes (e.g. mitophagy), as well as promote autophagosome-lysosome fusion.

Mitophagy is typically preceded by mitochondrial fission to segregate mitochondria into suitable sizes or to expel damaged mitochondrial fractions from the mitochondrial pool for engulfment by autophagosome^{341, 343}. In mammalian cells, mitophagy (selective autophagy of mitochondria) is either mediated by specific mitophagy receptors or via ubiquitin-autophagy adaptors, both of which are capable of binding with ATG8-family proteins (LC3, GABARAP) through conserved LC3-interacting region (LIR) motif to recruit autophagosomal membranes to selectively encapsulate mitochondria³⁴³ (**Fig.1-7**).

F.3.1 Receptor-mediated Mitophagy

Bcl-2 interacting protein 3 (BNIP3) and Bcl-2 interacting protein 3-like (BNIP3L, or NIX) are the most well-studied mitophagy receptors, and their pro-mitophagy role was first described in the maturation of erythrocytes/reticulocytes going through complete mitochondrial elimination. BNIP3 is expressed as an inactive monomer in the cytosol under basal condition, and the expression of which can be upregulated during hypoxia under the transcriptional factor HIF-1 α . During cellular stress, BNIP3 localises to the outer mitochondrial membrane (OMM) and homodimerizes to interact with LC3A/B and recruit autophagosomes through its LIR domain facing the cytoplasm; phosphorylation of serine residues 17 and 24 flanking the LIR motif was demonstrated to stabilise BNIP3/LC3 interaction and promote mitophagy³⁴⁶. As a Bcl-2 family protein, BNIP3 homodimer was also shown to promote apoptosis via BAK/BAD pore formation³⁴⁷⁻³⁴⁹, and BNIP3-mediated apoptosis could be suppressed through heterodimerisation with anti-apoptotic Bcl-2 or Bcl-XL³⁵⁰. On the other hand, BNIP3/Bcl-XL also potentiated BNIP3/LC3 interaction and BNIP3-dependent mitophagy³⁴⁶ and precluded the release of cytochrome c, highlighting the complex and differential regulation of BNIP3 on apoptosis-related pathways. BNIP3L shares 50% sequence homology with BNIP3³⁴⁹ such that exogenous BNIP3 could completely restore the mitophagy capacity in BNIP3L/NIX-deficient cells³⁵¹. BNIP3L/NIX similarly requires dimerisation and phosphorylation to engage ATG8-containing autophagosome and activate mitophagy^{352, 353}; phosphorylation of serine residues 34 and 35 within the LIR domain³⁵⁴ and of serine residue 212 at the c-terminal³⁵³ were demonstrated to be crucial to BNIP3L/NIX-dependent mitophagy.

The outer mitochondrial membrane additionally contains intrinsic LC3-receptor proteins to promote mitophagy. FUN14 domain-containing protein 1 (FUNDC1) is an OMM protein that engages with LC3 through its N-terminal LIR domain to induce mitophagy during hypoxia³⁵⁵. Unlike BNIP3 and BNIP3L, phosphorylation to the LIR suppresses FUNDC1-dependent mitophagy, whereby Src kinase was shown to phosphorylate FUNDC1 at tyrosine 18 to prevent FUNDC1/LC3 interaction and mitophagy under basal condition; during hypoxia, tyrosine 18 became quickly dephosphorylated to restore LC3 co-localisation with FUNDC1³⁵⁵. Phosphorylation status of the serine 13 residue of FUNDC1 also influences its capacity to induce mitophagy. Hypoxia or mitochondrial depolarisation was shown to activate the mitochondrial phosphatase phosphoglycerate mutase 5 (PGAM5) to dephosphorylate serine 13 to enhance FUNDC1/LC3 interaction,

and silencing of PGAM5 expression impaired cellular mitophagy in response to hypoxia/mitochondrial depolarisation³⁵⁶. Casein kinase 2 (CK2) is the corresponding kinase for FUNDC1 at serine 13 and antagonises PGAM5-induced and mitochondrial clearance. In this regard, CK2 and Src kinase were demonstrated to cooperatively phosphorylate FUNDC1 at serine 13 and tyrosine 18 to limit mitophagy in unstimulated cells, while hypoxia (or other stress) favours PGAM5 association with FUNDC1 to dephosphorylate both residues to promote mitophagy.

Bcl2-like protein 13 (Bcl2-L-13) is another OMM-localised protein with pro-mitophagy and pro-fission activities³⁵⁷. Sequence alignment revealed a functional LIR motif between residues W273A and I276A to facilitate Bcl2-L-13/LC3 interaction and mitophagy upon mitochondrial depolarisation. LIR motifs at W147A-L150A and W273A-I276A are required for Bcl2-L-13 dependent mitochondrial fragmentation, and Bcl2-L-13 may compensate for the pro-fission activity of Drp1. Moreover, phosphorylation of Bcl2-L-13 at serine residue 272 (S272) in proximity to the LIR domain was shown to be important for LC3 binding, as mitochondrial LC3 recruitment was impaired in S272A phosphor-mutant.

F.3.2 PINK1/Parkin-mediated Ubiquitination Pathway

Sporadic Parkinson's disease (PD) has a prevalence of 1-4% among those aged 65 years and above whereas familial PD resulting from hereditary mutations can have an earlier onset^{343, 358}. Dopaminergic neuronal loss/death in the substantia nigra of basal ganglia is believed to be accountable for the wide range of motor deficits characteristic to PD, such as tremor, bradykinesia, rigidity and postural instability. Non-motor phenotypes also include insomnia/sleeping difficulty, autonomic nervous disorder and neuropsychiatric dysfunction^{343, 358}. Despite the homogeneity in clinical presentations, the aetiologies of sporadic and familial PDs are likely different, whereby in the latter case mutations to specific gene loci have been identified³⁵⁸. Over the years, accumulating evidences have reinforced the concept that mitochondrial dysfunction contributes to PD. For example, mitochondrial complex I deficiency or mitochondrial DNA mutation was observed in the substantia nigra in patients with idiopathic/sporadic PD, genes that are mutated in familial PD encode mitochondrial proteins and that mitochondrial toxins drive PD in animals^{334, 358}. Even before the elucidation of their precise functions in mitophagy and mitochondrial quality control, *PINK1* (encoding PTEN-induced putative kinase 1)³⁵⁹ and *PARK2* (encoding Parkin)³⁶⁰ had been suggested as the causal genes for hereditary recessive cases of Parkinsonism with juvenile onset, and distinct mutations to *PINK1* (chromosome 1 p35) or *PARK2* (chromosome 6 q25) have been reported in these patients.

Genetic knockdown studies in *Drosophila* (flies) connected PINK1 and Parkin in the same pathogenesis pathway leading to PD^{361, 362}. *PINK1* or *PARK2* deficient flies showed flight muscle impairment, abnormal mitochondrial morphology and reduced ATP production along with diminished dopaminergic neurons, and Parkin overexpression effectively restored the abnormal phenotypes from PINK1 silencing, but not vice versa.

Moreover, double knockdown of PINK1 and PARK2 did not exacerbate the associated deficiencies further, and the collective findings would suggest that PINK1 and Parkin operate in the same pathogenesis pathway whereby PINK1 is upstream to Parkin.

PINK1 is a serine-threonine kinase synthesised in the cytosol and contains a mitochondrial-targeting sequence at N-terminal, which facilitates its importation into the mitochondrial compartment via the translocase of outer membrane (TOM) and translocase of inner membrane (TIM) complexes^{334, 358}. Imported PINK1 spans the outer and inner mitochondrial membranes and is cleaved by the inner membrane proteases mitochondrial processing peptidase (MPP) and presenilin-associated rhomboid-like protease (PARL), leading to release of truncated PINK1 into the cytoplasm for proteasome-mediated degradation³⁶³⁻³⁶⁶. In this regard, PINK1 has a rapid turnover rate under steady-state with healthy, polarised mitochondria, and upon mitochondrial damage or depolarisation, PARL-mediated cleavage is halted and PINK1 becomes stabilised on the outer mitochondrial membrane in association with the TOMs^{364, 367}. PINK1 stabilisation on the depolarised OMM is sufficient and necessary for Parkin recruitment and subsequent mitochondrial clearance^{368, 369}. PINK1 is activated through autophosphorylation and proceeds to phosphorylate ubiquitin molecules bound to OMM proteins, and the phosphorylated ubiquitin (phos-Ub) subsequently recruits Parkin from cytosol to associate with OMM^{370, 371}. PINK1 then phosphorylates mitochondria-localised Parkin to unleash its E3 ubiquitin ligase activity^{372, 373}, to enable Parkin-mediated ubiquitination of OMM proteins and to promote polyubiquitin chain formation. Parkin exists in a naïve, autoinhibited state, and PINK1 phosphorylation of serine 65 residue on the ubiquitin-like domain (Ubl) triggers intramolecular remodelling, to liberate the catalytic RING2 (really interesting new gene 2) domain from the suppression by unique parkin domain (UPD, or RING0) and to unblock the E2 interaction motif on RING1 domain. Such active conformation permits the catalytic transfer of ubiquitin from Parkin to its substrates³⁴³. The PINK1/Parkin interaction is reinforced and amplified through a positive feedforward mechanism, whereby PINK1 continues to phosphorylate the ubiquitin molecules aggregated by Parkin, and the phos-Ub tags will lead to further Parkin recruitment and activation³³⁴.

Parkin mediates two types of polyubiquitin chain formation, the lysine 48(K48) ubiquitin linkage is associated with proteasomal degradation, while lysine 27 or lysine 63 (K27/K63) linkage favours the autophagic degradation of mitochondria^{342, 374}. In mammalian cells, mitofusin 1 and 2 (MTF1/2) and voltage-dependent anion-selective channel protein 1 (VDAC1), which constitutes the mitochondrial permeability transition pore (mPTP), are the best-characterised Parkin substrates on the OMM. Quantitative proteomics analysis identified a total of 20 OMM proteins that are ubiquitinated by Parkin during mitochondrial depolarisation³⁷⁵, encompassing proteins involved in metabolism (malate dehydrogenase 1/2, MDH1/2; acetyl-CoA synthetase long chain family member 1/4, ACSL1/4), trafficking and fission/fusion (MTF1/2; mitochondrial Rho GTPase 1/2, Miro1/2), pore formation (VDAC 1-3) and protein translocation (TOMM70A, TOMM22). Importantly,

Parkin ubiquitinates the autophagy adaptor proteins sequestosome 1 (SQSTM1, or p62) and calcium binding and coiled-coil domain 2 (CALCO2, or NDP52) associated with OMM. These autophagy adaptor proteins bind to ubiquitinated proteins and also contain a LIR domain to interact with ATG8/LC3 on the autophagosome membranes, thus directing the autophagic machineries to specific ubiquitinated cargos for degradation.

The contribution of SQSTM1/p62 to PINK1/Parkin-mediated mitophagy remains debatable; while some found that Parkin-mediated mitophagy is dependent on SQSTM1/p62 recruitment to the ubiquitinated VDAC1 and the K27 polyubiquitin chain^{374, 376}, another study concluded that the p62/VDAC1 interaction is dispensable for mitophagy as p62 knockout does not compromise mitophagy, and that deletion of VDAC1/3 has not impede mitochondrial localisation of p62³⁷⁷. SQSTM1/p62 accumulation downstream to Parkin signalling also induces the perinuclear aggregation of dysfunctional mitochondria by polymerising through the PB1 domain³⁷⁷, however whether mitochondrial clumping is conducive for mitophagy requires further validation. The autophagy adaptor proteins NDP52 and optineurin (OPTN) have also been demonstrated to mediate PINK1/Parkin-activated mitophagy. The ubiquitin kinase activity of PINK1 is required to directly recruit NDP52 and OPTN to phosphorylated ubiquitin on OMM, while Parkin is not necessary but amplifies the ubiquitin phosphorylation signal to enhance receptor engagement³⁷⁸. In addition to LC3 interaction, NDP52 and OPTN could recruit upstream mediators for autophagosome membrane biogenesis, such as ULK1 and WIPI1, to the defective mitochondria to induce mitophagy³⁷⁸. The activity of autophagy adaptor proteins can be modified through phosphorylation to regulates mitophagy. Following PINK1/Parkin activation, TANK-binding kinase 1 (TBK1) is recruited to OMM and is phosphorylated on serine residue 172, which is dependent on ubiquitin-bound NDP52 and OPTN³⁷⁹. Phosphor-activated TBK1 then phosphorylates NDP52, p62 and OPTN, and OPTN phosphorylation at serine 177 and serine 473 was shown to facilitate LC3 association and strengthen binding with polyubiquitin chains on OMM, respectively³⁷⁸⁻³⁸⁰. Taken together, these findings support a positive feedback mechanism featuring TBK1-dependent phosphorylation and polyubiquitin-dependent recruitment of autophagy adaptor proteins to favour efficient mitophagy.

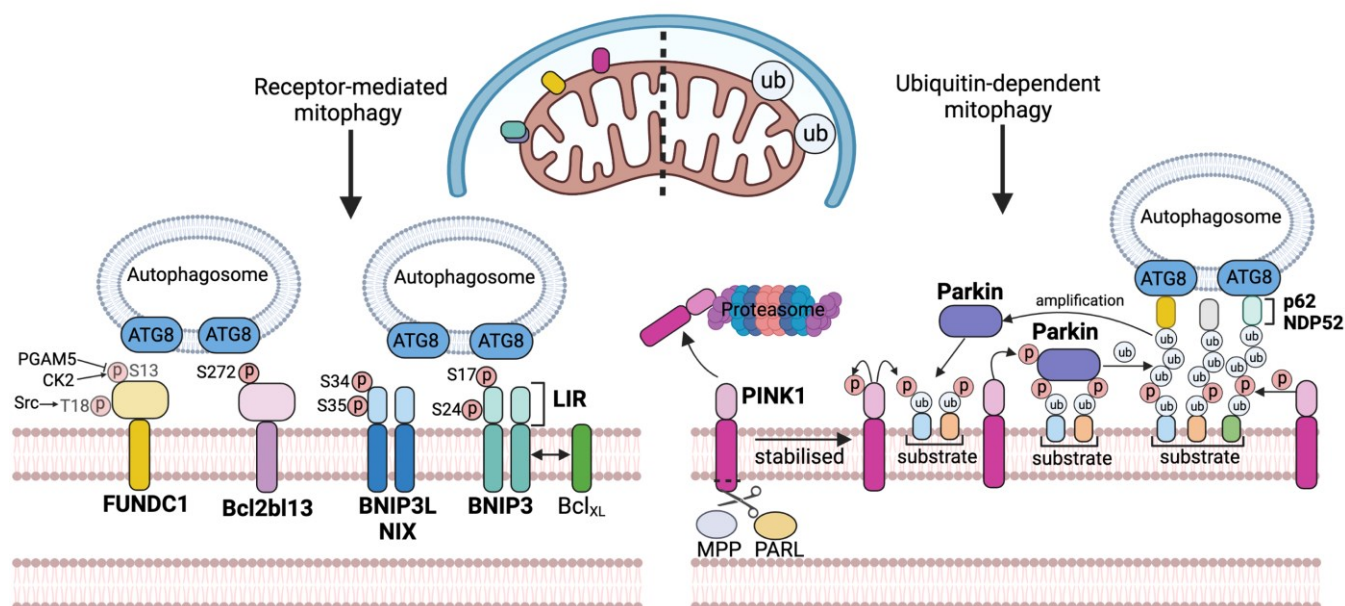


Figure 1-7. The Molecular Regulation of mitophagy. Mitophagy can be broadly divided into (A) receptor-mediated mitophagy or (B) ubiquitination-dependent mitophagy. (A) BNIP3 and BNIP3L/NIX are cytosolic proteins that translocate to the mitochondrial outer membrane to form homodimers during hypoxia, with the LC3-interacting region (LIR) motif oriented towards the cytoplasm to recruit ATG8/LC3-containing autophagosome to induce mitophagy. BNIP3 may also heterodimerise with Bcl_{xL} to promote mitophagy. BNIP3 phosphorylation at serine residues 17 and 24, and BNIP3L phosphorylation at serine residues 34 and 35 are necessary for LC3 interaction and subsequent mitophagy. As mitochondrial intrinsic LC3 receptor, FUNDC1 is negatively regulated by phosphorylation, whereby Src kinase and casein kinase 2 (CK2) phosphorylate tyrosine residue 18 and serine residue 13, respectively, to prevent FUNDC1/LC3 engagement through LIR. During hypoxia, dephosphorylation of the LIR residues is permissive for LC3 recruitment to FUNDC1, and PGAM5 was identified as the phosphatase for serine residue 13. Bcl2L13 is another OMM-localised mitophagy receptor that requires phosphorylation of serine 272 residue for LC3 recruitment and mitophagy. (B) PINK1 is subjected to basal degradation upon mitochondrial translocation, whereby PINK1 is first cleaved by IMM proteases mitochondrial processing peptidase (MPP) and presenilin-associated rhomboid-like protease (PARL) for cytosolic release and proteasome-mediated degradation. When mitochondria are damaged/depolarised, PINK1 processing by PARL is interrupted and PINK1 becomes stabilised on to the OMM. PINK1 is activated through autophosphorylation and then phosphorylates the OMM-bound ubiquitin molecules at serine 65 residue to promote Parkin recruitment. PINK1 similarly phosphorylates the serine 65 residue of ubiquitin-like domain of Parkin to cause Parkin conformational change and to activate its E3 ubiquitin ligase activity. Parkin ubiquitinates many OMM proteins and stimulates the formation of polyubiquitin chains that are recognised by autophagy adaptor proteins, such as sequestosome 1 (SQSTM1/p62), NDP52 and optineurin (OPTN). The ubiquitin-bound adaptor proteins also contain a LIR motif to drive the recruitment of LC3-containing autophagosome for the induction of mitophagy.

F.3.3 Measuring Mitophagy

Various methods have been developed for quantitative measurement of mitophagy and complementary methods could be used in conjunction to more accurately determine the extent of mitophagy. For example, colocalisation of mitochondrial probes (TOM20, COX-IV) with autophagy receptor or adaptor protein (LC3 or SQSTM1/p62) under fluorescence microscope is indicative of selective recruitment of autophagosome to mitochondria; in this instance, the number of LC3-positive puncta localised to mitochondria can be counted and the colocalisation coefficient can be determined^{381, 382}. *In vitro* live-cell imaging on LC3 localisation to mitochondria can be achieved through transfection of fluorescence-tagged LC3 reporter gene (e.g. GFP-LC3) followed by incubation with MitoTracker dye that selectively stain the mitochondria. Colocalisation of mitochondrial structure with lysosomal marker (lysosomal membrane protein 2, LAMP2) could be similarly visualised to reflect the delivery of mitochondria to lysosomal degradative compartment at the later phase of mitophagy; double-labelling cells with MitoTracker and LysoTracker dyes enable dynamic tracking of this process^{381, 382}. Engineered fluorescence probes (FP) offers a convenient and reliable option for mitophagy quantitation, and one of such FPs is mito-Keima with bimodal excitation wavelengths depending on the surrounding pH. Keima is a coral-derived acid-stable fluorescent protein that absorbs light at 440nm at neutral pH whereas at acidic pH (lysosomal compartment) it is excited at 586nm. Keima can be engineered to incorporate a mitochondrial-targeting sequence (MTS) to associate with mitochondria (mt-Keima), and upon delivery mitochondria to the acidic autolysosomes, an increase in the ratio of 586nm-excited (red) to 440nm-excited (green) mt-Keima is observed to indicate active mitophagy³⁸³. In addition to fluorescence microscopy, immunoblotting of conjugated LC3-II and adaptor p62 from mitochondrial fraction is also conventionally used for mitophagy quantitation, so is the determination of expression of mitophagy mediators (Parkin, BNIP3/BNIP3L, FUNDC1) in the cytosolic and mitochondrial compartments^{382, 384}.

F.3.4 Mitophagy in Hypoxic-Ischaemic Brain injury and HIE

Studies have consistently reported an increase in mitophagy along with upregulated expression of autophagy machineries in *in vitro* and *in vivo* models of ischaemia-reperfusion brain injury (i.e. stroke), with the most prominent increases seen during the reperfusion phase^{385, 386}. Importantly, inhibition of autophagy (3-methyladenine, bafilomycin A₁, Atg7 or Atg5 knockdown) or mitophagy (mdivi-1, knockdown of PARK2 or BNIP3L) during reperfusion aggravated neuronal death after oxygen-glucose deprivation (OGD) and enlarged infarct volume in mice subjected to transient middle cerebral artery occlusion (tMCAO)³⁸⁵. Impairing autophagy also prompted cytochrome c release from mitochondria to drive neuron apoptosis. On the other hand, blocking autophagy/mitophagy during OGD and in permanent MCAO (pMCAO) did not exacerbate neuronal injury and tissue loss³⁸⁵. These findings support mitophagy as a neuroprotective mechanism activated by cerebral reperfusion, which accords with the notion that mitophagy is an energy-consuming

process and unlikely to participate in hypoxia-ischaemia only brain injury. Parkin and BNIP3L/NIX-mediated mitophagy pathways are independently activated in cerebral ischaemia-reperfusion injury, and mice deficient in both pathways displayed the most extensive infarct compared to PARK2 or BNIP3L single-KO cohorts; forced expression of PARK2 effectively rescued neuronal viability and reduced infarct size in BNIP3L-KO subjects and vice versa³⁸⁶. Studies also attempted to exploit the salubrious nature of mitophagy in different neuroprotective paradigms. Administration of endoplasmic reticulum (ER) stress inducers tunicamycin and thapsigargin during reperfusion alleviated brain infarct and restored cerebral glucose uptake following tMCAO, which were associated with increased Parkin recruitment to mitochondria and elevated mitophagy³⁸⁷. Autophagy blockade (3-methyladenine, 3-MA or ATG7 knockdown) or PARK2 silencing abrogated the neuroprotective benefits of ER stress inducers, to suggest that mild ER stress-induced preconditioning is reliant on intact mitophagy pathway³⁸⁷. Acidic postconditioning (APC) by inhaling CO₂ during stroke thrombolysis (reperfusion and reoxygenation) was shown to potently trigger Parkin mitochondrial translocation and mitophagy compared to tMCAO alone³⁸⁸. Blocking mitophagy (mitochondrial division inhibitor-1, mdivi-1 or PARK2 deletion) or autophagy (3-MA, ATG7 deletion) abolished the neuroprotective benefits of APC, including reduced infarct and neuronal death and restored mitochondrial potential³⁸⁸.

In the immature brain, autophagy and mitophagy are strongly upregulated immediately after hypoxia-ischaemia insult, with increased LC3-II conversion and co-localisation between mitochondrial structural protein TOM20 and lysosome marker lysosomal membrane protein 2 (LAMP2)³⁸⁹⁻³⁹³. Enhanced autophagy in cerebral cortex and hippocampus was concluded to be neuroprotective in the injured hemisphere, as autophagy inhibitor 3-MA deteriorated brain injury and biased cell death towards necrosis, whereas autophagy inducer rapamycin (which attenuates mTORC1-mediated inhibition on autophagy initiation complex) reduced necrotic cell death and brain injury³⁸⁹. Correlation between autophagy and apoptosis also seems to be region-specific in the injured brain, whereby LC3 and caspase-3 are co-upregulated in the cortex but their expressions are inversely correlated in the hippocampus³⁹⁰. On the other hand, neonatal mice deficient in ATG7, a key autophagy mediator, were effectively protected from H/I brain injury with concomitant suppression of autophagosome formation and caspase-3 activation³⁹¹; in this case, it would be difficult to determine whether the neuroprotection is attributed to inhibition against apoptosis, autophagy or both. H/I-induced mitophagy was suggested to occur in two phases, with the first wave observed within 24 hrs concurrent with upregulation of PINK1/Parkin, NIX and FUNDC1, while the second mitophagy at 7 days post-H/I primarily features the PINK1/Parkin pathway³⁹³. It is plausible that the two episodes of mitophagy could differentially modulate mitochondrial function and injury outcomes, and such hypothesis will need be examined in the future.

F.4 Caseinolytic Protease CLpP and Mitochondrial Quality Control

In addition to mitophagy, mitochondrial quality control is also dependent on the appropriate and coordinated activities of the different proteases that are localised throughout all mitochondrial compartments (matrix, inner membrane, intramembrane space and outer membrane)³⁹⁴. Mitochondrial proteases perform diverse functions including degradation of misfolded/unfolded proteins, cleavage and maturation of imported proteins, and modulation of the half-lives of regulator proteins and transcriptional factors, to support the vigilant surveillance of mitochondrial protein turnover and the optimisation of mitochondrial function^{334, 394}.

To date 23 mitochondrial-intrinsic proteases have been identified and consist of four main classes to include ATP-dependent peptidases, processing peptidases, oligo peptidases and other mitochondrial peptidases. The ATP-dependent peptidases are located within mitochondrial matrix and primarily degrade unfolded, denatured or oxidised proteins from mitochondrial damage and oxidative stress³⁹⁵. ATP-dependent peptidases are central to unfolded protein response (UPR), whereby excessive accumulation of denatured proteins drives nuclear transcription to upregulate mitochondrial peptidases to eliminate misfolded proteins^{394, 395}. The ATP-dependent peptidases typically aggregate into multimeric complex and utilise energy from ATP hydrolysis to unravel and transport substrates into the proteolytic core for degradation. Substrates of ATP-dependent peptidases include respiratory chain subunits, translocases and other matrix and intermembrane proteins^{394, 395}. Caseinolytic mitochondrial matrix protease proteolytic subunit (CLpP) is a serine protease encoded by nuclear gene and trafficked into mitochondrial matrix following cytosolic synthesis. Seven CLpP monomers assemble into a heptameric ring through hydrophobic bonds, and two adjacent rings associate to form the complete tetradecamer CLpP complex, whereby each of the 14 subunits has an active, catalytic site³⁹⁵. Importantly, CLpP alone lacks effective proteolytic activity to digest full-length substrates as it does not contain an ATPase domain, and complexation with caseinolytic mitochondrial matrix protease chaperone subunit x (CLpX) is necessary to unleash the full proteolytic capacity of CLpP³⁹⁵. CLpX subunits assemble into hexameric ring and two rings of CLpX complement the CLpP tetradecamer at both ends to form a barrel-shaped CLpPX complex, which resembles the structure of cytoplasmic proteasome³⁹⁵.

The importance of CLpP and mitochondrial protein homeostasis is best illustrated by Cole et al^{396, 397}. CLpP overexpression in acute myeloid leukemia (AML) cells was linked to enhanced oxidative phosphorylation efficiency, which may be ascribed to CLpP-dependent degradation of misfolded metabolic enzymes, such as the respiratory chain complex II protein succinate dehydrogenase subunit A (SDHA)^{396, 397}. CLpP inhibition or knockdown impaired complex II activity, increased ROS production and potently induced AML cell death³⁹⁶. Paradoxically, overactivation of CLpP proteolysis results in excessive degradation of respiratory chain substrates and impairment of oxidative phosphorylation, to lead to substantial cancer cell lethality³⁹⁸. The collective evidences highlight the potential of CLpP-targeted strategy as next generation anti-cancer therapy.

Genetic study also found that autosomal recessive mutations to CLpP with single amino acid substitution, which disrupts the barrel-shaped chamber of CLpP for catalytic proteolysis, is associated with familial Perrault syndrome that leads to sensorineural hearing loss and ovarian failure³⁹⁹.

The role of CLpP beyond mitochondrial protein quality control remains largely elusive. CLpP was demonstrated to be one of the PINK1-processing peptidases along with PARL and MPP, and deficiency in any of these peptidases impeded PINK1 cleavage and promoted mitochondrial accumulation of full-length PINK1⁴⁰⁰. Such finding implies a potential role for CLpP in PINK1/Parkin-dependent mitophagy. Unpublished data from our group revealed CLpP as an important regulator of mitophagy in cancer cell lines, whereby knockdown of CLpP suppressed basal and hypoxia-stimulated mitophagy seen as reduced mitochondrial localisation of LC3-II signals. It remains unknown whether CLpP also participates in H/I or I/R-induced mitophagy in neurons and neuron-like cells.

G. Mitochondrion as the interface between necroptosis and mitophagy?

In addition to plasma membrane translocation, MLKL-containing necrosome was shown to localise to the mitochondrial membrane in cells undergoing necroptosis^{401, 402}, whereby MLKL/phos-MLKL shows affinity towards the mitochondrial lipid cardiolipin³¹². It was further illustrated that artificial liposomes comprising 15% cardiolipin, which mimics the lipid composition of mitochondrial membranes, were damaged by MLKL oligomers resulting in leakage of liposome content³¹². It was contemplated that mitochondrial-localised RIPK3/MLKL necrosome could act in a similar manner as pro-apoptotic BAX and BAD to cause mitochondrial permeabilisation and cell demise however such hypothesis is yet to be verified.

Studies have questioned the significance of mitochondrial translocation of necrosome, and more specifically the possibility of RIPK3-mediated phosphorylation of mitochondrial substrates as downstream mechanism precipitating necroptotic cell death, which may operate in parallel to MLKL-induced plasma membrane perforation. RIPK3 was shown to interact with and phosphorylate phosphoglycerate mutase 5 (PGAM5)⁴⁰³, which is a mitochondrial serine/threonine phosphatase that resides in inner mitochondrial membrane and exists in two splice isoforms (PGAM5-long, PGAM5_L and PGAM5-short, PGAM5_S). Interestingly, only RIPK3/PGAM5_S association and PGAM5_S phosphorylation were susceptible to MLKL inhibition by necrosulfonamide, which did not impair RIPK3/PGAM5_L dynamics, nonetheless both isoforms were required for TNF α -induced necroptotic cell death⁴⁰³. It was further demonstrated that RIPK3/MLKL/PGAM5_S activation precedes the mitochondrial recruitment of Drp-1 to enable Drp-1 dephosphorylation by PGAM5_S to trigger mitochondrial fission⁴⁰³, an event believed to predispose cells to necroptosis. Another study identified the mitochondrial metabolic complex pyruvate dehydrogenase complex (PDC) as the phosphorylation target of RIPK3⁴⁰⁴. PDC catalyses the conversion of pyruvate to acetyl-CoA through decarboxylation to shift glycolysis to Krebs cycle (aerobic respiration), and potentiation of PDC activity by RIPK3 resulted in excessive aerobic respiration and aggravated ROS production, which positively correlated with necroptotic cell death⁴⁰⁴. RIPK3/PDC association and RIPK3-mediated phosphorylation on PDC subunit E3 threonine 135 (T135) residue are dependent on MLKL-mediated necrosome translocation to the mitochondria⁴⁰⁴. The notion that RIPK1/RIPK3 is indispensable for TNF α -stimulated mitochondrial ROS synthesis is concurred by other studies^{405, 406}, however the necessity of mitochondrial translocation of necrosome was questioned⁴⁰⁶, to suggest the existence of cytosolic intermediates to relay RIPK1/RIPK3 activation to mitochondria. The mitochondrial permeable ROS scavenger butylated hydroxyanisole (BHA) attenuated TNF- α induced necrotic cell death^{405, 406} to implicate mitochondrial ROS as the downstream effector of RIPK1/RIPK3. The available evidences to date suggest MLKL primarily functions as a subcellular shuttle with the mere function of transporting necrosome to different membrane fractions, likely due to its lack of complete kinase activity like RIPK1 and RIPK3. Whether MLKL could interact with and modify membrane-bound substrates independent of the kinase activity of RIPK1/RIPK3 is an open question awaiting clarification. Future study along this

direction will enrich the current understanding on the execution mechanism of necroptosis, in addition to MLKL-mediated perforation of lipid bilayer membranes.

Few studies have attempted to investigate the molecular interaction between necroptosis and mitophagy with inconsistent findings. The dependence on mitochondrial ROS synthesis for the execution of necroptosis following RIPK1/RIPK3 activation^{290, 405, 406} provided the rationale for eliminating mitochondrial ROS through enhanced mitophagy in order to prevent necroptosis. Mitophagy can be artificially induced in cells with intact expression of mitophagy mediators (e.g. Parkin) by treatment with the mitochondrial potential uncoupler carbonyl cyanide chlorophenylhydrazone (CCCP), which resulted in near-complete depletion of mitochondria and prevented TNF- α induced ROS burst⁴⁰⁷. Nonetheless, mitochondria-deficient cells still responded to cell death inhibition by Nec-1s after TNF- α /caspase inhibitor treatment, and were not protected from cell death from forced RIPK3 dimerisation⁴⁰⁷, to suggest that RIPK1/RIPK3-dependent necroptosis does not require mitochondrial ROS as previously proposed^{405, 406} and is not altered by mitophagy. Another study demonstrated that the mitochondrial complex I inhibitor BAY87-2243 promoted mitochondrial permeability transition pore (mPTP) opening that induced simultaneous mitophagy and ROS production to precede RIPK1- and MLKL-dependent necroptosis⁴⁰⁸. Study on cigarette smoke-induced lung epithelial cell death revealed that mitophagy stimulates necroptosis, and implicated mitophagy and associated necroptosis in the pathogenesis of chronic obstructive pulmonary disorder (COPD)⁴⁰⁹. Cigarette smoke provoked PINK1-dependent mitophagy, and PINK1 deficiency or mitophagy inhibitor mdivi-1 significantly inhibited MLKL phosphorylation, mitochondrial dysfunction, epithelial cell death and airspace enlargement⁴⁰⁹, highlighting the therapeutic potential from inhibiting mitophagy and related pathways in COPD. The different pro-mitophagy stimuli across these studies could explain the discrepant findings, and the unique pathogenic mechanisms inherent to different diseases could make mitophagy protective or deleterious.

G.1 Open Questions and Novelties of the Current Study

As described in section above, the necrosome-mitochondria signalling axis upon necroptosis induction in cells also provides the theoretical basis for necroptosis to act upstream to regulate mitophagy, a hypothesis that has not been explicitly contested and would underscore the novelty of the present study (**Fig.1-8**). Studies published to date have only investigated the unidirectional regulation of necroptosis by mitophagy or more generally mitochondrial function, with a current lack of knowledge of whether the reciprocal regulation also exists. As discussed in F.3.4, provided that mitophagy (along with macro-autophagy) is neuroprotective in ischaemia-reperfusion brain injury, it is plausible for the necrosome to travel into mitochondrial compartment to promote mito-adaptive mitophagy to enhance neuronal survival, when coupled with enhanced mitochondrial biogenesis. It should be noted that the mitochondrial ROS response stimulates PINK1/Parkin-dependent mitophagy^{410, 411}, and taken together with RIPK1/RIPK3-inducible mitochondrial

ROS synthesis^{405, 406}, we speculate that necroptosis is positively linked to mitophagy. The reverse is equally feasible where necroptotic signal could inhibit mitophagy to lead to aggregation of dysfunctional mitochondria and prolonged energy failure, thus culminating to necroptotic cell death; blocking necroptosis subsequently alleviates the brake on mitophagy (disinhibition) to accelerate mitochondrial turnover in I/R-injured neurons. In addition to intermediate signalling through the ROS, necroptosis-exclusive mediators (RIPK3 and MLKL) may directly interact with mitophagy mediators when the necrosome is brought into the mitochondria. Understanding the precise molecular crosstalk between necroptosis and mitophagy will enrich the current understanding on the execution mechanism of necroptosis. Moreover, if mitophagy can be established as a downstream process of necroptosis, both pathways maybe simultaneously targeted to improve cell survival in hypoxic-ischaemic encephalopathy and in I/R injury of other organs.

G.2 Aims, Hypothesis and Objectives

The overarching aim of the current PhD project is to investigate the pathological involvement of necroptosis and mitophagy in hypoxic-ischaemic encephalopathy, with a specific focus on the molecular interaction between the two pathways. We hypothesise that hypoxia-ischaemia (H/I) and/or hypoxia-reperfusion (I/R) induces necroptosis as an upstream modulator of mitophagy in neurons of the immature brain (**Fig. 1-8**). The first objective is to verify the development of necroptosis *in vitro* in primary neurons and neuron-like cell lines following oxygen glucose deprivation (OGD, to model H/I) or OGD plus reperfusion (OGD/Rep, to model I/R), and to detect the successful translocation of necrosome proteins into mitochondrial compartment. Secondly, H/I or I/R-induced mitophagy and mitophagy-related proteins will be quantified *in vitro*. Third, necroptosis signaling will be blocked by pharmacological inhibitors or genetic silencing to determine whether the necroptotic mediators promote or suppress mitophagy *in vitro*. Fourth, I will examine mitochondrial function and cell survival after necroptosis blockade under basal and I/R-stimulated conditions *in vitro*. Fifth, the concurrent upregulation of necroptosis and mitophagy in susceptible brain regions will be confirmed in a murine model of neonatal hypoxic-ischaemic brain injury. Lastly, we would like to investigate whether necroptosis inhibition alters mitophagy and long-term behavioural outcomes mice subjected to HIE.

Objectives

- 1 – 1) To confirm MLKL phosphorylation as indicator of necroptosis, and 2) to verify translocation of phospho-MLKL, MLKL and RIPK3 in mouse primary neurons and Neuro-2A cells (mouse neuroblastoma) following OGD +/- reperfusion;
- 2 – 1) To quantify the cytosolic and mitochondrial expression of mitophagy mediators (e.g. PARKIN, CLpP), and 2) to quantify LC3A/B-II level in the mitochondrial fraction from primary cultured neurons and Neuro-2A cells, following OGD +/- reperfusion;

3 – 1) To quantify cytosolic and mitochondrial expression of mitophagy mediators and LC3A/B co-localisation to the mitochondria following OGD plus reperfusion, when necroptosis is blocked through pharmacological inhibition of RIPK1 or RIPK3 or genetic knockdown MLKL; 2) To examine MLKL interaction with mitophagy mediators through co-immunoprecipitation.

4 – To assess mitochondrial function and cell survival following OGD plus reperfusion, when necroptosis is blocked through 1) chemical inhibition of RIPK1 or RIPK3, or 2) genetic knockdown MLKL;

5 – Using a mouse neonatal hypoxic-ischaemic brain injury model, to 1) confirm neuronal necroptosis (phos-MLKL, phos-RIPK3) and 2) the concurrent upregulation of mitophagy markers (Parkin, BNIP3, CLpP and LC3A/B colocalisation with mitochondria) in susceptible brain regions;

6 – To evaluate the effect of RIPK1 inhibitor necrostatin-1 on mitophagy markers (Parkin, BNIP3, CLpP and LC3A/B colocalisation with mitochondria) and long-term motor and behavioural functions in HIE-injured mice.

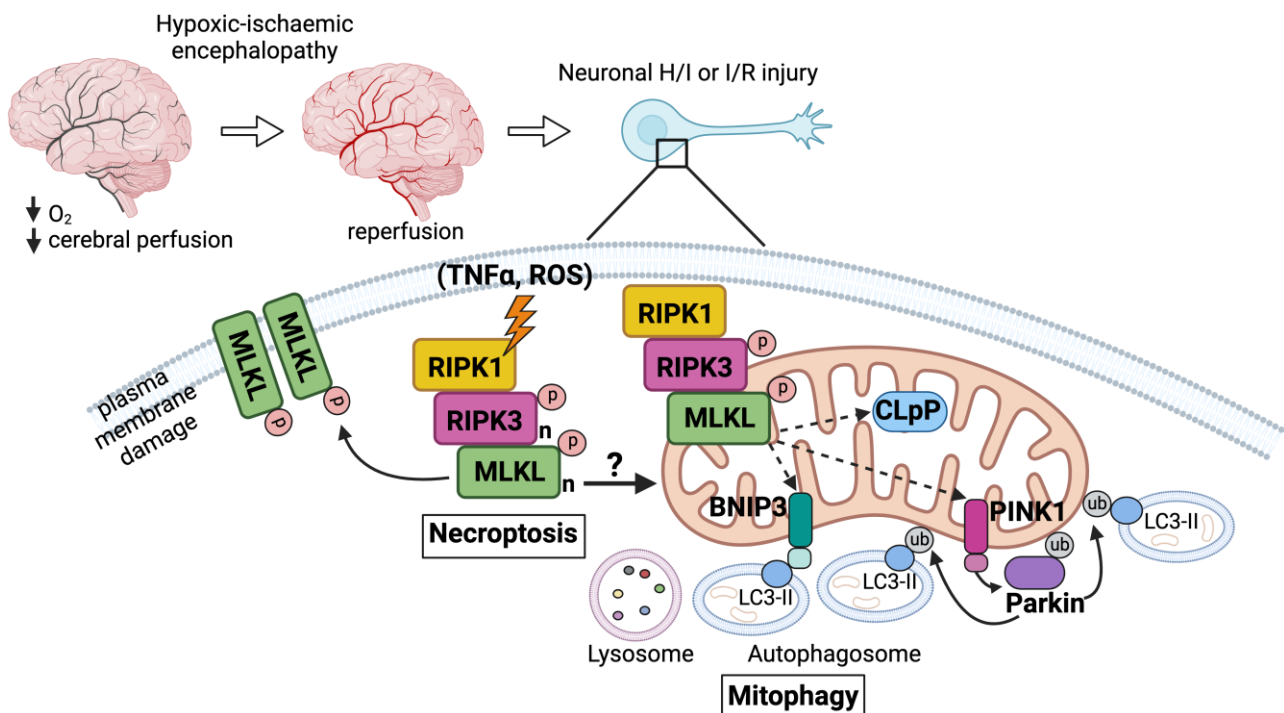


Figure 1-8. The hypothesised molecular regulation of necroptosis on mitophagy in the pathogenesis of hypoxic-ischaemic encephalopathy. Hypoxia-ischaemia and reperfusion promote necroptosis pathway to contribute to neuronal injury, with TNF-α dependent cell death receptor signaling and/or excessive reactive oxygen species regarded as the putative triggers for H/I or I/R-induced necroptosis. Mitochondrial translocation of RIPK1/RIPK3/MLKL necrosome may take place in neurons undergoing necroptosis (indicated by question mark). When localised to the mitochondrial compartment, the activated necrosome components may enhance or suppress mitochondrial clearance, possibly through direct interaction with known or potential mitophagy mediators (indicated as dashed lined), to alter mitochondrial function and cell viability following I/R injury. A better understanding of necroptosis-mitophagy crosstalk will enrich our knowledge on the execution mechanism of necroptosis, and to facilitate the identification of molecular target for future HIE therapy development.

CHAPTER TWO

Materials and Methods

2.1 Mouse Primary Neuronal Cell Culture

Cortical and hippocampal neuronal cells were harvested from gestational day17-18 mouse foetuses. In brief, day17-18 pregnant C57BL/6 dam was euthanised by a lethal dose of Euthasol (400mg/mL pentobarbital sodium), followed by isolation of foetuses from the embryonic sack and decapitation. For each foetus, bilateral cerebral cortices and hippocampi were dissected and digested with pre-warmed, sterile 0.25% trypsin (#25200072; Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) for 30 minutes at 37°C. Digestion was stopped with the addition of pre-warmed, sterile foetal bovine serum (A5256801; Gibco™, USA), and tissue was gently triturated through a flame-tipped, Pasteur glass pipette to liberate cells from connective tissue. Cell suspension was filtered through a 40µm cell strainer to remove tissue debris and centrifuged at 1000rpm for 5 minutes. Cell pellet was resuspended in neurobasal medium (#21103049; Gibco™, USA) supplemented with B-27 (#17504044; Gibco™, USA), 2µM L-glutamine (#25030081; Gibco™, USA) and 1% penicillin/streptomycin (#15140122; Gibco™, USA). Cell number was counted with a haemocytometer and cells were plated onto Nunclon™Δ-treated 60mm culture plate (#150326; Thermo Scientific, USA) or borosilicate cover glass (#48382-085; VWR International, Radnor, PA, USA) pre-coated with Poly-L-lysine (0.01% PPL; P4707; Sigma-Aldrich, St. Louis, MO, USA), at an approximate density of 5×10^5 cells/mL. Cells were maintained in a humidified incubator supplied with 5% CO₂ balanced with air at 37°C for 5 days prior to further manipulation. All embryos from a pregnant dam were counted as a single repeat.

2.2 Neuro-2a Cell Culture and Differentiation

Neuro-2a cells (#89121404, European Collection of Authenticated Cell Culture, Salisbury, UK) are neuroblastoma cells derived from a spontaneous brain tumour in mouse and adopt a neuronal and amoeboid stem cell-like morphology. Neuro-2a cells were cultured in Dulbeccos' Modified Eagle's Medium (31966021; Gibco, USA) supplemented with 10% heat-inactivated, foetal bovine serum (A5256801; Gibco™, USA) and 1% penicillin/streptomycin (#15140122; Gibco™, USA), and were maintained in humidified chamber filling with 5% CO₂ balanced with air at 37°C. Neuro-2a cells used in the present study had a passage number between 3 to 8 to ensure consistent biochemical and morphological characteristics.

Dibutyl-cyclic AMP (db-cAMP) was shown to be an effective differentiation agent for Neuro-2a cells to stimulate outgrowth of neurite and axon-like projections and dopamine expression^{412, 413}. db-cAMP differentiated Neuro-2a cells have been used a convenient tool for neurodevelopmental, neurodegenerative and neurotoxicity studies. Upon cell attachment, Neuro-2a cells were treated with 0.5mM N⁶,O^{2'}-Dibutyl cyclic adenosine 3,5-monophosphate (db-cAMP; D0627, Sigma-Aldrich) in complete DMEM medium for three consecutive days as described previously⁴¹², and were allowed to reach a confluency between 50-80% prior to experimentation.

2.3 Oxygen-Glucose Deprivation (OGD)

2.3.1 OGD in Primary Neuronal cell Culture

Oxygen-glucose deprivation (OGD) has been routinely used to simulate hypoxic-ischaemic (H/I) injury *in vitro*, and studies have tested OGD of different durations depending on the robustness of primary cell culture or specific cell lines studied^{322, 414, 415}. For primary neuronal culture to be examined herein, OGD was performed for 1hr at 8% O₂ instead of complete anoxia (0% O₂), in order to mirror the O₂ concentration (FiO₂ = 0.08) and insult duration commonly adopted for rodent model of hypoxic-ischaemic encephalopathy. The specific O₂ concentration and OGD duration were also suitable in view of the intolerance of primary neurons towards extended period of hypoxia or complete anoxia *in vitro*, which would make subsequent analysis unfeasible.

Provided that reperfusion triggers distinct injurious events and could aggravate H/I injury further, necroptosis and/or mitophagy maybe differentially regulated during pure OGD or OGD plus reperfusion. In a murine stroke model of transient middle cerebral artery occlusion (tMCAO), MLKL and RIPK3 phosphorylation was detected in the injured hemisphere within a brief reperfusion window of 1-2 hrs after 1hr MCAO to reflect a temporary induction of necroptosis²⁶⁶. Thus, to enable optimal detection of necroptosis and potentially related mitophagy pathway, primary neuronal culture will be subjected to 1hr OGD alone (OGD) or 1hr OGD plus 1hr reperfusion (OGD/Rep) in this set of experiment.

OGD or OGD/Rep in primary neuronal culture was induced on day5 *in vitro* (DIV5), whereby complete neurobasal medium was replaced with pre-warmed, deoxygenated Hank's balanced salt solution (HBSS), as previously described^{322, 416} (**Fig. 2-1**). HBSS was first placed in a Drechsel bottle and deoxygenated by bubbling N₂ (87%) and CO₂ (5%) gases through fine sintered glass bubbler to achieve an oxygen concentration of 8% (FiO₂=0.08), using an anaesthetic monitor (Datex-Ohmeda, Stirling, UK). After addition of 8% O₂ HBSS, neuronal cells were incubated in a purpose-built, airtight and temperature-controlled cell culture chamber, which was purged with N₂/CO₂ mixture to reduce ambient O₂ concentration to 8%. For OGD group, cells were incubated in 8% O₂ HBSS for 1hour; for OGD + reperfusion group, neuronal cells were allowed to recover in neurobasal medium/B-27 in humidified incubator with 5% CO₂ at 37°C for 1hr, after the removal of 8% O₂ HBSS.



Figure 2-1. Oxygen-glucose deprivation (OGD) and reperfusion in primary neurons and Neuro-2a cells, to model hypoxic-ischaemic (H/I) brain injury and ischaemia-reperfusion (I/R) brain injury *in vitro*.

As Neuro-2a cells are significantly more robust than primary neuron culture, our preliminary data suggests a minimum of 2hrs OGD (8% O₂) was required to produce noticeable cellular injury. As with primary neuronal culture, OGD and OGD/Rep groups were examined in parallel to compare and contrast the activation of necroptosis and mitophagy, with OGD/Rep more closely reflect the clinical course of HIE and should better recapitulate the pathological processes thereof.

Glucose-free DMEM medium (#11966025, Gibco™, USA) supplemented with 1% penicillin/streptomycin (#15140122; Gibco™, USA) was used to induce OGD in Neuro-2a cells. Prewarmed glucose-free DMEM was placed in Drechsel bottle and deoxygenated by bubbling N₂/CO₂ gas mixture through a fine sintered glass bubbler until the medium becomes hypoxic (8% O₂), while the oxygen concentration was continuously monitored using an anaesthetic monitor (Datex-Ohmeda, Stirling, UK). After the replacement of complete DMEM with glucose-free, hypoxic DMEM, Neuro-2a cells were placed into temperature-controlled, purpose-built gas chamber purged with N₂/CO₂ mixture with ambient oxygen concentration of 8% at 37°C (**Fig. 2-1**).

For pure OGD, Neuro-2a cells were kept in the gas chamber for various durations: 2hrs (O2), 6hrs (O6) or 24hrs (O24). For OGD plus reperfusion, OGD was initiated for 2hrs as above, before replacement with complete DMEM medium and returning cells to 5% CO₂ incubator at 37°C for 4hr (O2R4) or 22hr (O2R22). Details on experimental groups are provided in **Fig. 2-2A**. The time-matched groups (O6 vs. O2R4, O24 vs. O2R22) were intended to control for the total duration of insult between pure OGD and OGD plus reperfusion. Moreover, studies have reported that in cancer cell lines phosphor-activation of RIPK3 and MLKL is most strongly induced between 6hrs and 24hrs after indicated treatment, for example the necroptosis-inducing cocktail comprising of TNF- α , SMAC mimetic and pan-caspase inhibitor^{293, 296, 308, 311, 312}. Therefore, 6hrs (O6, O2R4) and 24hrs (O24, O2R22) were chosen to best capture MLKL phosphorylation and necroptosis activation in Neuro-2a cells.

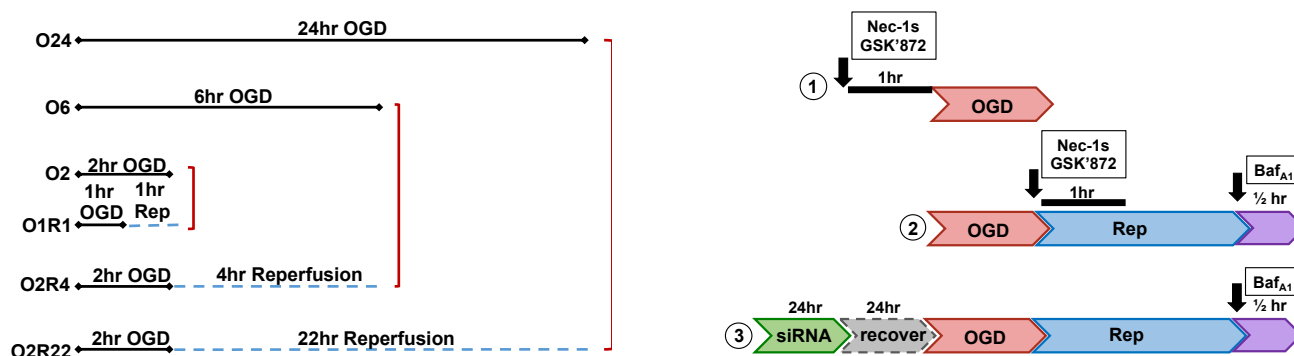


Figure 2-2. Timelines for oxygen-glucose deprivation, reperfusion and treatments. (A) Different durations of OGD (black solid line) and reperfusion (blue dashed line) and combinations thereof, red brackets indicating time-matched pure OGD and OGD + reperfusion groups. (B) Pharmacological treatments or small interfering RNA-mediated knockdown in cells subjected to pure OGD or OGD plus reperfusion: (1) pure OGD (+/-) inhibitor, (2) OGD plus reperfusion (+/-) inhibitors, or (3) OGD plus reperfusion (+/-) siRNA-mediated knockdown. Baf_{A1}, bafilomycin A1; Nec-1s, necrostatin-1; OGD, oxygen-glucose deprivation; siRNA, small interfering RNA.

2.4 Treatment with Pharmacological Inhibitors

2.4.1 Necroptosis Inhibition

For OGD plus reperfusion groups, primary neuronal cell culture or differentiated Neuro-2a cells were treated with RIPK1 inhibitor Nec-1s (#17802, Cell Signaling Technology, Danvers, MA, USA) or RIPK3 inhibitor GSK'872 (#2673-5, BioVision, Waltham, MA, USA) for 1 hour at the beginning of reperfusion at 37°C in 5% CO₂, (**Fig. 2-2B**). For pure OGD groups, cells were pre-treated with Nec-1s for 1 hour in complete, oxygenated culture medium prior to OGD (**Fig. 2-2B**). The working concentrations for Nec-1s (10μM) and GSK'872 (1μM) were chosen in view of previous studies showing effective and specific inhibition of RIPK1^{417, 418} and RIPK3 kinase activity⁴¹⁹, respectively. Nec-1s or GSK'872 was pre-dissolved in dimethyl sulfoxide (DMSO, D2650, Sigma-Aldrich) with a storage concentration of 10mM and 2mM, respectively, and were diluted to the desired working concentrations with complete culture medium.

2.4.2 Lysosome/autolysosome Inhibition

It is important to note that because mitophagy and autophagy are ongoing processes with constant turnover of the autophagosome marker LC3A/B-II by the functional autolysosome, the level of LC3A/B-II at any given timepoint available for detection is a product of total LC3A/B-II converted minus LC3A/B-II degraded by autolysosomes. In order to accurately measure real-time autophagic “flux” and LC3A/B-II conversion, either at a whole-cell level or within the mitochondrial fraction, lysosome inhibitor bafilomycin A1 (BafA1) could be administered to cells to disable lysosome-dependent LC3A/B-II degradation^{384, 420}. In this respect, Neuro-2a cells were incubated with 100nM Baf_{A1} (b0025-1; LKT Laboratories, St. Paul, MN, USA) for 30 minutes at the end of reperfusion (**Fig. 2-2B**). Baf_{A1} was pre-dissolved with DMSO to a storage concentration of 1μM, and was diluted with complete culture medium to the working concentration.

2.5 Small interfering RNA (siRNA)-mediated knockdown

Small interfering RNA (siRNA), along with short-hairpin RNA (shRNA), are commonly used RNA interference (RNAi) approaches that target specific messenger RNA (mRNA) sequences to lead to degradation of mRNA, thus enabling efficient silencing of specific genes at post-transcription level and preventing expression of the encoded protein⁴²¹. Small interfering RNA (siRNA) molecules are typically delivered as a double-stranded RNA (dsRNA) duplexes into the cytoplasm of cells, where they accumulate and are processed by the endonuclease dsRNA-specific RNase III enzyme Dicer into 20-30 nucleotides long siRNA molecule⁴²¹. The cleaved siRNA base pairs are then loaded onto the RNA-induced silencing complex (RISC), a ribonucleoprotein complex that can adopt versatile compositions and correspondingly suppresses gene expression through different mechanisms^{421, 422}. In the context of siRNA, siRNA duplex is first separated within the RISC to allow incorporation of only one strand as an anti-sense guide to the targeted, sense mRNA sequence through complementary base pairing. At the heart of RISC is the evolutionarily conserved Argonaute family protein (for example, Ago-2), which exhibits RNase-H like activity and strongly associates with the anti-sense, guide siRNA and its bound sense, targeted mRNA, to catalyse the cleavage of target mRNA sequence^{421, 422}.

Neuro-2a cells were incubated with small interfering RNA (siRNA) specific for mouse MLKL (target sequence: TACCATCAAAGTATTCAACAA; SI00868791; Qiagen, Germantown, MD, USA) or mouse CLpP (target sequence: CACGATGCAGTACATCCTCAA; SI00211785; Qiagen) in the presence of the transfection reagent Lipofectamine RNAiMAX (#13778030, Thermo Fisher Scientific, USA), as per the manufacturer's instructions. As negative control, additional group of Neuro-2a cells were incubated with AllStars Negative Control siRNAs (SI03650318; Qiagen) that are non-specific towards any known mammalian genes. Neuro-2a cells were plated in 35mm Nunclon™Δ coated cell culture plate (#153066, VWR International, USA) and were allowed to reach a confluency of 30-50%. siRNA and Lipofectamine RNAiMAX were individually pre-diluted with serum-free, antibiotics-free eagle's minimum essential medium (MEM, #11090081, Gibco™, USA), followed by incubation at room temperature for 10 minutes to form stable siRNA-lipid transfection complex. For a single transfection reaction in a 35mm plate, 10nM siRNA (from 10μM stock) and 5μL Lipofectamine RNAiMAX transfection reagent were added to Neuro-2a cells in total volume of 300μL of MEM. Neuro-2a cells were incubated with the transfection mixture for 24 hrs at 37°C with 5%CO₂, and were recovered in complete DMEM for 48 hrs prior to OGD/Rep as described above (**Fig. 2-2B**).

2.6 Immunofluorescence Staining

Overview of Immunofluorescence Staining

Immunofluorescence (IF) is a robust and informative molecular technique that allows visualisation as well as semi-quantification of different antigens across cellular components in cell or tissue samples. The principle of immunofluorescence detection relies on the specific interactions between target antigens and antibodies, whereby the antibodies are conjugated to a fluorophore/fluorochrome that emits fluorescence when excited by a shorter wavelength.

Appropriate and adequate fixation of cell/tissue samples is an essential prerequisite to immunofluorescence staining, as it prevents cell autolysis, putrefaction and protein degradation while preserves the overall cellular architecture and immunoreactivity of antigens/epitope sites on antigen. Cross-linking fixatives, such as glutaraldehyde, formalin and formaldehyde, are the most commonly used chemical fixatives by binding to and crosslinking the cellular proteins through the formation of intra- and intermolecular methylene links. Fixed tissues can either be embedded in optimal cutting temperature (OCT) solution for cryo-section or in paraffin wax for microtome sectioning. For paraffin-embedded tissue, the additional step of deparaffinisation by xylene is required and is followed by washes of ethanol and wash for tissue rehydration.

When the tissue samples are fixed with cross-linking chemicals and paraffin embedded, antigen retrieval is necessary to restore antigenicity and enhance antibody-antigen/epitope binding, as fixation creates crosslinks between amino acids within the antigen and the surrounding proteins that could distort the epitope conformation and mask the epitope sites. Either heat-induced epitope retrieval (HIER) or protease-induced epitope retrieval (PIER) can be performed to cleave the protein cross-links formed during fixative and to unmask the epitope site for antibody recognition.

Prior to antibody incubation blocking must be performed to prevent/minimise unspecific binding of the antibodies, with the most commonly used blocking agents for immunofluorescence being bovine serum albumin (BSA) solution and normal serum. Since the high concentration of proteins present in BSA will bind to all antigens present in the cell/tissue samples, non-specific binding by the primary antibody to non-target sites is reduced.

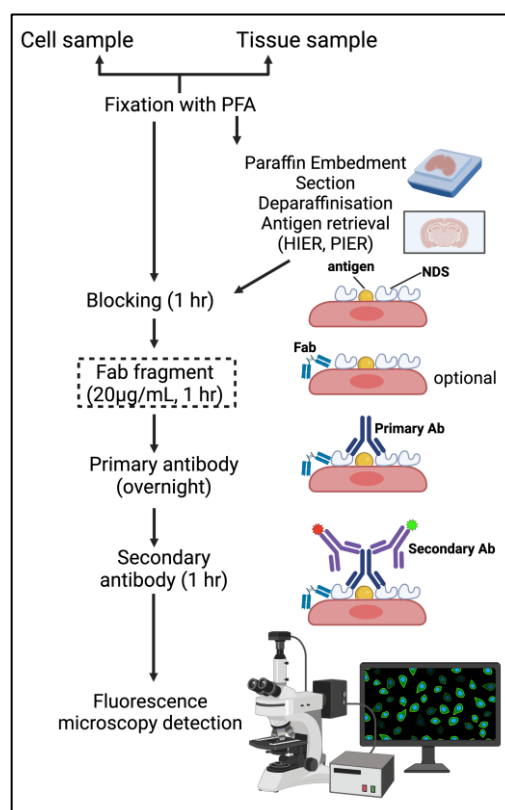


Figure 2.3. Work flow of immunofluorescence staining of cell and tissue samples.

Normal serum is derived from the same species as that of secondary antibody and is protein-rich with abundant antibodies. In addition to minimising non-specific primary antibody binding as with BSA, the antibodies from normal serum block the non-target reactive sites that secondary antibody would otherwise bind. Moreover, when the tissue sample and the primary antibody are from the species (e.g. mouse tissue incubated with mouse primary antibody), if the tissue expresses a high level of immunoglobulin, the secondary antibody cannot differentiate the endogenous immunoglobulins from the primary antibody, and binding of secondary antibody to endogenous immunoglobulins will generate high background fluorescence. To avoid this, the endogenous immunoglobulins can be blocked/masked with an unconjugated Fab fragment (20-40µg/mL) directed against the host species of the tissue (e.g. anti-mouse Fab fragment) before incubation with primary antibody, such that the secondary antibody introduced later will only recognise the bound primary antibody. Please refer to **Fig. 2-3** for an overview of the steps for immunofluorescence staining.

2.6.1 Immunofluorescence staining for cell sample

Primary neuronal culture or Neuro-2a cells were fixed with ice-cold 4% paraformaldehyde (PFA; sc-281696) for two cycles of 10 minutes. The fixed cells were then incubated with 5% normal donkey serum (NDS; D9663, Sigma-Aldrich) in phosphate-buffered saline with 0.3%-Tween 20 (PBS-Tween 20) at room temperature for 1hr, to permeabilise cell membrane and to prevent non-specific binding from the fluorescent donkey secondary antibodies. Cells were subsequently incubated with primary antibodies specific for necroptosis or mitophagy markers diluted in PBS-Tween 20 containing 2% NDS at 4°C overnight: rabbit anti-mouse phospho^{Ser345} MLKL (1:1000; Cell Signaling Technology), rabbit anti-LC3A/B (1:1000; Cell Signaling Technology), mouse anti-CLpP (1:200, Santa Cruz), mouse anti-TOM20 (1:200; Santa Cruz) and mouse anti-β tubulin III (1:500; Abcam). On day2, cells were rinsed with PBS-Tween 20 to remove unbound antibodies, before incubation with donkey anti-rabbit Alexa Fluo488 (1:1000; Invitrogen, Thermo Fisher Scientific), donkey anti-rabbit Alexa Fluo568 (1:1000; Invitrogen), donkey anti-mouse Alexa Fluo488 (1:1000; Invitrogen) or donkey anti-mouse Alexa Fluo568 (1:1000; Invitrogen) secondary antibody at room temperature for 1hr. Please refer to Table 2-1 for detailed information on the primary and secondary antibodies used in the present study. Cells were rinsed with 2 washes of PBS-Tween and 1 wash of PBS to remove unbound secondary antibodies, before mounting DAPI anti-fade mounting medium (H-1200, Vector Laboratories) and viewed under Zeiss Axio Observer widefield microscope.

2.6.2 Immunofluorescence staining for tissue sample

On the day of brain sample harvest, mice were anaesthetised by pentobarbital (50mg/kg, i.p.) before transcardial perfusion with 4% ice-cold paraformaldehyde (PFA) in phosphate-buffered saline (pH 7.4). Brains were harvested and further fixed in fresh 4% PFA in PBS overnight at 4°C, followed by serial dehydration in

10%, 20% and 30% sucrose in PBS. The brain samples were further dehydrated in increasing concentrations of ethanol (70-100%) and immersed in xylene before embedment in paraffin wax. The paraffin-embedded forebrains were sectioned coronally into 15-20 μm thick slices between -1.70mm and -2.46mm from bregma using a microtome. Brain sections were deparaffinised with three cycles of 5-minutes of xylene wash, and rehydrated in decreasing concentrations of ethanol (100%-50%) and lastly rinsed in deionised water. Heat-induced antigen retrieval was performed by heating slides containing the brain sections in 10mM sodium citrate buffer (pH 6.0; C9999, Sigma-Aldrich) for 15 minutes without boiling.

The antigen-recovered tissue samples were incubated in 5% NDS in PBS with 0.3% Triton X-100 (PBS-Triton X) for 1hr at room temperature to block unspecific binding from primary and secondary antibodies, as described above. Triton X-100 is a more potent surfactant than Tween20 to more effectively permeabilise cell membrane for antibody penetration into tissues. For samples that are to be reacted with mouse primary antibodies, the sections were additionally incubated with donkey anti-mouse monovalent Fab fragment (20 $\mu\text{g}/\text{mL}$ in PBS-Triton X; 715-007-003, Jackson Immuno Research, Ely, UK) to prevent secondary antibody-immunoglobulin interaction, as explained above. Tissue sections were then incubated in primary antibodies specific for necroptosis and mitophagy markers in 2% NDS in PBST at 4C overnight: rabbit anti-mouse phospho^{Ser345} MLKL (1:500), rabbit anti-mouse phospho^{Thr231/Ser232} RIPK3 (1:500), mouse anti-GFAP (1:500), mouse anti-Parkin (1:500), rabbit anti-BNIP3 (1:500), mouse anti-CLpP (1:200), rabbit anti-LC3A/B (1:500) and mouse anti-COX IV (1:500). On day2, the brain sections were further incubated with fluorescent secondary antibodies diluted in PBST with 2% NDS for 1hr at room temperature: donkey anti-rabbit Alexa FluorTM 488, donkey anti-mouse Alexa FluorTM 488, and/or donkey anti-mouse Alexa FluorTM 568. Please refer to **Table 2-1** for detailed information on the primary and secondary antibodies used in the present study. Stained tissues were mounted with DAPI anti-fade mounting medium (H-1200, Vector Laboratories) and viewed under Zeiss Axio Observer widefield microscope.

Antibody	Concentration	Catalogue	Host Species/Isotype	Company
Primary Abs				
Anti-BNIP3	WB: 1/1000	12396	Rabbit monoclonal IgG	Cell Signaling Technology, MA, USA
Anti- β tubulin III (Tuj)	IF: 1/500	Ab78078	Mouse monoclonal IgG2a	Abcam, Cambridge, UK
Anti-CLpP	IF: 1/500 (cells), 1/250 (tissue);	sc-271284	Mouse monoclonal IgG1	Santa Cruz, TX, USA
Anti-COX IV	IF: 1/500; WB: 1/2000	11967	Mouse monoclonal IgG1	Cell Signaling Technology, MA, USA
Anti-GAPDH	WB: 1/20,000	MAB374	Mouse monoclonal IgG1	Merck Millipore, MA, USA
Anti-GFAP	IF: 1/500	52522	Mouse monoclonal IgG1	Cell Signaling Technology, MA, USA
Anti-LC3A/B	IF: 1/1000 (cells), 1/500 (tissue); WB: 1/1000	12741	Rabbit monoclonal IgG	Cell Signaling Technology, MA, USA
Anti-mouse MLKL	WB: 1/2000	37705	Rabbit monoclonal IgG	Cell Signaling Technology, MA, USA
Anti-mouse phosphoSer345 MLKL	IF: 1/1000 (cells), 1/500 (tissue); WB: 1/2000	37335	Rabbit monoclonal IgG	Cell Signaling Technology, MA, USA
Anti-Parkin	WB: 1/1000	4211	Mouse monoclonal IgG2b	Cell Signaling Technology, MA, USA
Anti-RIPK3	WB: 1/2000	sc-374639	Mouse monoclonal IgG1	Santa Cruz, TX, USA
Anti-mouse phosphoThr231/Ser232 RIPK3	IF: 1/500 (tissue)	91702	Rabbit monoclonal IgG	Cell Signaling Technology, MA, USA
Anti-TOM20	IF: 1/200	sc-17764	Mouse monoclonal IgG2a	Santa Cruz, TX, USA
Anti-VDAC1	WB: 1/500	sc-390996	Mouse monoclonal IgG1	Santa Cruz, TX, USA
Secondary Abs				
Anti-rabbit IgG, HRP-conjugated	WB: 1/2000	7074	Donkey	Cell Signaling Technology, MA, USA
Anti-mouse IgG, HRP-conjugated	WB: 1/2000	7076	Donkey	Cell Signaling Technology, MA, USA
Anti-rabbit IgG (H+L), Alexa Fluor 488	IF: 1/1000	A-21206	Donkey	Invitrogen, MA, USA
Anti-rabbit IgG (H+L), Alexa Fluor 568	IF: 1/1000	A10042	Donkey	Invitrogen, MA, USA
Anti-mouse IgG (H+L), Alexa Fluor 488	IF: 1/1000	A-21202	Donkey	Invitrogen, MA, USA
Anti-mouse IgG (H+L), Alexa Fluor 568	IF: 1/1000	A10037	Donkey	Invitrogen, MA, USA

Table 2-1. List of primary and secondary antibodies used for immunofluorescence staining and western blot.

2.7 Cresyl-Violet Staining

Cresyl-violet is the gold-standard histological stain for neurons and is routinely used to map the distribution of neurons and to identify brain lesions. Cresyl-violet stains ribosomal RNA of the rough endoplasmic reticulum (i.e. Nissl substance) in the soma and dendrites to produce a violet-blue colour, and giving the cytoplasm a mottled appearance. At pH close to 3.0, cresyl-violet only stains the Nissl substance and nucleoli of neurons, with the nucleus of glial cells very weakly stained. The overall staining becomes darker when pH approaches 4.0 with the nerve fibres and glial cells co-stained.

Brain sections were deparaffinised with three cycles of 5-minutes of xylene wash, and rehydrated in decreasing concentrations of ethanol (100%-50%) and lastly in deionised water. Heat-induced antigen retrieval was performed by boiling glass slides containing the brain sections in 10mM sodium citrate buffer (pH 6.0; C9999, Sigma-Aldrich) for 15 minutes. The brain sections were incubated with cresyl violet staining solution with 0.1% cresyl violet acetate and 0.2% acetic acid at pH2.9 (Ab246816; Abcam) for 5-10 minutes in an oven heated to 37°C, differentiated with pure ethanol and cleared with xylene, before mounted with synthetic resin for microscopic examination.

2.8 Live-cell Imaging of Neuro-2a cells

Live-cell imaging was performed to visualise the effect of OGD/Rep and necroptosis inhibition on mitochondrial morphology. MitoTracker[®] Deep Red (M224226; Invitrogen, ThermoFisher Scientific, USA) is a mitochondrial probe that readily diffuses across mitochondrial membrane and becomes fluorescent upon oxidation by respiring mitochondria with an emission wavelength of 665nm. It also contains a mildly thiol-reactive chloromethyl moiety to react with the thiol groups of mitochondrial proteins to form conjugate and is sequestered within the mitochondria. Neuro-2a cells were grown in chambered coverslips, and after indicated periods of OGD/Rep and/or treatment or knockdown, were incubated with 100nM MitoTracker[®] Deep Red dissolved in complete DMEM medium for 30 minutes in dark at 37°C. The staining solution was replaced with serum-free DMEM medium and live cells were viewed under Leica SP8 inverted confocal microscope.

2.9 Western Blot

Overview of Western Blot

Western blot is a widely-employed molecular technique for detection of a specific protein from a mixture of proteins collected from cell culture or tissue sample, and is typically followed by quantification of the protein expression. With modifications to sample preparation procedures and buffer system, western blot can also be used to isolate multiprotein complexes for the study of protein-protein interaction, as will be discussed in more details below. Although the term western blot refers to the transfer of protein(s) from the gel onto a blotting membrane, the term is used to describe the entire process of protein detection from sample preparation, gel electrophoresis, protein transfer, antibody incubation and detection (**Fig. 2-4**).

After protein lysis, the concentration of protein lysate needs to be determined prior to loading sample for gel electrophoresis, such that equal loading will allow protein levels and expression differences between samples to be accurately reflected. Bradford assay is a convenient and colorimetric protein quantification assay that uses the dye Coomassie Blue, which changes colour from red (cationic, protonated form) to blue (anionic, unprotonated form) when it stably binds to protein.

During electrophoresis, proteins will migrate to the electrode of the opposite charge, and the speed and direction of protein movement is dictated by the size, conformation and overall charge of the protein. Polyacrylamide gel forms the gel matrix for proteins segregation, and the gel percentage (i.e. percentage of polyacrylamide monomer present) determines the gel pore size and can be customised depending on the size range of the proteins to be studied for optimised separation. In this regard, proteins of smaller size (lower molecular weight) will migrate faster than larger proteins (higher molecular weight) when placed in the same electrical field, and buffers of different compositions and pH or the addition of different detergents can alter the conformation and charge of protein to modify/facilitate protein separation through the aqueous gel.

The most commonly used type of gel electrophoresis incorporates the detergent sodium dodecyl sulphate (SDS) into the buffer system (e.g. lysis buffer, sample buffer, running buffer), whereby SDS acts as a denaturing agent to break the intra-protein disulphate bonds to unfold proteins into linear structures. Moreover, SDS is negatively charge and imparts an overall negative charge to proteins, which can uniformly migrate from the cathode (negative) end of the gel towards the anode (positive) end. The higher protein structures can be further reduced by including β -mercaptoethanol (β -ME) or dithiothreitol (DTT) into the lysis buffer/sample buffer.

Once proteins have been physically separated on the polyacrylamide gel, they can be transferred onto a hydrophobic blotting membrane (e.g. nitrocellulose, NC or polyvinylidene fluoride, PVDF) for antibody detection. Upon completion of protein transfer, the membrane is blocked with a protein-rich solution to minimise non-specific binding from primary antibody to non-target antigens, with skimmed milk (5% w/v) and BSA (1% w/v) being the most widely used blocking reagents. The blocked transfer membrane is ready for incubation with primary antibody overnight, before incubation with a conjugated secondary antibody for detection of the protein band of interest. Either fluorophore-conjugated secondary antibodies can be used for direct visualisation of the protein band, or more commonly, chemiluminescence detection of target protein is achieved with secondary antibody conjugated to a reporter enzyme, for example horseradish peroxidase (HRP) or alkaline phosphatase (AP). When the secondary antibodies recognise the primary antibody/antigen interaction, the conjugated enzymes will catalyse the chemical reaction to generate an immediate, transient and detectable light signal that is proportional to the expression level of the protein of interest. HRP-conjugation system represents the most sensitive detection option, whereby HRP catalyses the oxidation of luminol-based substrate in the presence of hydrogen peroxide to produce light of wavelength 425nm at the position of target protein band, which can be captured by a luminescent CCD camera and the light intensity can be subsequently quantified.

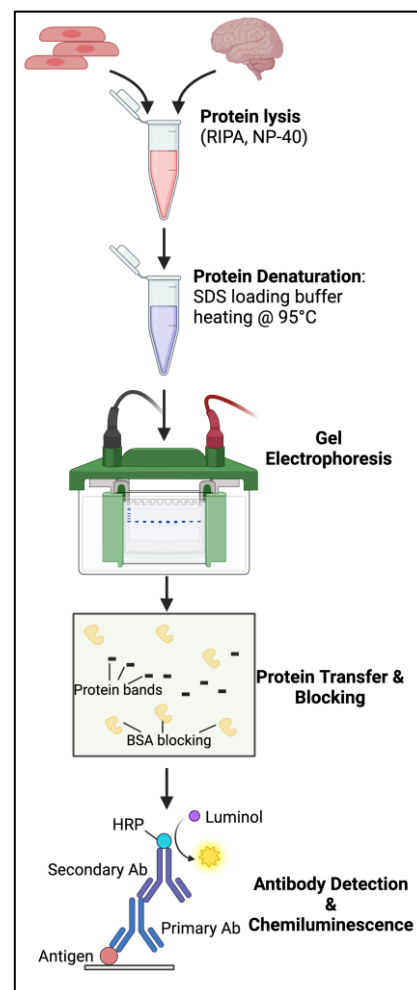


Figure 2.4. Overview of the key steps in western blot with cell or tissue samples.

2.9.1 Whole-cell Protein Lysis

Adherent primary neuronal cells or Neuro-2a cells were rinsed with ice-cold phosphate buffered-saline (PBS) and were lysed in RIPA buffer (R0278, Sigma-Aldrich) supplemented with protease inhibitor (#4693159001, Roche, Switzerland) and phosphatase inhibitor (#4906845001, Roche). For mouse brain samples, the brain tissues were snap-frozen liquid nitrogen (N₂), and were homogenised in protease inhibitor and phosphatase inhibitor-supplemented cell lysis buffer (2mL RIPA buffer per 100mg tissue in weight) on ice by a tissue sonicator. Cell and tissue samples were incubated on ice for 30 minutes with gentle shaking, before centrifugation at 12,000g for 15 minutes to collect the protein-rich supernatant.

The principle of mitochondrial isolation from cultured cells or tissue samples relies on a two-step differential centrifugation process^{423, 424}, which starts with low-speed centrifugation to remove intact cells, cellular debris and nuclei from the whole cell fraction, and is followed by high-speed centrifugation to enrich the mitochondrial fraction and separate mitochondria from other organelles. Studies have described differences in the dissociation/homogenisation process, buffer system and small variations to the centrifugation speed(s)⁴²³⁻⁴²⁵. Depending on the subsequent application using the mitochondrial preparation, e.g. western blot, cytochrome c release, ROS synthesis or ATP production, adjustments to the generic mitochondrial isolation protocol need to be made.

Mitochondrial proteins were extracted using mitochondria isolation kit (ab110170, Abcam, UK) as per the manufacturer's instructions, and all steps were performed on ice or at 4°C to minimise protein degradation. Adherent Neuro-2a cells were rinsed with ice-cold PBS (pH 7.4), detached with a cell scraper and centrifuged at 1,000g at 4°C for 5 minutes to pellet the cells. The cell pellet was re-suspended with Reagent A from the kit and dissociated with a dounce homogeniser with B pestle, whereby an approximate of 40-50 strokes per sample was performed to completely disrupt the cell pellet. The cell homogenate was centrifuged for 10 minutes at 1,000g at 4°C and the supernatant (SP1) was saved. The pellet was re-suspended with Reagent B from the kit, homogenised and centrifuged and as above, and the supernatant (SP2) was collected and combined with SN1. The above steps at low centrifugation speed remove cellular debris and extremely large cellular organelles (e.g nuclei). The combined supernatants were then centrifuged at 12,000g for 15 minutes to precipitate mitochondrial fraction from other organelles. The pelleted crude mitochondria fraction was further dissolved in Reagent C supplemented with protease inhibitor (#4693159001, Roche) and phosphatase inhibitor (#4906845001, Roche). Based on the number of cells to start with, the volumes of buffers used were scaled-up or -down from the manufacturer's protocol.

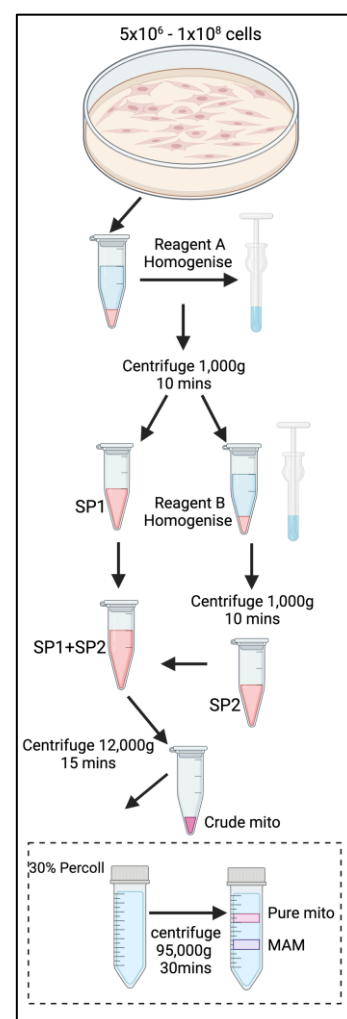


Figure 2.5. Overview of mitochondrial fraction extraction from cell samples.

2.9.3 Gel Electrophoresis, Membrane Transfer and Detection

The protein content from whole-cell lysate or mitochondrial extract was quantified with Bradford assay (#5000006, Bio-Rad, CA, US). Protein samples were prepared with NuPage LDS loading buffer (2165457,

Invitrogen) in a volume ratio of 4:1, and were heated to 97°C for 10 minutes under protein denaturation condition. Denatured protein samples were loaded onto 4-12% Bis-Tris NuPAGE gel (NP0321BOX, Invitrogen), followed by gel electrophoresis at 140V and 120mA for 1hr. Protein bands were transferred onto nitrocellulose membrane (LC2000, Invitrogen) using XCell^{III} system (EI9051, Invitrogen) at 30v for 90mins.

NC membrane was blocked in 5% non-fat milk (sc-2324, Santa Cruz, Dallas, TX, USA) in Tris-buffered saline with 0.1% Tween-20 (TBS-T) at room temperature for 1hr on a shaking platform. The blocked NC membrane was then incubated in primary antibodies in TBS-T overnight on a roller at 4°C: rabbit anti-mouse phospho^{Ser345} MLKL (1:2000; Cell Signaling Technology), rabbit anti-mouse MLKL (1:2000; Cell Signaling Technology), mouse anti-RIPK3 (1:500; Santa Cruz), mouse anti-Parkin (1:2000; Cell Signaling Technology), mouse anti-CLpP (1:200; Santa Cruz), rabbit anti-LC3A/B (1:1000; Cell Signaling Technology), mouse anti-VDAC1 (1:500; Santa Cruz) and mouse anti-GAPDH (1:20,000; Millipore). On day2, membrane was rinsed with TBS-T to remove unbound primary antibodies, and was further incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit (1:2000; Cell Signaling Technology) or donkey anti-mouse IgG antibody (1:2000; Cell Signaling Technology) in TBS-T for 1hr at room temperature on a shaking platform. Please refer to **Table 2-1** for detailed information on the antibodies used for western blot. The protein bands were developed with chemiluminescence detection reagent (Immobilon Western HRP Substrate; WBKLS0500, Millipore), visualised with Syngene imaging system (Cambridge, UK) and band intensity determined with ImageJ.

2.9.4 Co-immunoprecipitation

Co-immunoprecipitation (co-IP) is a specific application of western blot and is regarded as the gold standard method for studying interactions between proteins and functions of protein complexes. The principle of co-IP is that a primary antibody X recognising a target protein X as a constituent of a larger protein complex will precipitate protein X along with the entire protein multimer from the cell lysate, when the antibody X is conjugated to a solid bead support. The immuno-precipitated protein complex is eluted from the bead, separated by gel electrophoresis and blotted onto transfer membrane to be probed with antibodies against other constituents (Y, Z, etc.) of the protein complex, in order to reveal physical interactions between proteins X, Y and Z (**Fig. 2-6**). It should be emphasised that since the different components of the (potential) protein complex are held together by weak bonds/associations, cautions must be taken during protein lysis, beads incubation and elution steps, with extensive optimisations to preserve the delicate protein-protein interactions until the detection step. For example, denaturing agent (e.g. SDS) should be avoided during protein extraction and replaced with mild detergents/surfactants, as denaturing agent will disrupt inter-protein bonds/associations.

In order to establish whether MLKL exhibit physical interaction with Parkin, BNIP3 or CLpP during OGD/Rep, co-immunoprecipitation with MLKL specific antibody was performed to pull-down proteins, using the Pierce™ direct magnetic IP/Co-IP kit (88828; Thermo Scientific) as per manufacturer's instructions. In brief, the magnetic beads were incubated with rabbit anti-mouse MLKL antibody (3μg per reaction; 37705, Cell Signaling Technology) for 30 minutes at room temperature on a rotating platform. The antibody-coated magnetic beads were then incubated with Neuro-2a whole cell lysates for 2 hrs at room temperature on a shaking platform. Magnetic beads bound by antibody and target antigens were collected using a magnetic stand, before elution of the bound antigens for analysis by western blot. A non-specific rabbit IgG antibody was used as isotype control (3μg per reaction; #02-6102, Invitrogen) and was incubated with magnetic beads and cell lysate and eluted as above. Eluted samples were prepared for gel electrophoresis and protein transfer, as described above. The nitrocellulose membrane was blocked and incubated overnight at 4°C with rabbit anti-mouse MLKL antibody (1:2000; Cell Signaling Technology), mouse anti-Parkin antibody (1:2000; Cell Signaling Technology), rabbit anti-BNIP3 antibody (1:2000; Cell Signaling Technology), mouse anti-CLpP antibody (1:200; Santa Cruz) and mouse anti-GAPDH antibody (1:20,000; Millipore). On day2, the membrane was incubated with HRP-conjugated donkey anti-rabbit (1:2000; Cell Signaling Technology) or donkey anti-mouse IgG antibody (1:2000; Cell Signaling Technology), before membrane development with chemiluminescence detection reagent (Immobilon Western HRP Substrate; WBKLS0500, Millipore) and visualised with Syngene imaging system (Cambridge, UK).

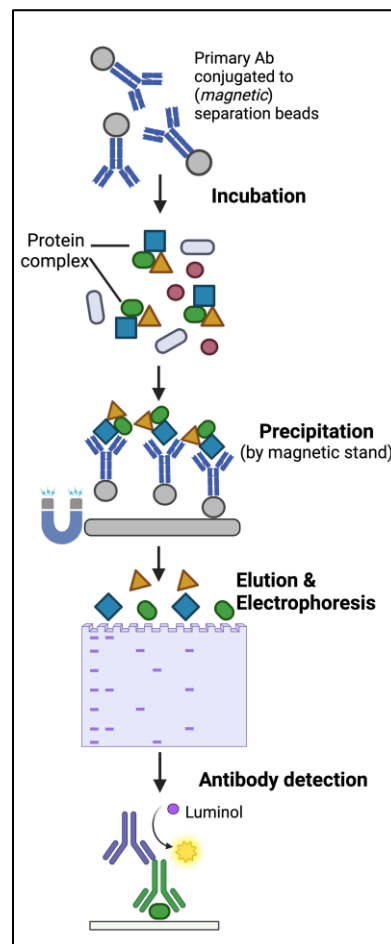


Figure 2.6. Principle and work flow of co-immunoprecipitation using magnetic separation beads.

2.10 Flow cytometry

The Principle of Flow Cytometry

A flow cytometer is a sophisticated and robust instrument that simultaneously and quantitatively measures complex physical and biochemical parameters (size, granularity, fluorescence level) of large number of cells as they flow through the instrument in a single suspension⁴²⁶⁻⁴²⁸. The principle of flow cytometry is underpinned by light scattering and deflection from the incoming cells, thereafter the scattered/deflected light is captured and converted into electrical signal then digital data for analysis. The working mechanics of flow cytometry rely on three key systems, namely the fluidics, the optics and the electronic network, as depicted in **Fig. 2-7**^{427, 428}.

The fluidics system consists of an “ambient” sheath fluid and a central core of sample stream with suspended cells, and the pressure difference between sheath fluid and sample stream aligns the cells in a single file fashion when they pass through the laser beam(s) to allow uniform illumination, termed as hydrodynamic focussing. The optical system of a flow cytometer consists of light source (laser) and excitation and collection optics in fixed positions. The excitation optics/lenses shape and focus the laser beam for illuminating the cells. The collection optics comprise of collection lens that collect light/fluorescence emitted by the cells, and a network of optical mirrors and filters to direct the specific wavelengths emitted to appropriate optical detectors. When laser strikes the incoming cell/particle, light is scattered forward (forward-scattered light, FSC) along the same axis as the laser beam and sideward (side-scattered light, SSC) that is perpendicular to the laser. FSC is detected by photodiode (PD) and the magnitude of FSC is proportional to cell size or surface area, whereas the weaker SSC is detected by photomultiplier tube (PMT) and gives a measure of granularity and intracellular complexity.

For the detection and quantification of fluorescence from fluorochrome-labelled cells/particles (fluorescent antibodies or dyes), appropriate lasers such as argon-ion laser (excitation wavelength 488nm), ultraviolet laser (excitation wavelength 300-400nm) and/or red diode laser (far-red excitation at 630nm) can be selected. When illuminated by the laser, the emitted fluorescence signals from cells/particles is captured by PMTs, and each PMT is fitted with an optical filter that only allows a narrow range of wavelength to reach the PMT detector, thus each PMT is assigned to a particular fluorescent signal with emission wavelength range that corresponds to spectral range of the optical filter. For example, the filter used for FITC detection is called a bandpass filter that transmits light within the band of 515-545nm.

Photodiodes (PD) and photomultiplier tubes (PMT) are photodetectors that convert the light and fluorescence signals into electrons. The electrons are then multiplied to generate an electrical current, before travelling to an amplifier to be converted into a voltage pulse, which will be proportional to the intensity of light or fluorescence. The size of the voltage pulse can be amplified by applying a voltage to the PMT (pre-

amplifier gain) or increasing the amplification gain, with either Log or Lin amplification. Finally, the Analog-to-Digital Converter (ADC) converts 0-10V voltage pulse into a digital number. In the analog system, each voltage pulse is given a digital number between 0-1000 channels, and the channel value is transferred to computer so the corresponding light signal is displayed at the correct position on the data plot.

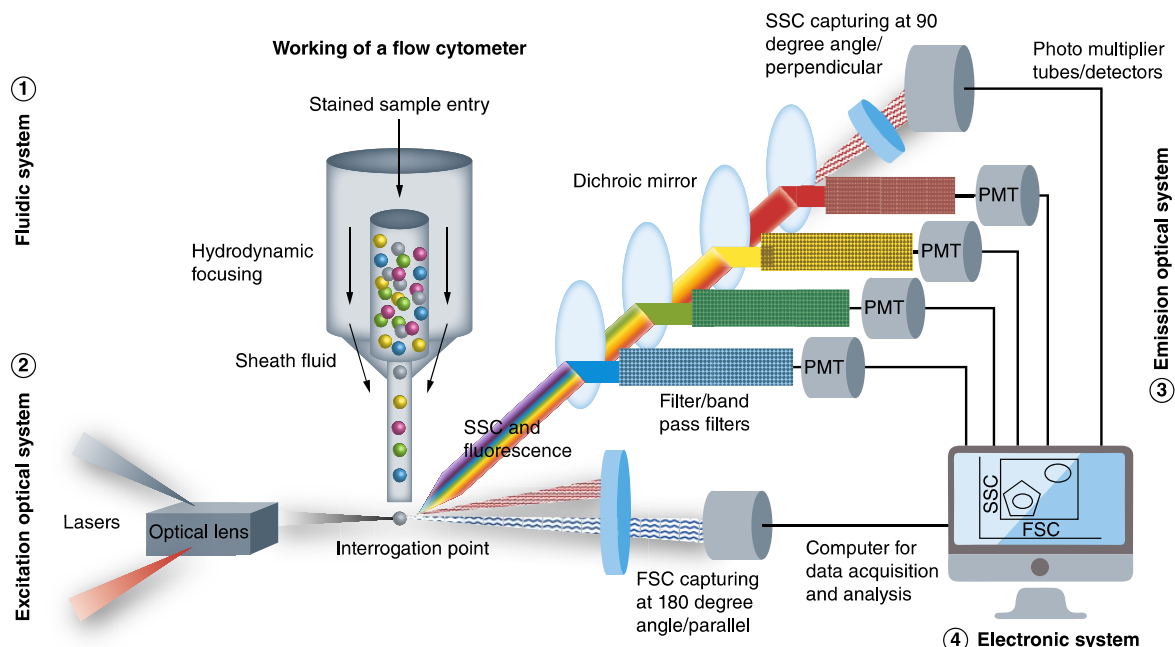


Figure 2-7. Principle of Flow Cytometry (Figure 1 from Manohar et al. Flow cytometry: principles, applications and recent advances⁴⁷⁰).

Cells in suspension are hydrodynamically focussed to pass through the laser beam(s) in a single file fashion to ensure uniform illumination. The light scattered and fluorescence emitted by the cells are captured by collection lenses and routed through a system of mirrors and optical filters before reaching the detectors. The light/fluorescence signals are first converted into electrical currents and then converted into digital information by the analog-to-digital converter to be displayed by the appropriate computer software. FSC: forward scatter; PMT: photomultiplier tube; SSC: side scatter.

2.10.1 JC-10 Detection of Mitochondrial Membrane Potential

JC-10 belongs to a class mitochondrial probes called carbocyanines and is an improved derivative from the better known 5,5,6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide (JC-1) with superior water solubility. JC-10 accumulates and forms J-aggregates in functional, polarised mitochondria to emit an orange-red fluorescence (emission wavelength 590nm), whereas in injured/depolarised mitochondria JC-10 remains as monomers to emit a green fluorescence (emission wavelength 525nm) when excited at 488nm (**Fig. 2-8**). In this respect, the shifts between orange/red and green fluorescence from collapse or recovery of mitochondrial membrane potential can be detected by flow cytometry with high degree of sensitivity.

Following indicated period of OGD/Rep with or without RIPK1 or RIPK3 inhibition or MLKL knockdown, Neuro-2a cells were detached from culture plate with Accutase detachment solution (00-4555-56; ThermoFisher Scientific). Neuro-2a cells were prepared and stained with JC-10 mitochondrial membrane potential assay kit (Ab112133; Abcam), as per the manufacturer's instructions. Cells pre-treated with 2 μ M mitochondrial uncoupler FCCP (Ab120081; Abcam) for 30 minutes were used as positive control. For each cell sample, approx. 5×10^5 cells were re-suspended in 500 μ L dye-loading solution with 2.5 μ L JC-10 dye and were incubated in dark at 37°C for 30 minutes.

JC-10 stained cells were analysed by FITC and PE channels on a flow cytometer (BD LSR-II; BD Biosciences), with PMT voltages set at 300 and 400 for FITC and PE, respectively. Naïve control (NC) cells and FCCP-treated positive control cells were used to establish the compensation threshold. This is because emission spectral overlap occurs between FITC and PE channels. The process of colour compensation involves subtracting a percentage of FITC signal from PE channel and vice versa. In the current study, FITC – PE compensation was set at 27% and PE – FITC compensation was set at 40% to enable sufficient separation of green JC-10 monomers from red-orange JC-10 aggregates. For each sample, the percentages of FITC-positive cells and PE-positive cells were determined, and the mitochondrial membrane potential was expressed as PE/FITC ratio.

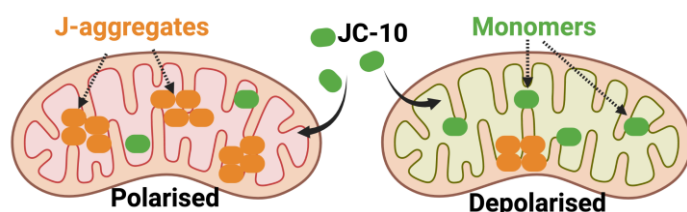


Figure 2.8. Principle of JC-10 detection of mitochondrial membrane potential.

2.10.2 TMRE Detection of Mitochondrial Membrane Potential

Tetramethylrhodamine ethyl ester (TMRE) is a positively-charged, lipophilic dye that could penetrate the mitochondrial membrane and is retained within the negatively-charged mitochondrial matrix⁴²⁹. TMRE can be excited at a wavelength of 488nm and emits an orange-red fluorescence at a wavelength of 575nm. In this regard, dissipation and depolarisation of mitochondrial membrane potential (i.e. less negative) from mitochondrial dysfunction or damage results in a reduced ability to retain TMRE whereas active, healthy mitochondria display strong TMRE fluorescence signals^{429, 430}.

Adherent Neuro-2a cells at 60-70% confluency were incubated with TMRE staining solution (working concentration 100nM; ab113852, Abcam) diluted in complete DMEM culture medium for 30 minutes in dark at 37°C. Neuro-2a cells were washed with phosphate-buffer saline (PBS, pH7.4) containing 0.2% bovine serum albumin (BSA), and were detached from culture plate by Accutase detachment solution (00-4555-56; ThermoFisher Scientific). Neuro-2a cells were suspended in PBS with 0.2% BSA, and TMRE fluorescence was

detected by PE channel (PMT 400) on a flow cytometer (BD LSR-II) and mean fluorescence intensity (MFI) was compared between groups.

2.10.3 Propidium Iodide and Annexin-V Staining

Propidium iodide (PI) and Annexin-V (ANV) double staining represents a routine flow cytometry application for differentiation between apoptotic and non-apoptotic cell populations. In brief, the apoptotic cells will be labelled by annexin V (ANV) that binds to the phosphatidylserine (PS) moiety expressed on the membrane surface of apoptotic cells^{431, 432}. Annexin V can be conjugated with FITC fluorochrome and ANV-positive cells will emit green fluorescence at 519nm when excited by the argon ion laser at 488nm. Propidium iodide is a nucleic acid dye with an emission wavelength of 615nm (orange-red) and can be detected by the PE-Texas Red channel on flow cytometer. PI only enters a cell when the plasma membrane is compromised, thus PI-stained cells are regarded as dead/dying cells. In this regard, apoptotic cells will be annexin V-positive or annexin V/PI-double positive, whereas non-apoptotic cells will only be stained by PI.

Neuro-2a cells were detached from cell culture plate using Accutase detachment solution (00-4555-56; ThermoFisher Scientific) before staining with the FITC Annexin V Apoptosis Detection Kit with PI (640914; BioLegend), as per the manufacturer's instructions. In brief, Neuro-2a cells were resuspended in Annexin V binding buffer (containing Ca^{2+} ion) at a concentration of approx. $1\text{-}5 \times 10^6/\text{mL}$, and 100 μL cell suspension was collected to incubate with 5 μL of FITC-Annexin V in dark at 37°C for 20 minutes. 10L propidium iodide (PI) was added into the reaction mix 5 minutes prior to flow cytometric analysis. Annexin V and PI were analysed by the FITC (PMT voltage 300) and PE-Texas Red (PMT voltage 450) channels on a flow cytometer (BD LSR-II). Percentages of PI-positive cells and FITC/PI-double positive cells were quantified as non-apoptotic and apoptotic populations, respectively.

2.11 Cell Viability by Cell Counting Kit-8 (CCK8)

Cell counting kit-8 (CCK-8) is a convenient and sensitive assay for determining overall cell viability and is routinely used for screening of cytotoxic reagents. CCK-8 kit comprises of the water-soluble tetrazolium salt WST-8, [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt, which upon reduction by the dehydrogenases of the electron transport chain produces an orange-coloured dye (formazan) that can be detected by colorimetric plate reader⁴³³. Therefore, the intensity of the orange dye produced is dependent on the quantity and activity of dehydrogenases in the incubated cells, which is proportional to the number of viable, respiring cells.

Neuro-2a cells were plated into 96-well cell culture plate at a density of 5×10^4 /mL (approx. 5000 cells per well) and were allowed to attach. 10 μ L of CCK-8 reagent was added into each well to reach a total reaction volume of 110 μ L (10 μ L CCK-8 + 100 μ L complete DMEM medium), and Neuro-2a cells were incubated with CCK-8 in dark at 37°C for 2 hrs until the end of reperfusion. Wells without cells were included as blank controls (BC), and 10 μ L of CCK-8 was added to 100 μ L DMEM medium and similarly incubated for 2 hrs. The development of colour is read with a microplate reader at 450nm, and readings from blank controls (BC) were averaged and subtracted from readings from all other wells. Thereafter, the values from naïve control (NC) group were averaged to represent 100% viability, and reading from OGD/Rep and treated groups were expressed as percentage viability relative to that of NC.

$$\% \text{ viability} = \frac{(\text{treatment} - \text{blank control})}{(\text{NC} - \text{blank control})}$$

2.12 Murine Model of Hypoxic-Ischaemic Encephalopathy (HIE)

2.12.1 Animals

The animal study was conducted in collaboration with Professor Xingrong Song from Guangzhou Children-Women Hospital, China and Professor Libing Zhou from Guangdong-Hongkong-Macau Institute of CNS Regeneration at Jinan University, Guangzhou, China. All animal experiments were carried out in accordance with the guidelines of the Animal Experiment Ethics Committees at Jinan University, Guangzhou, China and were performed in ways to minimise the animals' suffering and the number of animals used. Both sexes of wildtype C57BL/5 mouse pups on postnatal day 7 were used for this study. A litter size of six to eight per dam were used and housed with 12 hours light/dark cycle in a temperature and humidity-controlled room. The pups were housed with their dams until weaning on postnatal day 21, and thereafter were housed in a group of four per cage and separated by sex until postnatal day 30.

2.11.2 Animal Surgery and Hypoxia Exposure

Hypoxia-ischaemia was induced with modifications to the Rice-Vannucci's procedure. At postnatal day 7 (PND7), the mouse pups underwent right common carotid artery (CAA) coagulation followed by global hypoxia exposure to 8% O₂, with reference to previous studies⁹²⁻⁹⁵. Surgical anaesthesia was induced by placing the mouse pup into a sealed container with 3% isoflurane, and anaesthesia was maintained with 2% isoflurane delivered through a mouthpiece with minimal leakage, where isoflurane was balanced with 70% N₂O and 30% O₂. A vertical midline neck incision was performed to expose the right CCA, and a bipolar electrode forceps was used to coagulate the right CCA under a surgical microscope, with the total surgical time limited to 20 minutes. Coagulation was employed instead of ligation in view of the small size of the mouse pups and the brief surgery time, provided that both methods result in permanent disruption of blood flow into the ipsilateral hemisphere. Caution was taken to avoid damage to the vagus nerve that runs parallel to the CCA. The mice were allowed to recover and feed for 1.5 hrs with their dams.

To induce global hypoxia, the mice pups were then placed into a sealed plastic chamber partially submerged into a heated water bath with a constant temperature between 36-36.5°C, and the chamber was perfused with humidified 8% O₂ balanced with N₂ (FiO₂=0.08) for 45 minutes. **Fig.2-9A** depicts the CCA coagulation surgery and hypoxia procedures performed to elicit cerebral hypoxia-ischaemia (referred to as H/I). Sham-operated mice only received vertical midline neck incision and exposure of CCA under isoflurane anaesthesia, without CCA coagulation and hypoxia. After hypoxia the pups were returned to dams, and at 24 hrs and 72 hrs of reperfusion histological, biochemical and/or behavioural evaluations were performed to assess injury development. The described procedure of CCA coagulation plus 8% hypoxia carries an overall mortality of approx. 10% during or immediately after hypoxia, which we consider to be pragmatic and ethical to be adopted for subsequent studies.

2.12.3 Treatment with RIPK1 inhibitor 7-ClO-O-Nec-1/Nec-1s

To investigate the effect of RIPK1 inhibition on HIE in neonatal mice, additional group of H/I-injured mice were given the commercially available RIPK1 inhibitor 7-ClO-O-Nec-1/Nec-1s (CAS number 852391-15-2; Ab229184; Abcam, UK). 7-ClO-O-Nec-1 was pre-dissolved in DMSO to a stock concentration of 8mM, and for each mouse 1 μ L of 7-ClO-O-Nec-1 (8nmol) was administered into the ipsilateral hemisphere via intracerebrovascular (i.c.v) route^{323, 324}. For vehicle control, 1 μ L of DMSO was injected per mouse similarly through i.c.v. Intracerebrovascular injection was performed 15 minutes after hypoxia exposure and mice was re-anaesthetised by isoflurane, in accordance with steps outlined by Kim et al⁴³⁴. The injection site was identified as 2/5 the distance from lambda suture to the eye that is ipsilateral to CCA coagulation, before injecting the treatment solution (7-ClO-O-Nec-1 or DMSO) through a 10 μ L injection syringe with a 32-gauge needle (**Fig. 2-9B**).

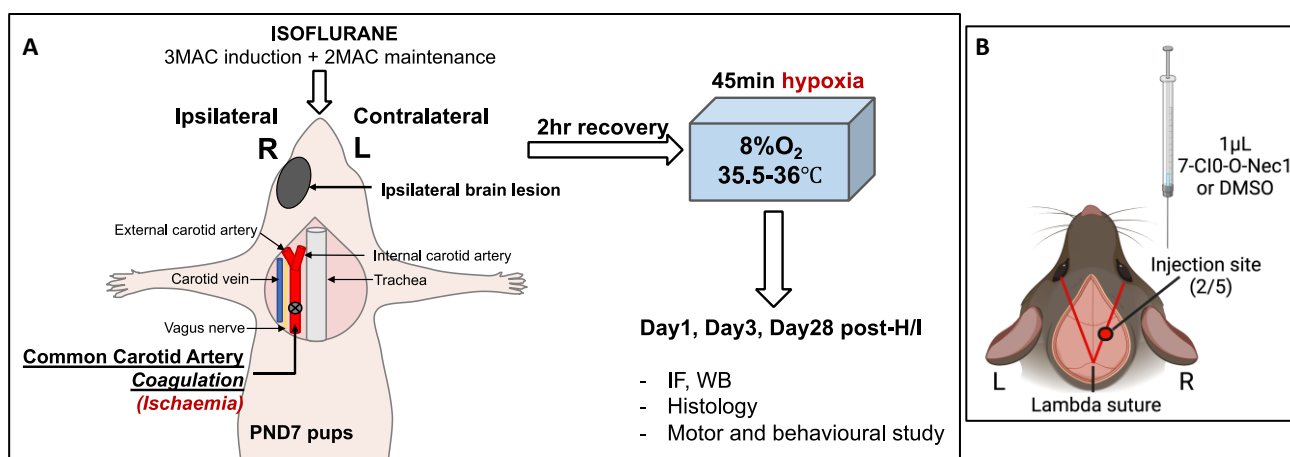


Figure 2-9. Induction of cerebral hypoxia-ischaemia in postnatal day 7 C57BL/6 mice and intracerebrovascular treatment. (A) Murine model of hypoxic-ischaemic encephalopathy (HIE), whereby the right common carotid artery is electro-coagulated before 45 minutes of hypoxia at 8% O₂ with an ambient temperature between 36°C to 36.5°C. **(B)** Intracerebrovascular (i.c.v) delivery of Nec-1s (80 μ mol) or the vehicle DMSO into the ipsilateral hemisphere, using the indicated landmarks to locate the delivery site.

2.12.4 Motor and behavioural assessments

Sensorimotor reflexes were assessed at day1 post-H/I (PND8) to confirm the development of cerebral H/I injury. Negative geotaxis¹¹⁶⁻¹¹⁸ (**Fig. 2-10A**) and cliff aversion^{116, 117} (**Fig. 2-10B**) are representative unlearned, automatic reflexes that are triggered by vestibular cues and require motor strength and coordinated movements. In this regard, negative geotaxis and cliff aversion performances were shown to be sensitive to cerebral H/I injury in murine models of HIE. During negative geotaxis, the rodent was placed head down on a 45° inclined board covered in rough clothes, and the mean time taken to rotate 180° to face up and climb the slope was recorded from three trials, with a maximum observation time of 60 seconds for each trial and trials were separated by 15 minutes. In pups with defective vestibular function to sense the downward slope

and/or significant motor impairment, they may fail to elicit directional movement (taxis) or even slide down the slope; maximum time (60s) was assigned if the mouse fails to display the reflex within the allocated time. For elicit the cliff aversion response, the pup was placed with snout and forepaws overhanging the cliff, and the mean time taken for the snout and paws to move away from the cliff edge is recorded from three trials, with each trial lasting a maximum of 60 seconds and trials separated by 15 minutes. For mouse that was unable to rotate away within the allocated time the maximum time was recorded.

At day21-23 post-H/I (correspond to PND28-30), coordinate motor movement and spontaneous locomotion were examined in juvenile mice with accelerating rota-rod test (**Fig. 2-10C**) and open field test (OPF; **Fig. 2-10D**), respectively. In the present study, young mice between PND28 and PND30 (day21 to day23 after cerebral H/I) underwent three consecutive days of accelerating rota-rod test, whereby on each day three accelerating trials were conducted with 20 minutes inter-trial breaks. For each trial, mice were placed on a rotating rod (Ugo Basile, Italy) with a starting speed of 4 rpm (rotation per minute) that accelerates to 40 rpm over the course of 5 minutes, as adapted from previous studies which demonstrated significantly shorter latency to fall in H/I-injured animals than their control cohorts^{100, 112-114}. In a single test, the time for mouse to fall off or to complete the first revolution (whilst holding the rod) was recorded, or until 5 minutes have elapsed if the mouse did not fall. The averaged latency to fall/to first revolution from day3 was quantified and presented.

Open field test was performed at day23 days post-H/I (PND30) to evaluate general locomotion and thigmotaxis, whereby mice were placed into the centre of an open-top, plexiglass container (50cm x 50cm x 40cm) consisting of 25 equal-sized squares, with the outer 16 squares designated as outer zone and the inner 9 squares designated as inner zone⁴³⁵. An increase in the distance travelled and or time spent in the outer zone relative to the inner zone is considered as increased thigmotaxis and is related to heightened anxiety-like behaviours⁴³⁵. The mice were allowed to travel freely inside the container for 15 minutes, and distances and traces were recorded by with a video camera and analysed using EthoVision XT 8.0 (Noldus, Wageningen, Netherlands). Total distance travelled, average velocity, distance travelled in the centre (inner zone) and distance travelled in the periphery (outer zone) during the 15 minutes period were recorded and compared between groups.

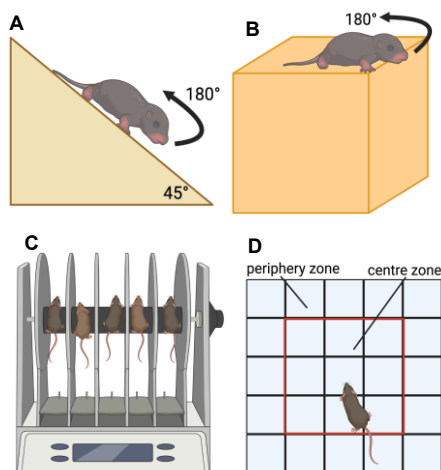


Figure 2-10. Motor and behavioural tests used to assess cerebral H/I injury. At day1 after H/I (postnatal day 8), sensorimotor reflexes such as (A) negative geotaxis and (B) cliff aversion were examined to confirm the development of cerebral H/I injury. At day21 to day23 after H/I (postnatal day 28 to 30), mice underwent (C) accelerating rota-rod and (D) open-field test to assess long-term motor and behavioural outcomes following HIE.

2.13 Statistical Analysis

For all experimental data, data from each experimental group was tested for normality and deviation from the Gaussian distribution using the D'Agostino-Pearson normality test, and a QQ-plot is generated for each group of data. The null hypothesis is that all data sampled from a population follows a Gaussian distribution. When $P > 0.05$, the null hypothesis is accepted, and the data is considered to be normally distributed; when $P < 0.05$, the null hypothesis is rejected, and the data is treated as non-normally distributed. If all groups of data from a single experiment follow a Gaussian distribution, parametric tests (Student t-test, One-Way ANOVA followed by Tukey's post-hoc test) will be used for comparison analysis between groups. If data from one or more groups fail to pass the normality test, non-parametric tests (Mann-Whitney U test, Kruskal-Wallis test with Dunn's post-hoc test) will be used for comparison analysis between groups. For behavioural test with repeated measurements over days, two-way ANOVA with mixed-effect analysis followed by Tukey's multiple comparison test was used. Normally-distributed data sets were expressed as mean $\pm$ standard deviation (SD) and dot plot, and non-normally distributed data sets were expressed as median $\pm$ interquartile range (IQR) and dot plot. A two-tailed p-value < 0.05 was considered to be statistically significant. All analysis was performed with Graphpad Prism 9.0 (La Jolla, USA).

For *in vitro* study, each n represents an independent cell experiment and at least 3 independent experiments ($n=3$) were performed for each set of data. For semi-quantitative analysis of *in vitro* immunofluorescence staining, each n corresponds to 3 replicates of the same experiment and represents the averaged value from 3 replicates. For *in vivo* study, each n represents an individual animal and each experiment included at least 4 animals ($n=4$) for statistical analysis.

CHAPTER THREE

Oxygen-Glucose Deprivation and Reperfusion (OGD/Rep) Upregulated Necroptosis and Mitophagy in Mouse Primary Neurons and Neuro-2a cells *in vitro*

3.1 Introduction

Necroptosis has been extensively implicated in a wide range of CNS disorders, from acute traumatic injury such as hypoxic/ischaemic injury and traumatic brain injury to chronic neurodegenerative, inflammatory conditions, as exemplified by multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD) and Parkinson's Disease (PD)^{276, 315, 316, 436-438}. As a non-apoptotic form of regulated cell death, necroptosis was firstly identified in a murine stroke model with permanent middle cerebral artery occlusion (MCAO)³¹⁷, and was succeeded by numerous studies confirming the pathogenic contribution of necroptosis in ischaemic/ischaemia-reperfusion injury of other solid organs^{271, 439}. Moreover, a murine transient MCAO study revealed that RIPK1 kinase mediates the sequential activation necroptosis and apoptosis in the ischaemic penumbra, with early phosphorylation of RIPK3 and MLKL in neurons and endothelial cells at 1-2hrs of reperfusion, followed by caspase 3 cleavage in neurons at 5-24 hrs of reperfusion²⁶⁶.

The small molecule inhibitor necrostatin-1 (Nec-1) was shown to confer remarkable therapeutic benefit in the initial MCAO/stroke studies with attenuated necroptotic neuronal death³¹⁷, and its mechanism of action was later revealed to be selective targeting of the receptor-interacting protein kinase 1 (RIPK1)³¹⁸. Studies on MS, ALS, AD and PD also corroborated that RIPK1 inhibition by Nec-1 is of great therapeutic potential by limiting necroptotic loss of neurons and oligodendrocytes (i.e. reduced demyelination and axonal degeneration), together with suppressed inflammatory responses from activated microglia^{276, 316, 437, 438}. Moreover, RIPK1 kinase-dead mutation (homozygous RIPK1^{D138N/D138N}) but not RIPK3 deficiency was shown to reduce ischaemic infarct and microgliosis in a murine model of transient MCAO (stroke); nonetheless, RIPK3 knockdown still prevented intracerebral haemorrhage and behavioural impairment as with RIPK1^{D138N/D138N}²⁶⁶. RIPK1 kinase-dead mutation, and to a lesser extent RIPK3 silencing, also suppressed neuroinflammatory responses (TNF- α , IL-6) in the ischaemic cortex²⁶⁶. Provided that RIPK1 activation drives both apoptosis and necroptosis, whereas RIPK3 exclusively promote necroptosis, inhibition of RIPK1 kinase activity represents a more effective and favourable therapeutic strategy in ischaemia/reperfusion brain injury. Furthermore, the upregulated expressions of RIPK1, RIPK3 and MLKL and the enhanced association between necrosome components from lesioned tissues from experimental models or patient samples of stroke, MS or ALS further corroborated the pathological involvement of necroptosis and related inflammatory pathway in these CNS conditions²⁷⁶.

Evidences are also emerging on the pathological involvement of necroptosis in hypoxic-ischaemic injury of the immature brain. In an *in vitro* model on HIE, where foetal primary hippocampal neuronal culture was subjected to 2hr of oxygen glucose deprivation (OGD) and reperfusion, neuronal expression of RIPK3 was strongly upregulated, and forced overexpression of RIPK1 or RIPK3 exacerbated neuronal loss that could be attenuated by the RIPK1 inhibitor Nec-1³²². In *in vivo* HIE study using PND7 mice, Nec-1 treatment

(intracerebrovascular injection) immediately after cerebral H/I was found to reduce cerebral, thalamic and hippocampal infarcts³²³, as well as suppressing NFκB-dependent pro-inflammatory cytokine synthesis (TNF-α, IL-1β, IL-6)³²³.

Attention should be drawn to the fact that some of the early studies had used the first-generation Nec-1, which has very poor metabolic stability and was later shown to additionally inhibit indoleamine-2,3-dioxygenase (IDO)^{417, 440}. Thus, caution must be exercised when interpreting results from studies using the original Nec-1, in view of the off-target inhibition on IDO and the limited bioavailability. The chemically improved Nec-1 analogue 7-Cl-O-Nec-1 or Nec-1s (Nec-1 stable) is exclusively selective towards RIPK1 and effectively prevented RIPK1/RIPK3 phosphorylation and necroptotic cell death, while exhibiting satisfactory metabolic stability and pharmacokinetics *in vivo* (plasma half-life $T_{1/2}$ ~1hr and plasma-to-brain penetration)^{417, 441}. Specifically, Nec-1s was shown to inhibit necroptosis with EC_{50} = 0.21μM compared to EC_{50} = 0.49μM of the original Nec-1 to demonstrate increased inhibitory activity⁴⁴¹. With reference to previous studies^{417, 418}, 10μM Nec-1s was chosen in the current study to investigate the effect of RIPK1 inhibition on mitophagy during OGD/Rep injury in Neuro-2a cells.

Small molecule screening has identified several RIPK3 inhibitor candidates such as GSK'840, GSK'843 and GSK'872 with favourable pharmacodynamics profiles. When compared to GSK'843, GSK'872 exhibited superior affinity towards recombinant human RIPK3 kinase domain (IC_{50} =1.8nM vs. IC_{50} = 8.6nM) and more effectively inhibited RIPK3 kinase activity in ADP-Glo assay (IC_{50} =1.3nM vs. IC_{50} =6.5nM)⁴¹⁹. Importantly, GSK'872 effectively blocked necroptotic cell death in a dose-dependent manner in both human and murine cells treated with TNF-α + caspase inhibitor (carbobenzoxycarbonyl-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone, zVAD), whereas GSK'840 was ineffective in murine cells despite having the best pharmacodynamic profiles towards human RIPK3 protein. GSK'872 was additionally shown to inhibit TRIF/RIPK3- and DAI/RIPK3-mediated necroptosis cell death that was not responsive to RIPK1 inhibition⁴¹⁹. Paradoxically, GSK'872 was cytotoxic at higher doses (3-10μM) by promoting the formation of the large ripoptosome complex comprising RIPK1, RIPK3, FADD and caspase-8 to trigger RIPK3-dependent apoptosis. In view of the foregoing, 1μM of GSK'872 was chosen to investigate the effect of RIPK3 inhibition on mitophagy in Neuro-2a cells during OGD/Rep.

Unlike its conventional kinase counterparts, the kinase-like domain (KLD) of MLKL lacks the key catalytic motif essential for phosphoryl transfer from ATP substrate⁴⁴². Upon recruitment into the necrosome, the C-terminal KLD of MLKL associates with the kinase domain of RIPK3 before phosphorylation of MLKL at Thr357 and Ser358 residues within its activation loop²⁹⁶ (Ser345 residue of mouse MLKL)³⁰⁷. Studies demonstrated that KLD phosphorylation would prompt MLKL disengagement from RIPK3⁴⁴³, and that phosphorylation-induced conformational change will cause dimerization between MLKL KLDs followed by self-assembly of the

internal coiled-coil domains in favour of MLKL oligomerisation⁴⁴⁴. The MLKL-selective inhibitor necrosulfonamide (NSA) was shown to be highly effective against necroptosis induced by the combination of TNF- α , SMAC mimetic and pan-caspase inhibitor in human cells^{296, 312}. Covalent binding of NSA to human MLKL at cysteine 86 residue was shown to halt the progression of necroptosis at the stage of RIPK3 puncta formation in the cytoplasm²⁹⁶, which concurs with later studies showing that NSA blocks plasma membrane translocation of MLKL oligomers^{312, 445, 446}. NSA may also block necroptosis by impairing the formation of high molecular-weight MLKL polymers⁴⁴⁷, which likely depends on the intact N-terminal domain that is targeted by NSA. Still further, it is unclear whether and to what extent NSA inhibits MLKL phosphorylation^{312, 446, 447}, a step that precedes the unfolding of N-terminal 4HBD domain.

On the other hand, NSA failed to attenuate TNF- α + caspase inhibitor-induced necroptosis in murine cells even when administered at 10-fold higher concentration than that used to inhibit necroptosis in human cells. It was then revealed that for mouse MLKL, the N-terminal Cys86 residue of the human homolog indispensable for binding is replaced by a tryptophan residue^{296, 448}, which renders murine MLKL untargetable by NSA. Small-silencing RNA mediated knockdown (siRNA) of MLKL offers an alternative to temporary elimination of MLKL activity especially in non-human cells. In this regard, knockdown of endogenous MLKL expression by siRNA oligos prevented necroptosis at comparable efficiency to NSA, and similarly arrested necroptosis at the step of RIPK3 puncta formation after TNF- α + caspase inhibitor treatment²⁹⁶. Therefore, we will introduce mouse MLKL-specific siRNA oligos to transiently downregulate the expression of MLKL in Neuro-2a cells, before subjecting the cells to OGD/Rep.

With respect to mitophagy, studies have predominantly reported a neuroprotective role of mitophagy (along with autophagy) in hypoxic-ischaemic injury of the adult brain, whereby pharmacological blockade of mitophagy (mdivi-1) or genetic silencing of Parkin and/or BNIP3 aggravated brain lesion. Upregulation of mitophagy and autophagy was similarly observed in the immature brain during the reperfusion phase after H/I insult. Efficient mitophagy eliminates defective mitochondria and prevents accumulation of ROS and release of pro-apoptotic factors, and in conjunction with increased mitochondrial biogenesis maintain a healthy pool of mitochondria to fulfil the energy requirement of neurons. Thus, alteration to mitophagy from manipulation of the different necrosome components is expected to have a direct impact on mitochondrial function and consequently also on neuron survival. The mitochondrial transmembrane potential ($\Delta\Psi_M$) has been routinely used as a marker of mitochondrial function, and a healthy mitochondrion typically maintains $\Delta\Psi_M$ at -180Vm with negatively charged matrix⁴⁴⁹. Dissipation of the mitochondrial potential seen as a less negative matrix charge therefore is indicative of mitochondrial dysfunction, as exemplified by electron transport chain inhibition or mitochondrial membrane permeabilisation⁴⁴⁹. Moreover, mitochondrial morphology is tightly linked to mitochondrial function, whereby the transition from an elongated, fibrous appearance to a granular and truncated phenotype is characteristic of H/I brain injury^{325, 326}, and we speculate

mitochondrial morphology to be malleable and sensitive to necroptosis inhibition and corresponding alterations to mitophagy.

Aims, hypothesis and objectives

In this Chapter, our first aim is to confirm the concurrent activation of necroptosis and mitophagy in mouse primary neurons and mouse neuroblastoma Neuro-2a cells following oxygen-glucose deprivation (OGD) as an *in vitro* model of HIE. Primary neurons or Neuro-2a cells will be subjected to either pure OGD or OGD plus reperfusion to allow comparison of different injury mechanisms, provided that reperfusion is associated with unique molecular events (e.g. oxidative stress, secondary energy failure) and exacerbated injury phenotype. we hypothesise that OGD or OGD/Rep would simultaneously stimulate necroptosis and mitophagy together with mitochondrial translocation of necrosome proteins, with the following specific objectives:

Objective 1 – 1) To confirm MLKL phosphorylation as indicator of necroptosis, and 2) to verify translocation of phos-MLKL, MLKL and RIPK3 in mouse primary neurons and Neuro-2A cells (mouse neuroblastoma) following OGD +/- reperfusion;

Objective 2 – 1) To quantify the cytosolic and mitochondrial expression of mitophagy mediators (e.g. PARKIN, CLpP), and 2) to quantify LC3A/B-II level in the mitochondrial fraction from primary cultured neurons and Neuro-2A cells, following OGD +/- reperfusion;

Our second aim is to individually investigate the effect of RIPK1 inhibition, RIPK3 inhibition or MLKL knockdown on OGD/Rep-induced necrosome translocation and upregulation of mitophagy mediators in Neuro-2a cells, using the pharmacological inhibition or siRNA-mediated silencing strategies described herein. We also aim to examine potential MLKL interaction with the mitophagy mediators Parkin, BNIP3 and CLpP. We hypothesise that necroptosis acts upstream to regulate I/R-induced mitophagy in Neuro-2a cells, and that MLKL exhibits direct interaction with mitophagy mediators. Our specific objective is as follow:

3 – 1) To quantify cytosolic and mitochondrial expression of mitophagy mediators and LC3A/B co-localisation to the mitochondria following OGD plus reperfusion, when necroptosis is blocked through pharmacological inhibition of RIPK1 or RIPK3 or genetic knockdown MLKL; 2) To examine MLKL interaction with mitophagy mediators (Parkin, BNIP3, CLpP) through co-immunoprecipitation.

Third, we aim to examine the effects of necroptosis inhibition on mitochondrial function and overall cell viability during OGD/Rep injury, with the specific objective:

4 – To assess mitochondrial membrane potential, mitochondrial morphology, cell survival and relative contribution of apoptotic and non-apoptotic cell death in Neuro-2a cells subjected to OGD plus reperfusion,

and when necroptosis is blocked through 1) chemical inhibition of RIPK1 or RIPK3, or 2) genetic knockdown MLKL;

3.2 Results

3.2.1. Oxygen-glucose deprivation plus reperfusion induced necroptosis and mitochondrial localisation of LC3A/B in mouse primary neurons

As seen under the light microscope, primary mixed cortical neuronal culture from embryonic day 17.5 mice foetuses developed extensive projections and inter-cluster connections. Brief OGD or OGD/Rep led to significant loss in cell number and neuronal projections and disrupted inter-cluster connections (**Appendix, Supplementary Fig.1**). Immunofluorescence staining demonstrated that OGD/Rep, but not OGD, significantly increased the number of phosphorylated-MLKL (p-MLKL) positive neurons (NC vs. OGD/Rep, $P<0.001$) and induced p-MLKL translocation to the plasma membrane as well as p-MLKL localisation with mitochondria (**Fig. 3-1A**, arrowheads; **Fig.3-1B**). Treatment with RIPK1 inhibitor necrostatin-1s (Nec-1s, 10 μ M) for 1hr upon reperfusion reduced MLKL phosphorylation and translocation (**Fig. 3-1A, 3-1B**; OGD/Rep vs. OGD/Rep+Nec-1s, $P=0.042$) and attenuated cell loss (**Appendix, Supplementary Fig.1**), to support that OGD/Rep induced RIPK1-dependent necroptotic cell death (necroptosis) in primary neuron culture. Provided that OGD alone did not induce MLKL phosphorylation and Nec-1s treatment still slightly reduced cell loss, it can be inferred that pure hypoxia/ischaemia does not induce neuronal necroptosis and that neuroprotection from RIPK1 inhibition is elicited through alternative mechanisms (e.g. reduced oxidative stress).

Double immunofluorescence staining was performed to visualise LC3A/B localisation to mitochondria to reflect the level of neuronal mitophagy, where mitochondria were labelled with antibody against the structural protein TOM20 (mitochondrial outer membrane transporter 20). The immunofluorescence intensity of the autophagosome marker LC3A/B was increased by OGD or OGD/Rep and corresponded to increased percentage of cells displaying LC3A/B and TOM20 co-localisation (**Fig. 3-2A, 3-2B**), which is indicative of enhanced mitophagy; compared with OGD/Rep, OGD was associated with greater LC3A/B increase and co-localisation (NC vs. OGD, $P=0.026$). Nec-1s treatment slightly decreased LC3A/B immunofluorescence intensity and LC3A/B co-localisation with TOM20 in OGD and OGD/Rep groups (**Fig. 3-2A, 3-2B**), to suggest RIPK1 as a positive regulator of mitophagy during H/I or I/R injury.

Primary neurons were additionally stained with the mitochondrial caseinolytic protease CLpP. As discussed in Chapter one, section F.3.4, the mitochondrial chaperone CLpP may also participate in mitophagy in addition to its more established role in the degradation of misfolded proteins. As expected, OGD and OGD/Rep significantly increased the immunofluorescence intensity of CLpP compared to NC (**Fig. 3-3A, 3-3B**; NC vs. OGD, $P=0.015$; NC vs. OGD/Rep, $P=0.012$), which likely reflects elevated mitochondrial stress and increased need for mitophagy. Moreover, Nec-1s was more effective at attenuating CLpP upregulation from OGD/Rep than from OGD (**Fig. 3-3A, 3-3B**; OGD/Rep vs. OGD/Rep+Nec-1s, $P=0.040$), to imply that CLpP response during OGD/Rep is more dependent on RIPK1 signalling than during pure OGD.

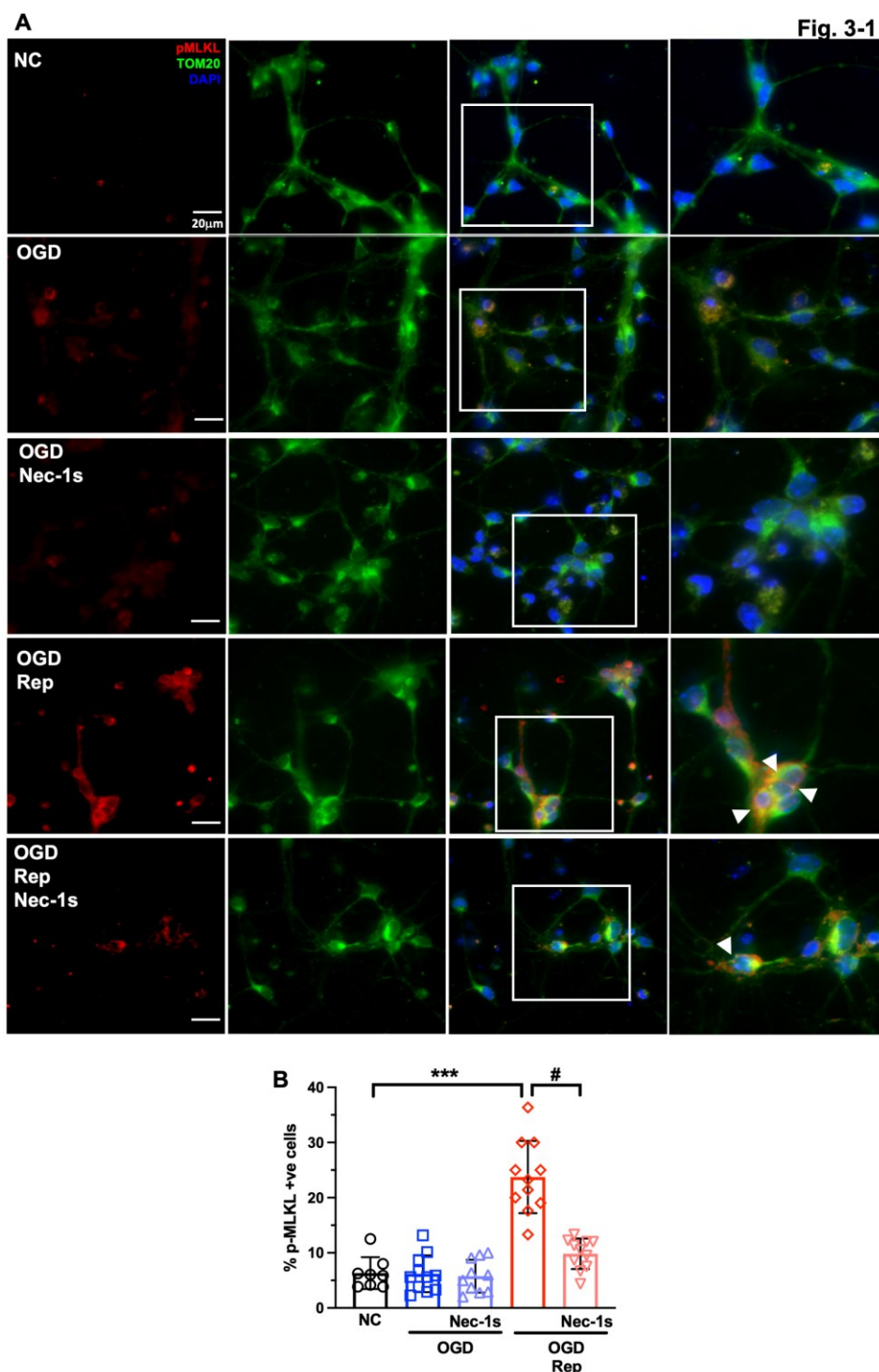


Figure 3-1. Immunofluorescence staining of phosphorylated MLKL in primary neurons. (A) Mouse primary neurons were subjected to 1hr oxygen-glucose deprivation (OGD) or 1hr OGD plus 1hr reperfusion (OGD/Rep) with or without necrostatin-1s (Nec-1s, 10 μ M) treatment, and were double labelled with phosphorylated mixed lineage kinase-domain like (p-MLKL, red) and the mitochondrial outer membrane transporter 20 (TOM20, green); boxed areas from merged photomicrographs were enlarged. Arrowheads: p-MLKL positive cells showing plasma membrane translocation and mitochondrial localisation of p-MLKL signals. Magnification x63, scale bar 20 μ m. **(B)** Percentage of p-MLKL positive neurons, data expressed as mean $\pm$ SD. NC vs. OGD/Rep: *** $P < 0.001$; OGD/Rep vs. OGD/Rep+Nec-1s: # $P < 0.05$. $n = 8-11$, each n represents an independent experiment, and each n is an average of 3 replicates.

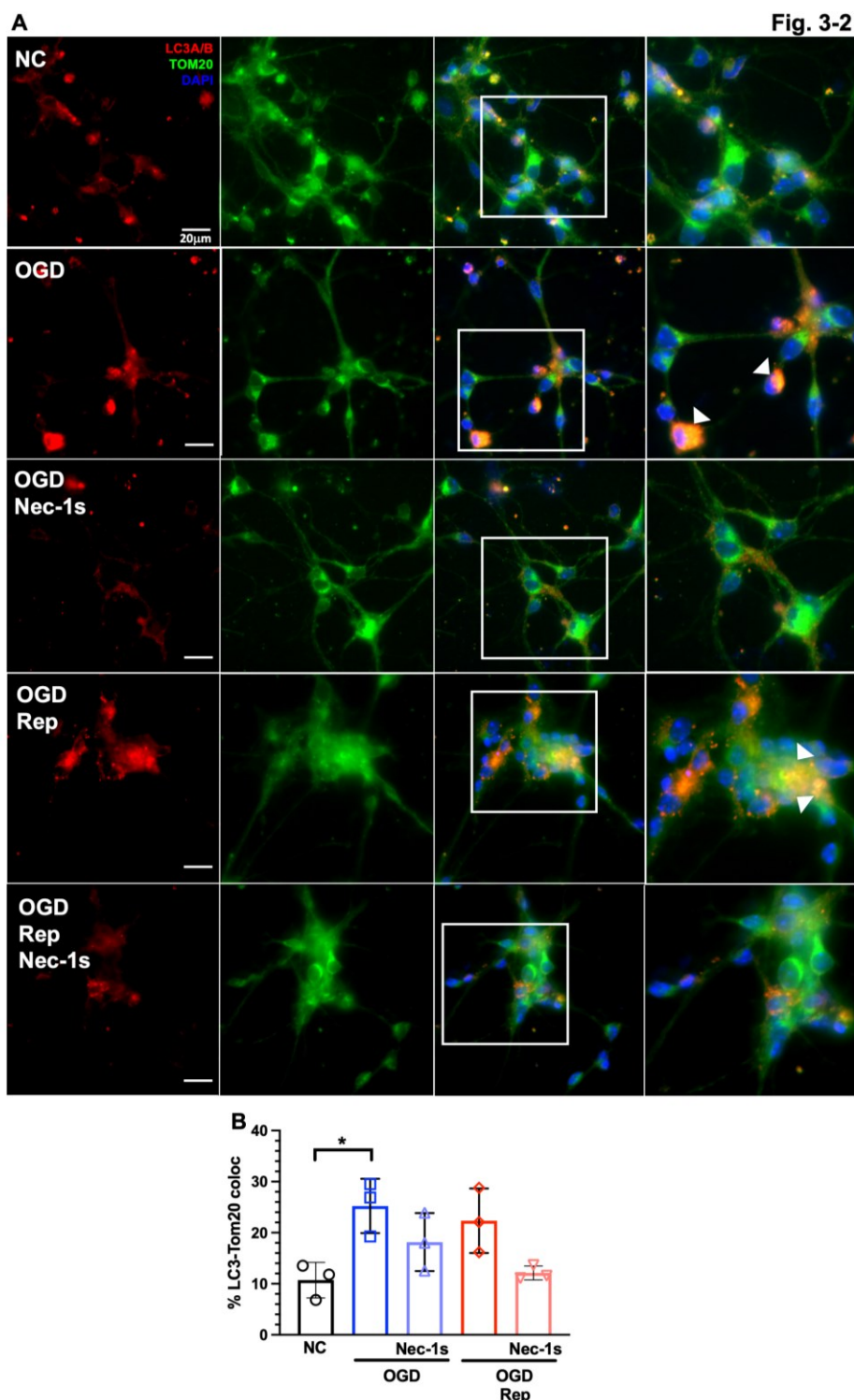


Figure 3-2. Double immunofluorescence staining of LC3A/B and TOM20 in primary neurons. (A) Mouse primary neurons were subjected to 1hr oxygen-glucose deprivation (OGD) or 1hr OGD plus 1hr reperfusion (OGD/Rep) with or without necrostatin-1s (Nec-1s, 10 μ M) treatment, and were double labelled with the autophagosome marker microtubule-associated protein light chain 3A/B (LC3A/B, red) and mitochondrial outer membrane transporter 20 (TOM20, green) to visualise LC3A/B localisation to mitochondria. Boxed area from merged photomicrographs were enlarged. Arrowheads: cells showing LC3A/B and TOM20 co-localisation to indicate mitophagy. Magnification x63, scale bar 20 μ m. (B) Percentage of primary neurons showing LC3A/B and TOM20 co-localisation, data expressed as mean $\pm$ SD. NC vs. OGD: * $P < 0.05$. $n = 3$, each n represents an independent experiment, and each n is an average from 3 replicates.

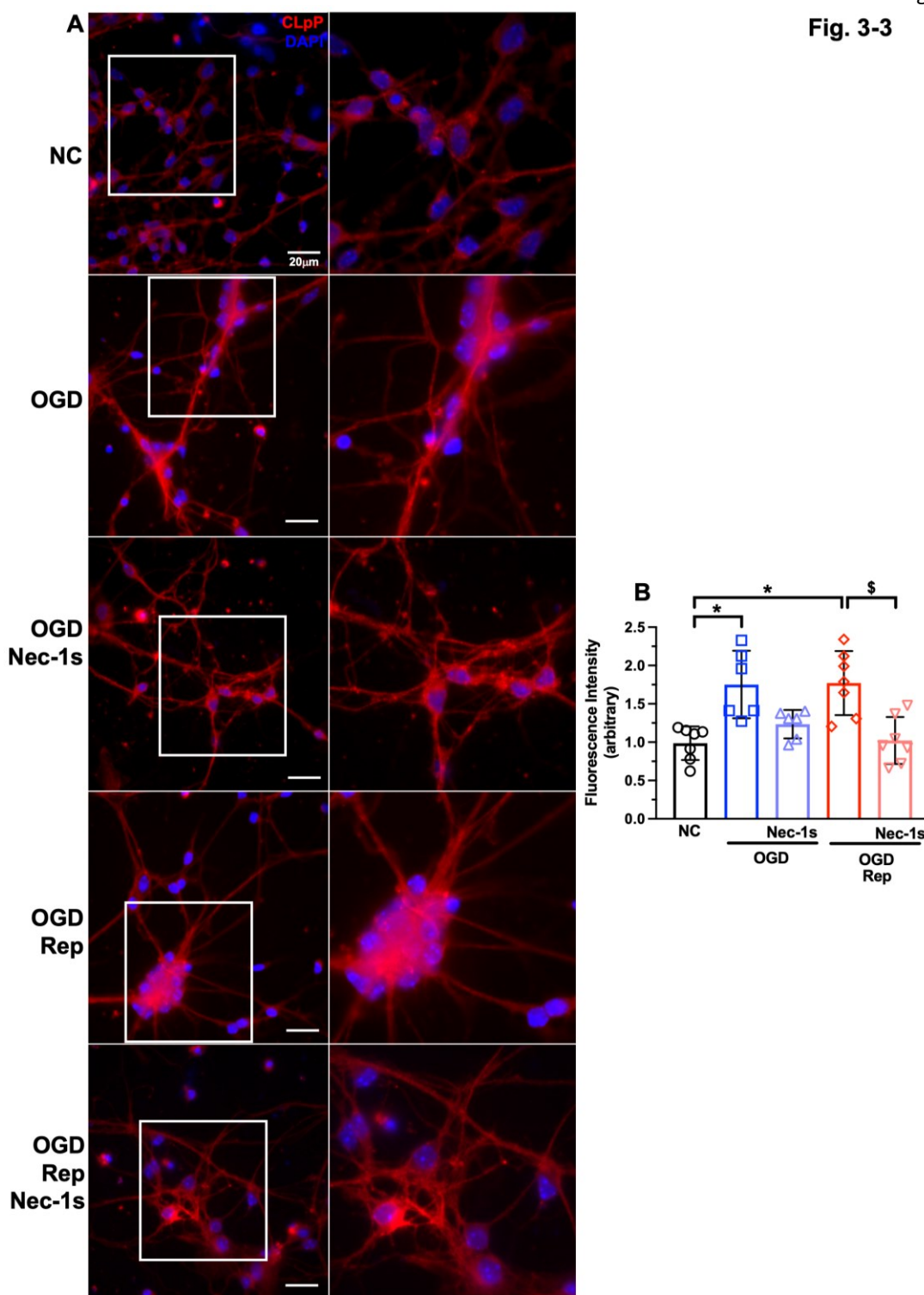


Figure 3-3. Immunofluorescence staining of CLpP in primary neurons. (A) Mouse primary neurons were subjected to 1hr oxygen-glucose deprivation (OGD) or 1hr OGD plus 1hr reperfusion (OGD/Rep) with or without necrostatin-1s (Nec-1s, 10μM) treatment, and were labelled with the mitochondrial caseinolytic protease proteolytic subunit (CLpP). Boxed areas from the photomicrographs were enlarged. Magnification x63, scale bar 20μm. (B) Immunofluorescence intensity of CLpP expressed as relative change to NC in arbitrary unit. Data expressed as mean±SD. NC vs. OGD, *P<0.05; NC vs. OGD/Rep, *P<0.05; OGD/Rep vs. OGD/Rep+Nec-1s, § P<0.05. n=6-7, each n represents an independent experiment, and each n is an average from 3 replicates.

Western blot analysis on primary neuron whole-cell lysate was performed to corroborate the findings from immunofluorescence staining. MLKL phosphorylation was markedly induced by OGD/Rep (Fig. 3-4A, 3-4B; NC vs. OGD/Rep, $P<0.001$). OGD/Rep-associated MLKL phosphorylation was attenuated by Nec-1s treatment (Fig. 3-4A, 3-4B; OGD/Rep vs. OGD/Rep+Nec-1s, $P=0.024$). Concurring with IF findings, CLpP expression was significantly elevated during OGD (Fig. 3-4A, 3-4C; NC vs. OGD, $P<0.001$) and OGD/Rep (NC vs. OGD/Rep, $P=0.023$), and Nec-1s was more effective at suppressing CLpP upregulation from OGD/Rep, while OGD-induced CLpP expression remained elevated in the presence of Nec-1s (NC vs. OGD+Nec-1s, $P<0.001$).

Our collective findings suggest the following: 1. OGD/Rep leads to a concomitant increase in necroptosis and mitophagy signalling pathways in primary neurons, 2. RIPK1 may act as a positive regulator of CLpP and mitophagy during OGD/Rep but not OGD, and 3) mitophagy and mitochondrial quality control are differentially regulated in OGD versus OGD/Rep by distinct upstream regulators (e.g. RIPK1 and necrosome in OGD/Rep).

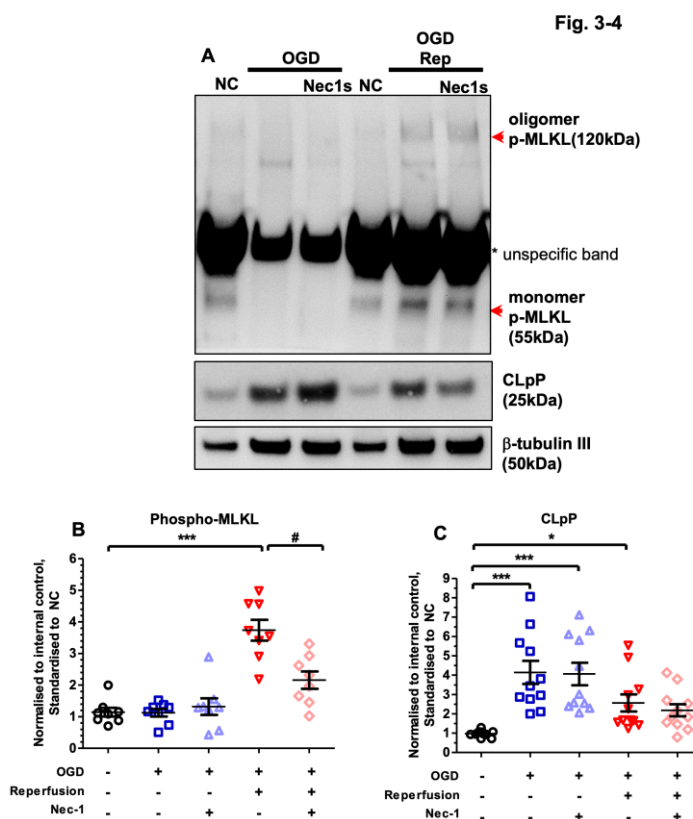


Figure 3-4. Western blot analysis of phosphorylated MLKL and CLpP in primary neurons. (A) Representative immunoblot of phosphorylated mixed lineage kinase-domain like (p-MLKL) and caseinolytic protease proteolytic subunit (CLpP) in primary mouse neurons subjected to 1hr OGD or 1hr OGD plus 1hr reperfusion (OGD/Rep), with or without necrostatin-1s (Nec-1s, 10 μ M) treatment. Neuron-specific cytoskeletal protein β -tubulin III was used as the internal control. Band intensity analysis of (B) phosphorylated mixed lineage kinase domain-like (p-MLKL), monomer and oligomer bands combined, and (C) mitochondrial caseinolytic protease proteolytic subunit (CLpP). Bands were normalised to internal control and standardised to NC. All data expressed as mean $\pm$ SD. [p-MLKL – *** $P<0.001$; OGD/Rep vs. OGD/Rep+Nec-1s, # $P<0.05$; $n=8$]. [CLpP – *** $P<0.001$; NC vs. OGD+Nec-1s, *** $P<0.001$; NC vs. OGD/Rep, * $P<0.05$; $n=11$]. Each n represents an independent experiment.

3.2.2. *Oxygen-glucose deprivation plus reperfusion induced necroptosis and upregulated mitophagy mediators in differentiated Neuro-2a cells*

We proceeded to verify the findings from primary neurons with mouse neuroblastoma cells Neuro-2a, which can be differentiated with dibutyryl cyclic AMP (db-cAMP) to adopt a neuron-like phenotype and have been routinely used as a reliable cell line for neuropharmacology and neurotoxicology studies⁴¹². After a 3-day differentiation protocol with 1mM db-cAMP, Neuro-2a cells developed extended protrusions that mimicked the axonal projections of neurons, with elevated expression of the neuron-specific cytoskeletal protein β -tubulin III (or tuji1) (Appendix, Supplementary Fig. 2).

As shown by western blot analysis on Neuro-2a whole cell lysate, O2R22 was associated with significant MLKL phosphorylation (**Fig. 3-5A, 3-5E**; NC vs. O2R22, $P=0.048$) and upregulation of the mitophagy mediator Parkin (**Fig. 3-5A, 3-5C**; NC vs. O2R22, $P=0.036$), along with mildly increased expression of RIPK3 and total MLKL (**Fig. 3-5A, 3-5B, 3-5D**). Immunofluorescence staining also revealed that MLKL phosphorylation and p-MLKL plasma membrane translocation were most prominent after O2R22 to indicate activation of necroptosis (**Fig. 3-5F, 3-5G**; NC vs. O2R22, $P=0.002$). Pure OGD (O2, O6 and O24) had no influence on the whole-cell expression of MLKL, phos-MLKL, RIPK3 and Parkin (**Fig. 3-5A to 3-5E**).

Neuro-2a mitochondrial fraction was extracted to determine the mitochondrial translocation of necrosome as well as the mitochondrial localisation of mitophagy mediators. As we have hypothesised, OGD/Rep induced translocation of RIPK3 (**Fig. 3-6A, 3-6B**), MLKL (**Fig. 3-6A, 3-6C**) and p-MLKL (**Fig. 3-6A, 3-6D**) into the mitochondrial compartment, which is most prominent in O2R22 (RIPK3: NC vs. O2R22, $P=0.005$; MLKL: NC vs. O2R22, $P=0.011$; p-MLKL, NC vs. O2R22, $P=0.026$) and to a lesser extent in the O2R4 group. OGD/Rep also stimulated mitochondrial accumulation of Parkin (**Fig. 3-6A, 3-6E**; NC vs. O2R22, $P=0.021$) and CLpP (**Fig. 3-6A, 3-6F**; NC vs. O2R22, $P=0.036$) and increased LC3A/B-II conversion (**Fig. 3-6A, 3-6G**; NC vs. O2R22, $P=0.041$) in a time-dependent manner to indicate activation of mitophagy.

On the contrary, pure OGD failed to promote necrosome translocation into the mitochondria and mildly increased mitochondrial levels of Parkin and CLpP (**Fig. 3-6A to 3-6F**). Immunofluorescence staining also demonstrated that O2R22 significantly induced whole-cell expression of CLpP (**Fig. 3-6H, 3-6I**; NC vs. O2R22, $P=0.012$). We note that unlike in primary neurons where OGD and OGD/Rep were equally effective at inducing CLpP, the CLpP response from pure OGD was dampened in Neuro-2a cells when compared to the time-matched OGD/Rep groups. It is plausible that as neuroblastoma cells, Neuro-2a are more resilient than primary neurons during pure OGD without the need to greatly enhance CLpP expression, however during OGD/Rep the aggravated mitochondrial damage would then stimulate CLpP expression to facilitate mitochondrial quality control. It is also worth highlighting that the significant LC3A/B mitochondrial

localisation and LC3A/B-II conversion from O24 appeared to exceed those from O2R22 (**Fig. 3-6A, 3-6G**; NC vs. O24, $P=0.006$), to suggest pure OGD as a more potent trigger for mitophagy than OGD/Rep.

Contingent with the observation made from primary neurons, we demonstrated that MLKL phosphorylation is exclusive to OGD/Rep in Neuro-2a cells to indicate activation of necroptosis, with O2R22 resulting in most significant MLKL phosphorylation. In addition, OGD/Rep-associated MLKL phosphorylation is accompanied by necrosome translocation into the mitochondrial compartment. As with primary neurons, both OGD and OGD/Rep promoted mitochondrial localisation of LC3A/B (LC3A/B-II) in Neuro-2a cells to indicate elevated mitophagy. OGD and OGD/Rep may be differentiated by their distinct recruitment dynamics of mitophagy mediators. Pure OGD led to more extensive mitochondrial localisation of LC3A/B-II, whereas OGD/Rep promoted mitochondrial accumulation of Parkin and CLpP along with a milder induction of LC3A/B-II. In view of the concomitant increase of necroptosis and mitophagy during OGD/Rep, we will investigate whether and how necroptosis regulates mitophagy in Neuro-2a cells in the next chapter.

Fig. 3-5

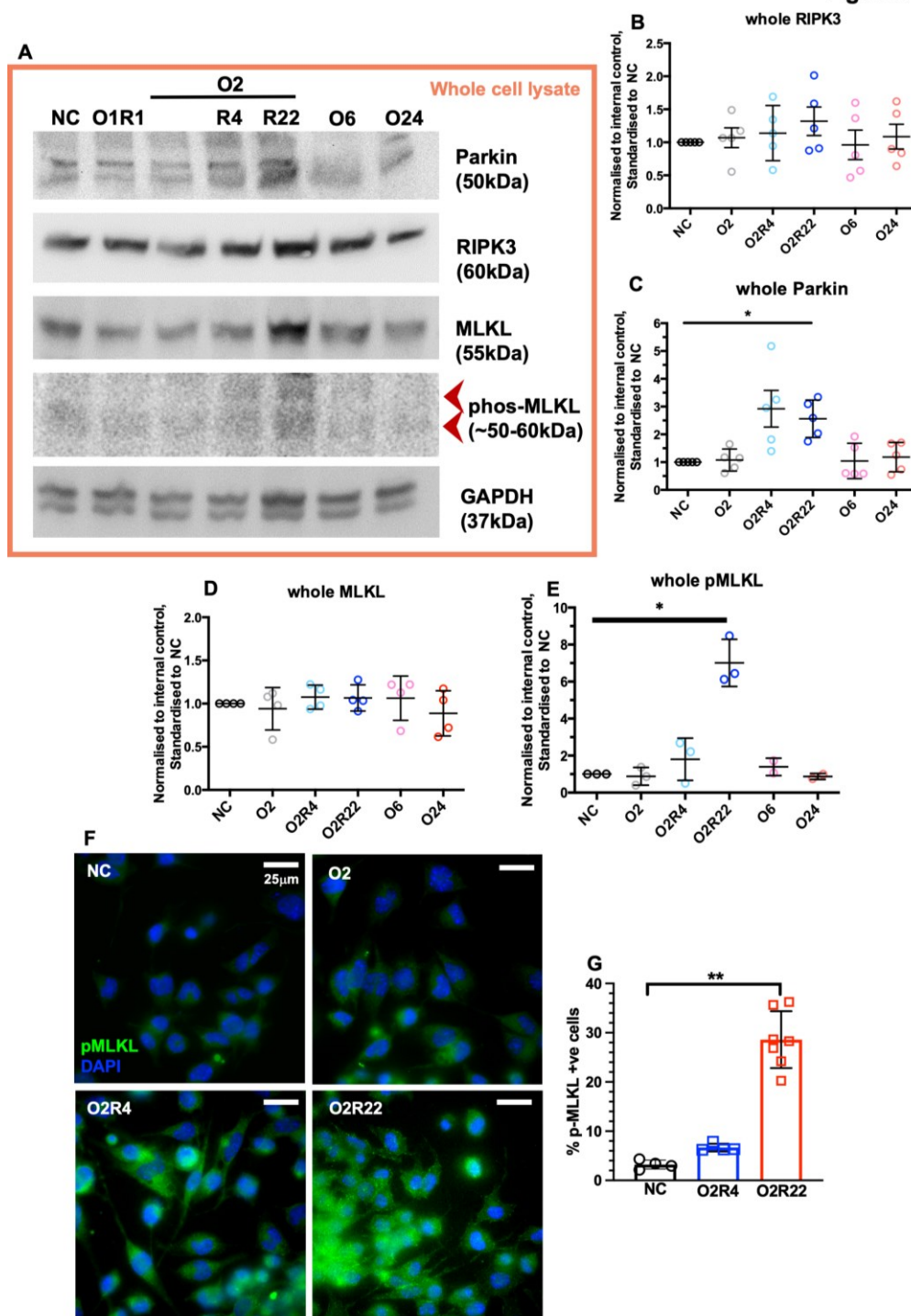


Figure 3-5. Whole-cell expression of necroptosis and mitophagy markers in Neuro-2a cells following OGD or OGD/Rep. (A) Representative immunoblot of Parkin, receptor interacting protein kinase 3 (RIPK3), mixed lineage kinase-domain like (MLKL) and phosphorylated MLKL (p-MLKL) from Neuro-2a whole cell lysates with indicated durations of oxygen glucose deprivation (OGD) or OGD plus reperfusion (OGD/Rep). GAPDH was used as the internal control. Band intensity analysis of (B) RIPK3, (C) Parkin, (D) MLKL and (E) p-MLKL, whereby bands were standardized to internal control and standardized to NC. All data were expressed as mean $\pm$ SD. [Parkin – NC vs. O2R22, * $P < 0.05$; $n = 5$]. [p-MLKL – NC vs. O2R22, * $P < 0.05$; $n = 3$]. (F) Immunofluorescence staining of p-MLKL in Neuro-2a cells; magnification $\times 63$, scale bar $25\mu\text{m}$. (G) Percentage of p-MLKL positive Neuro-2a cells, data expressed as mean $\pm$ SD. NC vs. O2R22, ** $P < 0.01$; $n = 4-7$, each n represents an independent experiment.

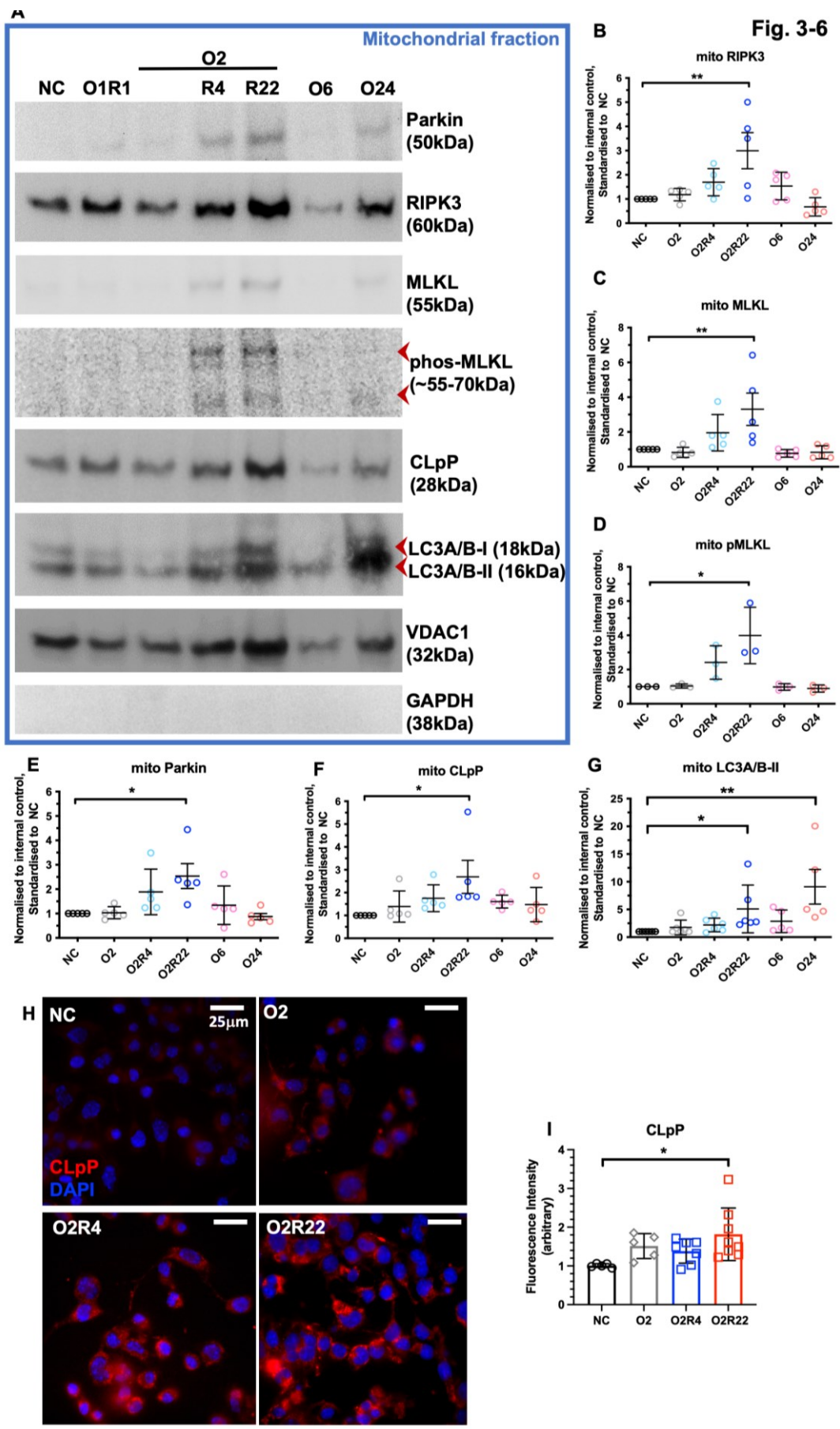


Figure 3-6. Mitochondrial expression of necrosome and mitophagy markers in Neuro-2 cells following OGD or OGD/Rep. (A) Representative immunoblot of Parkin, receptor interacting protein kinase 3 (RIPK3), mixed lineage kinase-domain like (MLKL), phosphorylated MLKL (p-MLKL), caseinolytic protease proteolytic subunit (CLpP) and microtubule-associated protein light chain 3A/B isoforms I and II (LC3A/B-I, LC3A/B-II) from Neuro-2a mitochondrial fraction with indicated durations of oxygen glucose deprivation (OGD) or OGD plus reperfusion (OGD/Rep). Mitochondrial outer membrane transporter protein voltage-dependent anion channel 1 (VDAC1) was used as the internal control. Band intensity analysis of (B) RIPK3, (C) MLKL, (D) p-MLKL, (E) Parkin, (F) CLpP and (G) LC3A/B-II, whereby bands were normalised to internal control and standardised to NC. All data were expressed as mean $\pm$ SD. [RIPK3 – NC vs. O2R22, * $P < 0.05$; $n = 5$]. [MLKL – NC vs. O2R22, * $P < 0.05$; $n = 5$]. [p-MLKL – NC vs. O2R22, * $P < 0.05$; $n = 3$]. [Parkin – NC vs. O2R22, * $P < 0.05$; $n = 5$]. [CLpP – NC vs. O2R22, * $P < 0.05$; $n = 5$]. [LC3A/B-II – NC vs. O2R22, * $P < 0.05$; NC vs. O24, ** $P < 0.01$; $n = 6$]. Each n represents an independent experiment. (H) Immunofluorescence staining of CLpP in Neuro-2a cells; magnification x63, scale bar = 25 μ m. (I) Immunofluorescence intensity of CLpP expressed as relative change to NC in arbitrary unit, data expressed as mean $\pm$ SD. NC vs. O2R22, * $P < 0.05$. $n = 5-8$, each n represents an independent experiment, and each n is an average of 3 replicates.

3.2.3. RIPK1 inhibitor necrostatin-1 suppressed necrosome translocation and mitophagy

Western blot analysis on Neuro-2a whole cell lysates revealed that Nec-1s effectively attenuated O2R22-induced MLKL phosphorylation (Fig. 3-7A, 3-7C; O2R22 vs. O2R22+Nec-1s, $P=0.006$) and Parkin expression (Fig. 3-7A, 3-7E; O2R22 vs. O2R22+Nec-1s, $P=0.025$), without appreciable effect on the expression of total MLKL and RIPK3 (Fig. 3-7A, 3-7B, 3-7D). The suppressive effect of Nec-1s in O2R4 group was not consistent, likely because O2R4 was not always associated with significant increases in p-MLKL and Parkin. Immunofluorescence staining also demonstrated that Nec-1s significantly reduced the number of p-MLKL positive Neuro-2a cells following O2R22 (Fig. 3-8A, 3-8B; O2R22 vs. O2R22+Nec-1s, $P=0.003$), reinforcing the notion that RIPK1 activity is required for MLKL phosphorylation.

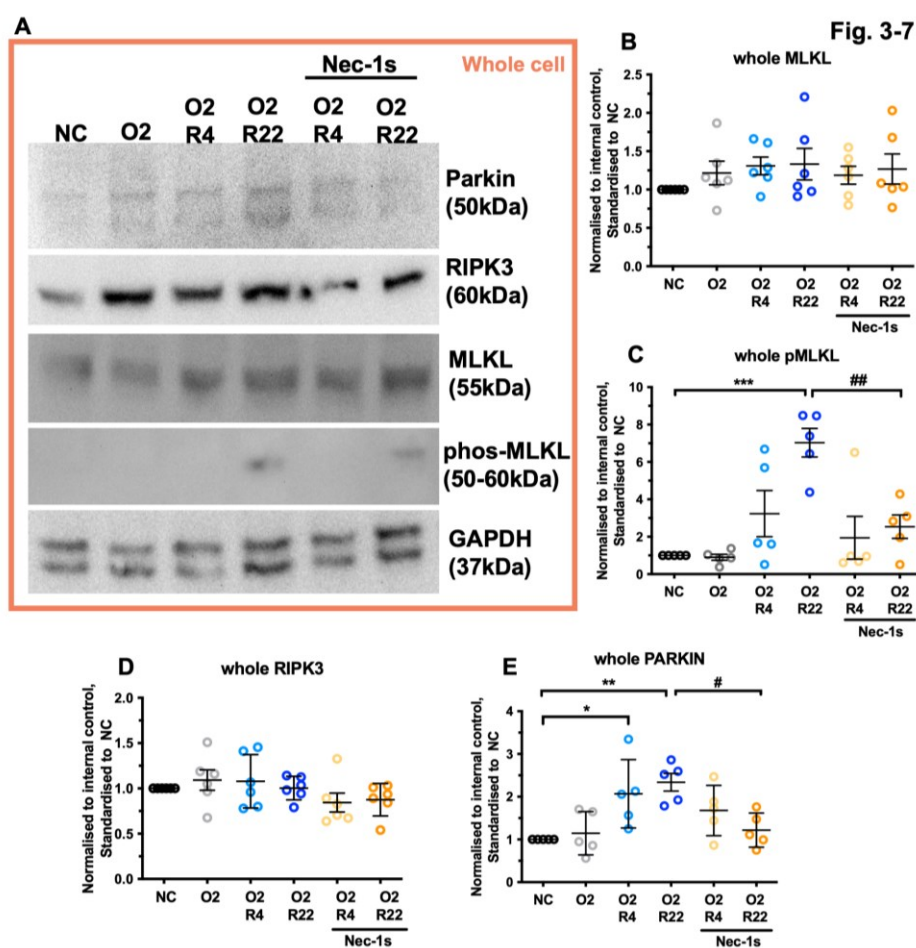


Figure 3-7. The effect of necrostatin-1s on Neuro-2a whole-cell expression of necroptosis and mitophagy markers following OGD/Rep. (A) Representative immunoblot of Parkin, receptor interacting protein kinase 3 (RIPK3), mixed lineage kinase-domain like (MLKL) and phosphorylated MLKL (p-MLKL) from Neuro-2a whole cell lysates after indicated periods of oxygen-glucose deprivation/reperfusion (OGD/Rep) with or without necrostatin-1s (Nec-1s, 10 μ M) treatment. GAPDH was used as the internal control. Band intensity analysis of (B) MLKL, (C) p-MLKL, (D) RIPK3 and (E) Parkin, whereby bands were normalised to internal control and standardised to NC. All data were expressed as mean $\pm$ SD. [p-MLKL, NC vs. O2R22, *** $P<0.001$; O2R22 vs. O2R22+Nec-1s, ## $P<0.01$; $n=5$]. [Parkin, NC vs. O2R4, * $P<0.05$; NC vs. O2R22, ** $P<0.01$; O2R22 vs. O2R22+Nec-1s, # $P<0.05$; $n=5$]; each n represents an independent experiment.

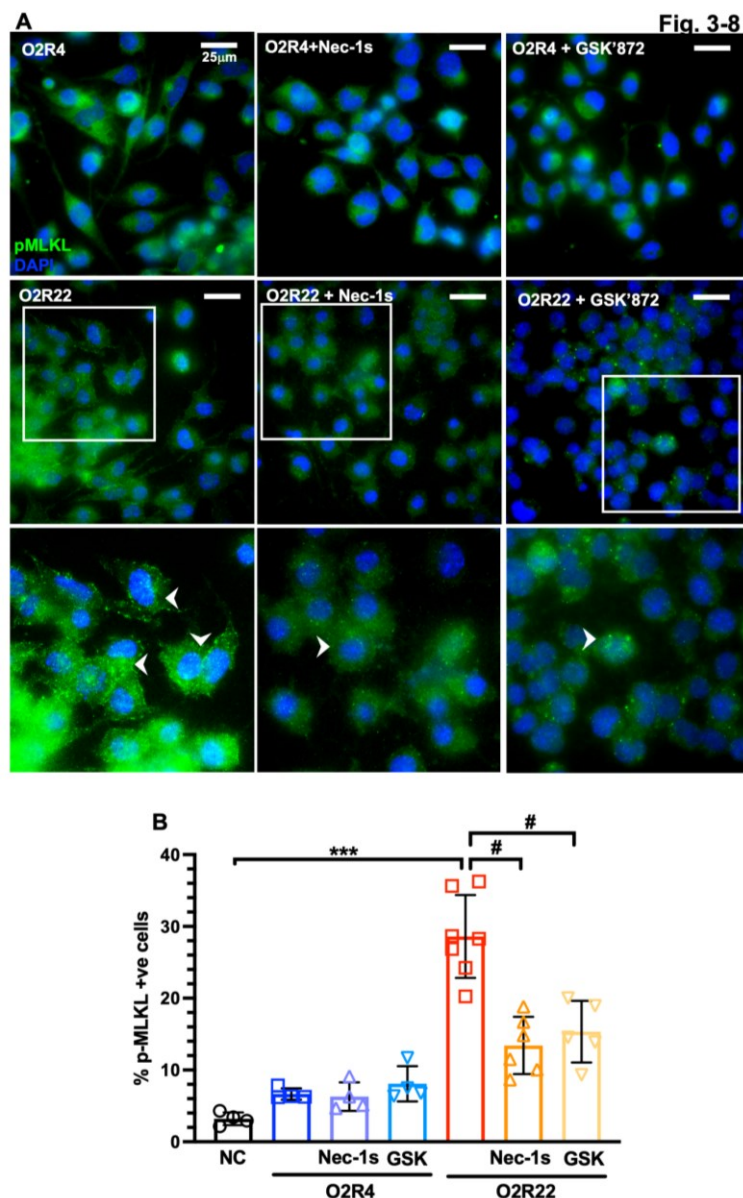


Figure 3-8. Immunofluorescence staining of phosphorylated MLKL in Neuro-2a cells following OGD/Rep with necroptosis inhibitor treatment. (A) Neuro-2a cells were subjected to 2hr oxygen-glucose deprivation (OGD) plus 4hr reperfusion (O2R4, upper) or 22hr reperfusion (O2R22, middle), with or without RIPK1 inhibitor necrostatin-1s (Nec-1s, 10 μ M) or RIPK3 inhibitor GSK'872 (1 μ M) treatment for 1hr, before detection of MLKL phosphorylation (green). Boxed area from O2R22 groups were enlarged (bottom), arrowheads indicate p-MLKL immunofluorescent puncta. Magnification x63, scale bar 25 μ m. **(B)** Percentage of p-MLKL positive Neuro-2a cells, data expressed as mean $\pm$ SD. NC vs. O2R22, *** P <0.001; O2R22 vs. O2R22+Nec-1s, # P <0.05; O2R22 vs. O2R22+GSK'872, # P <0.05; n =4-7, each n represents an independent experiment, and each is an average from 3 replicates.

We then proceeded to analyse the mitochondrial localisation of necrosome and mitophagy markers in the presence of Nec-1s. Importantly, O2R22-induced mitochondrial translocation of RIPK3 (**Fig. 3-9A, 3-9B**; O2R22 vs. O2R22+Nec-1s, $P=0.004$), MLKL (**Fig. 3-9A, 3-9C**; O2R22 vs. O2R22+Nec-1s, $P=0.015$) and p-MLKL (**Fig. 3-9A, 3-9D**) was diminished in cells treated with Nec-1s, to corroborate that RIPK1 kinase activity is required for mitochondrial translocation of the necrosome. Nec-1s also significantly attenuated mitochondrial accumulation of Parkin from O2R22 (**Fig. 3-9A, 3-9E**; O2R22 vs. O2R22+Nec-1s, $P=0.003$), along with decreased mitochondrial CLpP (**Fig. 3-9F**; O2R22 vs. O2R22+Nec-1s, $P=0.001$) and LC3A/B-II conversion (**Fig. 3-9G**, O2R22 vs. O2R22+Nec-1s, $P=0.04$). Immunofluorescence staining corroborated the decline in CLpP from Nec-1s treatment in O2R22 (**Fig. 3-10A, 3-10B**; O2R22 vs. O2R22+Nec-1s, $P=0.003$).

It should be emphasised that because mitophagy (as well as macro-autophagy) is an ongoing, dynamic process, the net availability of the active autophagosome marker LC3A/B-II at any given timepoint is the product of synthesis/lipidation minus constant autolysosomal degradation^{384, 420}. Therefore, provided that the autolysosome is functional, the amount of LC3A/B-II detected is not reflective of the real-time autophagic “flux” and may be underestimated due to constant degradation. To accurately quantify LC3A/B-II as a surrogate marker for autophagy, studies have used the lysosome inhibitor bafilomycin A1 (BafA1), which blocks the lysosomal H^+ ATPase to dissipate the acidic pH within lysosomes to lead to a build-up of sequestrants, including LC3A/B-II^{384, 420}. We consider lysosome inhibition to be also applicable to the study of mitophagy, and have thus treated separate groups of cells with BafA1 (100nM) for improved interpretation of LC3A/B-II mitochondrial localisation. As shown in **Fig. 3-9H**, mitochondrial LC3A/B-II conversion induced by O2R22 was attenuated by Nec-1s and co-treatment with BafA1 re-elevated mitochondrial LC3A/B-II level, to indicate 1) intact autolysosomal function following Nec-1s treatment 2) reduced LC3A/B-II from RIPK1 inhibition is due to decreased LC3A/B-II conversion. Moreover, Nec-1s alone (**Fig. 3-9H**, lanes 1-4) had little effect on the mitochondrial level of mitophagy markers in NC groups under basal condition.

Provided that Nec-1s attenuated OGD/Rep-induced mitophagy, RIPK1 normally would have promoted mitophagy. To compare and contrast the effect of different necrosome components on OGD/Rep-induced mitophagy, subsequent studies were performed with the RIPK3-selective inhibitor GSK'872 or small silencing RNA targeting MLKL, to compare and contrast with the findings obtained with RIPK1 inhibitor Nec-1s.

Fig. 3-9

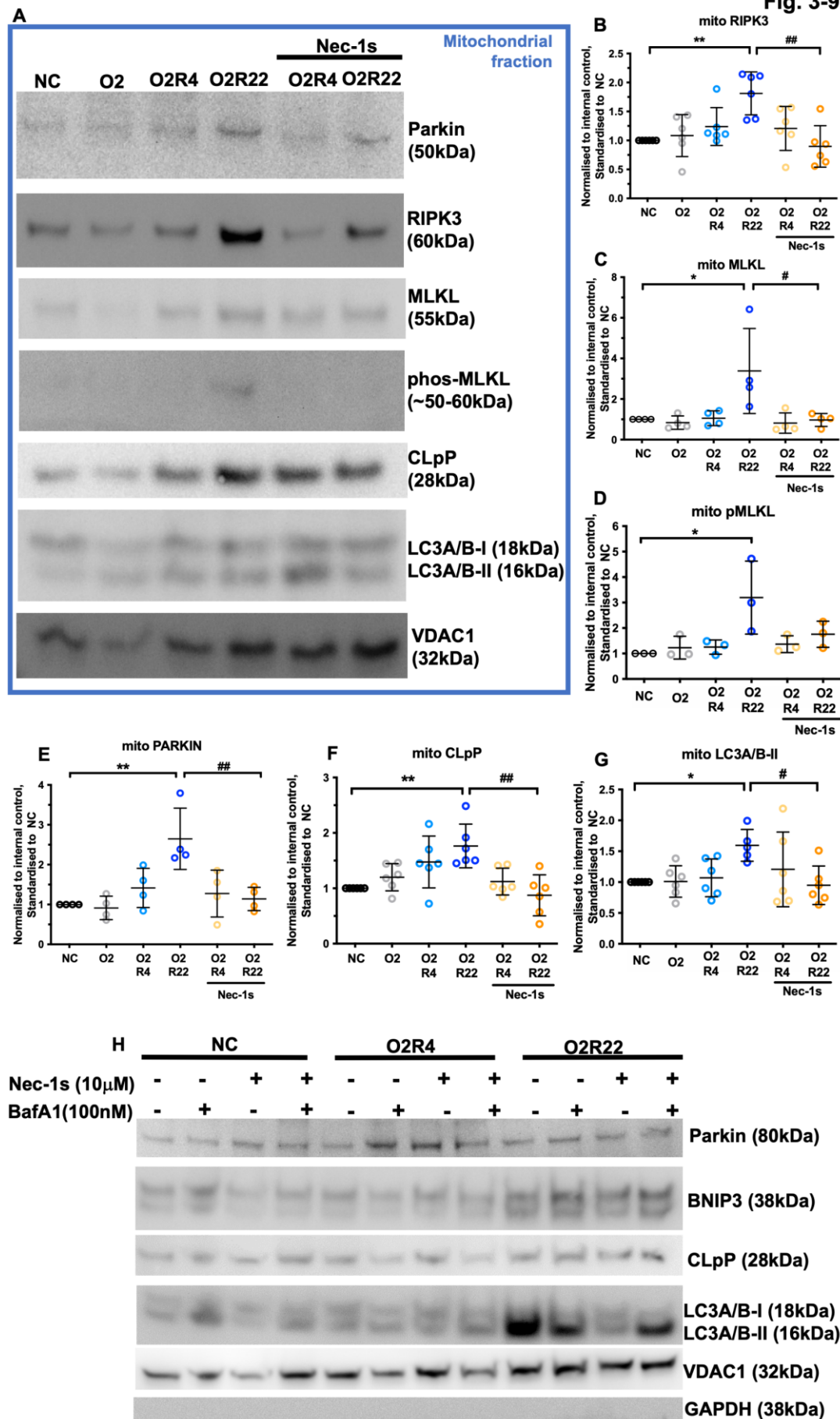


Figure 3-9. The effect of necrostatin-1s on mitochondrial expression of necrosome and mitophagy markers in Neuro-2a cells following OGD/Rep. **(A)** Representative immunoblot of Parkin, receptor interacting protein kinase 3 (RIPK3), mixed lineage kinase-domain like (MLKL), phosphorylated MLKL (p-MLKL), caseinolytic protease proteolytic subunit (CLpP) and microtubule-associated protein light chain 3A/B isoforms I and II (LC3A/B-I, LC3A/B-II) from Neuro-2a mitochondrial fraction with indicated periods of oxygen-glucose deprivation plus reperfusion (OGD/Rep), with or without necrostatin-1s (Nec-1s, 10 μ M) treatment. Mitochondrial outer membrane transporter protein voltage-dependent anion channel 1 (VDAC1) was used as the internal control. Band intensity analysis of **(B)** RIPK3, **(C)** MLKL, **(D)** p-MLKL, **(E)** Parkin, **(F)** CLpP and **(G)** LC3A/B-II, whereby bands were normalised to internal control and standardised to NC. All data were expressed as mean $\pm$ SD. [RIPK3 – NC vs. O2R22, **P<0.01; O2R22 vs. O2R22+Nec-1s, ###P<0.01; n=6] [MLKL – NC vs. O2R22, *P<0.05; O2R22 vs. O2R22+Nec-1s, #P<0.05; n=4] [p-MLKL – NC vs O2R22, *P<0.05; n=3] [Parkin – NC vs. O2R22, **P<0.01; O2R22 vs. O2R22+Nec-1s, ###P<0.01; n=4] [CLpP – NC vs. O2R22, **P<0.01; O2R22 vs. O2R22+Nec-1s, ###P<0.01; n=6] [LC3A/B-II – NC vs. O2R22, *P<0.05; O2R22 vs. O2R22+Nec-1s, #P<0.05; n=6]; each n represents an independent experiment. **(H)** Representative immunoblot of mitophagy-related markers (Parkin, BNIP3, CLpP and LC3A/B) in Neuro-2a mitochondrial fraction following OGD/Rep exposure and Nec-1s treatment, with or without the lysosome inhibitor bafilomycin A1 (100nM); not quantified.

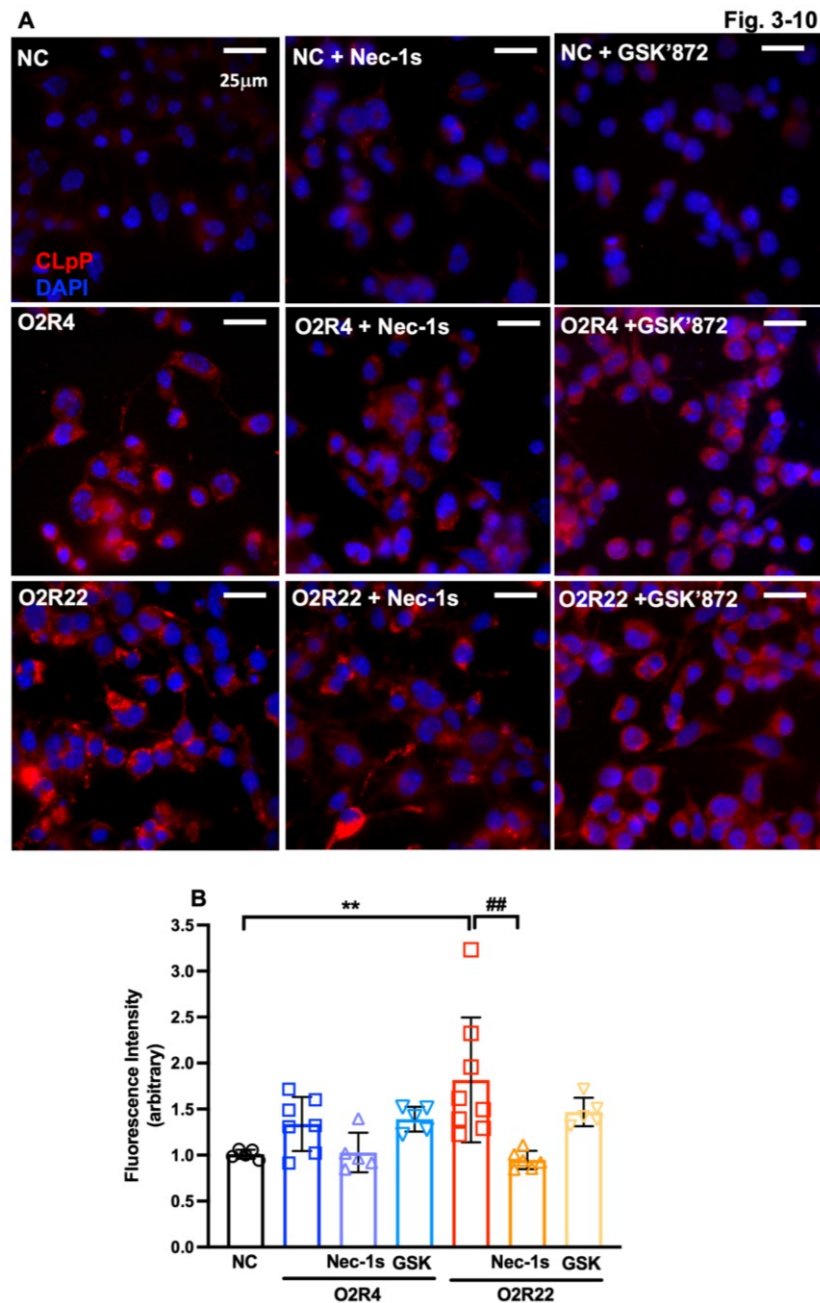


Figure 3-10. Immunofluorescence staining of CLpP in Neuro-2a cells following OGD/Rep with necroptosis inhibitor treatment. (A) Neuro-2a cells were subjected to 2hr OGD plus 4hr reperfusion (O2R4, upper) or 22hr reperfusion (O2R22, middle), with or without RIPK1 inhibitor necrostatin-1s (Nec-1s, 10 μ M) or RIPK3 inhibitor GSK'872 (1 μ M) treatment for 1hr, before detection of caseinolytic protease proteolytic subunit (CLpP, red). Magnification x63, scale bar 25 μ m. **(B)** Immunofluorescence intensity of CLpP with indicated OGD/Rep and treatments, expressed as relative change to NC in arbitrary unit. Data expressed as mean $\pm$ SD. NC vs. O2R22, **P<0.01; O2R22 vs. O2R22+Nec-1s, ###P<0.01. n=5-8, each n represents an independent experiment, and each n is an average of 3 replicates.

3.2.4 RIPK3 inhibitor GSK'872 suppressed necrosome translocation and mitophagy

As expected, GSK'872 treatment suppressed O2R22-induced MLKL phosphorylation (**Fig. 3-8A, 3-8B** – O2R22 vs. O2R22+GSK, $P=0.026$; **Fig. 3-11A**) to indicate inhibition of necroptosis, without significant alteration to whole-cell expression of RIPK3 and MLKL (**Fig. 3-11A**). GSK'872 treatment also attenuated O2R22-induced upregulation of Parkin (**Fig. 3-11A, 3-11B**) and BNIP3 (**Fig. 3-11A, 3-11C**; O2R22 vs. O2R22+GSK, $P=0.040$) to indicate decreased mitophagy, without altering whole-cell CLpP expression (**Fig. 3-11D**). Immunofluorescence staining also showed that GSK'872 only mildly suppressed CLpP intensity following O2R22 (**Fig. 3-10A, 3-10B**).

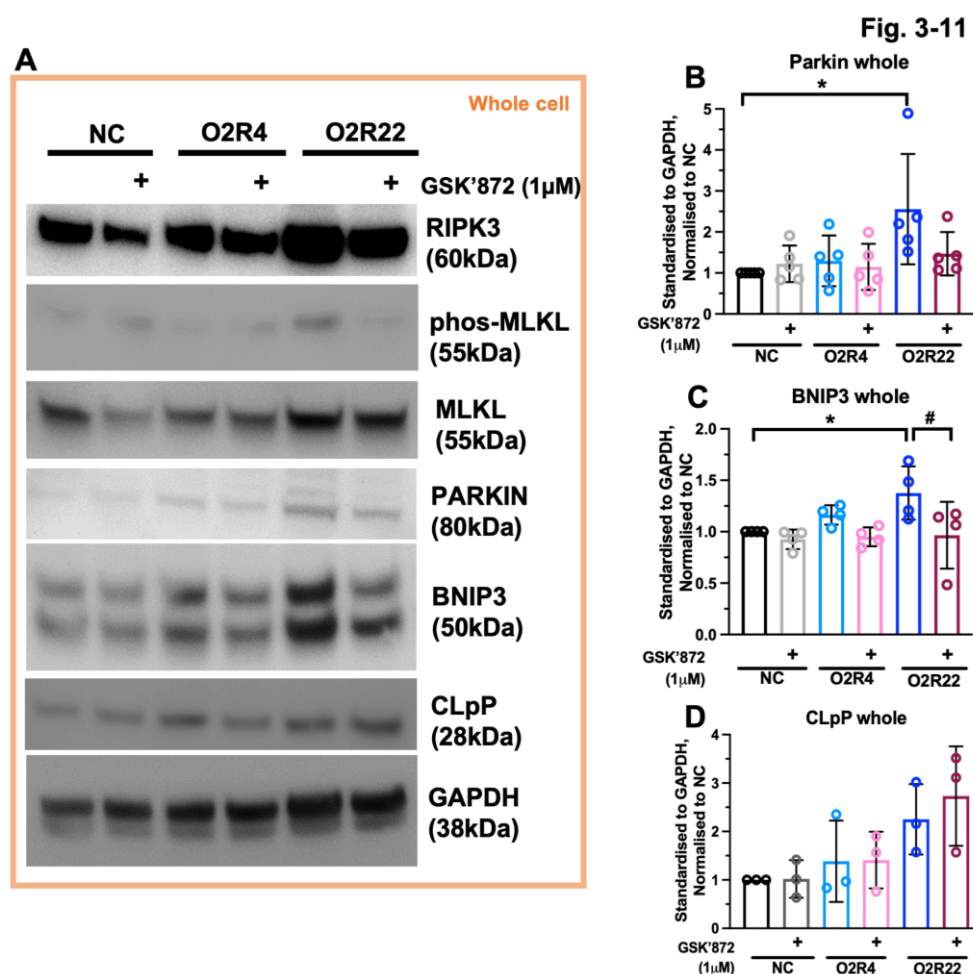


Figure 3-11. The effect of GSK'872 on Neuro-2a whole-cell expression of necroptosis and mitophagy markers following OGD/Rep. (A) Representative immunoblot of receptor interacting protein kinase 3 (RIPK3), mixed lineage kinase-domain like (MLKL), phosphorylated MLKL (p-MLKL), Parkin, Bcl2 interacting protein 3 (BNIP3), and caseinolytic protease proteolytic subunit (CLpP) from Neuro-2a whole cell lysates after indicated periods of oxygen-glucose deprivation (OGD/Rep), with or without GSK'872 (1μM) treatment. GAPDH was used as the internal control. Band intensity analysis of (B) Parkin, (C) BNIP3 and (D) CLpP, whereby bands were normalised to internal control and standardised to NC. All data were expressed as mean $\pm$ SD. [Parkin – NC vs. O2R22, $*P<0.05$; $n=5$] [BNIP3 – NC vs. O2R22, $*P<0.05$; O2R22 vs. O2R22+GSK, $\#P,0.05$; $n=4$] [CLpP – ns; $n=3$]; each n represents an independent experiment.

Mitochondrial fraction was analysed separately to determine mitochondrial translocation of necrosome and accumulation of mitophagy mediators. O2R22 promoted RIPK3 and MLKL localisation into the mitochondrial compartment, which could be reversed by RIPK3 inhibitor GSK'872 (**Fig. 3-12A**). As expected, GSK'872 also attenuated the mitochondrial accumulation of Parkin (**Fig. 3-12B**) and BNIP3 (**Fig. 3-12C**) and decreased LC3A/B-II conversion in the O2R22 group (**Fig. 3-12D**; O2R22 vs. O2R22+GSK, $P=0.032$). We note that GSK'872 appears to be less effective than Nec-1s at suppressing mitophagy activated by OGD/Rep. In agreement with the whole-cell analysis, GSK'872 treatment did not alter mitochondrial level of CLpP (**Fig. 3-12E**). Moreover, GSK'872-associated reduction in mitochondrial LC3A/B-II from O2R22 could be restored by Baf_{A1} treatment (**Fig. 3-12F**), to indicate that RIPK3 inhibitor decreases LC3A/B-II conversion and does not interfere with autolysosome function, as discussed in relation to RIPK1 inhibitor Nec-1s. In unstimulated NC cells, GSK'872 has no influence on basal LC3A/B-II conversion (**Fig. 3-12F**). Mirroring the findings from Nec-1s, the observation that GSK'872 inhibited OGD/Rep-induced mitophagy suggests a pro-mitophagy role of RIPK3.

Fig. 3-12

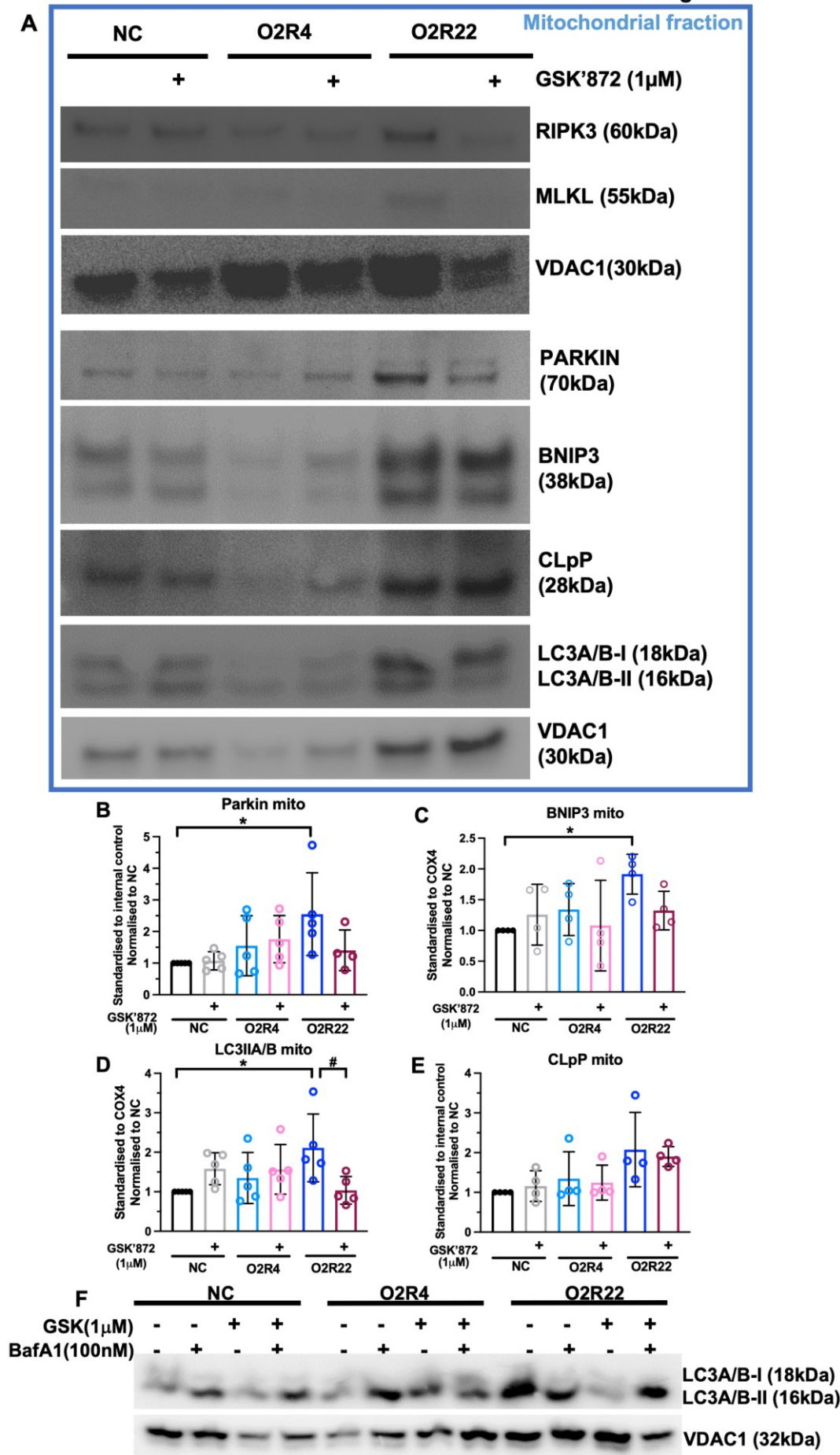


Figure 3-12. The effect of GSK'872 on Neuro-2a mitochondrial expression of necrosome and mitophagy markers following OGD/Rep. (A) Representative immunoblot of receptor interacting protein kinase 3 (RIPK3), mixed lineage kinase-domain like (MLKL), phosphorylated MLKL (p-MLKL), Parkin, Bcl2-interacting protein 3 (BNIP3), caseinolytic protease proteolytic subunit (CLpP) and microtubule-associated protein light chain 3A/B isoforms I and II (LC3A/B-I, LC3A/B-II) from Neuro-2a mitochondrial fraction after indicated periods of oxygen-glucose deprivation and reperfusion (OGD/Rep), with or without GSK'872 (1 μ M) treatment. Mitochondrial outer membrane transporter protein voltage-dependent anion channel 1 (VDAC1) was used as the internal control. Band intensity analysis of (B) Parkin, (C) BNIP3, (D) LC3A/B-II and (E) CLpP, whereby bands were normalised to internal control and standardised to NC. All data were expressed as mean $\pm$ SD. [Parkin – NC vs. O2R22, *P<0.05; n=5] [BNIP3 – NC vs. O2R22, *P<0.05; n=4] [LC3A/B-II – NC vs. O2R22, *P<0.05; O2R22 vs. O2R22+GSK, #P<0.05; n=5] [CLpP – ns; n=4]; each n represents an independent experiment. (F) Representative immunoblot of LC3A/B-I and LC4A/B-II in Neuro-2a mitochondrial fraction following OGD/Rep exposure and GSK'872 treatment, with or without the lysosome inhibitor bafilomycin A1 (100nM); not quantified.

3.2.5 MLKL knockdown prevented necrosome translocation and enhanced mitophagy

The above findings from RIPK1 or RIPK3 inhibition suggest the necrosome components as positive regulators of mitophagy during OGD/Rep. We then proceeded to investigate the effect of MLKL blockade on mitophagy in Neuro-2a cells.

Western blot analysis showed that incubation with MLKL-targeting siRNA led to specific and efficient knockdown of whole-cell MLKL expression in Neuro-2a cells under basal and OGD/Rep conditions (**Fig. 3-13A, 3-13B**; NC vs. NC + MLKL siRNA, $P=0.020$; O2R4 vs. O2R4 + MLKL siRNA, $P=0.029$; O2R22 vs. O2R22 + MLKL siRNA, $P=0.001$), along with diminished phos-MLKL signal in the O2R22 group. Non-specific scrambled siRNA was used as negative control (-ve siRNA) and did not downregulate MLKL expression. MLKL knockdown did not result in a compensatory increase in the expression of whole-cell RIPK3 (**Fig. 3-13C**), while appeared to have increased whole-cell Parkin and BNIP3 expressions in O2R4 and O2R22 groups to suggest transcriptional or translational upregulation of mitophagy mediators (**Fig. 3-13C to 3-13E**). The induction of Parkin and BNIP3 contrasts with previous observations from RIPK1/RIPK3 inhibition, as treatment with Nec-1s or GSK'872 suppressed whole-cell Parkin and BNIP3 levels.

In isolated mitochondrial fraction, we demonstrated that MLKL-specific siRNA treatment abolished OGD/Rep-induced mitochondrial translocation of MLKL (**Fig. 3-14A to 3-14C**; O2R22 vs. O2R22 + siMLKL, $P=0.012$) and significantly attenuated the mitochondrial localisation of RIPK3 (**Fig. 3-14B, 3-14D**; O2R22 vs. O2R22 + siMLKL, $P=0.020$). MLKL knockdown also promoted the mitochondrial accumulation of Parkin (**Fig. 3-14A, 3-14B, 3-14E**; $P=0.034$) and BNIP3 (**Fig. 3-14B, 3-14F**) in O2R22, to correspond to whole-cell upregulation of mitophagy mediators. Increased mitochondrial Parkin and BNIP3 from MLKL silencing corresponded to enhanced mitochondrial LC3A/B-II conversion (**Fig. 3-14A, 3-14B, 3-14G**; O2R22 vs. O2R22 + siMLKL, $P=0.048$) to imply potentiation of OGD/Rep-induced mitophagy. Mitochondrial CLpP level was mildly decreased with MLKL knockdown (**Fig. 3-14A, 3-14B, 3-14H**), possibly reflecting amelioration of mitochondrial stress response from enhanced mitophagy. Immunofluorescence staining supported the attenuation of O2R22-induced CLpP by MLKL knockdown (**Fig. 3-15A, 3-15B**; O2R22 vs. O2R22 + siMLKL, $P=0.016$).

Separate groups of cells were treated with BafA₁ for improved interpretation of mitochondrial LC3A/B-II. As shown in **Fig. 3-14I**, BafA₁ elevated O2R22-induced LC3A/B-II conversion and amplified mitochondrial LC3A/B-II accumulation from MLKL silencing. As discussed in relation to autophagy, where a treatment/stimulus increases LC3A/B-II band intensity relative to LC3A/B-I (increased "flux"), it could either be that LC3A/B-I to LC3A/B-II conversion is increased, or that LC3A/B-II degradation by autolysosome is suppressed by the treatment/stimulus^{384, 420}. In the case of the latter, the presence of the lysosomal inhibitor BafA₁ would be unable to further increase LC3A/B-II band intensity as the autolysosomal/lysosomal function

PhD Thesis Lingzhi Wu
is compromised. As demonstrated here, the fact that BafA₁ amplified mitochondrial LC3A/B-II signal in O2R22, with further potentiation from MLKL-siRNA treatment suggests 1) intact autolysosomal degradative function from OGD/Rep and following MLKL knockdown, and 2) OGD/Rep and MLKL silencing synergistically promote mitochondrial LC3A/B-II conversion and mitophagy.

In concert, the observation that MLKL knockdown potentiates OGD/Rep-induced mitophagy suggests that MLKL suppresses mitophagy and acts in opposition to its upstream regulators RIPK1 and RIPK3.

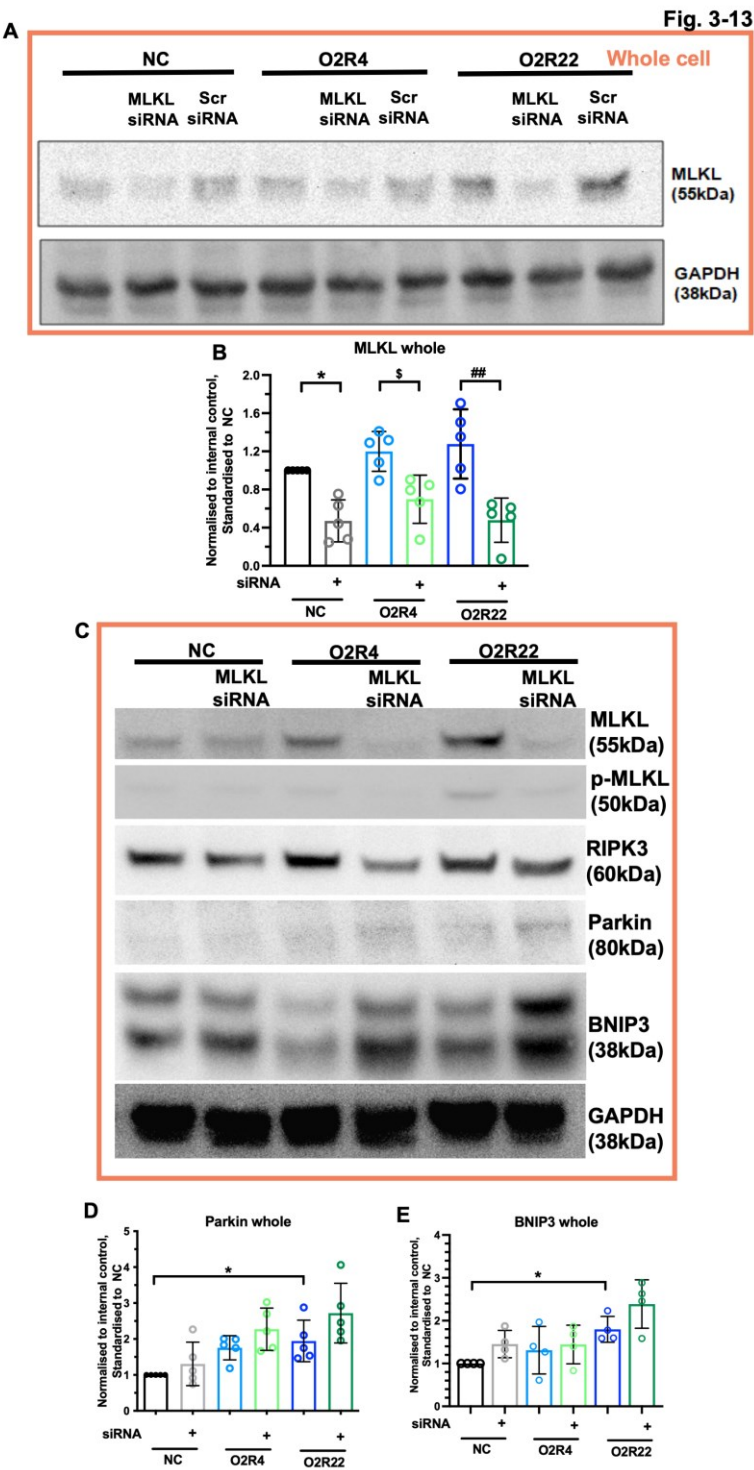


Figure 3-13. The effect of small silencing RNA-mediated MLKL knockdown on the expression of necroptosis and mitophagy markers following OGD/Rep. **(A)** Representative immunoblot of mixed lineage kinase-domain like (MLKL) from Neuro-2a whole cell lysates treated with MLKL-specific small silencing RNA (MLKL siRNA, 10nM) or scrambled siRNA as negative control (Scr siRNA, 10nM), followed by indicated periods of oxygen-glucose deprivation and reperfusion (OGD/Rep). **(B)** Band intensity analysis of whole-cell MLKL expression. **(C)** Representative immunoblot of MLKL, phosphorylated MLKL (p-MLKL), receptor interacting protein kinase 3 (RIPK3), Parkin and Bcl2-interacting protein 3 (BNIP3) from Neuro-2a whole cell lysates treated with MLKL siRNA and followed by indicated periods of OGD/Rep. GAPDH was used as the internal control. Band intensity analysis of **(D)** Parkin and **(E)** BNIP3 from whole-cell lysates, whereby bands were normalised to internal control and standardised to NC. All data were expressed as mean $\pm$ SD. [MLKL – NC vs. NC + MLKL siRNA, * $P < 0.05$; O2R4 vs. O2R4 + MLKL siRNA, \$ $P < 0.05$; O2R22 vs. O2R22 + MLKL siRNA, ### $P < 0.01$; n=5.] [Parkin – NC vs. O2R22, * $P < 0.05$; n=5]. [BNIP3 – NC vs. O2R22, * $P < 0.05$; n=4]; each n represents an independent experiment.

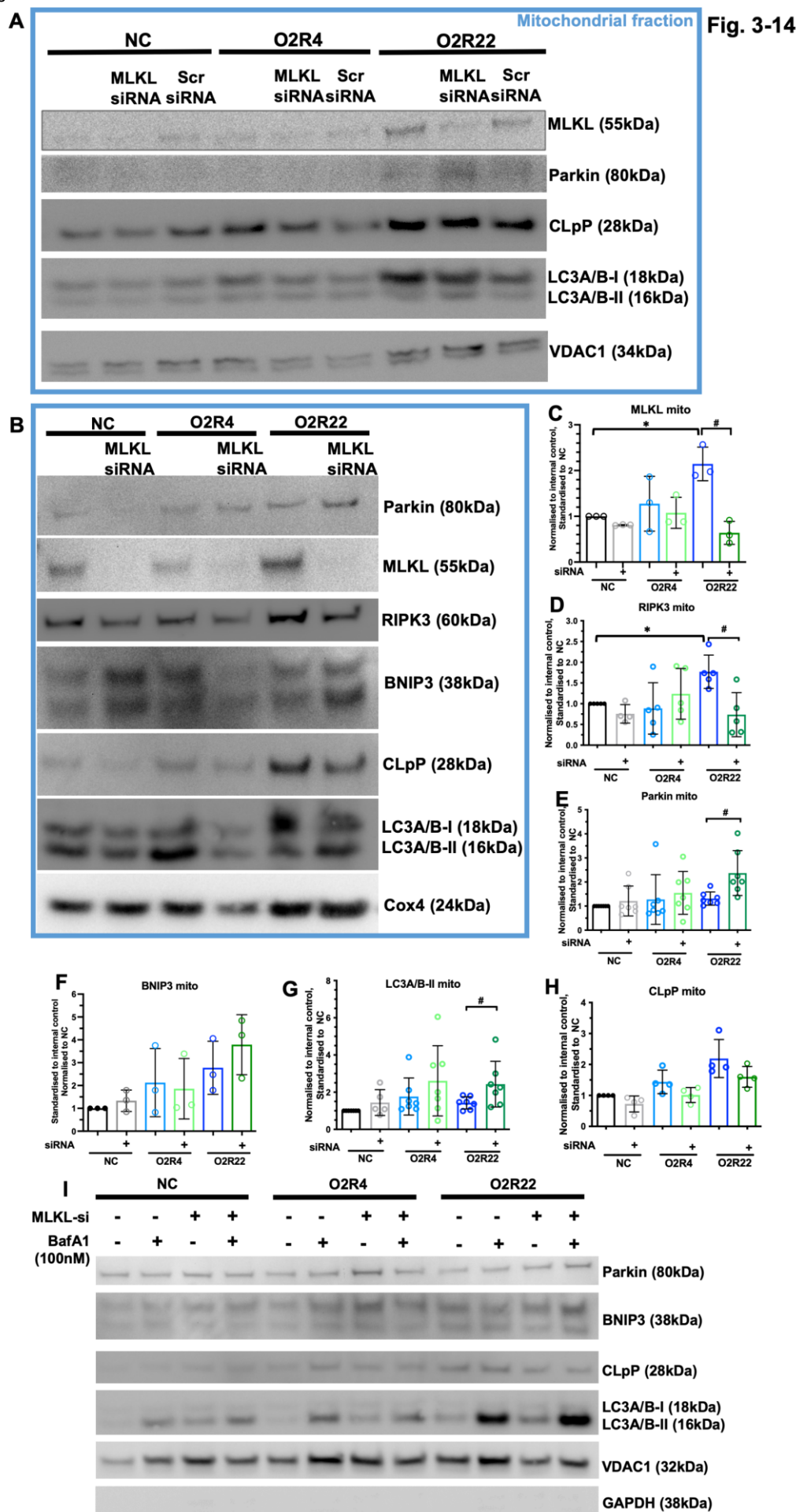


Figure 3-14. The effect of small silencing RNA-mediated MLKL knockdown on the mitochondrial expression of necrosome and mitophagy markers following OGD/Rep. (A) Representative immunoblot of mixed lineage kinase-domain like (MLKL), Parkin, Bcl2 interacting protein 3 (BNIP3), caseinolytic protease proteolytic subunit (CLpP) and microtubule-associated protein light chain 3A/B isoforms I and II (LC3A/B-I, LC3A/B-II) from Neuro-2a mitochondrial fraction, where cells were treated with MLKL-specific small silencing RNA (MLKL siRNA, 10nM) or scrambled siRNA as negative control (Scr siRNA, 10nM), followed by indicated periods of oxygen-glucose deprivation and reperfusion (OGD/Rep). Mitochondrial outer membrane transporter protein voltage-dependent anion channel 1 (VDAC1) was used as the loading control. (B) Representative immunoblot of Parkin, MLKL, RIPK3, Bcl2-interacting protein 3 (BNIP3), CLpP and LC3A/B (I and II) from Neuro-2a mitochondrial fraction, where cells were treated with MLKL-specific small silencing RNA (MLKL siRNA) and followed by indicated periods of OGD/Rep. Mitochondrial inner membrane electron transport chain enzyme cytochrome c oxidase 4 (COX4) was used as the internal control. Band intensity analysis of (C) MLKL, (D) RIPK3, (E) Parkin, (F) BNIP3, (G) LC3A/B-II and (H) CLpP from mitochondrial fraction, whereby bands were normalised to internal control and standardised to NC. All data were expressed as mean $\pm$ SD. [MLKL – NC vs. O2R22, * P <0.05; O2R22 vs. O2R22 + MLKL siRNA, # P <0.05; n =3] [RIPK3 – NC vs. O2R22, * P <0.05; O2R22 vs. O2R22 + MLKL siRNA, # P <0.05; n =4-5] [Parkin – O2R22 vs. O2R22 + MLKL siRNA, # P <0.05; n =7] [LC3A/B-II – O2R22 vs. O2R22 + MLKL siRNA, # P <0.05; n =5-7]; each n represents an independent experiment. (I) Representative immunoblot of mitophagy-related markers (Parkin, BNIP3, CLpP and LC3A/B) in Neuro-2a mitochondrial fraction following OGD/Rep exposure and siRNA-mediated MLKL knockdown, with or without the lysosome inhibitor bafilomycin A1 (100nM); not quantified.

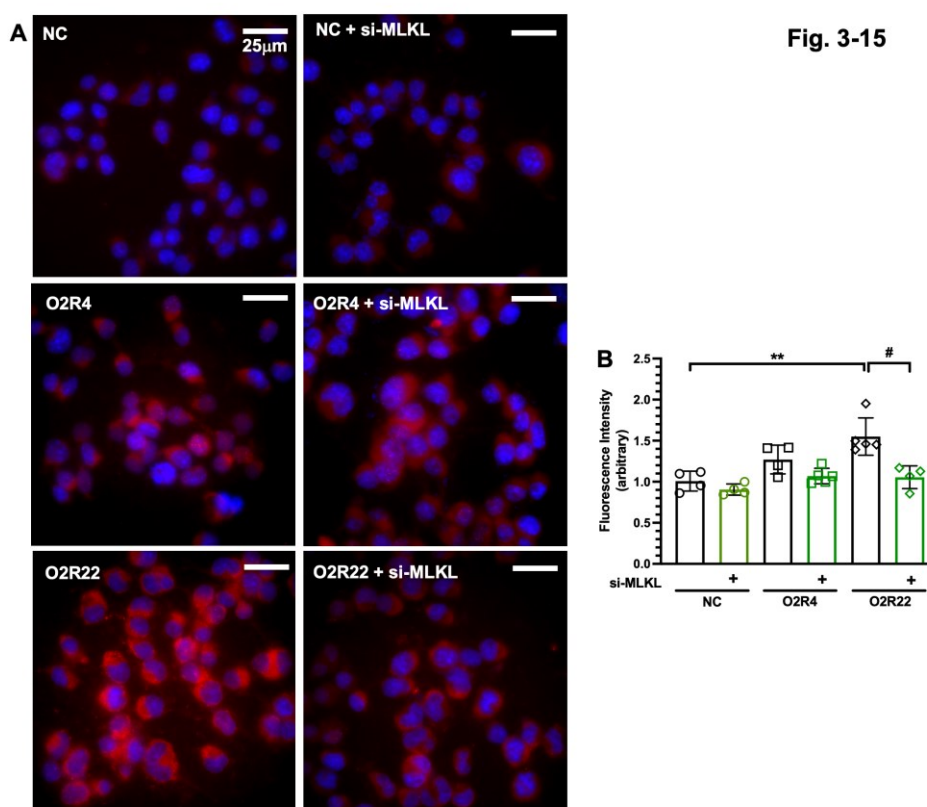


Figure 3-15. Immunofluorescence staining of CLpP in Neuro-2a cells with small silencing RNA-mediated MLKL knockdown and following OGD/Rep. (A) Neuro-2a cells were incubated with MLKL-specific siRNA prior to 2hr OGD plus 4hr reperfusion (O2R4) or 22hr reperfusion (O2R22), before detection of caseinolytic protease proteolytic subunit (CLpP, red). Magnification $\times 63$, scale bar 25 μ m. (B) Immunofluorescence intensity of CLpP, expressed as relative change to NC in arbitrary unit. Data expressed as mean $\pm$ SD. NC vs. O2R22, ** P <0.01; O2R22 vs. O2R22 + MLKL siRNA, # P <0.05; n =4-5, each n represents an independent experiment, and each n is an average of 3 replicates.

3.2.6 Interaction between MLKL and mitophagy mediators

In view of the suppressive effect of MLKL on mitophagy, it is plausible for MLKL to directly interact with mitophagy mediators to modulate the rate of mitochondrial turnover. To investigate the potential interaction between MLKL and mitophagy mediators, we performed co-immunoprecipitation on Neuro-2a whole-cell lysates following OGD/Rep (**Fig. 3-16A**). In brief, MLKL-specific antibody was used to pull-down MLKL and associated proteins from NC and OGD/Rep-challenged cells. Under basal condition, MLKL was shown to interact with BNIP3 and the interaction strengthens with prolonged OGD/Rep at O2R22 (**Fig. 3-16B**, left, lanes 1-3). We did not detect MLKL interaction with Parkin or CLpP under basal or OGD/Rep conditions (**Fig. 3-16B**). Isotype control rabbit IgG antibody did not pull-down MLKL or associated proteins (**Fig. 3-16B**, lane 4).

Unpublished data from our group suggested CLpP as a potential regulator of basal and hypoxia-induced mitophagy in cancer cells. Provided that Co-IP experiment did not demonstrate physical interaction between MLKL and CLpP, we performed siRNA-MLKL and siRNA-CLpP double knockdown to explore a possible link between MLKL and CLpP in the context of mitophagy. siRNA-MLKL or siRNA-CLpP alone enhanced mitochondrial LC3A/B-II conversion in Baf_{A1}-treated NC and O2R22 cells (**Fig. 3-16C**), with siRNA-CLpP led to more prominent accumulation of LC3A/B-II, Parkin and BNIP3. siRNA-MLKL/CLpP double knockdown did not further alter the level of mitophagy mediators when compared to siRNA-MLKL, but appeared to decrease mitophagy mediators from siRNA-CLpP.

Fig. 3-16

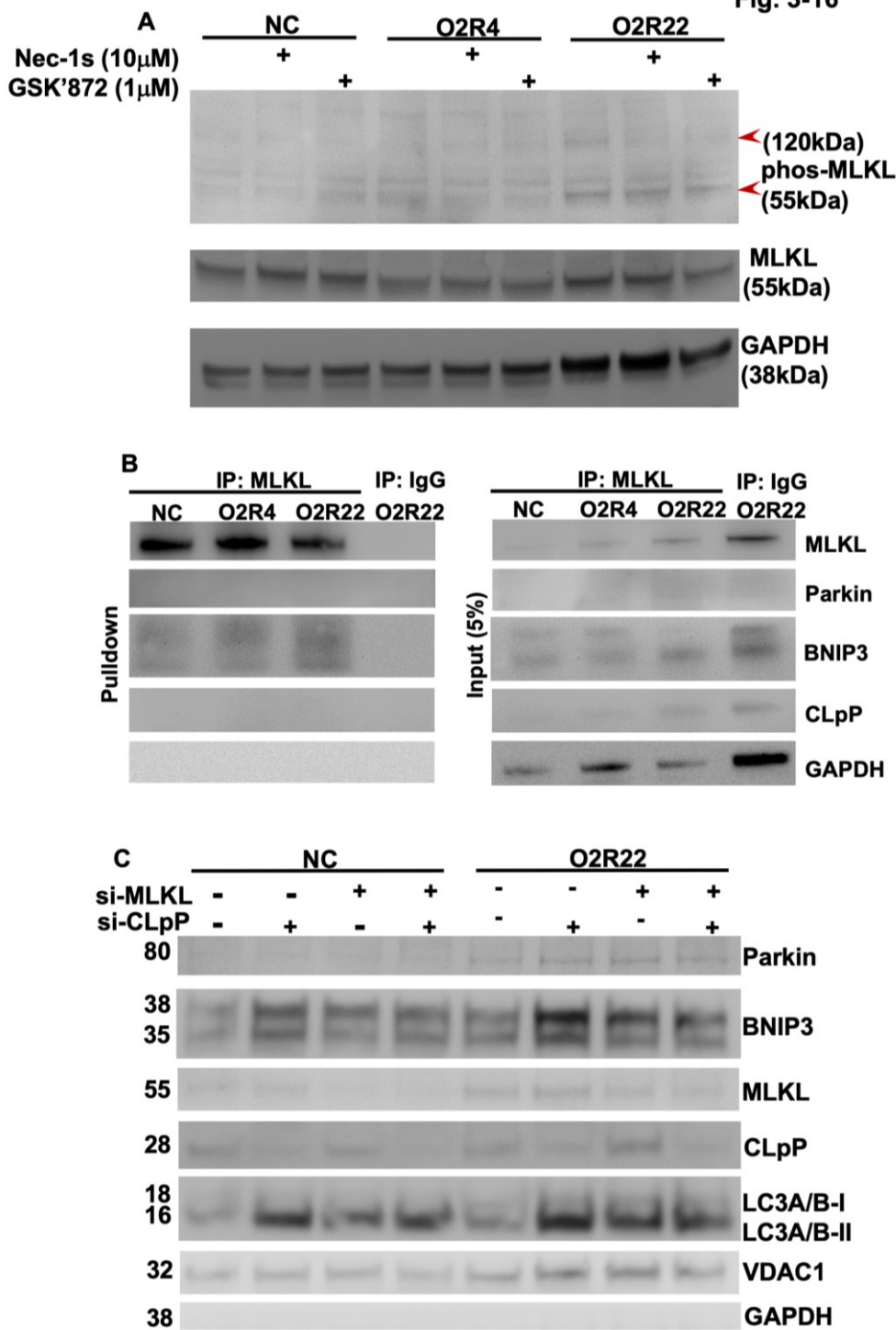


Figure 3-16. Potential interaction between MLKL and mitophagy-related proteins in Neuro-2 cells. (A) Representative immunoblot of mixed lineage kinase domain like (MLKL) and phosphorylated MLKL from Neuro-2a cells after oxygen-glucose deprivation and reperfusion (OGD/Rep) with or without necroptosis inhibitor treatment. **(B)** Co-immunoprecipitation immunoblot showing pulldowns from MLKL-specific antibody (IP:MLKL) or isotype control antibody (IP:IgG), left; co-IP reaction lysate was diluted to 5% as input control, right, showing endogenous expression of MLKL, Parkin, Bcl2-interacting protein 3 (BNIP3) and caseinolytic protease proteolytic subunit (CLpP); not quantified. **(C)** Representative immunoblot of mitophagy-related markers from Neuro-2a mitochondrial fractions with MLKL (MLKL-si) or CLpP (CLpP-si) knockdown or double knockdown, in NC or O2R22; not quantified.

3.2.7 RIPK1 inhibition, RIPK3 inhibition or MLKL knockdown restored mitochondrial membrane potential

Using flow cytometry, we tracked changes to mitochondrial potential in Neuro-2a cells subjected to OGD/Rep. In gated single Neuro-2a cell population (**Supplementary Fig. 3A**), we observed a left-shift in TMRE mean fluorescence intensity (MFI) in O2R22, and to a lesser extent in O2R4, when compared to NC; FCCP treatment led to a more significant TMRE left shift (**Supplementary Fig. 3B**). JC-10 staining revealed that in comparison to NC (Fig. 18C, top left), OGD/Rep significantly reduced PE-positive population and correspondingly increased FITC-positive population in a time-dependent manner (**Supplementary Fig. 3C; Fig. 3-17A, 3-17B**; NC vs. O2R22, $P=0.006$), and FCCP was associated with the most prominent suppression of PE signal (**Supplementary Fig. 3C**). When referenced to changes induced by FCCP, the TMRE left shift and decreased red (PE)/ green (FICT) JC-10 ratio are indicative of a decline in mitochondrial potential following OGD/Rep, especially in the O2R22 group.

We proceeded to examine the effect of RIPK1 and RIPK3 inhibition on mitochondrial potential under basal and OGD/Rep conditions. In unstimulated Neuro-2a cells, Nec-1s or GSK'872 treatment led to small yet significant decreases in PE/FITC ratio compared to NC (**Fig. 3-17A**, top panel; **Fig. 3-17B**; NC vs. NC+Nec-1s, $P=0.018$; NC vs. NC+GSK'872, $P=0.047$), whereas in the O2R22 cohorts Nec-1s or GSK'872 partially restored the depressed PE/FITC ratio (**Fig. 3-17A**, bottom panel; **Fig. 3-17B**; O2R22 vs. O2R22+Nec-1s, $P=0.032$; O2R22 vs. O2R22+GSK'872, $P=0.047$). In the O2R4 groups, the reduced PE/FITC ratio did not respond to RIPK1 or RIPK3 inhibition (**Fig. 3-17A**, middle panel; **Fig. 3-17B**). Our findings herein suggest that inhibiting RIPK1 or RIPK3 under basal condition impairs mitochondrial function whereas blocking RIPK1 or RIPK3 activity becomes improves mitochondrial function during OGD/Rep challenge. Moreover, the relatively milder decrease in PE/FITC ratio in O2R4 compared to that from O2R22 could have masked any beneficial effect from Nec-1s or GSK'872 treatment.

In a parallel set of experiment, we investigated the effect of MLKL knockdown on mitochondrial function under basal versus OGD/Rep conditions. siRNA-mediated MLKL silencing did not alter the JC-10 PE/FITC ratio in unstimulated cells (**Fig. 3-18A**, top panel; **Fig. 3-18B**). Remarkably, selective knockdown of MLKL reversed O2R4- (**Fig. 3-18A**, middle panel; **Fig. 3-19B**; O2R4 vs. O2R4+MLKL-si, $P=0.008$) or O2R22-induced (**Fig. 3-18A**, bottom panel; **Fig. 3-18B**; O2R22 vs. O2R22+MLKL-si, $P=0.007$) decrease in PE/FITC ratio to levels comparable to that of NC; the rescue of PE/FITC ratio is specific to MLKL downregulation as the PE/FITC ratio remained depressed in cells treated with scrambled siRNA (**Fig. 3-18A**). Drawing on the findings, MLKL knockdown restored mitochondrial potential and conferred significant mitochondrial protection during OGD/Rep, without impairing mitochondrial function in unstimulated cells.

Fig. 3-17

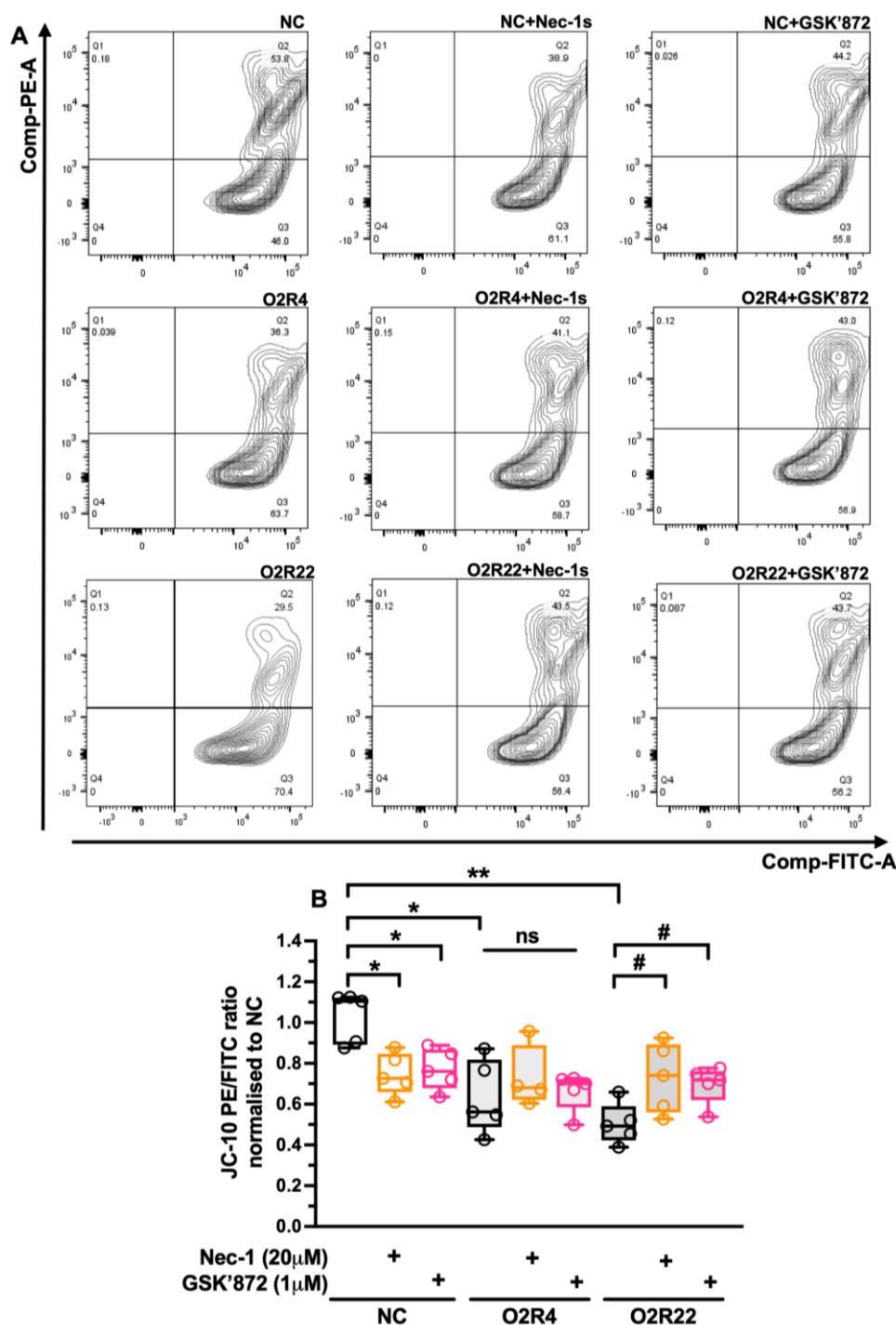


Figure 3-17. The effect of necroptosis inhibitors on mitochondrial membrane potential in Neuro-2a cells. **(A)** Flow cytometric JC-10 analysis of Neuro-2a mitochondrial membrane potential following oxygen-glucose deprivation and reperfusion (OGD/Rep), with or without the RIPK1 inhibitor necrostatin-1s (Nec-1s, 10μM) or RIPK3 inhibitor GSK'872 (1μM), fluorescence intensities shown as compensated FITC and PE channels. **(B)** JC-10 FITC to PE fluorescence ratio analysis, ratio normalised to naïve control (NC). Data expressed as median $\pm$ interquartile range. NC vs. NC+Nec-1s, * P <0.05; NC vs. NC+GSK, * P <0.05; NC vs. O2R4, * P <0.05; NC vs. O2R22, ** P <0.01; O2R22 vs. O2R22+Nec-1s, # P <0.05; O2R22 vs. O2R22+GSK, # P <0.05; n =5, each n represents an independent experiment.

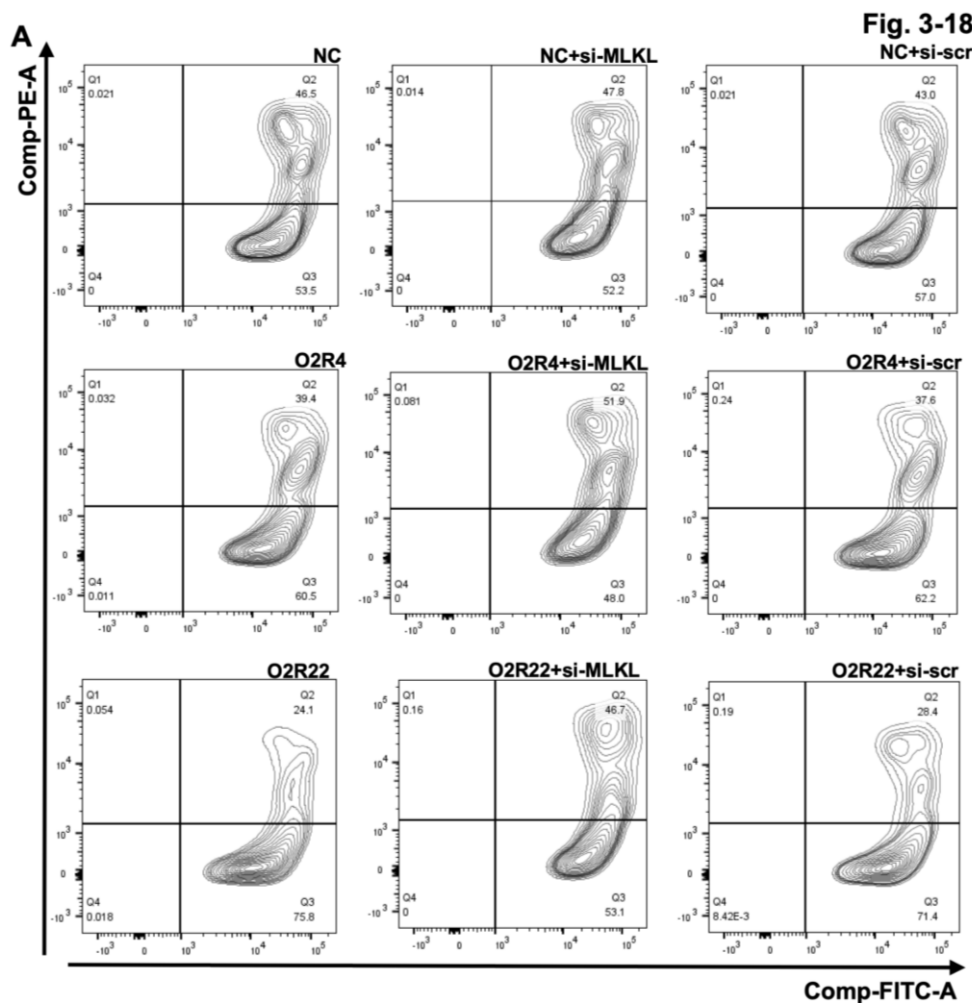


Figure 3-18. The effect of MLKL knockdown on mitochondrial membrane potential in Neuro-2a cells. (A) Flow cytometric JC-10 analysis of Neuro-2a mitochondrial membrane potential following indicated periods of oxygen-glucose deprivation (OGD/Rep), with or without small interfering RNA-mediated MLKL knockdown (si-MLKL), fluorescence intensity shown as compensated FITC and PE channels. **(B)** JC-10 FITC to PE fluorescence ratio analysis, ratio 148 ormalized to naïve control (NC). Data expressed as median $\pm$ interquartile range. NC vs. O2R4, * $P < 0.05$; NC vs. O2R22, ** $P < 0.01$; O2R4 vs. O2R4 + si-MLKL, \$ $P < 0.05$; O2R22 vs. O2R22 + si-MLKL, # $P < 0.05$; $n = 5$, each n represents an independent experiment.

3.2.8 RIPK1 inhibition or MLKL knockdown improved mitochondrial morphology

Unstimulated NC cells displayed a highly-interconnected, filamentous mitochondrial network (**Fig. 3-19A**) that becomes severely swollen and truncated following prolonged OGD/Rep (**Fig. 3-19B**, arrowheads), which correlates to a decline in mitochondrial potential as detected by TMRE and JC-10 assay (**Fig. 3-19A, 3-19B**). In the O2R22 groups, siRNA-mediated MLKL knockdown appeared to be more effective at restoring the elongated, reticular appearance of the mitochondrial network than RIPK1 inhibition by Nec-1s (**Fig. 3-19B**), which was commensurate with the magnitude of increase in PE/FITC ratio (**Fig. 3-19B, Fig. 3-19B**). Within the NC cohorts, Nec-1s treatment but not MLKL silencing was associated with a minor disruption to mitochondrial morphology (**Fig. 3-19A**) to correspond to the small fall in PE/FITC ratio (**Fig. 3-17A, Fig. 3-17B**). Cells treated with FCCP were used as positive control and demonstrated a complete annihilation of mitochondrial structure and network (**Fig. 3-19A**; asterisks). Taken together, the functional improvement from necroptosis inhibition is accompanied and substantiated by a corresponding restoration of mitochondrial structure and morphology.

3.2.9 Cell viability

Provided that disruption of necrosome signalling leads to improved mitochondrial function and morphology during OGD/Rep, we proceeded to examine whether the mitochondrial protection correlates with enhanced cell survival. In this regard, OGD/Rep resulted in significant and time-dependent reduction in overall cell viability when compared to NC (**Fig. 3-20A**; NC vs. O2R4, $P=0.021$; NC vs. O2R22, $P=0.005$). Treatment with Nec-1s or GSK'872, or siRNA-mediated MLKL knockdown, appeared to mildly protect Neuro-2a cells from OGD/Rep without reaching statistical significance. Moreover, RIPK1/RIPK3 inhibition or MLKL silencing seemed to be associated with a small decrease in cell viability under basal condition.

Although we did not detect a significant effect of necrosome blockade on overall cell viability, we continued to explore how RIPK1/RIPK3 inhibition and MLKL knockdown would affect apoptotic and non-apoptotic cell death. As expected, prolonged OGD/Rep from O2R22 significantly increased the PI-positive (Q3, **Fig. 3-20B, Fig. 3-20C**; $P=0.001$) and Annexin V/PI-positive cells (Q2, **Fig. 3-20B, Fig. 3-20D**; $P=0.007$) compared to NC to reflect increased necrosis and late apoptosis, respectively; O2R4 did not significantly elevate the proportions of necrotic and apoptotic cells. Within the O2R22 groups, MLKL knockdown significantly decreased the number of PI-positive necrotic cells (O2R22 vs. O2R22 + MLKL-si, $P=0.006$), and a milder suppressive effect was also observed in the presence of Nec-1s or GSK'872 (**Fig. 3-20B, 3-20C**). On the other hand, neither RIPK1/RIPK3 inhibitor nor MLKL silencing attenuated the increase in Annexin V/PI-positive cells in the O2R22 cohorts (**Fig. 3-20B, 3-20D**). In this regard, PI-positive cell death reversible by RIPK1/RIPK3 inhibition or MLKL knockdown likely corresponds to a population of necroptotic cells following OGD/Rep challenge, and it is

unsurprising that manipulation to necrosome components did not affect OGD/Rep-induced apoptosis that is primarily dependent on caspase activation.

In unstimulated NC cells, we noticed that RIPK1 inhibition slightly increased the percentage of PI-positive cells (**Fig. 3-20B, 3-20C**; NC vs. NC+Nec-1s, $P=0.018$), while MLKL knockdown expanded both the PI-positive (NC vs. NC+MLKL-si, $P=0.028$) and Annexin V/PI-positive (NC vs. NC+MLKL-si, $P=0.014$) populations. In light of the fact that RIPK1 serves as an upstream master modulator of regulated cell death (necroptosis and apoptosis) as well as the pro-survival inflammatory pathway, inhibition of RIPK1 activity under basal condition may preferentially disable the pro-survival signalling cascade to slightly elevate cell death. Moreover, Nec-1s induced necrosis could be related to the mild mitochondrial dysfunction in NC (**Fig. 3-17A, 3-17B**) shown above. Regarding MLKL, it is plausible that MLKL exerts basal inhibition on apoptosis and non-apoptotic regulated cell death pathways, which become “disinhibited” upon MLKL knockdown to manifest as increased Annexin-V/PI positive and PI positive populations, respectively.

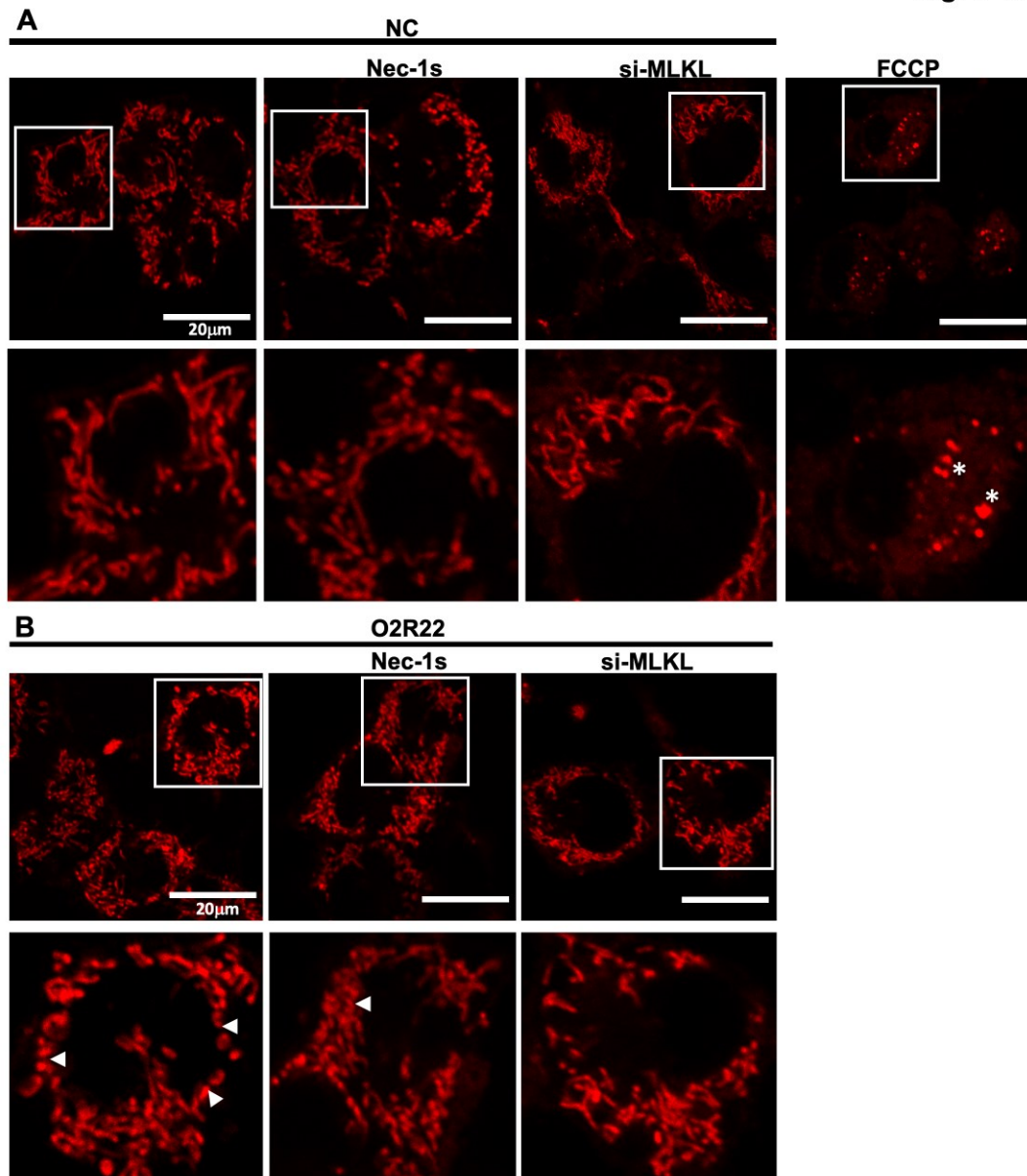
Fig. 3-19

Figure 3-19. Confocal microscopy of mitochondrial morphology in Neuro-2a cells. Representative confocal photomicrograph of Neuro-2a cells labelled with the mitochondrial dye MitoTracker-Deep Red in **(A)** NC and **(B)** O2R22 (2hr oxygen-glucose deprivation plus 22hr reperfusion) groups, treated with RIPK1 inhibitor necrostatin-1s (Nec-1s, 10μM) or MLKL-specific small silencing RNA (si-MLKL, 10nM). Boxed areas were enlarged in the lower panel, arrowheads pointing to swollen, truncated mitochondrial structures. The mitochondrial potential uncoupler FCCP was used as positive control with complete disruption of mitochondrial network (asterisks). Magnification x180, scale bar 20μm.

Fig. 3-20

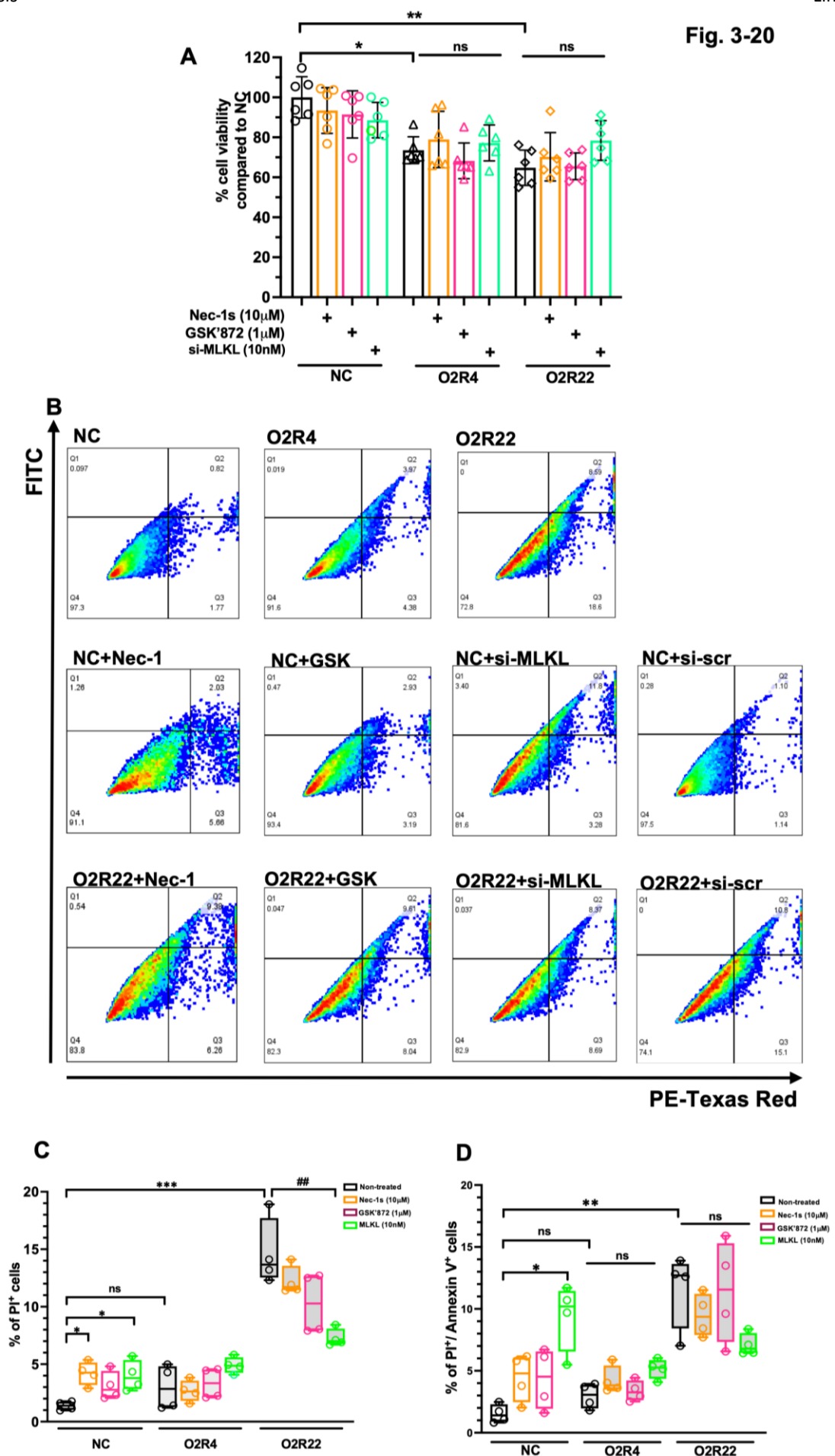


Figure 3-20. The effect of oxygen-glucose deprivation and reperfusion (OGD/Rep) on Neuro-2a cell viability.

(A) Cell-counting kit 8 (CCK8) assay on Neuro-2a cell viability following oxygen-glucose deprivation and reperfusion (OGD/Rep), with or without RIPK1 inhibitor necrostatin-1s (Nec-1s, 10 μ M), RIPK3 inhibitor GSK'872 (1 μ M) or MLKL-specific small silencing RNA (si-MLKL, 10nM). Cell viability normalised to NC, and data expressed as mean $\pm$ SD. NC vs. O2R4, *P<0.05; NC vs. O2R22, **P<0.01; n=6, each n represents an independent experiment. **(B)** Flow cytometric analysis of Annexin V/propidium iodide (PI) staining of Neuro-2a cells following OGD/Rep,, with or without RIPK1 inhibitor necrostatin-1s (Nec-1s, 10 μ M), RIPK3 inhibitor GSK'872 (1 μ M) or MLKL-specific small silencing RNA (si-MLKL, 10nM). **(C)** Percentage of PI-positive cells and **(D)** Percentage of Annexin V/PI double-positive cells. Data expressed as median $\pm$ interquartile range. [PI-positive, NC vs. NC+Nec-1s, *P<0.05; NC vs. NC+si-MLKL, *P<0.05; NC vs. O2R4, **P<0.01; NC vs. O2R22, ***P<0.001; O2R22 vs. O2R22 + si-MLKL, ##P<0.01; n=4]. [Annexin V/PI-positive – NC vs. NC+si-MLKL, *P<0.01; NC vs. O2R4, **P<0.01; NC vs. O2R22, **P<0.01; n=4]; each n represents an independent experiment.

3.3 Discussion

3.3.1 Concurrent activation of necroptosis and mitophagy in primary neurons and Neuro-2a cells from OGD and reperfusion

In stark contrast to passive necrosis, regulated cell death is considered to be energy-dependent and requires specific molecular mediators and intricate molecular pathways for execution. Necroptosis, or regulated necrosis is stimulated by TNFSF ligands and is orchestrated by the RIPK1/RIPK3/MLKL necrosome^{290, 291, 296, 312}. The sequential phosphor-activation of RIPK1, RIPK3 and MLKL ultimately leads to plasma membrane perforation by phosphor-MLKL oligomers, the putative mechanism by which necroptosis contributes to cell death and produces the characteristic membrane damage that is reminiscent of necrosis. It was further demonstrated that MLKL-mediated plasma membrane damage can be rescued by the intrinsic membrane repair machinery comprising the ESCRT-III complex³¹³, which coordinates the enclosure and shedding of perforated membrane for release as extracellular vesicles. A few studies have also observed the translocation of MLKL along with RIPK1/RIPK3 into the mitochondrial membrane^{312, 401, 402}, however the implication of mitochondrial necrosome translocation remains poorly understood.

In this Chapter, we first demonstrated that oxygen glucose deprivation plus reperfusion (OGD/Rep) induced MLKL phosphorylation in primary neurons and neuron-like cells to indicate activation of necroptosis. For the first time, our study reported that OGD/Rep-induced necroptosis also promoted mitochondrial translocation of necrosome, which can be distinguished from previous studies showing necrosome translocation after stimulation with the necroptosis-inducing cocktail that comprised of tumour necrosis factor- α , a SMAC mimetic (to inhibit cIAP1/2 and release RIPK1 from complex I) and a pan-caspase inhibitor (to prevent caspase-8 activation and favour necrosome formation)^{296, 312, 402}. In this regard, we consider findings derived from our OGD/Rep protocol to be more relevant to human pathophysiology than from stimulation by compounds (SMAC mimetic, pan-caspase inhibitor) that do not naturally occur. We further hypothesised that necroptosis activation, possibly through mitochondrial localisation of necrosome, may function upstream to regulate mitophagy rate during OGD/Rep. In this respect, we observed induction of mitophagy-related markers in the whole-cell and mitochondrial fractions along with necrosome activation, to indicate concurrent upregulation of necroptosis and mitophagy in neurons and neuron-like cells during OGD/Rep.

3.3.2 Necrosome components exert opposing effects on OGD/Rep-induced mitophagy

RIPK1 inhibition by Nec-1s (10 μ M) effectively prevented OGD/Rep-induced MLKL phosphorylation and mitophagy in Neuro-2a cells, to suggest that RIPK1 activation during OGD/Rep could promote mitophagy in ischaemia-reperfusion brain injury. Nec-1s reduced both the whole-cell and mitochondrial expression of Parkin, to imply amelioration of mitochondrial stress and decreased need for transcription, synthesis and mitochondrial localisation of Parkin. Inhibition of RIPK1 activity also attenuates necrosome translocation into the mitochondria that may account for depressed mitophagy, provided mitochondrial-translocated necrosome would normally enhance mitophagy during OGD/Rep stress.

In support of such notion, studies have demonstrated that oligomerised MLKL could perforate artificial liposomes with similar lipid composition (10-15% cardiolipin) to mitochondria³¹². In this regard, RIPK1 activation and subsequent mitochondrial localisation of MLKL may aggravate the mitochondrial damage acquired through OGD/Rep, to cause further accumulation of the mitophagy mediators and to potentiate OGD/Rep-induced mitophagy. Moreover, as discussed in chapter one, section G, mitochondrial translocation of RIPK3, presumably downstream to RIPK1 activation, could phosphorylate PGAM5⁴⁰³ and PDC⁴⁰⁴ to drive mitochondrial fission and ROS production to promote mitophagy. In this respect, fragmentation of mitochondrial networks may favour mitophagy, whereby the mitochondrial fission protein Drp-1 interacts with the mitophagy receptors BNIP3, FUNDC1 and Bcl2-like protein 13 (Bcl2-L-13)^{450, 451}, and that increased mitochondrial ROS is conducive for PINK1/Parkin-dependent mitophagy^{410, 411}. Alternatively, it is also plausible that necroptosis could regulate mitophagy independent of necrosome translocation, and an example of which would be that RIPK1/RIPK3-induced mitochondrial ROS synthesis does not always necessitate RIPK1/RIPK3 mitochondrial localisation⁴⁰⁶.

Moreover, we have demonstrated that RIPK3 inhibition by GSK'872 (1 μ M) at concentration known to prevent necroptosis effectively diminished mitochondrial localisation of necrosome and mitophagy mediators, to indicate that RIPK3 inhibition prevents mitophagy during OGD/Rep injury. On this basis, we speculate that RIPK3 normally would promote mitophagy in neuron-like cells undergoing ischaemia/reperfusion, in conjunction with RIPK1 as an integral, active complex. As hypothesised above, mitochondrially-localised RIPK3 could exert its kinase activity on substrates such as PGAM5s and PDC to accelerate mitophagy^{403, 404}, or RIPK1/RIPK3 may promote mitochondrial ROS synthesis⁴⁰⁶ and subsequently mitophagy with or without mitochondrial translocation.

The observation that mitophagy suppression from RIPK3 inhibition is less prominent than from RIPK1 inhibition implies that OGD/Rep-induced mitophagy maybe more dependent on RIPK1 activity; it is plausible that RIPK1 may signal through effectors other than RIPK3 to promote mitophagy. Moreover, in view of its cytotoxicity at higher doses (3-10 μ M)⁴¹⁹, the effective therapeutic window of GSK'872 likely falls within 1-

3 μ M for *in vitro* studies, and future mechanistic studies will require careful titration of GSK'872 concentration for optimal inhibition of RIPK3 activity and downstream pathways. *In vivo* studies have reported much higher concentrations of GSK'872 for achieving therapeutic efficacy in global cerebral ischaemia/reperfusion injury (~26mM; 260nmol per animal)⁴⁵² and traumatic brain injury (~25mM; 150nM per animal)⁴⁵³ with intracerebrovascular administration, which was associated with reduced tissue loss, improved water maze performance and dampened cytokine expression.

MLKL knockdown in Neuro-2a cells prevents mitochondrial necrosome translocation yet unexpectedly stimulated mitophagy, to suggest that MLKL activation suppresses mitophagy and mitochondrial turnover during OGD/Rep injury. Such conjecture is in stark contrast to conclusions derived from studies on Nec-1s and GSK'872 that imply RIPK1 and RIPK3 as potential positive regulators of mitophagy. Based on the collective findings reported herein, it appears that ischaemia-reperfusion concomitantly upregulates necroptosis and mitophagy, whereby mitophagy is dependent on RIPK1/RIPK3 activation. Subsequent phosphor-activation of MLKL facilitates the mitochondrial translocation of all necrosome components, while the activated MLKL exerts its suppressive effect on mitophagy. Moreover, we found siRNA-MLKL was associated with a decrease in whole-cell and mitochondrial CLpP level that is in line with cells treated with Nec-1s or GSK'872, despite the divergent findings on other mitophagy mediators. Thus, CLpP may not directly contribute to OGD/Rep-induced mitophagy but rather should be treated as a surrogate marker of mitochondrial stress. The decreased CLpP, therefore, can be considered to reflect improved mitochondrial function from RIPK1/RIPK3 inhibition or MLKL knockdown.

As discussed previously, studies have primarily focussed on the significance of RIPK3 phosphor-activation and RIPK3 mitochondrial localisation in the context of necroptosis execution and downstream pathways, and have identified different mitochondrial substrates as phosphorylation targets of RIPK3^{403, 404}. RIPK1/RIPK3-dependent mitochondrial ROS synthesis, whether requiring mitochondrial transportation of the necrosome or not^{405, 406}, also represents a feasible mechanism by which RIPK1/RIPK3 triggers mitochondrial dysfunction to drive necroptosis. As a pseudo-kinase lacking kinase activity, activated MLKL is unlikely to phosphorylate downstream substrates but rather accomplishes necroptosis through plasma membrane perforation, an execution mechanism that has become the consensus in the field of necroptosis research. On the other hand, the purpose of mitochondrially translocated MLKL remains unclear, other than acting as a "cargo" to facilitate RIPK3 interaction with mitochondrial substrates. Our studies surprisingly revealed that MLKL inhibition produces opposite effects to RIPK1/RIPK3 blockade on mitophagy, to suggest that MLKL is uncoupled from RIPK1/RIPK3 and assumes a distinct role in mitochondrial regulation during OGD/Rep injury. Moreover, preliminary evidence from Wang et al. showed MLKL as capable of perforating artificial "mitochondrion-like" liposomes that is reminiscent of mitochondrial permeabilisation from pro-apoptotic BAX/BAD³¹². In this

regard, mitochondrial localised MLKL would cause membrane damage to stimulate mitophagy, yet our result highlighted MLKL deficiency as a more potent inducer of mitophagy.

Nonetheless, it should be noted that the absence of MLKL may exert different effect on mitophagy than having the protein in an inactive state (i.e. pharmacological inhibition). Although MLKL deficiency and inhibition would both prevent necrosome translocation, it is unclear whether the inhibited/inactive MLKL is still capable of interacting with mitophagy-related factors. In a parallel study design, human neuroblastoma cell SY-5-SHY could be treated with MLKL inhibitor NSA, and OGD/Rep-induced mitophagy could be compared with Neuro-2a cells with MLKL knockdown. Alternatively, MLKL-KO Neuro-2a cells could be reconstituted with phospho-dead or N-terminal modified MLKL mutants^{296, 312, 447}, which are incapable of phosphor-activation by RIPK3 or membrane translocation, and the mitophagy responses between MLKL-mutant and WT MLKL cells following OGD/Rep can be compared.

The precise mechanism by which MLKL inhibits mitophagy remains elusive, and it is also unclear how the respective changes to mitophagy (from RIPK1/3 inhibition or MLKL knockdown) would affect mitochondrial function and cell survival in OGD/Rep-challenged Neuro-2a cells. In the preliminary experiment below, we attempt to explore the potential interaction between MLKL and mitophagy mediators during OGD/Rep insult. The effect of RIPK1/RIPK3 inhibition and MLKL manipulation on mitochondrial function and cell survival will be examined in the next chapter.

Our preliminary Co-IP data highlights a physiologically relevant interaction between MLKL and the mitophagy mediator BNIP3 during OGD/Rep injury, and suggests a potential mechanism for MLKL to directly associate and inhibit BNIP3-dependent mitophagy, within or outside of the mitochondrial compartment. Moreover, it remains plausible that MLKL could indirectly signal to Parkin or CLpP to regulate mitophagy and mitochondrial quality control. Furthermore, siRNA knockdown study supports CLpP as a more potent negative regulator of mitophagy than MLKL under basal and OGD/Rep conditions. Unexpectedly, we were unable to observe an additive effect on mitophagy from double knockdown, which maybe attributable to an intrinsic braking mechanism to prevent excessive mitophagy. Moreover, CLpP knockdown did not cause a compensatory increase in MLKL and vice versa to imply that MLKL and CLpP do not collaboratively modulate mitophagy. We speculate that stimulation of mitophagy from CLpP silencing represents a secondary response resulting from impaired mitochondrial protein homeostasis and subsequent ROS accumulation.

3.3.3 Necroptosis inhibition restores mitochondrial function decline from OGD/Rep challenge

OGD/Rep is associated with significant and time-dependent decrease in mitochondrial potential and impairment to mitochondrial function and morphology in Neuro-2a cells, especially in the O2R22 group. Pharmacological inhibition of RIPK1 or RIPK3, or siRNA-mediated silencing of MLKL all led to improvement in mitochondrial potential from prolonged OGD/Rep (O2R22), with MLKL knockdown resulted in almost full restoration of mitochondrial function and the filamentous, reticular appearance of mitochondrial networks. In contrast to their mito-protective effect during OGD/Rep, RIPK1 or RIPK3 blockade slightly impaired mitochondrial function and morphology under basal condition, which was not mirrored by MLKL downregulation.

In view of the previous studies supporting mitophagy as neuroprotective and that RIPK1/RIPK3 inhibition suppresses OGD/Rep-induced mitophagy (Chapter Four), we hypothesised that Nec-1s or GSK'872 treatment would further deteriorate mitochondrial function following I/R. However, treating Neuro-2a cells with either inhibitor at the respective concentration results in partial recovery of mitochondrial potential from O2R22. In the present study, prolonged, severe OGD/Rep causes significant mitochondrial depolarisation and dysfunction to trigger mitophagy for the removal of defective mitochondria. OGD/Rep-induced activation of RIPK1/RIPK3 likely promotes a range of mitochondrial events in conjunction with increased mitophagy, such as ROS production, mitochondrial fission and other unidentified processes, to collectively contribute to mitochondrial dysfunction. Therefore, attenuation of RIPK1/RIPK3 activity would antagonise these processes with improved mitochondrial function as an overall outcome. Thus, it is still feasible that RIPK1/RIPK3-associated mitophagy is mito-protective, however its beneficial effect could have been masked by the other mito-destructive mechanisms co-activated by RIPK1/RIPK3. It may also be the case that the decline in mitophagy following RIPK1/RIPK3 inhibition is secondary to and reflective of improved mitochondrial function, such that there is a reduced need for mitochondrial quality control via mitophagy. In view of the available data, we cannot conclude whether RIPK1/RIPK3-associated mitophagy is mito-protective or not during OGD/Rep, yet selective targeting of RIPK1/RIPK3 still holds promise for OGD/Rep injury in neuron-like cells as it prevents mitochondrial dysfunction.

On the other hand, our data suggests that RIPK1/RIPK3 is required for the maintenance of mitochondrial function under normoxia (NC cells), which becomes compromised upon RIPK1/RIPK3 inhibition. Provided that RIPK1 and RIPK3 are not activated under basal condition and lack kinase activities, it is unclear how RIPK1/RIPK3 would modulate mitochondrial function and whether it is achieved through mitophagy regulation. In this regard, the decline in mitochondrial potential from RIPK1/RIPK3-inhibited NC cells is not affiliated with altered expression of mitophagy mediators. Moreover, as Nec-1s and GSK'872 are highly

specific inhibitors for RIPK1 and RIPK3 at the concentrations administered, it is unlikely that the depressed mitochondrial function in NC cells is due to off-target inhibition of other molecules/mediators.

With respect to MLKL, the observation that MLKL knockdown significantly improved mitochondrial function following OGD/Rep aligns neatly with the notion that mitophagy is neuroprotective, when taken together with our data demonstrating that MLKL silencing upregulated all mitophagy-related markers. In this regard, if MLKL phosphor-activation normally suppresses mitophagy during necroptosis, MLKL downregulation would remove such brake to potentiate the “mitoprotective” mitophagy. Unlike the uncertainties concerning RIPK1/RIPK3-induced mitophagy discussed above, a correlation between increased mitophagy and enhanced mitochondrial function can be established in the context of MLKL knockdown. In future studies, double knockdown of MLKL and Parkin or BNIP3 could be performed to further substantiate the link between necroptosis-associated mitophagy and mitochondrial function. If the absence of Parkin or BNIP3 abolishes the improvement of mitochondrial function following MLKL knockdown, one may conclude that MLKL activation impairs Parkin and/or BNIP3-dependent mitophagy to contribute to mitochondrial dysfunction during OGD/Rep. Taken together, our findings indicate that pharmacological inhibition of RIPK1/RIPK3 or genetic downregulation of MLKL restores mitochondrial function and network morphology during OGD/Rep, and in the case of MLKL knockdown the salubrious effect is likely achieved through amplification of mitoprotective mitophagy. Selective targeting of MLKL during OGD/Rep maybe preferred over RIPK1/RIPK3-targeted strategies as maximal restoration of mitochondrial function is evident in the former.

Mitochondrial dysfunction was repeated shown to be a core feature of necroptotic cell death triggered by a wide range of necroptotic stimuli, and the perplexing question is whether mitochondrial dysfunction represents a cause or a result, or both, of necroptosis. Prior to the identification of MLKL as the effector substrate of RIPK1/RIPK3 and the executor of necroptosis, mitochondrial ROS production and accumulation stimulated by RIPK1/RIPK3 activation was initially regarded the execution mechanism of necroptosis^{290, 405}. The molecular mechanism that necroptosis promotes mitochondrial ROS synthesis may be attributed the coordinated ROS stimulatory activities from RIPK3 phosphor-activation of pyruvate dehydrogenase complex (PDC)⁴⁰⁴ and RIPK3-dependent mitochondrial enrichment of NADPH oxidase 4 (NOX4)⁴⁵⁴, and likely also via other unidentified mitochondrial substrates. Other than oxidative stress, mitochondrial dysfunction during/accompanying necroptosis could manifest as decreased ATP synthesis, fall in oxygen consumption rate (OCR), mitochondrial membrane depolarisation and downregulated expression of respiratory chain complex I, III and V proteins, which are also dependent on the particular necroptosis triggers involved (e.g. conventional TNF- α /pan-caspase inhibitor combination⁴⁵⁵, respiratory chain inhibitor⁴⁰⁸, or disease-specific models: septic kidney injury⁴⁵⁴, cadmium hepatotoxicity⁴⁰¹, cigarette-smoke induced lung injury⁴⁰⁹).

Moreover, we have demonstrated herein I/R injury significantly decreased cell viability in Neuro-2a cells by promoting Annexin-V/PI positive apoptosis and PI positive necrosis. Within I/R-induced necrotic cell population, cell death that was preventable by RIPK1/RIPK3 inhibition or MLKL knockdown are suggestive of necroptosis, while OGD/Rep-induced apoptosis remained largely unaffected by RIPK1/RIPK3 inhibition and MLKL silencing. It should be noted that since OGD/Rep activates multiple regulated cell death (RCD) pathways, antagonising individual pathway, for example necroptosis inhibition as shown here, may not lead to satisfactory improvement to overall cell viability. Therefore, a combinatorial approach targeting multiple, parallel RCD pathways will be needed to confer significant protection against OGD/Rep or I/R challenge.

CHAPTER FOUR

Activation of Necroptosis and Mitophagy in a Murine Model of Hypoxic-Ischaemic Encephalopathy

4.1 Introduction

The development of appropriate and robust rodent models for hypoxic-ischaemic encephalopathy has provided substantial insights into the molecular pathophysiology of neonatal HI brain injury and enabled investigators to examine the potential salubrious effects of an array of neuroprotective therapies. Inter-species comparison on neuroanatomy, neurogenesis, synaptogenesis, myelination, immune maturation and other biochemical signatures has correlated postnatal day 7 to day 10 (PND7-10) in rodents to 36- to 40-week gestation in human^{83, 84}. In this regard, PND 7-10 rodents are deemed suitable for studying HIE in near-term or termed infants, whereas immature rodents of PND 1-5 are routinely used to model pre-term HI injury predominated by white matter lesion.

The adapted Rice-Vannucci procedure (unilateral common carotid artery ligation plus global hypoxia) in postnatal day 7-10 mice represents a reliable and convenient model for examining the molecular perturbations and behavioural dysfunctions from hypoxic-ischaemic encephalopathy. The initial Rice-Vannucci model developed for PND7 rats involved unilateral carotid artery ligation (i.e. ischaemia) followed by 8%O₂ hypoxia (FiO₂=0.08) for an extended duration for 3.5 hrs⁸⁸, and other studies have used at least 90 minutes of hypoxia to produce moderate-to-severe injury in neonatal rats. Neonatal mice on the other hand are significantly less resistant to global hypoxia, and the duration/intensity of hypoxia needs to be carefully titrated to elicit sufficient brain injury without inflicting excessive death. In addition, surgical microscope is required to perform carotid artery ligation in much smaller mouse pups. In this regard, studies have demonstrated that unilateral common carotid artery ligation with 1 – 2 hrs recovery before 40-70mins of 8% O₂ hypoxia (FiO₂ = 0.08)⁹²⁻⁹⁵ can be well-tolerated by mice, and the duration of hypoxia maybe extended up to 2 hrs when O₂ concentration is set at 10%^{109, 206}. Studies have also noted considerable variations in susceptibility and injury scores between different mouse strains. When comparing CD1, C57BL/6 and 129Sv PND7 mice and cross-strain breeds, 129Sv exhibited the highest mortality (up to 50%) with 30mins hypoxia when no death was reported from the other strains, whereas CD1 background harboured more severe neuropathology following 30 minutes hypoxia, however the inter-strain injury variation diminished with prolonged hypoxia⁹⁶.

Histopathological examination of the lesioned hemisphere from mice pups revealed the cortex, hippocampus and striatum to be highly susceptible to the Rice-Vannucci manipulation. As evident from cresyl-violet stained brain sections, mild injury could manifest as focal or isolated group of shrunken/pyknotic neurons or dead cells, to moderate injury including columnar neuronal loss in cortical laminar layers II – IV, confluent infarction in the cortex, and more extensive cell loss in the hippocampus, striatum and thalamus, and finally severe injury that presents as cystic infarction of the cortex, severe atrophy of hippocampus, striatum and thalamus (>50% involvement) and extensive gliosis^{92-95, 109, 456}. The lesions of the ipsilateral hemisphere can

be scored by sub-region or aggregated into a total injury score, while the uninjured contralateral hemisphere serves as a reference for comparison. Total volumetric loss from infarction can also be quantified for more severe form of HIE²⁰⁶. Scoring the histopathology has become the gold-standard method for assessing the therapeutic potential of candidate treatments for HIE, and it is more informative to evaluate lesions in the short-term (from one week) as well as the long-term (to one month) as certain therapies only delayed the onset of injury but did not translate into a reduction of neuronal/cell loss.

The murine HIE model also enabled long-term study of motor, behavioural and cognitive impairments that are underpinned by neuronal loss/atrophy across the cortex, hippocampus and deep grey matter. These functional tests allow researchers to correlate biochemical and neuropathological changes with functional disturbances for a more in-depth, comprehensive understanding of HIE, and to determine whether potential therapeutic intervention is as effective at improving functional outcomes as biochemical and pathological outcomes⁸². However, it must be emphasised that the immature murine brain by no means recapitulates the highly compartmentalised, complex structure of human brain with extended window of neurogenesis and immune maturation, which collectively render the human response to HIE a more sophisticated, interconnected process⁹⁸. Nonetheless, functional testing in rodents is still integral to HIE study and adds on to the translational value of HIE research. In this regard, sensorimotor reflexes emerging from day 7-8 of age are early, sensitive indicators for verifying the successful establishment of HIE in mouse pups. In juvenile mice at around PND30, accelerating rota-rod represents a particularly informative and pragmatic test for assessing long-term motor movement and limb coordination, and the three-day training component also requires a certain degree of learning/acquisition.

The RIPK1 inhibitor necrostatin-1 (Nec-1) and its chemically-improved analogue necrostatin-1s (necrostatin-1 stable, or 7-Cl-O-Nec-1) were shown to attenuate cell death in OGD-challenged primary neuronal culture⁴⁵⁷, and reduce infarct volume in adult^{317, 457} and developing brains^{323, 324} following H/I injury with an extended therapeutic window of up to 6hrs post-insult. Additionally, blocking RIPK1 activity in the H/I-injured neonatal brain appeared to bias cell death towards “apoptotic” phenotype, attenuate oxidative stress, improve mitochondrial morphology and re-elevate ATP production at 24hrs³²⁴. Our findings from Nec-1s treated Neuro-2a cells further corroborated RIPK1 inhibition as a neuroprotective strategy, whereby RIPK1 inhibition restored mitochondrial membrane potential and filamentous morphology following OGD plus reperfusion.

The existence of a necroptosis-mitochondria signalling axis has been much speculated even before the identification of MLKL as the executioner of necroptosis, with studies showing that RIPK1/RIPK3 promotes mitochondrial ROS synthesis and mitochondrial dysfunction and suggesting this mechanism to directly underpin RIPK1/RIPK3-dependent necrotic cell death; the observations that Nec-1 mediated RIPK1 blockade prevented oxidative stress and reduced lesion size in the immature brain post-H/I further reinforced the

notion that RIPK1 antagonism is neuroprotective. Whether mitochondrial ROS is a prerequisite for RIPK1/RIPK3-dependent necroptosis is questioned by others, as mitochondria-depleted cells were still susceptible to cell death from forced RIPK3 overexpression and sensitive to Nec-1 cytoprotection following TNF- α + caspase inhibitor treatment.

In the present study, we proposed a novel concept that necroptosis could act upstream to mitophagy to modulate mitochondrial function during I/R brain injury, with the activated necrosome possibly serving as the signal transducer when it travels into the mitochondrial fraction. As demonstrated in OGD/Rep-challenged Neuro-2a cells, pharmacological inhibition of RIPK1 or RIPK3 obstructed the mitochondrial translocation of necrosome (RIPK3, MLKL) along with depressed mitophagy, to suggest that RIPK1/RIPK3 signalling would normally stimulate mitophagy during I/R. Moreover, the attenuation of mitophagy from Nec-1 or GSK'872 treatment coincided with partial restoration of mitochondrial membrane potential after OGD plus reperfusion, to corroborate the therapeutic potentials of RIPK1 and RIPK3 inhibitors. Paradoxically, necroptosis inhibition by MLKL knockdown potentiated I/R-induced mitophagy with complete reversal of mitochondrial dysfunction and recovery of mitochondrial morphology, to indicate that the anti-mitophagy activity of MLKL is "uncoupled" from the pro-mitophagy property of RIPK1/RIPK3, and suggest MLKL blockade as an equally promising therapeutic strategy as RIPK1/RIPK3 inhibition against I/R brain injury.

In this Chapter, we aim to demonstrate the successful induction of ipsilateral HI brain injury from unilateral common carotid artery coagulation (as a more pragmatic alternative to ligation for the small mouse pups) plus global hypoxia at 8% in PND7 C57BL/6 mice, with established histopathological lesions and impairments to sensorimotor reflexes and rota-rod performance. Moreover, we aim to investigate the effect of 7-Cl-O-Nec-1/Necrostatin-1 stable (Nec-1s) on mitophagy in the described murine model of HIE, and to assess whether RIPK1 inhibition is associated with functional improvements following cerebral H/I injury. Our specific objectives are as follow:

5 – To confirm neuronal necroptosis (phos-MLKL, phos-RIPK3) and concurrent upregulation of mitophagy markers (Parkin, BNIP3, CLpP and LC3A/B colocalisation with mitochondria) in susceptible brain regions in a neonatal murine model of HIE;

6 – To evaluate the effect of RIPK1 inhibitor necrostatin-1 on mitophagy markers (Parkin, BNIP3, CLpP and LC3A/B colocalisation with mitochondria) and long-term motor and behavioural functions in HIE-injured mice.

4.2 Results

4.2.1 The development of brain lesion and motor dysfunction following HIE

In the present study, right common carotid artery (CCA) coagulation was performed in C57BL/6 mice at postnatal day 7 surgical microscopy to produce unilateral hemispheric “ischaemia”. After 2hrs of recovery with the dams, hypoxia is commenced by placing the operated mice in a humidity and temperature (36°-36.5°C) controlled chamber with 8% O₂ (FiO₂=0.08) balanced by a constant flow of N₂ gas for 45 minutes.

We first examined whether our hypoxia-ischaemia procedure is capable of inducing detectable brain lesion in C57BL/6 mice, given that previous studies had opted for longer duration of hypoxia (up to 70mins). As evident from gross morphology at day1 and day3 post-injury, right CCA cauterisation plus 45mins hypoxia (hereafter referred to as H/I) led to unilateral cerebral oedema (**Fig. 4-1A**), a hallmark of acute ischaemic stroke that is attributable to capillary endothelial dysfunction and subsequent BBB breakdown; by day21 post-injury (postnatal day 28), dramatic brain volume loss was observed in the ipsilateral hemisphere of the H/I group (**Fig. 4-1A**). Cresyl violet staining was performed to visualise the pattern of brain injury at day3 post-H/I. When compared to age-matched sham, H/I injured mice displayed significant neuron loss within the ipsilateral (right) hippocampus and cortex (**Fig. 4-1B**, right), with marked decrease in pyramidal neurons and granular neurons in hippocampal cornu ammonis 1-3 (CA1-CA3) region (**Fig. 4-1C**, bottom row, red arrowheads), and also to a lesser extent cell death in the dentate gyrus (DG) and the cortical laminar layers II-IV.

In addition to histological examination, we also assessed sensorimotor reflexes and motor functions to confirm the development of hypoxic-ischaemic brain injury. As described in Introduction, Section C.1.3, age-specific sensorimotor reflexes in rodents are sensitive to HIE and impairment to early sensorimotor reflexes correlates well with cognitive deficit later on in life. At 24hrs (PND8 mice), we found that negative geotaxis (**Fig. 4-1D**; Sham vs. H/I, $P<0.001$) and cliff aversion (**Fig. 4-1E**; Sham vs. H/I, $P<0.001$) were impaired in the H/I group compared to the sham, where H/I-injured mice took significantly longer to face up the slope and turn away from the cliff edge. Between PND28 to PND30, mice were subjected to accelerated rota-rod test for 3 consecutive days to assess long-term motor function, and latency to fall or to first complete revolution within a 5-minutes interval was recorded. As expected, the averaged latency on day30 was significantly shorter in H/I-injured mice than in the sham controls (**Fig. 4-1F**; Sham vs. H/I, $P=0.038$) to support the development of motor dysfunction.

Fig. 4-1

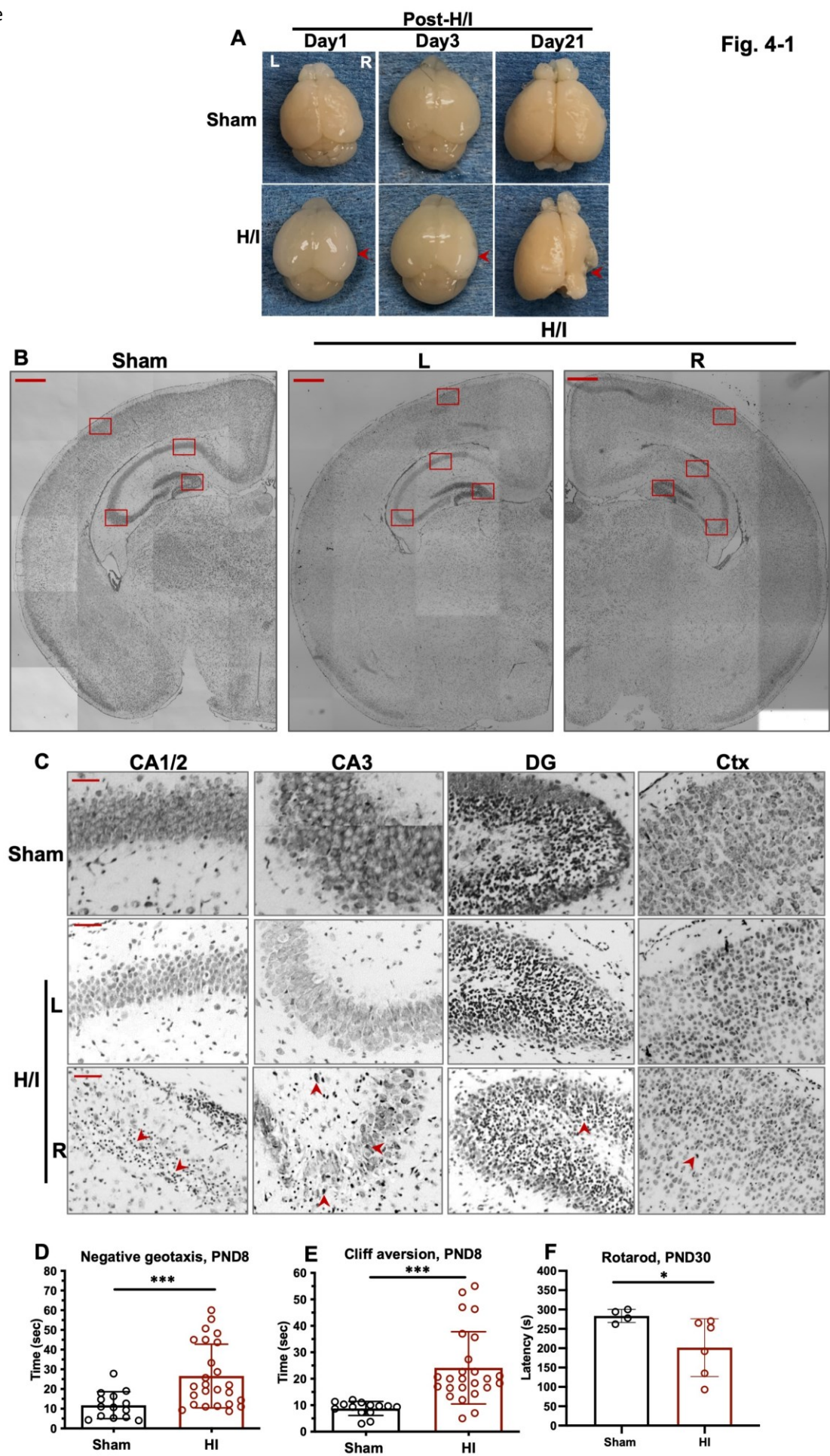


Figure 4-1. The development of brain lesion in a murine model of hypoxic-ischaemic encephalopathy (HIE) in postnatal day7-10 C57BL/6 mice. (A) Representative images showing the gross morphology of brain from sham and mice subjected to cerebral hypoxia/ischaemia (H/I) at day1, day3 and day28 post-injury. **(B)** Representative cresyl violet staining of sham (left hemisphere) and H/I-injured brain (left and right hemispheres) at day3. Magnification 10x, scale bar 500µm. **(C)** Enlarged hippocampal and cortical regions from **(B)**, red arrowheads showing neuronal loss in the right (ipsilateral) hemisphere of H/I-injured mice. Magnification 20x, scale bar 50µm. Impairment to sensorimotor reflex was assessed by **(D)** negative geotaxis and **(E)** cliff aversion at day 1 after cerebral H/I (equivalent of postnatal day 8 – PND8), time taken to turn up the slope or turn away from the cliff was recorded. **(F)** Long-term motor function was assessed by accelerating rota-rod performance on day23 after cerebral H/I (equivalent of postnatal days30 – PND30), and the latency to fall or to first complete revolution was recorded. All data expressed as mean ± SD. [Negative geotaxis – Sham vs. H/I, ***P<0.001; n=14-25] [Cliff aversion – Sham vs. H/I, ***P<0.001; n=14-25] [Rota-rod – Sham vs. H/I. *P<0.05; n=6]; each n represents an independent experiment.

4.2.2. Upregulated expression of necroptosis markers following HIE

Based on cresyl violet staining, it appears that the ipsilateral hippocampus sustained the most extensive neuronal loss from H/I (**Fig. 4-1B, 4-1C**), and thus we have focussed on the hippocampal region for subsequent analysis on necroptosis and mitophagy markers. At day1 post-H/I, a large number of phosphorylated-MLKL positive neurons/cell bodies were detected in the ipsilateral hippocampal CA1 to CA3 regions (**Fig. 4-2A, 4-2B**), which was accompanied by an acute astrogliosis response. Weaker signal of MLKL phosphorylation may be detected in the contralateral hippocampus post-H/I with considerable between-subjects variation. Western blot on isolated hippocampal tissue confirmed phosphorylation of MLKL in the ipsilateral (H/I-R), and to a lesser extent, the contralateral hippocampus (H/I-L) at day1 post-H/I (**Fig. 4-2C**). The number of phosphorylated RIPK3 (phos-RIPK3) positive cell bodies displayed a similar pattern, whereby phos-RIPK3 was most elevated in the ipsilateral hippocampal CA1 to CA3 regions and minimally increased in the contralateral hippocampus (**Fig. 4-3A, 4-3B**) at day1 post-injury; western blot corroborated H/I-induced RIPK3 phosphorylation in the ipsilateral hippocampus (**Fig. 4-3C**; H/I-R). The concurrent upregulation of phos-MLKL and phos-RIPK3 in the ipsilateral hippocampus supports the activation of necroptosis at day1 following H/I injury. The number of cleaved-caspase 3 positive neurons was also markedly increased in the CA regions as well as in the dentate gyrus (DG) of the ipsilateral hippocampus at day1 after H/I (**Fig. 4-4A, 4-4B**), to indicate increased apoptotic neuron death, whereas the contralateral and the sham hippocampi showed negligible level of cleaved caspase 3 (**Fig. 4-4A, 4-4B**).

Moreover, H/I-induced phos-MLKL, phos-RIPK3 or cleaved caspase-3 immunofluorescence intensity persisted into day3 post-insult, where phos-MLKL (**Fig. 4-5A**), phos-RIPK3 (**Fig. 4-5B**) or cleaved caspase-3 (**Fig. 4-5C**) positive neurons were confined to the same regions in the ipsilateral hippocampus as in day1 but the number of positive neurons appears to be decreased. In view of the immunofluorescence findings, we conclude that the current H/I procedure potently induced necroptosis and apoptosis in the ipsilateral hippocampus at least 3 days post-injury in neonatal mice.

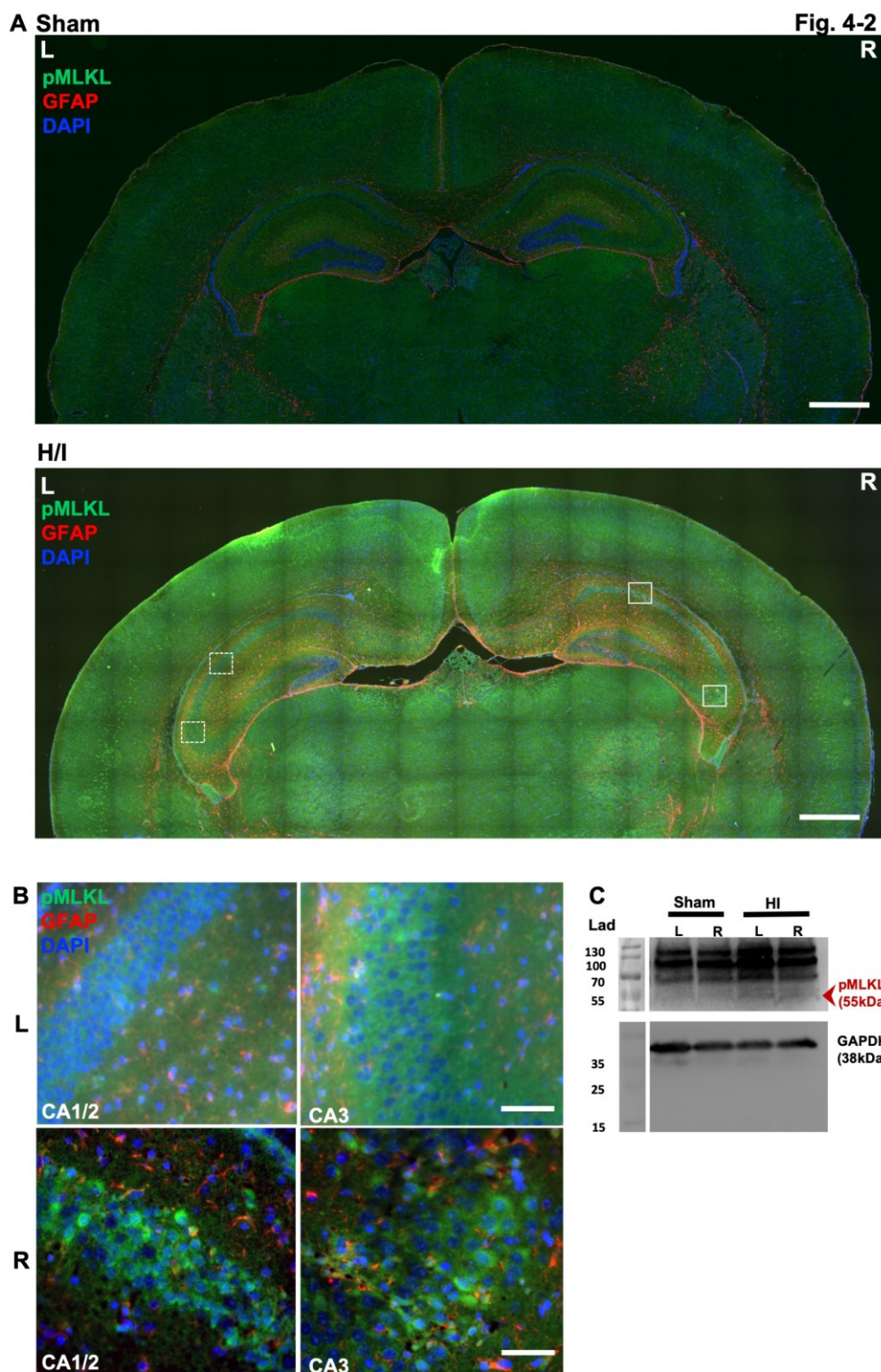


Figure 4-2. MLKL phosphorylation in a murine model of hypoxic-ischaemic encephalopathy (HIE) at day1. (A) Immunofluorescence staining of phosphorylated mixed lineage kinase-domain like (p-MLKL, green) and glial fibrillary-associated protein (GFAP, red) in sham and cerebral hypoxia-ischaemia (H/I) subjects (bregma -1.82mm to -2.46mm). Magnification x20, scale bar 200µm. (B) Boxed hippocampal regions cornu ammonis 1/2 (CA1/2) and cornu ammonis 3 (CA3) from H/I group in (A) were enlarged; left hemisphere, dashed line, upper panel; right hemisphere, solid line, lower panel. Magnification x40, scale bar 50µm. (C) Representative immunoblot of p-MLKL from left and right hemispheres of sham and H/I-injured animals at day1.

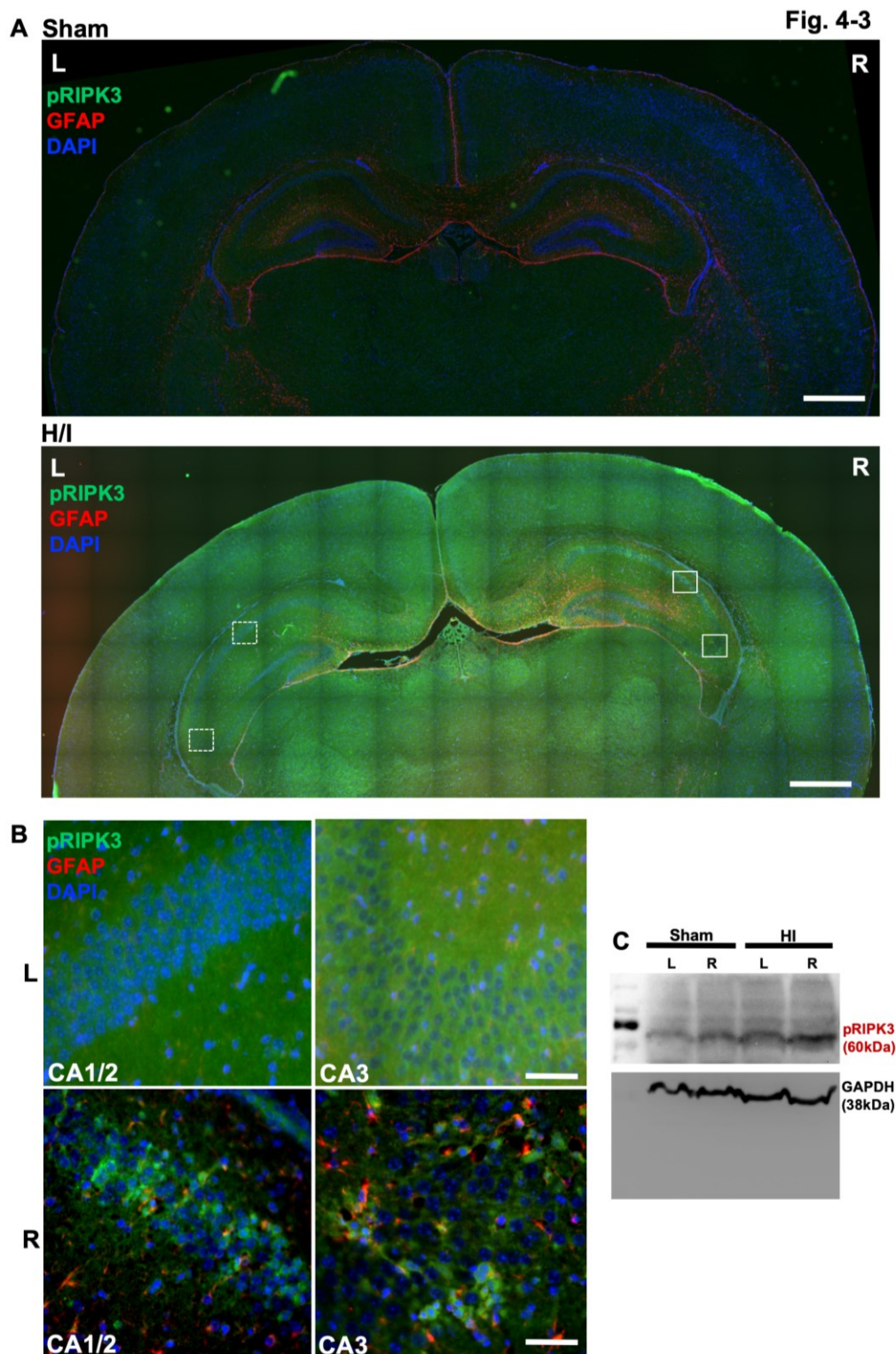


Figure 4-3. RIPK3 phosphorylation in a murine model of hypoxic-ischaemic encephalopathy (HIE) at day1. (A) Immunofluorescence staining of phosphorylated receptor-interacting protein kinase 3 (p-RIPK3, green) and glial fibrillary-associated protein (GFAP, red) in sham and cerebral hypoxia-ischaemia (H/I) subjects (bregma -1.82mm to -2.46mm). Magnification x20, scale bar 200µm. (B) Boxed hippocampal regions cornu ammonis 1/2 (CA1/2) and cornu ammonis 3 (CA3) from H/I group in (A) were enlarged. Magnification x40, scale bar 50µm; left hemisphere, dashed line, upper panel; right hemisphere, solid line, lower panel. (C) Representative immunoblot of p-RIPK3 from left and right hemispheres of sham and H/I-injured animals at day1.

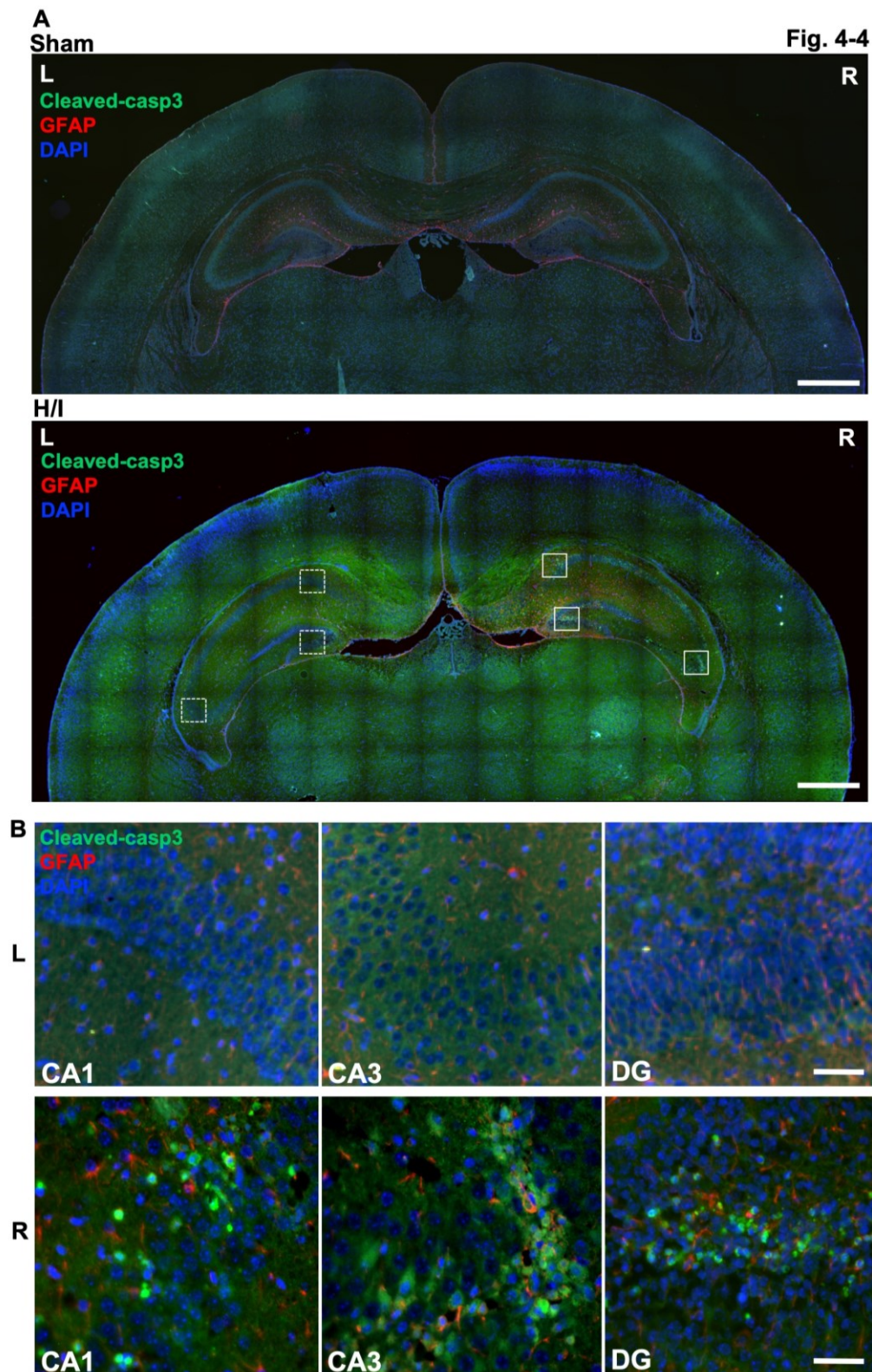


Figure 4-4. Cleavage of caspase 3 in a murine model of hypoxic ischaemic encephalopathy at day1. (A) Immunofluorescence staining of cleaved caspase 3 (green) and glial fibrillary-associated protein (GFAP, red) in sham and cerebral hypoxia-ischaemia (H/I) subjects (bregma -1.82mm to -2.46mm). Magnification x20, scale bar 200µm. **(B)** Boxed hippocampal regions of cornu ammonis 1 (CA1), cornu ammonis 3 (CA3) and dentate gyrus (DG) from H/I group in (A) were enlarged; left hemisphere, dashed line, upper panel; right hemisphere, solid line, lower panel. Magnification x40, scale bar 50µm.

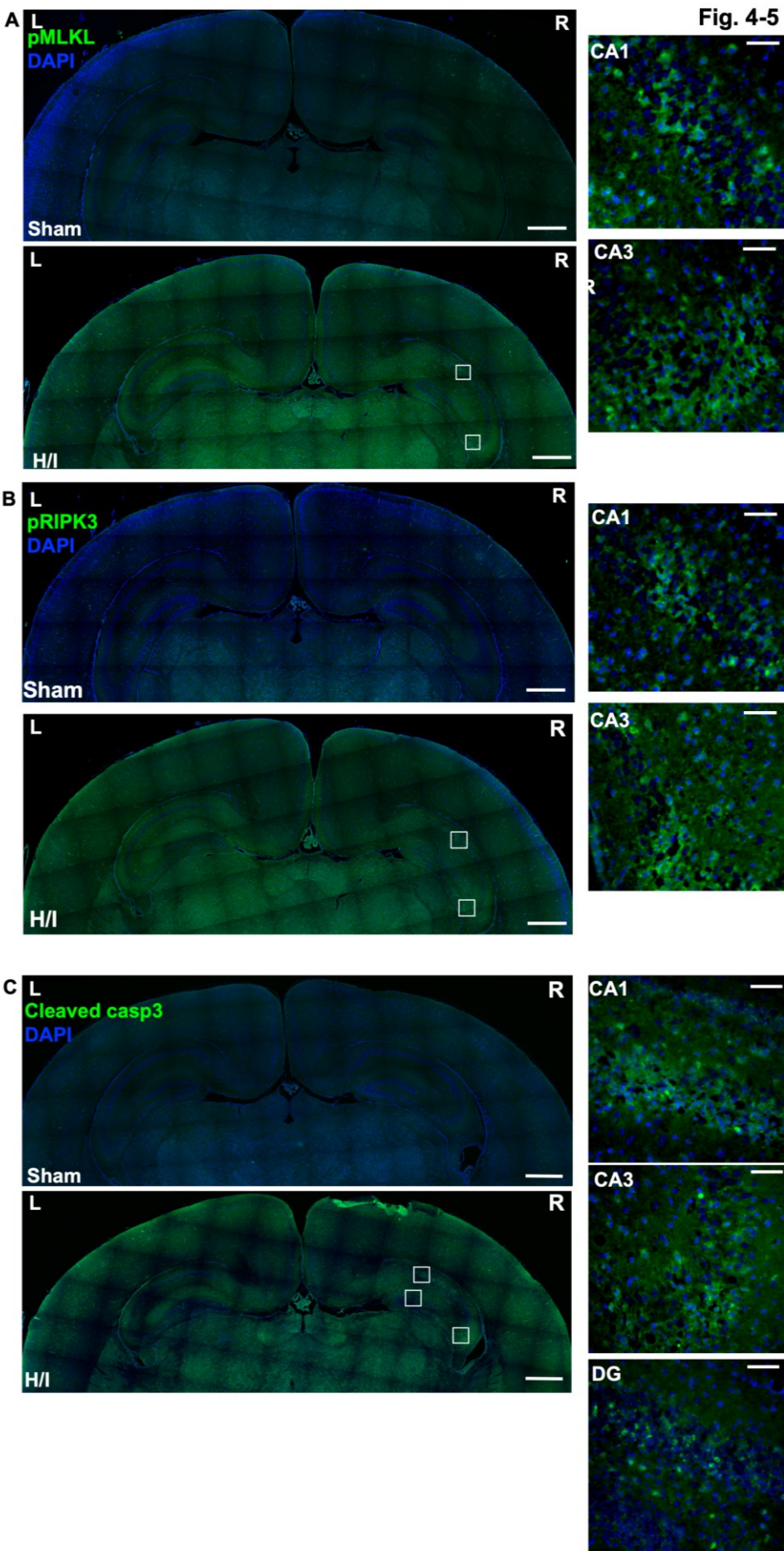


Figure 4-5. Expression of necroptosis and apoptosis markers in murine model of hypoxic ischaemic encephalopathy at day3. Immunofluorescence staining of (A) phosphorylated mixed lineage kinase-domain like (p-MLKL, green), (B) phosphorylated receptor-interacting protein kinase 3 (RIPK3, green) and (C) cleaved caspase 3 (cleaved-casp3, green) in sham and cerebral hypoxia-ischaemia (H/I) subjects (bregma -2.18mm to -2.46mm). Magnification x20, scale bar 200µm. Boxed hippocampal regions cornu ammonis 1 (CA1), cornu ammonis 3 (CA3) and dentate gyrus (DG) of the injured hemisphere from H/I were enlarged on the right. Magnification x40, scale bar 50µm.

4.2.3. Upregulated expression of mitophagy markers following HIE

As our *in vitro* findings indicate a concurrent activation of necroptosis and mitophagy during OGD/reperfusion, we proceeded to examine whether mitophagy is similarly upregulated in regions of the neonatal brain where necroptosis is evident following H/I challenge. At day1 post-H/I, we observed a significant upregulation of mitophagy markers Parkin (**Fig. 4-6A, 4-6B**) and BNIP3 (**Fig. 4-7A, 4-7B**) in the ipsilateral hippocampus, where Parkin or BNIP3-positive neurons were most concentrated in the CA1-CA3 regions. Parkin (**Fig. 4-6A, 4-6B**) and BNIP3 (**Fig. 4-7A, 4-7B**) immunofluorescence intensities in the contralateral hippocampus were not noticeably increased from the baseline levels of sham. H/I also induced CLpP expression in the ipsilateral hippocampus at day1, and CLpP-positive cell bodies can be seen across the CA areas (**Fig. 4-8A, 4-8B**); as with Parkin and BNIP3, CLpP level in the contralateral hippocampus remained comparable to that of sham. We double-labelled the neonatal brain with LC3A/B and the mitochondrial marker COX4 to visualise autophagosome localisation to mitochondria as a measure of mitophagy. In this regard, LC3A/B immunofluorescence intensity was significantly elevated in the ipsilateral hippocampus following H/I along with considerable COX4 co-localisation from CA1 to CA3 regions (**Fig. 4-9A, 4-9B**; arrowheads). In the contralateral hippocampus, LC3A/B level and LC3A/B and COX4 co-localisation were not apparent when compared to the sham.

At day 3 post-H/I, the ipsilateral hippocampus still displayed enhanced immunofluorescence intensity for Parkin and BNIP3 compared to the contralateral hippocampus or the sham (**Fig. 4-10A, 4-10B**), however the signals have weakened since day 1. The upregulated mitophagy markers corresponded to modest increases of LC3A/B level and LC3A/B and COX4 double-positive neurons in the ipsilateral hippocampus at day3 post-H/I (**Fig. 4-10C**), which have declined from the levels seen at day 1. It is also worth noting that COX4 immunofluorescence intensity appears to be mildly elevated in the ipsilateral hippocampus at day 3, which is suggestive of increased mitochondrial biogenesis in response to enhanced mitophagy. Based on these findings, H/I likely promotes mitophagy in the ipsilateral hippocampus of neonatal mice for at least 3 days post-injury.

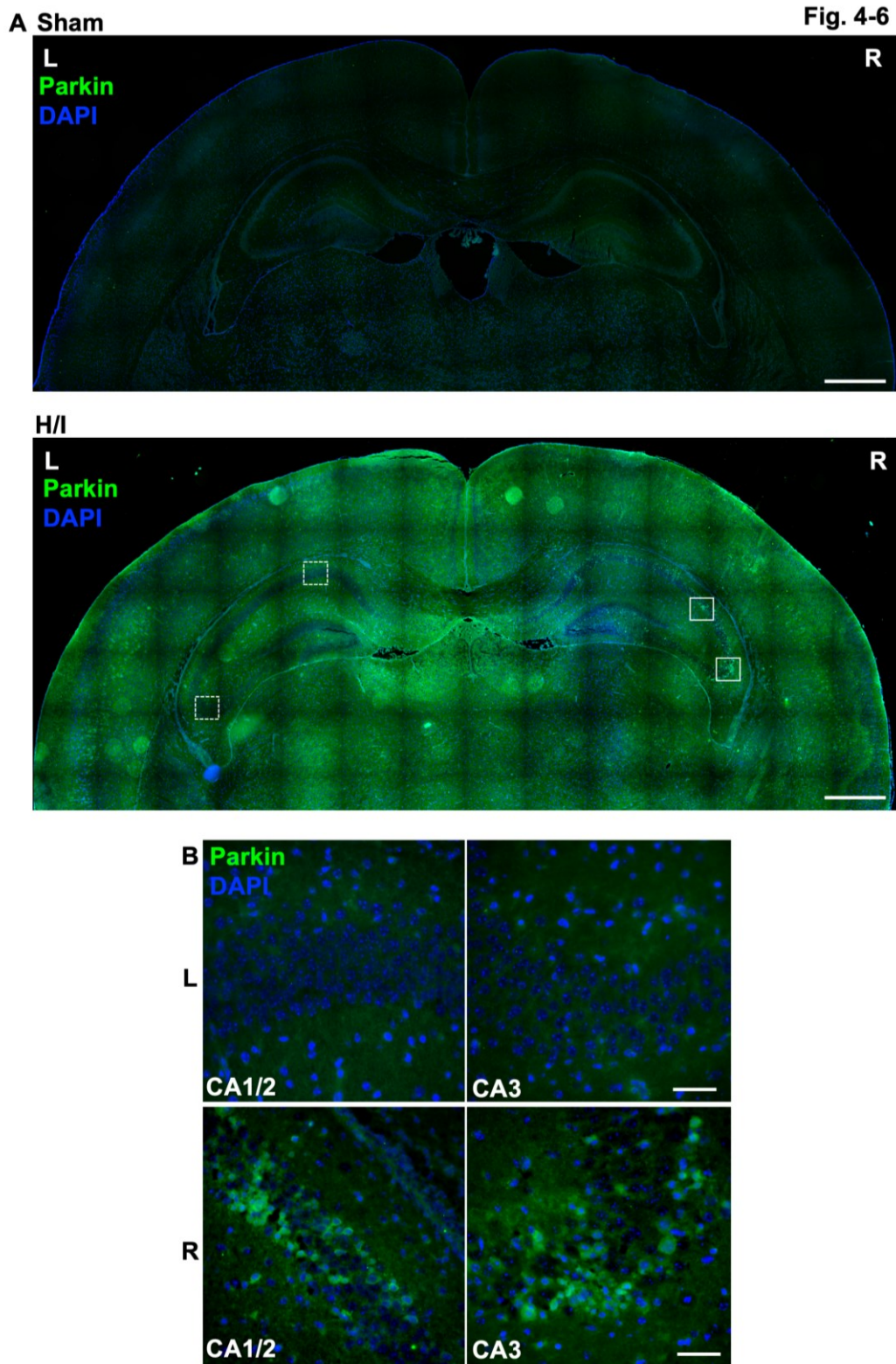


Figure 4-6. Upregulation of mitophagy mediator Parkin in a murine model of hypoxic ischaemic encephalopathy at day1. (A) Immunofluorescence staining of Parkin (green) in sham and cerebral hypoxia-ischaemia (H/I) subjects (bregma -1.82mm to -2.46mm). Magnification x20, scale bar 200µm. **(B)** Boxed hippocampal regions cornu ammonis 1/2 (CA1/2) and cornu ammonis 3 (CA3) from H/I group in (A) were enlarged; left hemisphere, dashed line, upper panel; right hemisphere, solid line, lower panel. Magnification x40, scale bar 50µm.

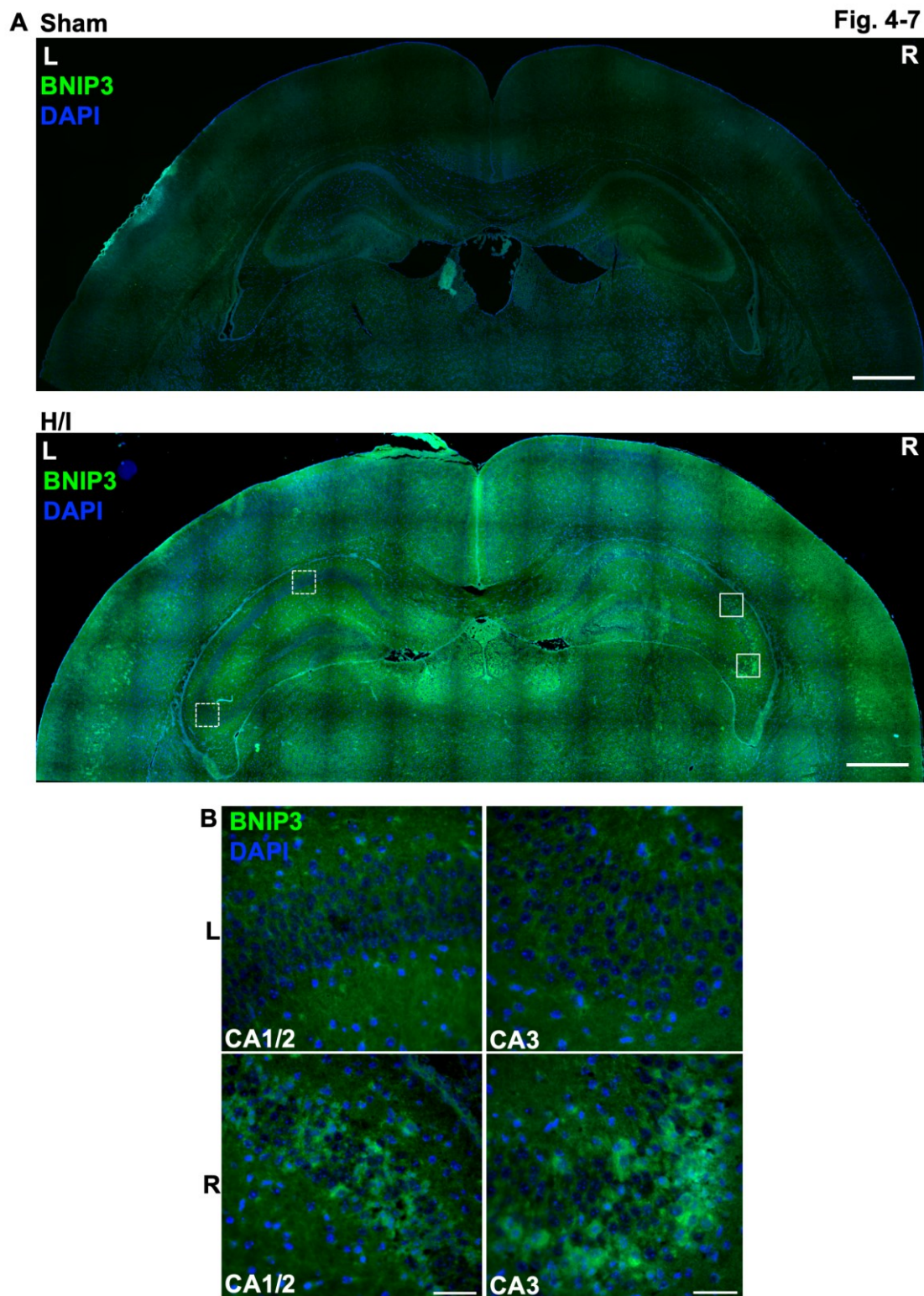


Figure 4-7. Upregulation of mitophagy mediator BNIP3 in a murine model of hypoxic ischaemic encephalopathy at day1. (A) Immunofluorescence staining of Bcl2-interacting protein 3 (BNIP3, green) in sham and cerebral hypoxia-ischaemia (H/I) subjects (bregma -1.82mm to -2.46mm). Magnification x20, scale bar 200 μ m. (B) Boxed hippocampal regions cornu ammonis 1/2 (CA1/2) and cornu ammonis 3 (CA3) from H/I group in (A) were enlarged; left hemisphere, dashed line, upper panel; right hemisphere, solid line, lower panel. Magnification x40, scale bar 50 μ m.

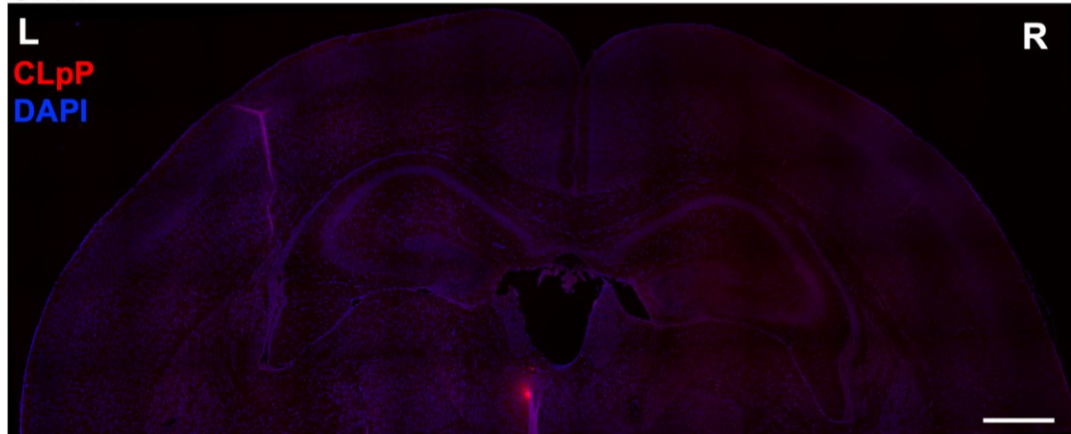
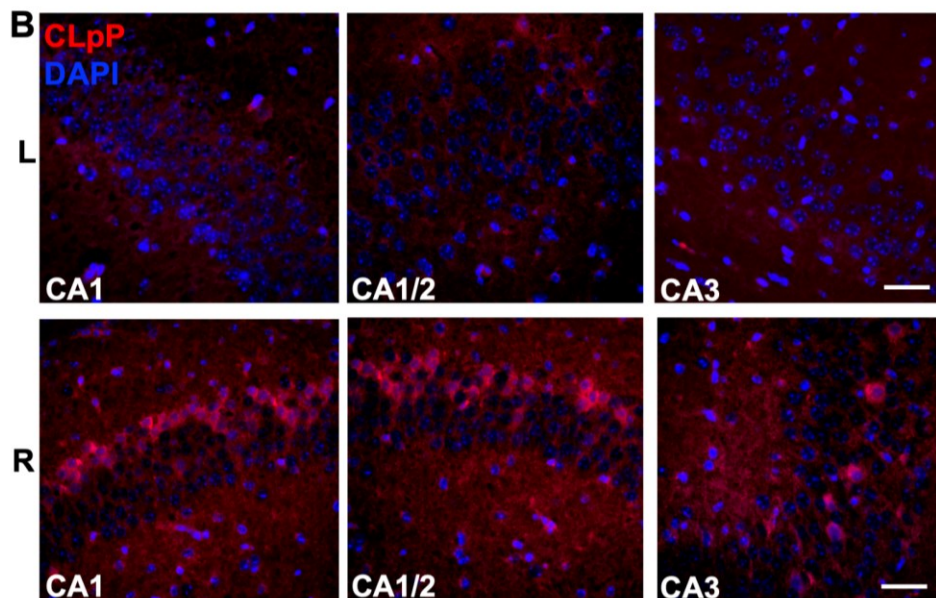
A Sham**Fig. 4-8****H/I**

Figure 4-8. Upregulation of CLpP in a murine model of hypoxic ischaemic encephalopathy at day1. (A) Immunofluorescence staining of caseinolytic protease proteolytic subunit (CLpP, red) in sham and cerebral hypoxia-ischaemia (H/I) subjects (bregma -1.70mm to 1.82mm). Magnification x20, scale bar 200 μ m. **(B)** Boxed hippocampal regions cornu ammonis 1 (CA1), cornu ammonis 2 (CA2) and cornu ammonis 3 (CA3) from H/I group in (A) were enlarged; left hemisphere, dashed line, upper panel; right hemisphere, solid line, lower panel. Magnification x40, scale bar 50 μ m.

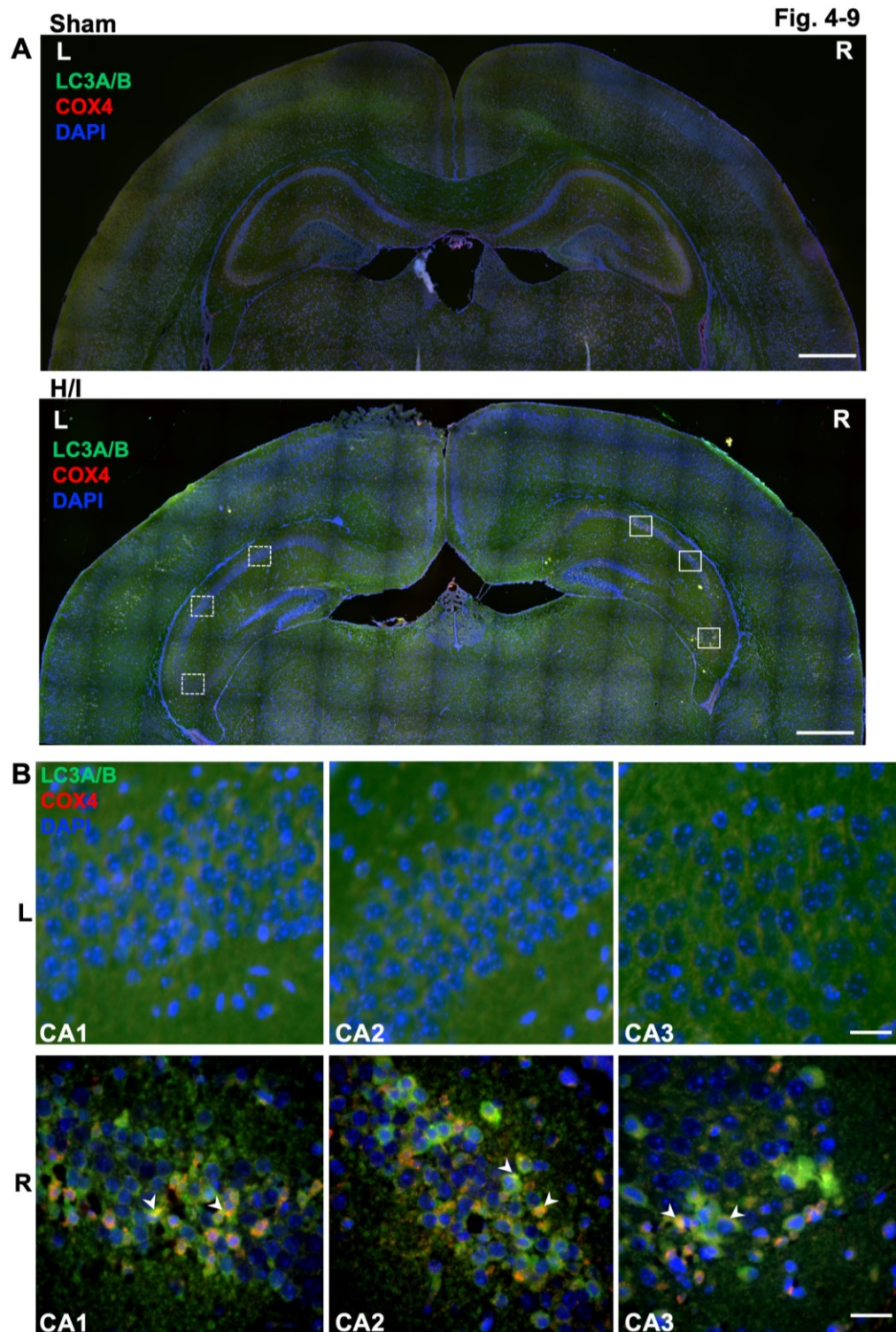


Figure 4-9. Increased mitochondrial localisation of LC3A/B in a murine model of hypoxic ischaemic encephalopathy at day1. (A) Double immunofluorescence staining of the autophagosome marker microtubule-associated light chain 3A/B (LC3A/B, green) and the mitochondrial electron transport chain enzyme cytochrome c oxidase subunit 4 (COX4, red) in sham and cerebral hypoxia-ischaemia (H/I) subjects (bregma -1.82mm to -2.46mm). Magnification x20, scale bar 200 μ m. **(B)** Boxed hippocampal regions cornu ammonis 1 (CA1), cornu ammonis 2 (CA2) and cornu ammonis 3 (CA3) from H/I group in (A) were enlarged; left hemisphere, dashed line, upper panel; right hemisphere, solid line, lower panel. Arrowheads point to neurons showing LC3A/B co-localisation with COX4. Magnification x60, scale bar 25 μ m.

Fig. 4-10

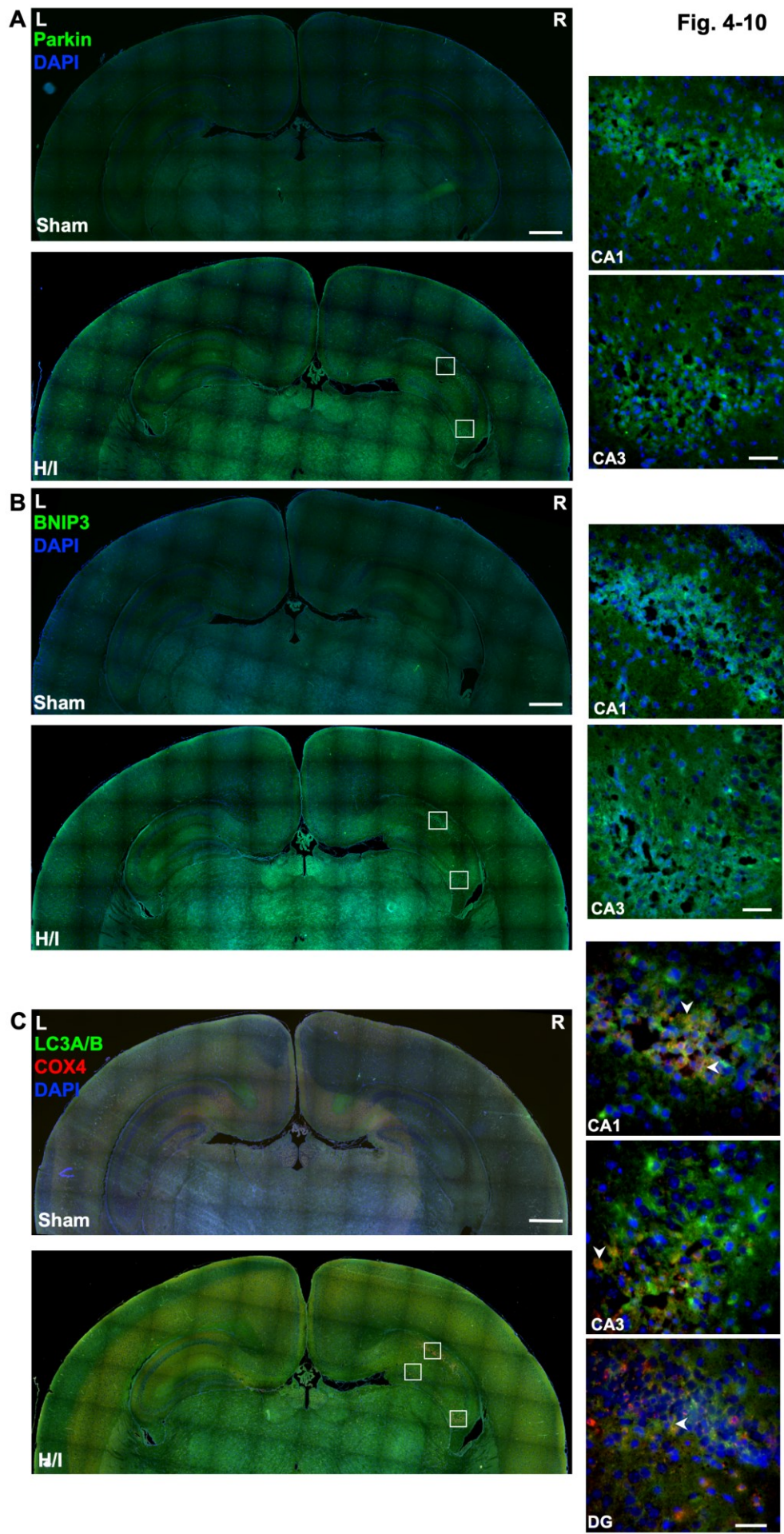


Figure 4-10. Upregulation of mitophagy-related markers in a murine model of hypoxic ischaemic encephalopathy at day3. Immunofluorescence staining of **(A)** Parkin (green), **(B)** Bcl2 interacting protein kinase 3 (BNIP3, green) and **(C)** microtubule-associated light chain 3A/B (LC3A/B, green) and mitochondrial electron transport chain enzyme cytochrome c oxidase subunit 4 (COX4, red) co-localisation in sham and cerebral hypoxia-ischaemia (H/I) subjects (bregma -1.82mm to -2.46mm). Magnification x20, scale bar 200µm. Boxed hippocampal regions cornu ammonis 1 (CA1), cornu ammonis 3 (CA3) and dentate gyrus (DG) of the injured hemisphere from H/I were enlarged on the right. Arrowheads point to neurons showing LC3A/B co-localisation with COX4. Parkin and BNIP3: Magnification x40, scale bar 50µm; LC3A/B with COX4: Magnification x60, scale bar 25µm.

4.2.4 Necrostatin-1s prevents MLKL and RIPK3 phosphorylation in the hippocampus after cerebral I/R

As a potent RIPK1 selective inhibitor, necrostatin-1s effectively prevented MLKL phosphorylation in OGD/Rep-challenged Neuro-2a cells to indicate suppression of necroptosis, as demonstrated in Chapter three, section 3.2.3. We proceeded to examine whether Nec-1s could similarly prevent phosphor-activation of necroptosis mediators MLKL and RIPK3 *in vivo* in neonatal mice subjected to HIE. We observed a significant reduction in the percentage of phosphorylated MLKL (phos-MLKL, green) positive cell bodies throughout hippocampus CA1-CA3 regions in Nec-1s treated pups (8nmol/pup) at day1 post-H/I, when compared to untreated H/I or vehicle DMSO-treated cohorts (**Fig. 4-11 A-B, Fig. 4-16A**; H/I vs. H/I + Nec-1s, $P=0.041$; H/I + Veh vs. H/I + Nec-1s, $P=0.010$). Nec-1s treated pups also displayed reduced number of phosphorylated RIPK3 (phos-RIPK3, green) positive cell bodies in hippocampal CA1-CA3 regions at day 1, in comparison to the DMSO-treated or untreated H/I pups (**Fig. 4-12 A-B, Fig. 4-16B**; H/I vs. H/I + Nec-1s, $P=0.016$; H/I + Veh vs. H/I + Nec-1s, $P=0.010$). It is also evident that Nec-1s treatment preserved the morphological integrity of ipsilateral hippocampus at day1 along with reduced astrocytic infiltration (**Fig. 4-11, 4-12**).

4.2.5 Necrostatin-1s attenuates mitophagy in the hippocampus after cerebral I/R

Provided that Nec-1s downregulated whole-cell and mitochondrial expression of Parkin and mitochondrial localisation of autophagosome marker LC3A/B in OGD-challenged Neuro-2a cells, we proceeded to verify whether Nec-1s also exerts an “anti-mitophagy” effect in the immature brain subjected to HIE. As shown by immunofluorescence staining, Nec-1s i.c.v administration markedly reduced the percentage of Parkin-positive cell bodies in the ipsilateral hippocampus at day1, especially in the CA1 and CA3 regions, when compared to the ipsilateral hippocampi of vehicle-treated and untreated pups (**Fig. 4-13A-B, Fig. 4-16C**; H/I vs. H/I + Nec-1s, $P=0.047$; H/I + Veh vs. H/I + Nec-1s, $P=0.044$). The mean fluorescence intensity of BNIP3 was also semi-quantified, and Nec-1s treatment dampened BNIP3 immunofluorescence reactivity in the injured hippocampus compared to H/I and H/I + Veh (**Fig. 4-14A-B, Fig. 4-16D**; H/I vs. H/I + Nec-1s, $P=0.031$). When LC3A/B and COXIV co-localisation was used as a surrogate marker for mitophagy (i.e. autophagosome translocation to mitochondria), we observed a significant decrease in the number of LC3A/B and COXIV double-positive cell bodies in the ipsilateral hippocampus from levels seen in the H/I and H/I + DMSO cohorts (**Fig. 4-15A-B, Fig. 4-16E**; H/I vs. H/I + Nec-1s, $P=0.031$; H/I + Veh vs. H/I + Nec-1s, $P=0.048$).

Thus, in accordance with the “anti-mitophagy” effect of Nec-1s in OGD-challenged Neuro-2a cells, we demonstrated that RIPK1 inhibition effectively downregulated Parkin and BNIP3 and prevented LC3A/B COXIV co-localisation in the neonatal brain following cerebral H/I.

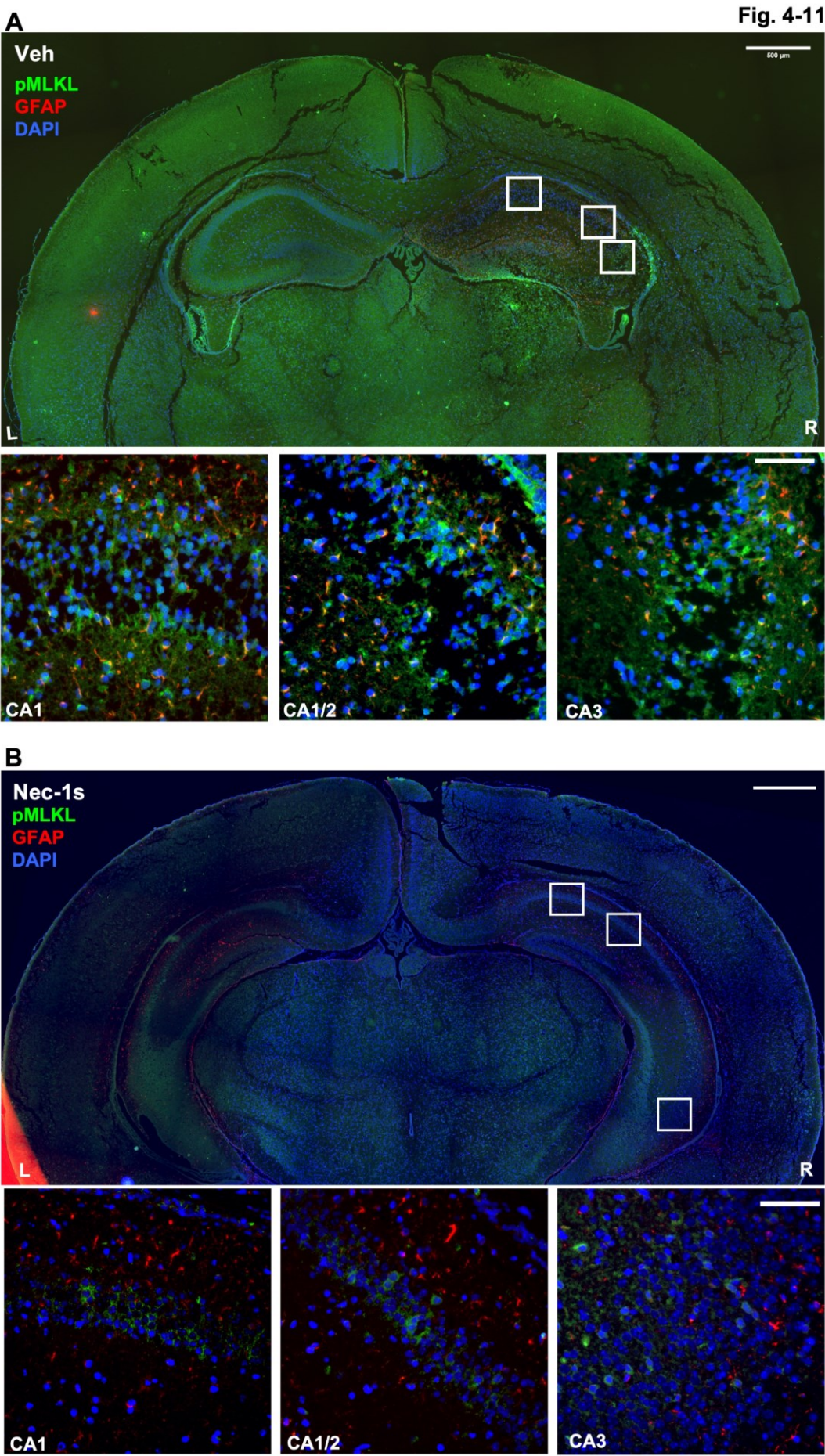


Figure 4-11. MLKL phosphorylation in a murine model of hypoxic-ischaemic encephalopathy (HIE) at day1 after Necrostatin-1s treatment. (A) Immunofluorescence staining of phosphorylated mixed lineage kinase-domain like (p-MLKL, green) and glial fibrillary-associated protein (GFAP, red) in vehicle (DMSO)-treated, H/I-injured mice (bregma -1.82mm to -2.46mm). Magnification x20, scale bar 200µm. (B) Ipsilateral hippocampal regions cornu ammonis 1, cornu ammonis 1/2 (CA1/2) and cornu ammonis 3 (CA3) from boxed areas in (A) were enlarged. Magnification x40, scale bar 50µm. (C) Immunofluorescence staining of p-MLKL (green) and GFAP (red) in Nec-1s-treated (8nmol), H/I-injured mice (bregma -1.82mm to -2.46mm). Magnification x20, scale bar 200µm. (D) Ipsilateral hippocampal regions cornu ammonis 1, cornu ammonis 1/2 (CA1/2) and cornu ammonis 3 (CA3) from the boxed areas in (C) were enlarged. Magnification x40, scale bar 50µm.

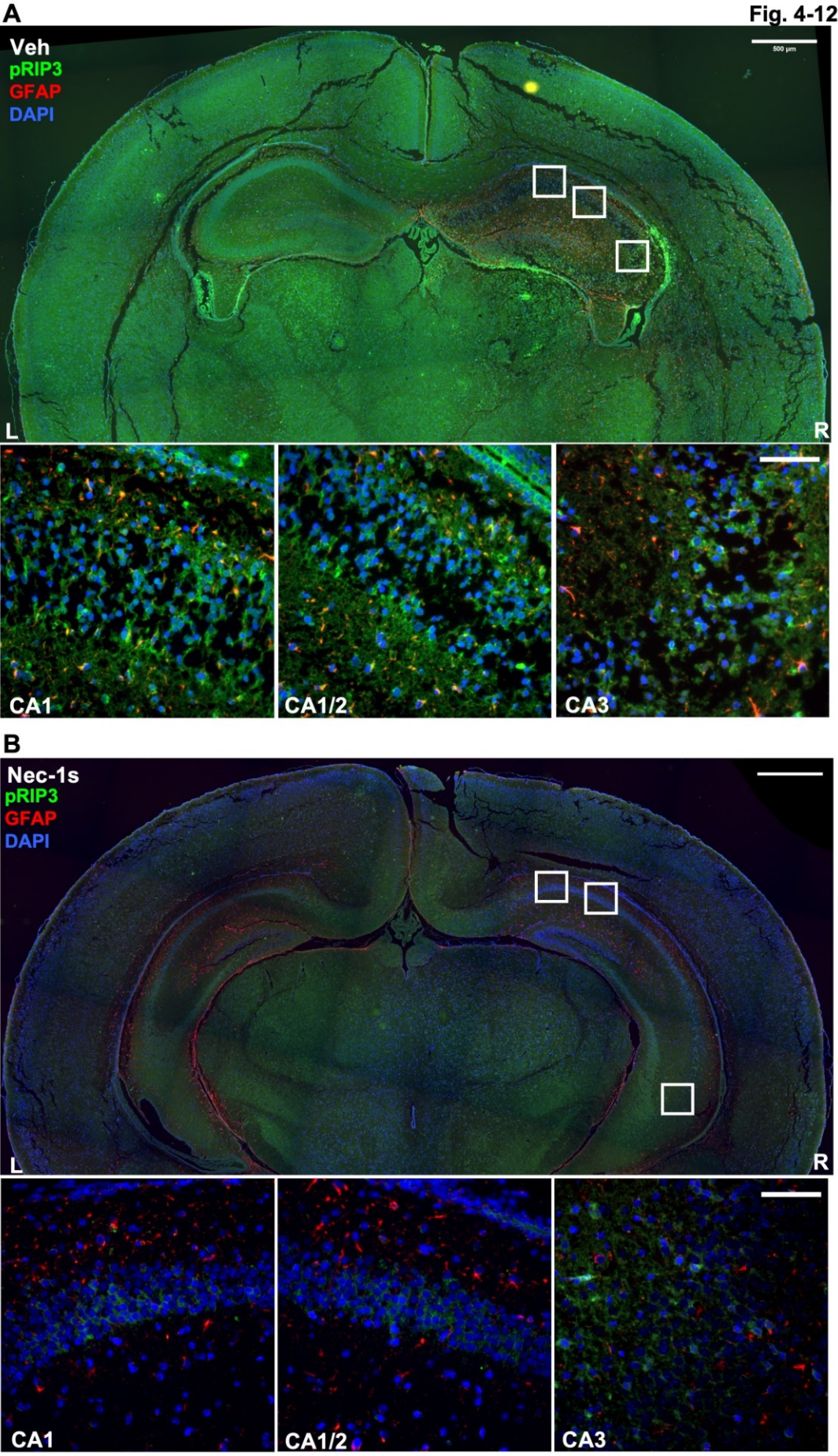


Figure 4-12. RIPK3 phosphorylation in a murine model of hypoxic-ischaemic encephalopathy (HIE) at day1 after Necrostatin-1s treatment. (A) Immunofluorescence staining of phosphorylated receptor-interacting protein kinase 3 (p-RIPK3, green) and glial fibrillary-associated protein (GFAP, red) in vehicle (DMSO)-treated, H/I-injured mice (bregma -1.82mm to -2.46mm). Magnification x20, scale bar 200µm. (B) Ipsilateral hippocampal regions cornu ammonis 1, cornu ammonis 1/2 (CA1/2) and cornu ammonis 3 (CA3) from boxed areas in (A) were enlarged. Magnification x40, scale bar 50µm. (C) Immunofluorescence staining of p-RIPK3 (green) and GFAP (red) in Nec-1s-treated (8nmol), H/I-injured mice (bregma -1.82mm to -2.46mm). Magnification x20, scale bar 200µm. (D) Ipsilateral hippocampal regions cornu ammonis 1, cornu ammonis 1/2 (CA1/2) and cornu ammonis 3 (CA3) from the boxed areas in (C) were enlarged. Magnification x40, scale bar 50µm.

Fig. 4-13

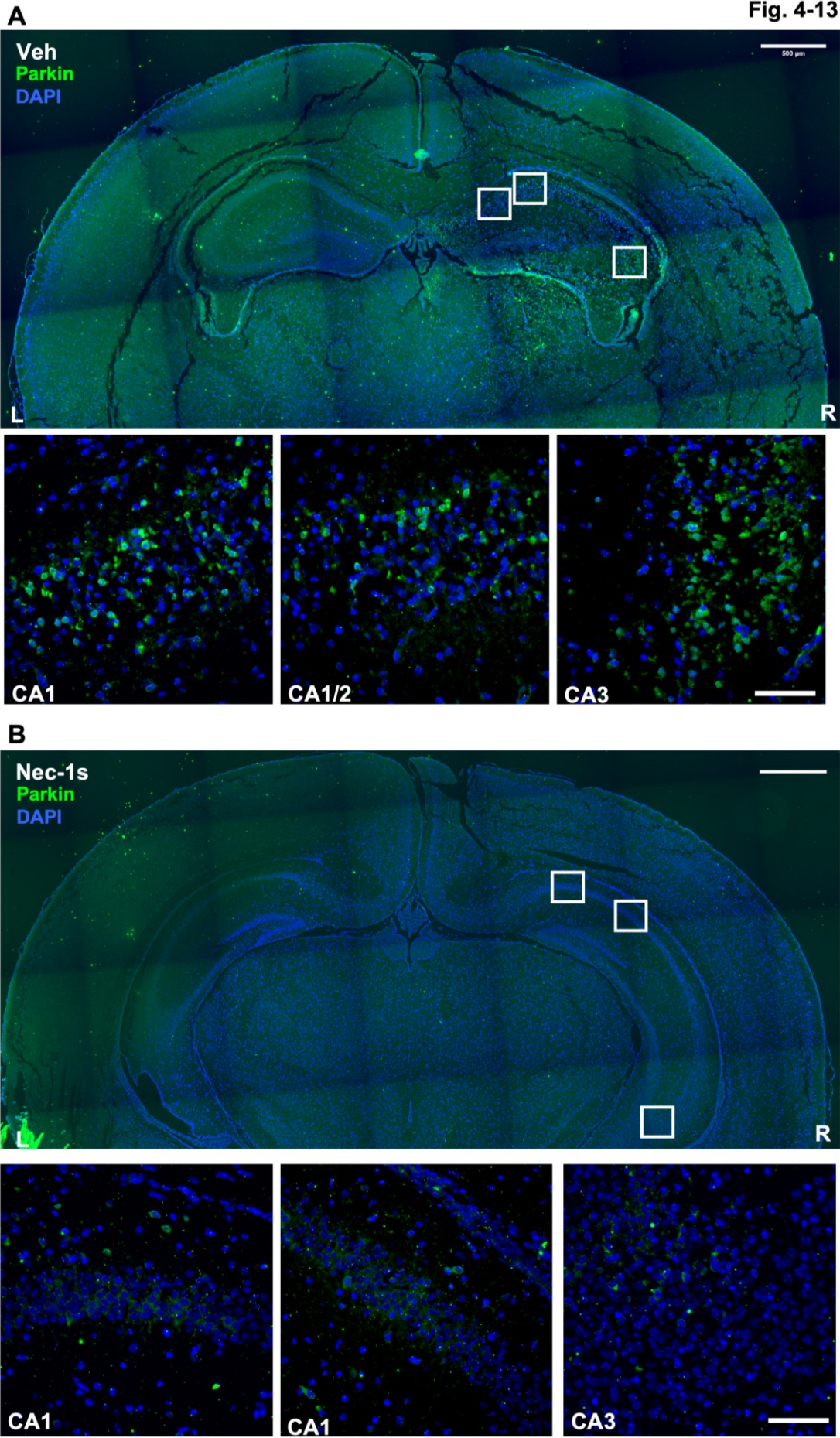


Figure 4-13. Expression of mitophagy mediator Parkin in a murine model of hypoxic-ischaemic encephalopathy (HIE) at day1 after Necrostatin-1s treatment. (A) Immunofluorescence staining of Parkin (green) in vehicle (DMSO)-treated, H/I-injured mice (bregma -1.82mm to -2.46mm). Magnification x20, scale bar 200µm. **(B)** Ipsilateral hippocampal regions cornu ammonis 1, cornu ammonis 1/2 (CA1/2) and cornu ammonis 3 (CA3) from boxed areas in (A) were enlarged. Magnification x40, scale bar 50µm. **(C)** Immunofluorescence staining of Parkin (green) in Nec-1s-treated (8nmol), H/I-injured mice (bregma -1.82mm to -2.46mm). Magnification x20, scale bar 200µm. **(D)** Ipsilateral hippocampal regions cornu ammonis 1, cornu ammonis 1/2 (CA1/2) and cornu ammonis 3 (CA3) from the boxed areas in (C) were enlarged. Magnification x40, scale bar 50µm.

Fig. 4-14

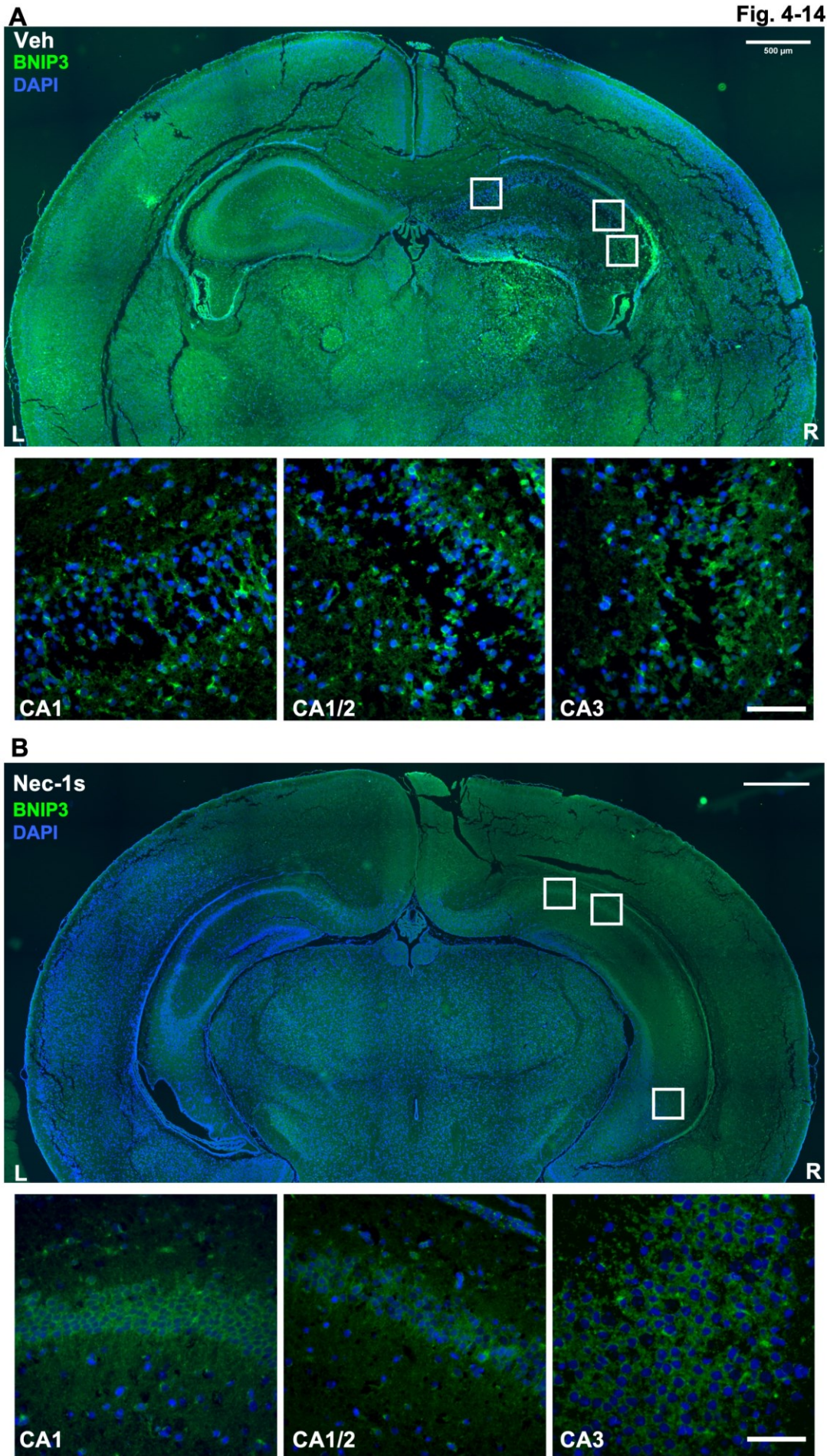


Figure 4-14. Expression of mitophagy mediator BNIP3 in a murine model of hypoxic-ischaemic encephalopathy (HIE) at day1 after Necrostatin-1s treatment. (A) Immunofluorescence staining of BNIP3 (green) in vehicle (DMSO)-treated, H/I-injured mice (bregma -1.82mm to -2.46mm). Magnification x20, scale bar 200µm. (B) Ipsilateral hippocampal regions cornu ammonis 1, cornu ammonis 1/2 (CA1/2) and cornu ammonis 3 (CA3) from boxed areas in (A) were enlarged. Magnification x40, scale bar 50µm. (C) Immunofluorescence staining of BNIP3 (green) in Nec-1s-treated (8nmol), H/I-injured mice (bregma -1.82mm to -2.46mm). Magnification x20, scale bar 200µm. (D) Ipsilateral hippocampal regions cornu ammonis 1, cornu ammonis 1/2 (CA1/2) and cornu ammonis 3 (CA3) from the boxed areas in (C) were enlarged. Magnification x40, scale bar 50µm.

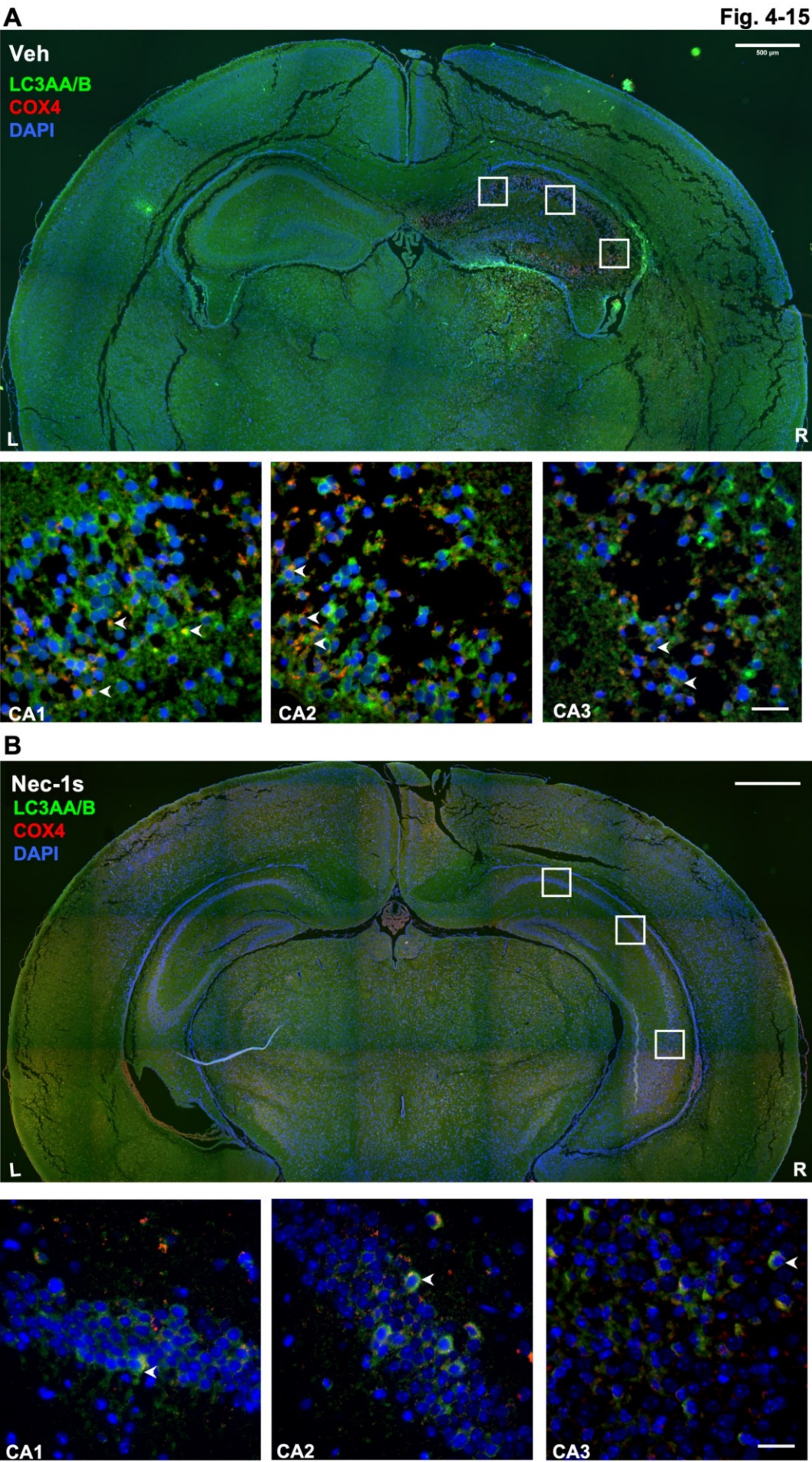


Figure 4-15. Mitochondrial localisation of LC3A/B in a murine model of hypoxic ischaemic encephalopathy at day1 after Necrostatin-1s treatment. **(A)** Double immunofluorescence staining of the autophagosome marker microtubule-associated light chain 3A/B (LC3A/B, green) and the mitochondrial electron transport chain enzyme cytochrome c oxidase subunit 4 (COX4, red) in vehicle (DMSO)-treated, H/I-injured mice (bregma -1.82mm to -2.46mm). Magnification x20, scale bar 200µm. **(B)** Ipsilateral hippocampal regions cornu ammonis 1 (CA1), cornu ammonis 2 (CA2) and cornu ammonis 3 (CA3) from the boxed areas in (A) were enlarged. Arrowheads point to neurons showing LC3A/B co-localisation with COX4. Magnification x63, scale bar 25µm. **(C)** Double immunofluorescence staining of LC3A/B and COX4 in Nec-1s-treated (8nmol), H/I-injured mice (bregma -1.82mm to -2.46mm). Magnification x20, scale bar 200µm. **(D)** Ipsilateral hippocampal regions cornu ammonis 1 (CA1), cornu ammonis 2 (CA2) and cornu ammonis 3 (CA3) from the boxed areas in (C) were enlarged. Arrowheads point to neurons showing LC3A/B co-localisation with COX4. Magnification x63, scale bar 25µm.

Fig. 4-16

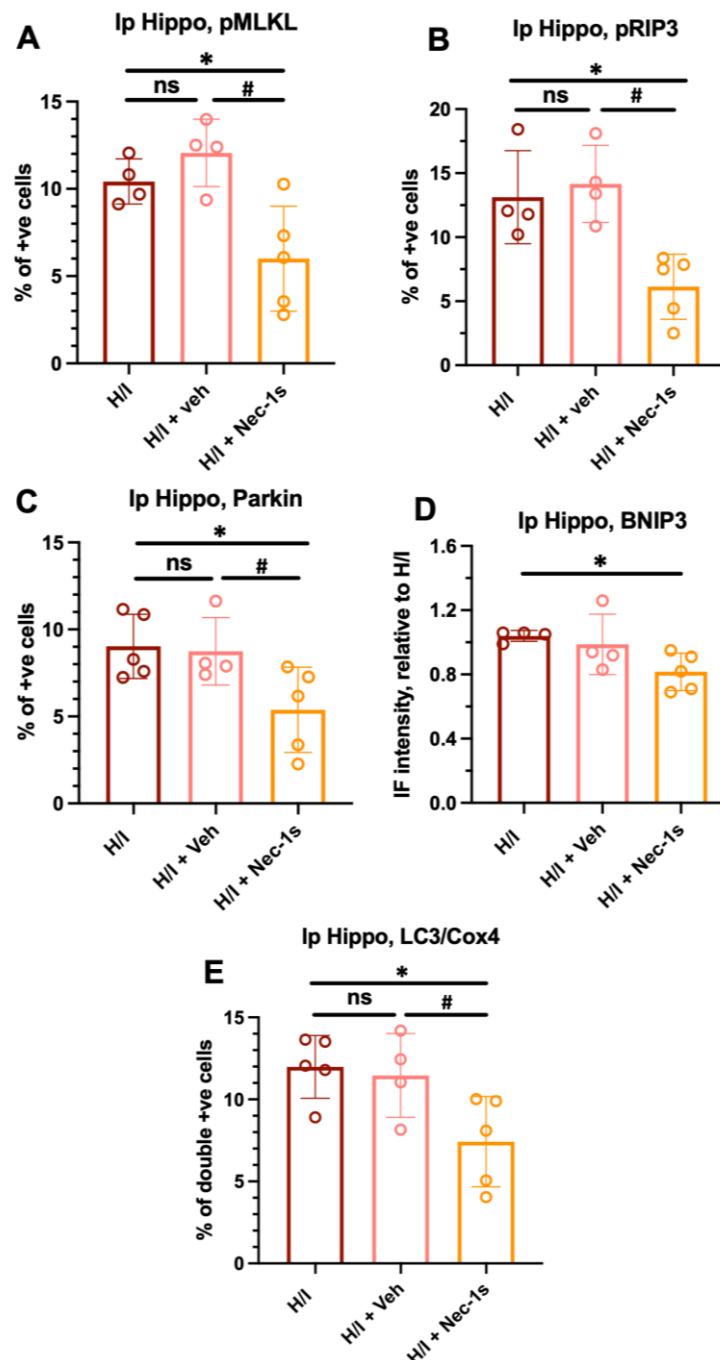


Figure 4-16. Analysis of immunofluorescence staining in a murine model of hypoxic ischaemic encephalopathy at day1 after Necrostatin-1s treatment. Comparison of **(A)** Percentage of phos-MLKL positive cell bodies, **(B)** Percentage of phos-RIPK3 positive cell bodies, **(C)** Percentage of Parkin-positive cell bodies, **(D)** Immunofluorescence intensity of BNIP3, and **(E)** Percentage of LC3A/B and COX IV double-positive cell bodies in ipsilateral hippocampus between H/I-injured mice, vehicle-treated (Veh, DMSO) and Nec-1s-treated (8nmol) mice. Data expressed as mean $\pm$ SD. H/I vs. H/I + Nec-1s, * $P < 0.05$; H/I + veh vs. H/I + Nec-1s, # $P < 0.05$; H/I vs. H/I + veh, ns; $n = 4-5$, each n represents an independent experiment/animal.

4.2.6 The effect of Necrostatin-1s treatment on long-term motor and behavioural functions

Motor and behavioural tests were performed on PND28 to PND30 juvenile mice (corresponding to day21 to day23 post-H/I) to examine the long-term effect of Nec-1s treatment following cerebral hypoxia-ischaemia. As detailed in Chapter one, section C.1.3, accelerating rota-rod is a convenient and sensitive test for detecting motor impairment and poor coordination in rodents subjected to HIE. A consecutive three-day rota-rod test is used in the present study. On each day, each mouse had 3 accelerating trials (from 4rpm to 40rpm in 5 minutes) with inter-trial breaks and the latency to fall/first revolution from 3 trials was averaged, and the latency data was recorded for three days (**Fig. 4-17A**).

Two-way ANOVA analysis revealed a significant difference between injury/treatment ($F_{3,31}=5.180$; $P=0.005$) but not over time ($F_{1,789, 25.05} = 2.075$; $P=0.1505$), and there was no interaction between injury/treatment and time ($F_{6,28}=0.668$; $P=0.676$). When compared to sham controls, H/I-injured mice (day 1: $P=0.011$; day2, $P=0.028$; day3, $P=0.002$) and H/I-injured, DMSO-treated mice (day 1: $P=0.019$; day 2: $P=0.010$; day 3: $P=0.001$) displayed significantly shorter latency to fall/first revolution on rota-rod than sham on all three days (**Fig. 4-17A, 4-17B**). Nec-1s treated mice performed worse than sham cohorts on day1 ($P=0.030$), but not on day2 (ns, $P=0.597$) and day3 (**Fig. 4-17A**, ns, $P=0.083$; **Fig. 4-17B**, ns, $P=0.103$). Moreover, latency to fall/first revolution did not differ between H/I, H/I + DMSO and H/I + Nec-1s groups across three days (**Fig. 4-17A, 4-17B**). Our data suggests that Nec-1s treatment maybe associated with long-term amelioration of impaired rota-rod performance from cerebral H/I.

On PND30, spontaneous locomotor activity and thigmotaxis (preference for periphery over centre) were examined by open-field test. The total distanced travelled within the 15 minutes recording window was significantly reduced in the H/I ($P=0.014$) and H/I + DMSO ($P=0.011$) when compared to the NC to suggest an overall hypoactivity after injury (**Fig. 4-17C**). On the other hand, the total distance travelled by Nec-1s treated animals was slightly reduced from that of NC without reaching statistical significance ($P=0.061$; **Fig. 4-17C**). When compared to NC, H/I ($P=0.041$) and H/I + DMSO ($P=0.046$) animals also travelled significantly less in the central zone (**Fig. 4-17D**), however the percentage of central distance over the total distance travelled was comparable between all groups (ns; **Fig. 4-17E**). The net peripheral distance covered and as a percentage to total distance explored were also not significantly different between the groups (**Fig. 4-17F, Fig. 4-17G**). Taken together, our findings suggest that long-term locomotor activity is impaired following neonatal cerebral H/I injury, which was reflected as a decrease of net movement in the central zone, yet the percentage distance covered in the central versus peripheral zones remained unchanged. Moreover, Nec-1s treatment maybe associated with an amelioration of H/I-induced hypoactivity without altering the thigmotaxis behaviour.

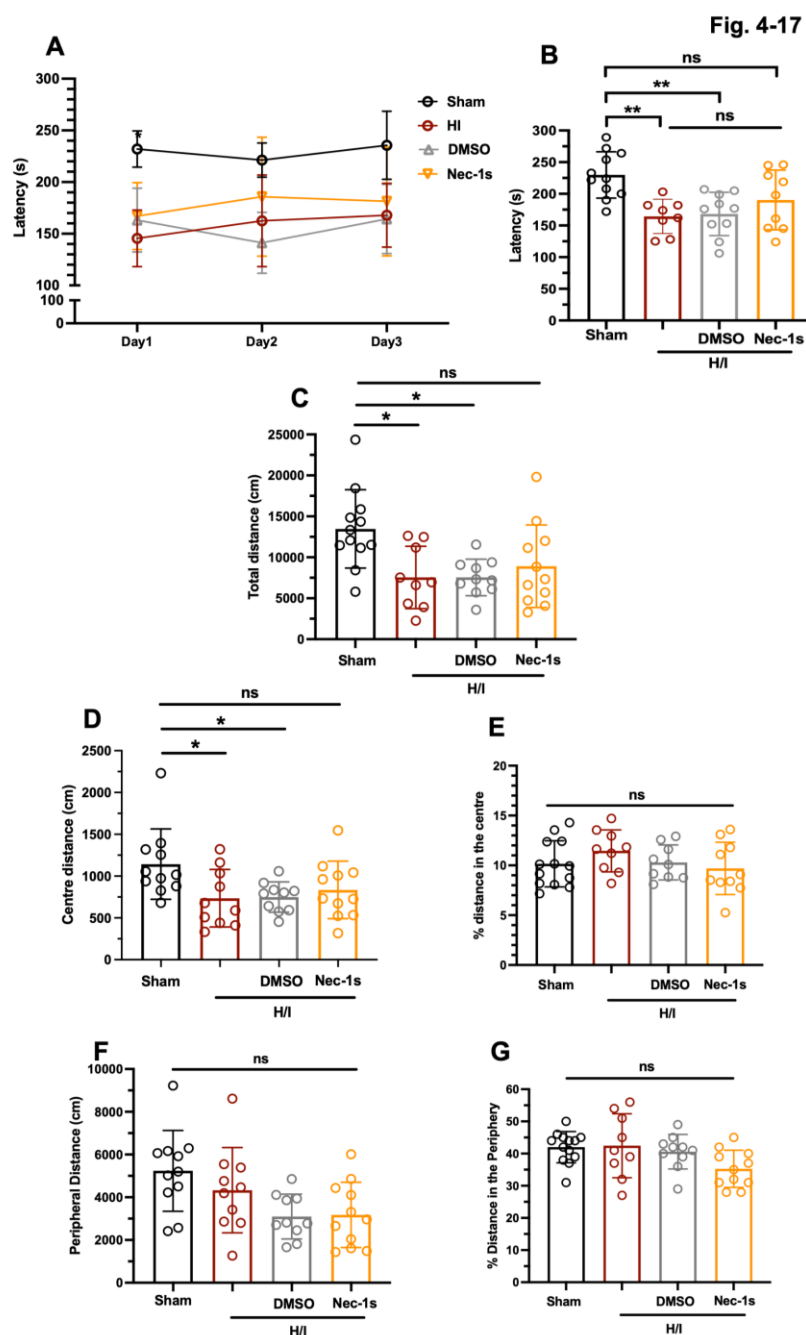


Figure 4-17. The effect of necrostatin-1s on long-term motor and behavioural outcomes in a murine model of hypoxic-ischaemic encephalopathy. (A) Three-day accelerating rota-rod performance in PND28 to PND30 mice, latency to fall or to first complete revolution in seconds. (B) Day 3 accelerating rota-rod performance in PND30 mice, latency to fall or to first complete revolution in seconds. (C) Total distance travelled in centimetres (cm), (D) Distance travelled in the centre in cm, (E) Distance travelled in the centre as a percentage of total distance travelled, (F) Distance travelled in the periphery in cm, and (G) Distance travelled in the periphery as a percentage of total distance travelled in open-field test in PND30 mice. All data expressed as mean $\pm$ SD. [Day 3 accelerating rota-rod: Sham vs. H/I, $**P < 0.01$; Sham vs. H/I + DMSO, $**P < 0.01$; n=8-11] [OFT total distance: Sham vs. H/I, $*P < 0.05$; Sham vs. H/I + DMSO, $*P < 0.05$; Sham vs. H/I + Nec-1s, ns; n=9-12] [OFT centre distance: Sham vs. H/I, $*P < 0.05$; Sham vs. H/I + DMSO, $*P < 0.05$; Sham vs. H/I + Nec-1s, ns; n=10-11] [OFT % distance in centre, ns; n=9-13] [OFT periphery distance: ns; n=10-11] [OFT % distance in periphery: ns; n=9-13]; each n represents an independent experiment/animal.

4.3 Discussion

4.3.1 Activation of regulated cell death during hypoxic-ischemic encephalopathy

As detailed in Chapter one, section D.2, during brain ischaemia-reperfusion injury, hypoxic-ischaemic insult of sufficient severity results in sudden depletion of high-energy phosphates (e.g. ATP) with uncontrolled, necrotic cell death. The restoration of blood flow and oxygenation to H/I-injured brain tissues initiate a “secondary”, delayed phase of energy failure that is characterised by mitochondrial electron transport chain failure and reactive oxygen species production. Reperfusion-associated metabolic disturbances, oxidative stress and the ensuing pathophysiological processes would culminate to regulated cell death in neurons, oligodendrocytes and other susceptible cell types. In addition to apoptosis as the most extensively studied form of regulated cell death (RCD), the pathological contribution of necroptosis as an alternative RCD mechanism to HIE is rapidly gaining recognition, with both forms of RCD significantly upregulated between 1-24 hrs post-reperfusion in rodent models of HIE. Necroptosis and apoptosis overlap at the level of membrane-bound death receptor (e.g. TNFRSF: TNF receptor, Fas) stimulation by extracellular death receptor ligands (e.g. TNF- α , Fas ligand), as described in details in Chapter one, section E.1. On the other hand, apoptosis and necroptosis can be distinguished by their requirement for mitochondria-originated injurious signals, with mitochondrial outer membrane permeabilisation (MOMP) and mitochondrial release of cytochrome C and apoptosis-inducing factor (AIF) established as indispensable triggers for intrinsic apoptosis, whereas evidences on mitochondrial contribution to necroptosis initiation were conflicting.

In neonatal rodents subjected to cerebral H/I, apoptotic cells identified by cleaved-caspase 3 staining and/or TUNEL-positive staining (DNA fragmentation) were evident in the cerebral cortex^{177, 458, 459}, striatum^{191, 459}, thalamus³²⁵ and hippocampus^{416, 460} of the hemisphere ipsilateral to CCA ligation, accompanied by increased caspase-3 cleavage activity (DEVD-AFC as substrate) in the ipsilateral hemisphere that could persist up to one week post-H/I^{461, 462}. In this regard, suppression of caspase-3 cleavage and activation in the injured hemisphere has become a standard endpoint for quantifying the therapeutic efficacy of potential neuroprotective agents. In the present study, cleaved caspase-3 positive cell bodies were concentrated in the ipsilateral hippocampal CA1, CA3 and DG regions at day1 (**Fig. 4-4**) and day3 (**Fig. 4-5**) after unilateral CCA coagulation plus 45 minutes of 8% hypoxia, to suggest that the immature hippocampus is particularly susceptible to apoptosis from cerebral I/R injury.

The observation that administration of RIPK1 inhibitor necrostatin-1 post-H/I reduced cerebral and hippocampal infarcts suggests necroptosis as a crucial pathological event of HIE³²³. The upregulated expression of the necroptosis mediators RIPK1 and RIPK3 and the enhanced RIPK1/RIPK3 interaction in the H/I-challenged immature brain or foetal neurons further corroborated the activation of necroptosis during neonatal H/I brain injury^{322, 323}. In an attempt to map the distribution of necroptotic neurons, RIPK3-positive

cells were shown to populate the peri-infarct zone instead of the ischaemic core of the injured cortex³²¹, and our group previously reported increased RIPK1 and RIPK3 immunofluorescence reactivity in the ipsilateral hippocampus in a PND7 rat model of HIE⁴⁶³. Nonetheless, one should bear in mind the multifaceted role of RIPK1 as a master regulator of apoptosis, necroptosis and inflammation (please refer to Chapter one, section E.1.2 for a detailed discussion) when interpreting studies showing increased RIPK1 expression from H/I insult and injury amelioration from RIPK1 inhibition, as these studies cannot be used to conclusively demonstrate the involvement of necroptosis in HIE. In view of the foregoing, we have selected phosphorylated MLKL (p-MLKL) and phosphorylated RIPK3 (p-RIPK3) as necroptosis-exclusive markers to be studied at day1 and day3 after unilateral CCA coagulation plus hypoxia. As shown in **Fig.4-2** and **Fig.4-3**, H/I led to prominent increase in the number of p-MLKL and p-RIPK3 positive cell bodies throughout the cornu ammonis (CA) regions of ipsilateral hippocampus at day1, with western blot showing upregulated expression of p-MLKL and p-RIPK3 from cortical and hippocampal brain lysates; the number of p-MLKL and p-RIPK3 positive neurons remained elevated in CA1 and CA3 until day3 post-H/I (**Fig. 4-5**). Our findings indicate that necroptosis is preferentially activated in the hippocampal CA region when immature brain is subjected to H/I injury. Unlike cleaved caspase-3, we did not detect increased MLKL and RIPK3 phosphorylation in dentate gyrus of the ipsilateral hippocampus to imply differential activation of regulated cell death pathways by different brain (sub)regions upon hypoxia-ischaemia challenge. In this regard, the different availabilities of inhibitor of apoptotic proteins (IAPs – XIAP, cIAP), SMAC/Diablo, caspases and RIPKs/MLKL, together with differential mitochondrial susceptibility between brain (sub)regions could dictate the sensitivity towards one form of RCD over the other during H/I injury. Future studies are needed to better map, compare and contrast the expression of apoptosis and necroptosis markers in the developing, H/I-injured brain.

4.3.2 Enhanced mitophagy during hypoxic-ischemic encephalopathy

In addition to perturbed energy metabolism and heightened oxidative stress, mitochondrial dysfunction during H/I brain injury also encompasses dysregulation to mitochondrial life cycle to impact mitochondrial fission/fusion, biogenesis and clearance by mitophagy, as detailed in Chapter one, sections F.1 to F.3. In this regard, increased mitochondrial fission and fragmentation was evident in primary neurons challenged with OGD plus reperfusion⁴⁶⁴, and a reduction in mitochondrial size was similarly observed in brain of neonatal rats subjected to cerebral H/I³⁹². The disrupted fission and fusion balance may be ascribed to aberrant degradation/cleavage of the mitochondrial fusion protein OPA1 into fusion-incompetent truncated form, which was detected OGD/R-injured neurons as well as in H/I-injured brain of PND9 mouse pups^{464, 465}. Along this line, suppression of mitochondrial fission by genetic silencing of the mitochondrial fission mediator dynamin-related protein 1 (Drp1) or pharmacological inhibition of Drp1 (mdiviA, mdiviB) protected hippocampal neuron-derived HT-22 cells from OGD injury and attenuated mitochondrial depolarisation, and reduced cerebral infarct in mice subjected to transient MCAO⁴⁶⁶.

The opposing processes of mitophagy and mitochondrial biogenesis are carefully balanced to maintain an adequate population of healthy, functional mitochondria to meet the high energy demand of neurons. In the event of hypoxia/ischaemia, neuronal adaptation or maladaptation to mitophagy and mitochondrial biogenesis will be crucial in determining the extent of neuronal injury. Animal and cellular studies have observed upregulation of mitophagy along with macro-autophagy in I/R injury of the adult brain, in particularly during the reperfusion phase^{385, 386}. The current available evidences support mitophagy as neuroprotective, provided that inhibition mitophagy (mdivi-1, knockdown of PARK2 or BNIP3L), or suppression of autophagy (3-methyladenine, bafilomycin A₁, Atg7 or Atg5 knockdown), during reperfusion exacerbated neuronal death and brain infarct post-injury³⁸⁵.

In the immature brain, increased LC3-II conversion and co-localisation with mitochondrial structural protein TOM20 or increased mitochondria/lysosome co-localisation (TOM20 with LAMP2, lysosomal membrane protein 2) was detected in the ipsilateral hemisphere immediately after H/I insult³⁸⁹⁻³⁹³ to indicate enhanced mitophagy. Using the mt-Keima reporter mice, whereby mt-Keima shifts its excitation wavelength from 440nm (green) to 586nm (red) when mitochondrion is delivered into acidic lysosome (as detailed in Chapter one, section F.3.3), mitophagy was shown to occur in two waves post-H/I, with the first wave induced immediately upon reperfusion and the second wave taking place at one week³⁹³. Studies also reported induction of mitochondrial biogenesis in the postnatal brain following H/I³⁴⁰, with increased mtDNA content and upregulated expression of mitochondrial transcription factors (NRF-1, nuclear respiratory factor 1; TFAM, mitochondrial transcriptional factor A) and respiratory enzyme (COX4, cytochrome c oxidative IV) in the ipsilateral cortex.

In the present study, our findings concurred with those demonstrated previously whereby unilateral CCA plus hypoxia induced mitophagy in the ipsilateral hemisphere, in particular in the hippocampus, with increased number of cell bodies staining positive for Parkin and BNIP3 and displaying LC3A/B and COXIV co-localisation at day1 (**Fig. 4-6, 4-7, 4-9**) and day3 (**Fig. 4-10**) post-H/I. Moreover, increase in hippocampal CLpP immunofluorescence reactivity (**Fig. 4-8**) was evident following H/I, which accords with *in vitro* findings on OGD-challenged primary neurons and Neuro2A cells and likely reflects an accumulation of mitochondrial stress and dysfunction.

It remains debatable whether elevated mitophagy is neuroprotective in H/I-challenged neonatal brain, and direct evidence is lacking as studies have concluded primarily in view of findings from inhibition of macro-autophagy instead of specific blockade of mitophagy. In this regard, treatment with autophagy inhibitor 3-MA deteriorated cerebral and hippocampal lesions and biased cell death towards necrosis, whereas autophagy inducer rapamycin reduced necrotic cell death and brain injury³⁸⁹. Moreover, when using TOM20/LAMP2 co-localisation as a marker of mitophagy, female pups displayed significantly higher level of

mitophagy and lesser neuronal loss following H/I challenge, and the male vulnerability was suggested to be linked to inefficient mitophagy and removal of ubiquitinated mitochondria³⁹². In contrast, other groups demonstrated that 3-MA pre-treatment reduced neuronal death in OGD-challenged hippocampal slice⁴⁶⁷ and H/I-injured neonatal rats⁴⁶⁸, as well as improving Morris water maze performance later on in life. Moreover, genetic deletion of the autophagy mediator ATG7 was found to reduce brain lesion and prevent caspase-3 activation in H/I-injured neonatal mice³⁹¹. In one study using BNIP3-KO neonatal mice, brain mitophagy, apoptosis and infarct volume from cerebral H/I were significantly suppressed along with a compensatory increase in macro-autophagy⁴⁶⁹. In the upcoming chapter, we will investigate the effect of RIPK1 inhibition by necrostatin-1s on H/I-induced mitophagy.

4.3.3 The effect of RIPK1 inhibition on necroptosis and mitophagy markers

In agreement with the *in vitro* findings from Neuro-2a cells (Chapter four, section 4.3.1), we demonstrated herein that the RIPK1-selective inhibitor Nec-1s prevented hypoxia-ischaemia induced phosphorylation of necroptosis mediators MLKL (**Fig. 4-11**) and RIPK3 (**Fig. 4-12**) in the ipsilateral hemisphere in a murine model of HIE, when administered immediately after the H/I insult. Specifically, the ipsilateral hippocampus as the most severely lesioned brain region in our HIE model showed significant decrease in the number of phos-MLKL and phos-RIPK3 positive cell bodies in H/I-injured, Nec-1s-treated pups, when compared H/I-injured and H/I-injured, vehicle-treated cohorts. Previous studies also demonstrated effective blockade of MLKL phosphorylation, and subsequent improvement in retinal ganglion cell and oligodendrocyte survival, by the first-generation RIPK1 inhibitor necrostatin-1 (Nec-1) in murine models of excitotoxicity-induced glaucoma⁴⁷⁰ and transient focal cerebral ischaemia⁴⁷¹, respectively. Phosphorylation of RIPK3 and MLKL in neurons and endothelial cells was also prevented in mice expressing the kinase-dead RIPK1 mutant RIPK1^{D138N/D138N} following transient MCAO²⁶⁶. Taken together, current and previous evidence unanimously support that RIPK1 kinase activity is indispensable for activating the RIPK1/RIPK3/MLKL signalling axis to drive necroptosis in neurons and susceptible cell types, in particularly during H/I or I/R brain injury.

In the current study, we also discovered that Nec-1s treatment abolished the number of Parkin-positive (**Fig. 4-13**) and LC3A/B and COXIV-double positive cell bodies (**Fig. 4-15**) as well as BNIP3 immunofluorescence intensity (**Fig. 4-14**) in the ipsilateral hemisphere at day1 post-H/I. The suppression of mitophagy from inhibition of RIPK1 kinase activity implies that RIPK1 signalling would normally promote Parkin-dependent and BNIP3-dependent mitophagy pathways in the immature brain during H/I and reperfusion injury. Our *in vivo* study aligned with the *in vitro* findings derived from Neuro-2a cells, whereby Nec-1s prevented OGD/Rep-induced whole-cell and mitochondrial upregulation of mitophagy markers (Chapter Four, section 4.3.1). Only a handful of studies have examined the molecular crosstalk between necroptosis and mitophagy

with discrepant findings, and studies have primarily focussing on mitochondrial dysfunction, exemplified by ROS burst and/or aberrant mitophagy, as likely effector of necroptotic cell death, as discussed in Introduction, section G. In lung epithelial cells exposed to cigarette smoke, enhanced PINK/Parkin-dependent mitophagy was shown to activate RIPK1/RIPK3/MLKL signalling and promote necroptosis⁴⁰⁹. Moreover, mitophagy and mitochondrial ROS production triggered by the respiratory chain complex I inhibitor BAY87-2243 were accompanied by increased RIPK1- and MLKL-dependent necroptosis⁴⁰⁸. On the contrary, mitochondrial depletion from excessive mitophagy following mitochondrial uncoupler FCCP treatment failed to prevent necroptosis induced by forced RIPK3 dimerisation or TNF- α plus pan-caspase inhibitor treatment⁴⁰⁷.

On the other hand, it remains elusive whether RIPK1/RIPK3/MLKL signalling could directly regulate mitophagy during I/R. Evidences to date support the recruitment of RIPK1/RIPK3/MLKL necrosome into mitochondrial fraction upon induction of necroptosis by TNF- α and pan-caspase inhibitor. Within the mitochondrial compartment, mitochondrial enzymes phosphoglycerate mutase 5 (PGAM5) and pyruvate dehydrogenase complex (PDC) were identified as the phosphorylation targets of RIPK3 kinase, and phosphorylation of PGAM5 and PDC was associated with potentiation of mitochondrial fission and ROS production, respectively, to mediate necroptotic death downstream to RIPK1/RIPK3 signalling (refer to Chapter one, section G for more detailed discussion). In a separate study, however, Lu et al. unveiled PGAM5 as cytoprotective by promoting mitochondrial accumulation of PINK1 and PINK1-dependent mitophagy during necroptosis triggered by TNF- α and pan-caspase inhibitor⁴⁷². Moreover, PGAM5 deficiency impaired mitochondrial clearance and aggravated cell death, and PGAM5-KO mice displayed greater heart and brain lesions following ischaemia/reperfusion injury⁴⁷². It is feasible for PGAM5 (or other mitochondrial enzymes) to relay RIPK1/RIPK3/MLKL signalling to downstream mitophagy during I/R injury shown here, and please refer to Chapter Eight, section 8.3.2 for a more in-depth discussion.

4.3.4 The effect of RIPK1 inhibition on long-term motor and behavioural outcomes

Provided that RIPK1 inhibition by Nec-1/Nec-1s effectively reduced ipsilateral lesion in adult and developing brains following cerebral H/I injury, we proceeded to evaluate whether Nec-1s treatment could also improve long-term motor and behavioural outcomes. As expected, hypoxia/ischaemia insult at PND7 (H/I group) significantly impaired accelerating rota-rod performance and spontaneous locomotor activity in PND28 to PND30 mice (**Fig. 4-17**). On the other hand, we did not observe alteration to thigmotaxis (preference for periphery over centre zones) in OPF to indicate that neonatal H/I injury does not induce anxiety-like behaviours in our HIE model. Intracerebrovascular administration of Nec-1s immediately after H/I exposure (H/I + Nec-1s) may have led to slight improvements in rota-rod performance and spontaneous locomotion, as both activities were not significantly impaired when compared to the NC unlike the H/I and H/I + DMSO groups. We acknowledge that when compared to some of the other studies^{112, 113}, accelerating rota-rod is

performed at a relative younger age in our study (PND28-30 versus PND40-50), and it is possible that the salubrious effect of Nec-1s on rota-rod performance will become more apparent as the mouse matures.

Accelerating rota-rod

Accelerating rota-rod remains a popular functional test for assessing motor coordination in rodents as it is sensitive to graded deterioration and graded improvement of motor functions from different types of injury/intervention. Magnetic imaging resonance (MRI) study identified brain regions that are engaged and activated during rota-rod training, including regions that are directly related to running/balancing (primary/secondary motor cortex, cerebellum, striatum) and regions involved in general learning (hippocampus, frontal cortex), with the volume of motor cortex and the fractional anisotropy (FA, a measure of microstructural integrity) of hippocampus showing the strongest positive correlation to rota-rod performance⁴⁷³. The hippocampus was suggested as the brain structure most malleable to repeated rota-rod training, with the CA1 molecular layer showing the greatest volume expansion and FA increase amongst all sub-regions to reflect increased apical dendritic growth of CA1 pyramidal neurons⁴⁷³. The hippocampus – motor behaviour correlation is concurred by our study, whereby the substantial neuronal loss (**Fig. 4-1**) and activation of necroptosis (**Fig. 4-3, 4-4**) and mitophagy pathways (**Fig. 4-7 to 4-9**) in the ipsilateral hippocampus, in particular in the CA1 and CA3 sub-regions, are predictive of shorter latency on rota-rod following neonatal cerebral H/I injury. Therefore, the attenuated impairment to rota-rod performance in Nec-1s treated cohorts could be explained by the suppression of necroptosis and mitophagy in the ipsilateral hippocampus, to preserve hippocampus-dependent motor learning and memory. Moreover, increased neuronal c-Fos expression was detected in the motor cortex and striatum of mice undergoing rota-rod training⁴⁷⁴, along with increased spine formation in the motor cortex to correlate with better motor performance⁴⁷⁵. In the future study, activation of necroptosis and mitophagy pathways in the other rota-rod relevant regions, such as the motor cortex and striatum, needs to be investigated using the murine HIE model described herein to better correlate anatomical lesions with motor deficits.

On this note, the striatum and associated basal ganglia structures are particularly relevant nodes for future investigation of hypoxic-ischaemic encephalopathy. The basal ganglia nuclei (caudate, putamen, globus pallidus, subthalamic nuclei and substantia nigra) are essential in modulating the motor cortex outputs and shaping the descending motor pathways⁴⁷⁶. The striato-cortical movement circuitry starts with the striatal neurons receiving afferent inputs from the motor and somatosensory cortices, followed by inhibitory innervation into the globus pallidus that also sends inhibitory projections into the anterolateral/ventrolateral thalamic nuclei, which then relay information back to the cerebral cortex to enable processing and coordination of motor movements (the *direct* pathway)⁴⁷⁷. The circuit is subjected to further excitatory glutamatergic input from the subthalamic nucleus (the *indirect* pathway) and the dually excitatory/inhibitory

dopaminergic input substantia nigra (the *nigrostriatal* pathway)⁴⁷⁷. Lesion anywhere along this complex motor control circuitry will result in distinctive motor syndromes, for example, striatum and substantia nigra lesions were shown to directly contribute to the characteristic motor disorders of Huntington's disease and Parkinson's disease, respectively^{476, 477}. Moreover, in children survived from HIE, MRI study revealed hyperintensities in basal ganglia/thalamus (BGT) along with diminished signal in the posterior limb of internal capsule (PLIC), the white matter fibrous tract separating caudate and putamen, and abnormal PLIC intensity was shown to be a strong and positive predictor of cerebral palsy^{478, 479}. In view of the motor deficits/hypoactivity in rota-rod performance and open-field test (discussed below) observed herein, future study will need to comprehensively evaluate necroptosis, mitophagy and overall histopathology across basal ganglia structures to better correlate anatomical lesion with functional impairments.

Open-field test

With regard to open-field test (OFT), the significant reduction in total distance travelled observed is indicative of weakened overall mobility and locomotion in our murine model of HIE, which disagreed with previous studies reporting a lack of effect on general locomotor activity from neonatal cerebral H/I injury⁹⁹⁻¹⁰¹. It should be noted that the earlier studies were performed on slightly older pups (PND9-10) and/or assessed open-field behaviours at an older age (6-8 weeks after injury). It is possible that the susceptibilities of brain structures responsible for general locomotion (e.g. motor cortex, basal ganglia) differ within the postnatal period, or that overall hypoactivity only manifests at a younger age (3 weeks after injury, as studied here) and does not persist into more developed stage. As with accelerating rota-rod, Nec-1s treatment maybe associated with a slight improvement in spontaneous locomotion that was no longer significantly less than the total distance travelled by the NC controls (**Fig. 4-17C**). Thus, the impaired general locomotor activity at PND30 may explain the poor rota-rod performance from neonatal H/I exposure.

Our study also differed from previous reports in that we did not detect changes in thigmotaxis as a surrogate measure for anxiety-like behaviours in H/I-injured subjects, however the previous studies also reached divergent findings in rats or mice. When thigmotaxis is stratified by sex following H/I injury, increased preference for the periphery over centre zones by female mice but not male mice at PND30 was detected to suggest increase in anxiety-like behaviours⁹⁹, whereas in rats both sexes showed increased tendency to move into the centre zones at PND28 to indicate anxiolytic-like behaviours¹⁰⁰. In addition to not stratifying subjects by sex, our study used different parameters to evaluate thigmotaxis from previous studies and therefore results may not be directly comparable. As explained in Chapter Two, section 2.12.4, in the current study thigmotaxis is determined by the respective distance travelled in the centre or periphery zones and as percentages to total distance travelled, whereas the other groups measured time spent in the centre/periphery or number of entries into the centre zones^{99, 100}.

As a complex emotional state, anxiety is characterised by increased autonomic and behavioural arousals and increased avoidance to uncertain, unpredictable and unfamiliar environmental situations⁴⁸⁰; in the case of OFT, the periphery/edge of the testing box is deemed to be “safer” than the open, exposed centre that mice with anxious phenotype will display increased avoidance than controls. An interconnected neural circuitry encompassing different forebrain and midbrain structures have been shown to initiate anxiety and anxiety-like behaviours, to include the medial prefrontal cortex (mPFC), septo-hippocampus system, bed nucleus of the stria terminalis (BNST) and amygdala, before recruitment of brainstem and hypothalamic nuclei to drive physiological, vigilant and defensive responses^{480, 481}. Amygdala is central to elicitation of anxiety and related behaviours by receiving afferents from and projecting afferents into BNST, septo-hippocampal system and mPFC thus to form a circular signalling loop. The basolateral amygdala (BLA) within amygdala is responsible for receiving sensory stimuli from the sensory cortices and attaches emotional value to the sensory stimuli, before transmitting sensory information onward to central amygdala (CeA), BNST, hippocampus and mPFC (forward) and eventually to the effector nuclei in the hypothalamus and periaqueductal grey⁴⁸⁰. Sensory information also flows backward from mPFC and hippocampus into BNST and BLA, and in the context of open-field test, retrograde tracing identified afferent inputs from the ventral hippocampus CA1 region, subiculum and entorhinal cortex into the BLA to activate BLA neurons with increased c-Fos expression⁴⁸¹. In view of the above, the inconsistent findings on thigmotaxis following neonatal H/I insult maybe ascribed to the degree by which amygdala/BLA is lesioned and/or damage to the bilateral projections between BLA and associated regions, depending on the specific H/I protocol and the postnatal day when H/I is induced.

To summarise, in this Chapter we demonstrated that Nec-1s treatment in PND7, H/I-injured mice inhibited necroptosis and mitophagy pathways in the ipsilateral hippocampus that is most vulnerable to H/I insult, corroborating our in vitro data obtained from OGD/Rep-challenged Neuro-2a cells. Moreover, Nec-1s mediated attenuation of necroptosis and mitophagy maybe associated with long-term improvements in motor function, and additional functional tests that assess coordinated movements, exploratory activity and social behaviours can be performed, such as CatWalk for gait analysis and novel objection recognition, to more comprehensively assess the neuroprotective potential of Nec-1s in HIE.

Final Discussion

5.1 Rationale of the Study

In both developed and developing countries, neonatal encephalopathy resulting from birth hypoxia-ischaemia, or hypoxic-ischaemic encephalopathy (HIE), represents a significant cause of mortality and morbidities in termed infants. Those surviving from perinatal HIE will likely continue to suffer from life-long cerebral palsy, learning difficulties, sensorimotor impairments, mental disorders and physiological disabilities with substantial economic, emotional and societal burdens. The advent of therapeutic hypothermia has effectively reduced mortality and long-term morbidity and has become the standard of care for moderate to severe cases of HIE. However, the clinical benefit of therapeutic hypothermia does not extend to all cases moderate/severe HIE, which calls for ongoing research into novel therapy that can be administered in conjunction with hypothermia to achieve synergistic neuroprotection. To this end, a better understanding of the pathophysiological mechanisms underlying HIE will hopefully identify molecular targets for therapeutic development.

Following the hypoxia/ischaemia phase, the reperfusion phase of HIE injury is characterised by activation of regulated cell death (RCD) pathways and secondary energy failure from mitochondrial dysfunction. Apoptosis as the most widely studied RCD mechanism was believed to be primarily responsible for neuronal injury following HIE, with evidences now support that different RCD programmes co-exist in parallel to collectively contribute to ischaemia-reperfusion brain injury. Necroptosis, or regulated necrosis, was shown to mediate hypoxic-ischaemic (H/I) and ischaemia-reperfusion (I/R) brain injury in the adult³¹⁷ and immature^{323, 324} brains. Necroptosis is mediated by the receptor interacting kinase 1 and 3 (RIPK1 and RIPK3) and downstream effector molecule mixed lineage kinase-domain like (MLKL), and blocking RIPK1/RIPK3 activity was shown to attenuate mitochondrial dysfunction³²⁴.

Mitochondrion has been regarded as the signalling “hub” during ischaemia-reperfusion brain injury, for one, the damaged mitochondrion serves as a source of reactive oxygen species (ROS) and pro-apoptotic factors¹³². During I/R insult, the intrinsic processes fundamental to mitochondrial health and function are disturbed, including fusion/fission, biogenesis and mitophagy; adaptation/maladaptation in these processes will dictate recovery or decline of mitochondrial function, and subsequently cell survival, following I/R injury¹³².

Studies have attempted to address the link between necroptosis and mitochondrial dysfunction without reaching a unifying conclusion. The observation that activated RIPK1/RIPK3/MLKL complex undergoes mitochondrial translocation during necroptosis^{312, 401-403} provides the theoretical basis for necroptosis to directly influence mitochondria function, and implies mitochondrial dysfunction as a downstream effector mechanism to necroptotic cell death. In the current study, we hypothesise that activation of necroptosis during I/R brain injury could modulate the rate of mitophagy and in turn regulate mitochondrial function. Using a combination of *in vitro* model of oxygen-glucose deprivation (OGD) plus reperfusion and a murine

model of HIE, the necroptosis-mitophagy association uncovered herein are considered to be relevant to I/R brain injury of the immature and the adult brains. In this regard, elucidation of the pathophysiological pathways downstream to necroptosis activation will justify the development of selective necroptosis inhibitor as novel treatment for HIE, and for I/R brain injury conditions alike.

5.2 Summary of the Findings

In Chapter Three, we addressed *Aims 1 and 2* of the present study as stated in Chapter One, Section G.1. Using oxygen-glucose deprivation (OGD) and reperfusion as established *in vitro* model of ischaemia-reperfusion (I/R) brain injury, we detected MLKL phosphorylation, Parkin and CLpP upregulation, LC3A/B mitochondrial localisation and LC3A/B-II mitochondrial conversion in mouse primary neurons and mouse neuroblastoma Neuro-2a cells, suggesting concurrent activation of necroptosis and mitophagy. We also found translocation of RIPK3, MLKL and phosphor-MLKL into the mitochondrial compartment during OGD/Rep insult. In comparison to OGD/Rep, pure OGD insult did not induce MLKL phosphorylation or necrosome translocation, but was associated with more extensive LC3A/B recruitment and LC3A/B-II conversion in the mitochondrial compartment. Our comparison study highlighted that necroptosis activation and necrosome translocation are exclusive to I/R injury, and that H/I and I/R brain injuries can be distinguished by different mitophagy phenotypes.

In Chapter Four, we explored *Aim 3* of the present study as stated in Chapter One, Section G.1. We examined whether and how necroptosis regulates mitophagy during I/R brain injury by manipulating the individual components of necrosome (RIPK1, RIPK3 and MLKL) in Neuro-2a cells. Inhibiting RIPK1 kinase activity with Nec-1s prevented MLKL phosphorylation and mitochondrial translocation of necrosome, and downregulated mitochondrial levels of Parkin, CLpP and LC3A/B-II to suggest suppression of mitophagy. Treatment with the RIPK3 selective inhibitor GSK'872 yielded comparable results, with attenuated mitochondrial translocation of RIPK3 and MLKL, decreased expression of Parkin and BNIP3, and reduced mitochondrial localisation of autophagosome marker LC3A/B-II. Small silencing RNA-mediated MLKL knockdown similarly prevented mitochondrial translocation of RIPK3, yet in stark contrast to RIPK1/RIPK3 inhibition, potentiated OGD/Rep-induced mitophagy (upregulated Parkin and BNIP3, increased mitochondrial conversion of LC3A/B-II). Moreover, preliminary co-immunoprecipitation study identified a potential MLKL-BNIP3 association during OGD/Rep. Collectively, our findings suggest that the individual necrosome components exert opposing effects on mitophagy during I/R brain injury.

In Chapter Five, we investigated *Aim 4* of the present study as indicated in Chapter One, Section G.1. We studied the functional implications of necroptosis inhibition and the associated changes to mitophagy by assessing mitochondrial function, mitochondrial morphology and cell viability. RIPK1 inhibitor Nec-1s or RIPK3 inhibitor GSK'872 partially restored mitochondrial dysfunction and ameliorated mitochondrial fragmentation following OGD/Rep, and MLKL silencing resulted in a near-complete recovery in mitochondrial function and mitochondrial morphology. We did not detect significant improvement to overall cell viability in OGD/Rep-challenged Neuro-2a cells from RIPK1/RIPK3 inhibition or MLKL silencing, nonetheless necroptotic cell death is reduced. Drawing on the findings, compared to pharmacological inhibition of

RIPK1/RIPK3, MLKL knockdown appears to confer greater mitochondrial protection during I/R brain injury, which may be attributed to an upregulation of mito-protective mitophagy.

In Chapter Six, we addressed *Aim 5* of the present study as indicated in Chapter One, Section G.1, by investigating the activation of necroptosis and mitophagy pathways in a murine model of hypoxic-ischaemic encephalopathy. In postnatal day 7 (PND7) mice, unilateral common carotid artery coagulation plus hypoxia at 8% O₂ (hypoxia-ischaemia) resulted in significant neuronal loss in the ipsilateral hippocampus, and in conjunction with increased phosphorylation of MLKL and RIPK3 point to increased necroptosis from HIE. We also detected a concurrent upregulation of Parkin and BNIP3 and increased mitochondrial localisation of LC3A/B, indicative of enhanced mitophagy, in the ipsilateral hippocampus. The concurrent activation of necroptosis and mitophagy in the injured hippocampus were evident from PND8 to PND10 and predicts impaired rota-rod performance at PND30.

In Chapter Seven, we addressed *Aim 6* of the present study as stated in Chapter One, Section G.1, by examining the effect of RIPK1 inhibitor Nec-1s on mitophagy and long-term functional recovery in a murine model of HIE. Administration of Nec-1s after H/I, but not the vehicle, prevented MLKL and RIPK3 phosphorylation in the ipsilateral hippocampus, and attenuated Parkin expression and LC3A/B localisation to mitochondria, to indicate simultaneous inhibition of necroptosis and mitophagy. Nec-1s treatment also mildly improved long-term rota-rod performance and spontaneous locomotion. The *in vivo* findings concurred with *in vitro* data to support RIPK1 as a positive regulator of mitophagy during I/R brain injury, and the functional improvement maybe ascribed to enhanced mitochondrial function from RIPK1 inhibition.

Collectively, the current study demonstrated concurrent activation of necroptosis and mitophagy in *in vitro* model of ischaemia-reperfusion brain injury and in murine model of hypoxic-ischaemic encephalopathy (**Fig. 5-1**). For the first time, the individual contribution of necrosome components to mitophagy was investigated. Our findings highlighted RIPK1 and RIPK3 as positive upstream regulators of I/R-induced mitophagy, as inhibiting the kinase activity of RIPK1 or RIPK3 decreased mitophagy. On the other hand, MLKL knockdown potentiated mitophagy during I/R to suggest an anti-mitophagy role of MLKL. Although targeting different necrosome components produced contrasting effects on mitophagy, mitochondrial protection *in vitro* is evident regardless of interventions, with MLKL manipulation possibly as a more promising mito- and neuroprotective strategy during I/R brain injury. Moreover, RIPK1 inhibition and subsequent blockade of mitophagy by Nec-1s may be associated with long-term motor and functional improvement in HIE-injured mice. Mitochondrial translocation of necrosome during I/R might be conducive for necroptotic regulation over mitophagy, and future studies are needed to elucidate the precise mechanisms underpinning the necroptosis-mitophagy axis.

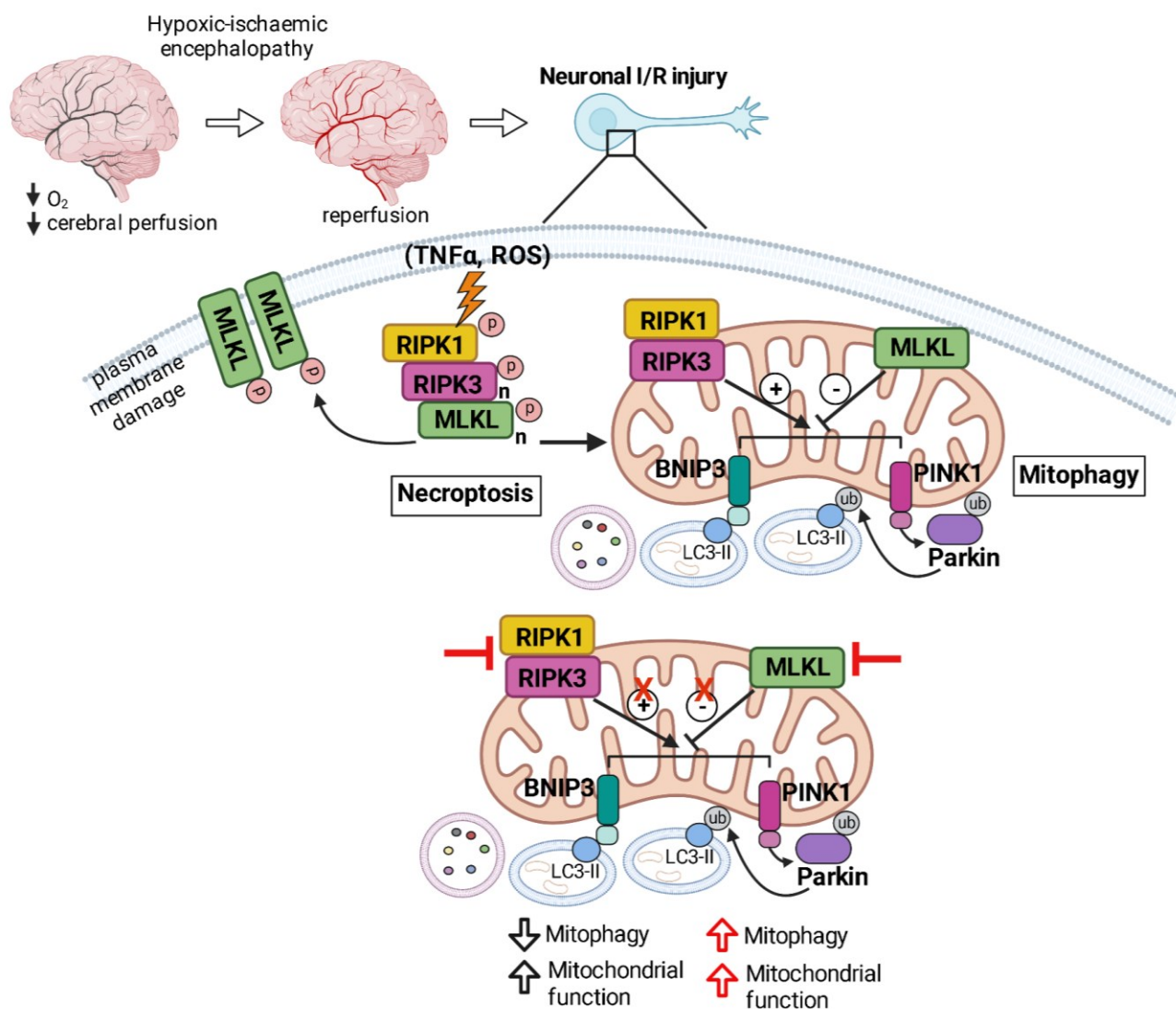


Figure 5-1. Schematic summary of necroptosis regulation over mitophagy during hypoxia-ischaemia (H/I) and reperfusion brain injury in hypoxic-ischaemic encephalopathy (HIE). Perinatal H/I and reperfusion (I/R) challenge in HIE stimulates necroptotic signaling in neurons and also other susceptible cell types, via tumour necrosis factor- α (TNF- α), oxidative stress and other molecular pathways. Formation and activation of the necrosome complex comprising receptor-interacting protein kinase 1/3 (RIPK1/3) and mixed lineage kinase domain-like (MLKL), and subsequently phosphor-activation and oligomerisation of MLKL, enables translocation of necrosome proteins into cell membrane fractions including the mitochondria, in neurons and neuron-like cells. Through selective targeting of individual necrosome components, our data suggest RIPK1 and RIPK3 as positive regulators of Parkin- and BNIP3-mediated mitophagy during neuronal I/R injury, whereas MLKL would suppress I/R-induced mitophagy to highlight “uncoupling” of mechanism of action between the necrosome components. Despite the opposing effects on mitophagy, inhibiting RIPK1/RIPK3 or MLKL resulted in improvements of mitochondrial function and morphology following I/R. Necroptosis and associated mitophagy pathway collectively could represent a promising therapeutic target for HIE and other H/I or I/R brain injury alike.

5.3 Scientific and Translational Implications

Our study offered novel and intriguing insights into necroptosis and mitophagy crosstalk during I/R brain injury, and at the same time unveiled the knowledge gaps and inconsistencies with reference to previous studies in the relevant fields. In this section, we will discuss the scientific implications of our findings in view of the current understanding of necroptosis and mitophagy, and the potential translational impact of our study in developing novel treatment for I/R brain injury.

5.3.1 Mitochondrial Translocation of Necrosome

With the identification MLKL as the phosphorylation substrate and downstream signalling partner of RIPK1/RIPK3, studies demonstrated translocation of RIPK1/RIPK3/MLKL into mitochondrial compartment in cells treated with the necroptosis-inducing cocktail (TNF- α , SMAC mimetic and pan-caspase inhibitor)^{296, 312, 403}. Phosphor-activation of MLKL by RIPK3 is essential for targeting MLKL and RIPK1/RIPK3 as an integral necrosome complex into the membrane fractions of cells, including the plasma membrane and the mitochondrial membrane. MLKL binds to mitochondrial membrane lipids (phosphatidylinositol and cardiolipin) through its coiled-coil domain, and MLKL binding leads to perforation of artificial liposomes within similar lipid composition to mitochondrial membrane³¹². It is therefore speculated that mitochondrial-localised MLKL may cause mitochondrial membrane perforation/disruption that is analogous to proapoptotic BAX/BAK. Studies to date have not looked into MLKL beyond its “cargo” function for membrane targeting, likely because its “kinase-like” domain lacks complete kinase activity, and it remains elusive whether the mitochondrial-localised MLKL could interact with other proteins to influence mitochondrial function. Mitochondrial-targeted RIPK3, on the other hand, was shown to phosphorylate PGAM5⁴⁰³ and pyruvate dehydrogenase complex (PDC)⁴⁰⁴ to promote Drp1-dependent mitochondrial fission and excessive ROS production, respectively.

In the present study, for the first time, we demonstrated that ischaemia-reperfusion insult also promoted mitochondrial translocation of RIPK3, MLKL and phosphorylated MLKL in neuron-like cells. In this regard, activation of necroptosis from I/R or from chemical stimulation TNF- α /SMAC mimetic/pan-caspase inhibitor is reflective of different disease processes/modalities (e.g. inflammatory conditions in the latter case), and our findings herein more directly support mitochondrial translocation of necrosome as a signature event during the development of I/R injury.

5.3.2 Necroptosis and Mitochondrial Dysfunction

Mitochondrial Reactive Oxygen Species

In the pioneering studies of necroptosis and before the identification of MLKL as the executor molecule of necroptosis through plasma membrane perforation, TNF- α induced RIPK1/RIPK3 activation was shown to promote mitochondrial ROS synthesis²⁹⁰. The observation that scavenging ROS by butylated hydroxyanisole (BHA) treatment⁴⁰⁵ or curbing ROS production from complex I by knockdown the complex I accessory protein NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8 (NDUFB8)⁴⁰⁶ prevented cell death from RIPK1/RIPK3 activation, initially supported that mitochondrial oxidative stress is necessary for necroptotic cell death. Moreover, inhibition of RIPK1 kinase activity or RIPK3 knockdown in necroptotic cells (e.g. TNF- α treatment) also reduced cellular ROS content^{405, 406, 454}. Later study argued that mitochondrial ROS is dispensable for necroptosis, as widespread mitochondrial depletion and ROS elimination from excessive mitophagy, induced by mitochondrial uncoupler CCCP, failed to suppress necroptosis from forced dimerization of RIPK3, while the mitochondria-deficient cells were sensitive to cytoprotection by Nec-1s against TNF- α /SMAC mimetic/pan-caspase inhibitor induced necroptosis⁴⁰⁷, highlighting that mitochondria-depleted cells are still necroptosis-competent.

RIPK3 interaction with metabolic enzymes could be accountable for enhanced ROS production during necroptosis. Specifically, RIPK3 was shown to activate cytosolic glycogen phosphorylase (PYGL) and glutamate-ammonia ligase (GLUL) to drive glycolysis and glutamine catabolism, as well as mitochondrial glutamate dehydrogenase 1 (GLUD1)²⁹⁰ and pyruvate dehydrogenase complex (PDC)⁴⁰⁴, which collectively accelerate the mitochondrial TCA cycle (aerobic respiration) and overall energy production with increased ROS as by-product. During necroptosis, RIPK3 could also promote the mitochondrial translocation of NADPH oxidase 4 (NOX4)⁴⁵⁴, which catalyses the synthesis of superoxide and other downstream ROS species by transferring electrons from NADPH to oxygen, to further aggravate mitochondrial oxidative stress. A positive feedforward loop between mitochondrial ROS and necrosome signalling was identified, whereby mitochondrial ROS oxidises cysteine residues 257, 268 and 586 on RIPK1 to favour the formation of high molecular weight RIPK1 oligomers through disulphate bonds, which then facilitate RIPK1 serine 161 residue autophosphorylation required for RIPK1 kinase activity and RIPK1/RIPK3 necrosome assembly⁴⁸².

With respect to the current study, provided that mitochondrial ROS stimulates PINK1/Parkin-dependent mitophagy^{410, 411}, RIP1/RIPK3 activation during necroptosis could indirectly upregulate mitophagy by promoting mitochondrial ROS response. The positive correlation between RIPK1/3 activation and mitophagy is concurred by our *in vitro* and murine model I/R brain injury, whereby RIPK1 and RIPK3 inhibition attenuates I/R-induced mitophagy with improved mitochondrial function, possibly reflecting a decline in mitochondrial ROS level although was not explicitly tested. It remains elusive whether/how MLKL contributes to

necroptosis-associated ROS response, which could be addressed by quantifying mitochondrial ROS content in OGD/Rep-challenged, MLKL-silenced/inhibited cells in the future studies.

Mitochondrial Phosphatase PGAM5 as the Linkage between Necroptosis and Mitophagy?

Phosphoglycerate mutases (PGAM) are a family of enzymes that catalyse the conversion of 3-phosphoglycerate (3PG) to 2-phosphoglycerate (2PG) in the glycolysis reaction through the conserved catalytic C-terminal PGAM domain. Unlike the other members of PGAM family that function as phosphotransferases/phosphohydrolases for small molecule substrates, phosphoglycerate mutase 5 (PGAM5) exhibits serine/threonine phosphatase activity⁴⁸³ and displays extensive protein-protein interaction with different mitochondrial substrates to regulate mitochondrial function and homeostasis⁴⁸⁴. Alternative splicing generates the PGAM5-long (PGAM5_L) and PGAM5-short (PGAM5_S) isoforms, both of which are targeted into the mitochondrial compartment where they form stable transmembrane dimers or decamers⁴⁸⁴. PGAM5-dependent dephosphorylation of inhibitory sites on protein substrates leads to “dis-inhibition”/recovery of substrate activity, as exemplified by dynamin-related protein 1 (Drp1).

In TNF- α treated cells, a RIPK3/PGAM5/Drp1 axis was identified to stimulate mitochondrial fission and mitochondrial dysfunction as the candidate effector mechanisms of necroptotic cell death, whereby PGAM5 dephosphorylates serine 637 on Drp1 to enhance its GTPase activity⁴⁰³. PGAM5 was also shown to be pro-mitophagic during mitochondrial dysfunction or oxidative stress, and mitophagy induction could be achieved through phosphatase-dependent or phosphatase-independent means. PGAM5 directly counteracts casein kinase 2 (CK2) and Src kinase activities by dephosphorylating serine 13 residue on the mitophagy mediator FUNDC1, to promote FUNDC1-LC3A/B interaction and mitochondrial recruitment of autophagosomes³⁵⁶. Mechanistically, the phosphatase activity of PGAM5 is inhibited by the anti-apoptotic protein Bcl2-like 1 (Bcl2L1)/Bcl_{XL} through direct binding during, to prevent FUNDC1 activation and suppress FUNDC1-mediated mitophagy under basal condition; during hypoxia, degradation of Bcl2L1/ Bcl_{XL} releases PGAM5 to dephosphorylate FUNDC1 to promote mitophagy⁴⁸⁵. Furthermore, PGAM5 contains a N-terminal cleavage site for the mitochondrial protease presenilin-associated rhomboid-like (PARL), which also processes the mitophagy mediator PTEN-induced putative kinase 1 (PINK1) as substrate⁴⁸⁶. Therefore, PGAM5 would compete with PINK1 as PARL cleavage substrate, and increased PGAM5 will stabilise PINK1 on the mitochondrial outer membrane to recruit Parkin and promote mitophagy⁴⁸⁷.

It remains debatable whether PGAM5 activation is cytotoxic or cytoprotective depending on the mitochondrial processes it regulates. With respect to PGAM5/Drp1-mediated mitochondrial fission, PGAM5 knockdown attenuated whereas PGAM5 overexpression accelerated TNF- α /SMAC mimetic/pan-caspase inhibitor induced necroptotic cell death, to suggest that activation of PGAM5 and associated mitochondrial fission as cytotoxic during necroptosis⁴⁰³. Others concluded PGAM5 activation to be cytoprotective, whereby

PGAM5 knockdown disabled PINK1-mediated mitophagy and elevated mitochondrial ROS content with increased cell death from TNF- α /SMAC mimetic/pan-caspase inhibitor treatment^{472, 488}. PGAM5-deficient mice also exhibited greater heart and brain infarct following ischaemia-reperfusion injury⁴⁷², and dopaminergic neuron loss in substantia nigra along with Parkinsonian-like movement deficits at 1 year of age⁴⁸⁸. There has also been report demonstrating that knockdown of PGAM5, Drp1 or PINK1 did not attenuate cell death induced by TNF- α /pan-caspase inhibitor, to challenge the involvement of PGAM5-related pathways in regulated cell death⁴⁵⁵. Moreover, it should be noted that mitochondrial fission and mitophagy are intricately linked processes. Studies suggested mitochondrial fission to be permissive for mitophagy as the smaller, fragmented mitochondria are more susceptible to autophagosome engulfment⁴⁸⁹. Others argued that mitochondrial fission prevents excessive PINK1/Parkin-mitophagy to achieve selective elimination of damaged mitochondrial subdomains, as fission inhibition from Drp1 knockdown enhanced Parkin recruitment to fused mitochondrial network with reduced selectivity for damaged subdomains⁴⁹⁰. Taken together, PGAM5 likely assumes a complex role in regulating mitochondrial function and homeostasis, in part through mitochondrial fission and mitophagy control, and PGAM5 activation downstream to RIPK1/RIPK3 signalling may simultaneously induce detrimental and cytoprotective processes in a necroptotic cell.

With respect to the current study, it is plausible for PGAM5 to mediate the necroptosis-mitophagy crosstalk in our *in vitro* and *in vivo* models of I/R brain injury, whereby RIPK1/RIPK3 activation from I/R would promote PGAM5-dependent mitophagy, seen as upregulated mitochondrial expression of Parkin, BNIP3 and LC3A/B-II, and inhibition of RIPK1 or RIPK3 kinase activity would suppress PGAM5-mediated mitophagy. Provided the RIPK1/RIPK3/PGAM5 mitophagy axis operates in our model of I/R brain injury, nonetheless, our findings would challenge the concept that PGAM5-dependent mitophagy is mito/cytoprotective, as RIPK1/RIPK3 inhibition and consequently impaired PGAM5 activity in fact improved mitochondrial function and mitochondrial morphology. Therefore, it is reasonable to speculate the existence of alternative/additional mitochondrial substrates that could mediate the effect of RIPK1/RIPK3 activation on mitophagy and account for the necroptotic regulation over mitochondrial function. With respect to MLKL, evidence supported MLKL as indispensable for PGAM5 phosphor-activation by RIPK3⁴⁰³, presumably dependent on MLKL-mediated mitochondrial translocation of RIPK3-containing necrosome to enable RIPK3-PGAM5 interaction. It remains unclear whether MLKL could directly interact with PGAM5 and influence PGAM5-associated mitophagy independent from RIPK1/RIPK3. Our preliminary co-immunoprecipitation study uncovered a potential interaction between MLKL and BNIP3 during I/R exposure, to imply that necroptotic machineries may directly associate with mitophagy receptors to influence the rate of mitophagy. In view of the above, future studies may focus on 1) dissecting out the precise contribution of PGAM5 in necroptosis-associated mitophagy, 2) examining MLKL-PGAM5 interaction, and 3) identifying alternative RIPK3 or MLKL-interacting mitochondrial

proteins other than PGAM5, to further enrich the molecular understanding of necroptosis-mitophagy crosstalk during I/R injury.

5.3.3 Mitophagy as a Neuroprotective Mitochondrial Quality Control Mechanism

As detailed in Chapter one, sections F.3.1 and F.3.2, two main modalities of mitophagy have been identified to date, one being ubiquitin-driven mitophagy that requires mitochondrial Parkin recruitment by PINK1 and the E3 ubiquitin ligase activity of Parkin, while receptor-dependent mitophagy is achieved by the direct interaction between mitochondria-localised LC3 receptors (e.g. BNIP3, FUNDC1) and autophagosomes containing active, PE-conjugated LC3. Mitophagy plays a crucial role in physiological as well as pathophysiological processes. The mitophagy receptor Bcl2/adenovirus E18-interacting protein 3-like (BNIP3L, or NIX), a mitochondrial outer membrane protein that shares 50% homology with BNIP3 and directly interacts with LC3 through its N-terminal LC3-interacting region (LIR), was shown to be indispensable for mitochondrial clearance during reticulocyte maturation. Mitophagy is also implicated in the removal of paternal mitochondrial DNA during fertilisation and the underlying mechanisms await further elucidation.

In experimental settings, mitochondrial dysfunction and stress in the form of ROS accumulation, membrane potential depolarisation or aggregation of misfolded protein can be induced by hypoxia-ischaemia challenge, treatment with mitochondrial potential uncoupler, or forced expression of insoluble mitochondrial proteins, to subsequently provoke mitophagy *in vitro*^{334, 341}. BNIP3 was shown to mediate hypoxia-induced mitophagy and transcriptional upregulation of BNIP3 by hypoxia-inducible factor 1 α (HIF-1 α) during hypoxia is evident⁴⁹¹. In this regard, BNIP3 silencing was associated with increased mitochondrial ROS and cell death from prolonged hypoxia, and BNIP3-mediated mitophagy was suggested as a mito-adaptive, cytoprotective mechanism during hypoxia challenge⁴⁹². PINK1/Parkin-mediate mitophagy could be induced by mitochondrial depolarisation *in vitro*, and impairment to PINK1/Parkin mitophagy pathway has been implicated in neurodegeneration conditions with compromised mitochondrial function, to imply PINK1/Parkin-dependent mitophagy as a crucial adaptive and quality control mechanism during mitochondrial stress and damage⁴⁹³. The most well-studied example is Parkinson's Disease (PD), whereby loss-of-function mutation to the *PARK2* gene that encodes Parkin, is strongly associated with a form of juvenile, autosomal recessive PD^{360, 494}. Homozygous mutation to the PINK1 gene, *PARK6*, was also identified to predispose to familial, early-onset PD^{359, 495}. Parkin-mediated mitophagy is also implicated in Alzheimer's disease, whereby the brain samples from AD patients showed depletion of Parkin over disease progression and aberrant accumulation of dysfunctional mitochondria⁴⁹⁶, suggesting impaired Parkin-dependent mitophagy as a pathological event leading to AD.

In the context of ischaemia-reperfusion brain injury, upregulation of mitophagy along with macro-autophagy is deemed to be an intrinsic neuroprotective mechanism within the adult and immature brains, whereby

pharmacological inhibition of mitophagy or autophagy^{385, 389} or genetic silencing of PARK2³⁸⁷ or BNIP3L³⁸⁶ was shown to worsen neuronal injury and aggravate brain infarct in cerebral I/R injury. In the present study, we also observed enhanced mitophagy in *in vitro* OGD challenge and *in vivo* HIE murine model along with increased expression of Parkin and BNIP3, and we attempted to address whether necroptosis signalling could interfere with this endogenous mito-protective and neuroprotective mechanism during I/R brain injury. To our surprise, inhibition of RIPK1/RIPK3 kinase activity and knockdown of MLKL expression yielded opposite findings, with RIPK1/RIPK3 blockade suppressing and MLKL knockdown potentiating I/R-induced mitophagy, yet improvement to mitochondrial function and morphology is evident regardless of the treatment/intervention.

With respect to RIPK1/RIPK3, provided that RIPK3 targets multiple mitochondrial substrates to precipitate mitochondrial dysfunction, such as ROS overproduction from RIPK3/PDC signalling⁴⁰⁴ and mitochondrial fission from RIPK3/PGAM/Drp1 axis⁴⁰³, RIPK1/RIPK3 inhibition would eliminate the array of mitochondrial damages that would have otherwise acted as triggers for mitophagy. This way, reduced mitophagy from RIPK1/RIPK3 inhibition likely reflects a dampened need for mitophagy, which corresponds to decreased level of mitochondrial chaperone protein CLpP and is accompanied by restored mitochondrial membrane potential and network morphology. RIPK1/RIPK3 blockade may also directly attenuate PGAM5-dependent mitophagy coordinated by the RIPK3/PGAM5/FUNDC1 or RIPK3/PGAM5/PINK1/Parkin axis (refer to discussion in the section above), however provided that PGAM5-dependent mitophagy is cytoprotective/mitoprotective^{472, 488}, this hypothesis would be in discord with the improved mitochondrial function and morphology observed herein with RIPK1/RIPK3 inhibition. Further still, RIPK3 could target alternative mitochondrial substrates or intermediates, or even directly interact with mitophagy receptors to influence the rate of mitophagy during I/R injury, and the possibility of which will need to be investigated in future studies. Nonetheless, even with the precise molecular mechanisms linking RIPK1/RIPK3 activity to mitophagy remaining elusive, RIPK1/RIPK3 inhibition still represents a promising therapeutic strategy for I/R brain injury such as HIE.

With respect to MLKL, we demonstrated that MLKL knockdown resulted in an almost complete recovery of mitochondrial membrane potential and restoration of mitochondrial tubular networks following OGD/Rep challenge, with MLKL silencing appeared to have achieved greater mitochondrial protection than pharmacological inhibition of RIPK1/RIPK3. Our findings would suggest MLKL as a negative regulator of mitophagy during mitophagy, and the restoration of mitochondrial function and morphology following MLKL knockdown align with the concept that mitophagy is neuroprotective as proposed by others. Therefore, as with RIPK1/RIPK3 inhibition, we consider MLKL inhibition and MLKL-targeted strategy as promising candidates for therapeutic development for HIE and other I/R brain injury alike, to achieve simultaneous blockade of necroptotic cell death and improvement of mitochondrial function.

To our knowledge, we are the first study group to specifically investigate the mechanistic link between MLKL and mitophagy, and contrary to our expectation, enhancement to I/R-induced mitophagy from MLKL silencing was in stark contrast to and was uncoupled from the suppression of mitophagy from RIPK1/RIPK3 inhibition. Other than translocation to and perforation of the plasma membrane, studies have rarely looked into the implication and consequence of MLKL translocation into intracellular membrane fractions. MLKL-containing necrosome was shown to localise to mitochondrial membrane³¹² the lysosomal/autolysosomal membrane⁴⁹⁷ during necroptosis. Using artificial liposomes with lipid compositions resembling the mitochondrion, Wang et al. demonstrated that phosphor-activated, oligomerised MLKL could perforate the “mitochondrion-like” liposomes³¹², which would suggest MLKL-dependent mitochondrial damage during necroptosis to upregulate mitochondrial quality control mechanism, such as mitophagy. According to study by Frank et al., activated, translocated MLKL may also disrupt the lysosomal/autolysosomal membranes, dissipate the interior acidic pH environment within and eventually impair macro-autophagy, and increased LC3B-II clearance from MLKL silencing was regarded as restoration of autophagic flux and autolysosomal function⁴⁹⁷. In view of the findings by Frank et al., it is possible that MLKL activation during OGD/Rep (I/R) studied here could also jeopardise lysosomal/autolysosomal function to indirectly attenuate mitophagy and oppose the pro-mitophagy activity of RIPK1/RIPK3, and MLKL knockdown therefore removes such brake to accelerate mitophagy. However, it should be noted that macro-autophagy and mitophagy are interlinked yet distinct cellular processes, and the TNF- α /SMAC mimetic/caspase inhibitor combination used by Frank et al. and OGD plus reperfusion employed herein are different triggers for necroptosis. Moreover, the increase of mitochondrial LC3A/B-II from MLKL knockdown shown here is accompanied by mitochondrial and whole-cell upregulation of mitophagy mediators Parkin and BNIP3, to indicate that potentiation of mitophagy from MLKL silencing does not merely result from preservation of lysosomal/autolysosomal function. Moreover, the observation that increased mitochondrial LC3A/B-II conversion in MLKL-KO, OGD/Rep-challenged cells could be further elevated by the lysosomal inhibitor BafA1 (**Fig. 4-8I**) further supports that MLKL activity does not, at least not entirely, obstruct mitophagy through disruption of lysosomal function, but rather antagonises mitophagy through alternative mechanisms. On this point, our preliminary Co-IP experiment revealing a potential interaction between MLKL and BNIP3 (**Fig. 4-10B**) suggests that MLKL could directly bind to and inhibit BNIP3 as a novel regulatory mechanism for mitophagy during I/R injury, and more rigorous Co-IP and protein-protein interaction studies will be needed in the future to substantiate this hypothesis.

5.3.4 Targeting Necroptosis and Mitophagy for Treating I/R Brain Injury

Previous studies have separately demonstrated that inhibition of necroptosis^{317, 323, 324} or enhancement of mitophagy^{385, 387, 388} significantly reduced brain infarct following brain I/R injury, as detailed in Chapter one, sections E.2.1 and F.3.4. For neonatal mice, intracerebrovascular delivery of RIPK1 inhibitor Nec-1s immediately after cerebral hypoxia-ischaemia also resulted in improvement of mitochondrial function with reduced oxidative stress and restored ATP production. The present study concurred that RIPK1 inhibition is mito-protective and neuroprotective, as we demonstrated that Nec-1s treatment suppressed necroptosis, restored mitochondrial membrane potential and mitochondrial morphology *in vitro*, and was associated with long-term improvement in motor function in postnatal day 28 to 30 mice. Inhibiting RIPK1 activity by Nec-1s suppressed Parkin- and BNIP3-mediated mitophagy along with downregulated expression of Parkin and BNIP3, which may reflect improved mitochondrial health with reduced need for mitochondrial clearance, or alternatively from inhibition of the pro-mitophagy RIPK3/PGAM5 pathway, as discussed in section 8.3.2. On the other hand, elevated mitophagy and upregulated expression of Parkin and BNIP3 from MLKL knockdown experiment also predicted mitochondrial recovery *in vitro*, which was in accord with studies showing interventions that stimulated mitophagy^{387, 388} (e.g. acidic post-conditioning, endoplasmic reticulum stress, PARK2 overexpression) resulted in decreased lesion size and improved neurological score in adult mice subjected to transient middle cerebral artery occlusion (tMCAO). To date, studies have yet to examine the therapeutic potential from inducing mitophagy in I/R injury of the immature brain, and moving forward from the present study, *in vivo* MLKL knockdown/inhibition can be performed in the current murine model of HIE with the intention to recapitulate the pro-mitophagy and mito-protective effects observed *in vitro*. In conclusion, our study corroborates RIPK1/3 inhibition and MLKL silencing as promising neuroprotective strategies with simultaneous necroptosis inhibition and mitochondrial protection during I/R brain injury as exemplified by HIE, and justifies development for more efficacious and metabolically more stable inhibitors of RIPK1, RIPK3 or MLKL that are suitable for peripheral administration and with extended therapeutic window.

5.4 Limitations and Future Works

As discussed in Chapter Five, Sections 5.2 and 5.3, the current study offers valuable insights into the molecular crosstalk between necroptosis and mitophagy, and at same time exposes important knowledge gaps and raises new research questions that need addressing. Our study is not without limitations. In this section, we would like to discuss the limitations and potential future experiments that can be conducted to make the current study design more rigorous and to advance the mechanistic elucidation of necroptosis-mitophagy axis.

5.4.1 Main limitations

A major limitation of the study design is we did not compare and contrast the susceptibilities of different cell types or subtypes from cerebral hypoxia-ischaemia in the context of HIE. Nonetheless, we anticipate a potential difference in cell type/subtype-specific susceptibilities to various cell death modalities, and we acknowledge that deepened understanding of the differential susceptibilities will enable optimised and selective targeting of cell death pathways within the vulnerable cell populations. As discussed in Introduction, section D.4, neurons and oligodendrocyte lineage have been shown to be the most susceptible to H/I and reperfusion injury in the immature and the adult brains, with documented susceptibilities across different neuronal subtypes including GABAergic, dopaminergic, cholinergic and the neural stem cells in the striatum and subventricular zone. Future study will investigate the cell type/subtype-specific activation of necroptosis and mitophagy using the in vitro and in vivo HIE models described herein (please refer to section 5.4.2 for study details).

With respect to experiments, we admit that antibody specificity should be further validated in the present study, in particular regarding experiments using the rabbit anti-mouse phosphor-MLKL Ser³⁴⁵ antibody (37335, Cell Signaling Technology), which generated unspecific, confounding western blot bands within a range of band sizes, as seen from Fig. 3-4 to Fig. 3-16. One option to validate the antibody is to include a positive control known to stimulate necroptosis in mouse cells and record the corresponding band size, for example the necroptosis-inducing cocktail comprising of TNF- α , SMAC mimetic and pan-caspase inhibitor^{293, 296, 308, 311, 312}, before confirming the phos-MLKL band in the subsequent experiments. On a different note but also relating to antibody-based detection, quantification of immunofluorescence intensities from cell culture and mouse brain sections should eliminate confounding signals from background fluorescence, to allow normalisation and improve batch-to-batch comparison between stainings/ experiments. Substrating background fluorescence can be particularly critical for intensity quantification of brain sections due to high or variable tissue autofluorescence. Inclusion of primary antibody- or secondary antibody-only controls will allow us to better discern “genuine” stainings from background signals.

Lastly, due to time limitation, we were unable to conduct comprehensive studies on mitochondrial morphology and functions, as well as long-term behavioural and cognitive assessments on H/I-mice to correlate hippocampal lesion and functional deficits. In the following sections, we will detail the *in vitro* and *in vivo* experiments that could be performed in the future to complement the available datasets and to address the listed limitations.

5.4.2 *In vitro* experiments

Exploring differential activation of regulated cell death pathways between cell types and subtypes

Provided that susceptibility to necroptosis and related pathways including mitophagy could differ by neuronal subtypes, primary neuronal cultures obtained from foetal mouse cortex/hippocampus can be co-labelled with neuron subtype-specific markers together with necroptosis (phos-MLKL, phos-RIPK3) or apoptosis markers (cleaved caspase-3), to uncover subtype-specific sensitivity towards a particular regulated cell death (RCD) modality following the oxygen-glucose deprivation and reperfusion challenge described herein. Examples of neuron subtype-specific markers are shown below for a primitive, generalised classification of cortical and hippocampal neurons as projecting neurons or interneurons, and within interneurons common sub-populations can be further distinguished by neuropeptide expressions; the table by no means intends to reflect the complete molecular signatures that distinguishes each and every neuronal sub-population within the cortex and hippocampus. Moreover, purified oligodendrocyte precursor cells (OPC) can be cultured *in vitro* and differentiated into mature oligodendrocytes (OGs) to study OG/OPC-specific vulnerability towards the various RCD⁴⁹⁸.

Projecting neurons	Interneurons			Neural stem cells	
Glutamatergic	GABAergic (GAD67, GAT1)			V-SVZ	SGZ
vGLUT1, vGLUT2	PV	SST	Htr3A	Sox2, Nestin	

GAD67, glutamate decarboxylase 67kDa isoform; GAT1, GABA transporter 1; Htr3A, serotonin receptor 3A; PV, parvalbumin; SGZ, subgranular zone; Sox2, (sex determining region Y)-box transcription factor 2; SST, somatostatin; vGLUT1/vGLUT2, vesicular glutamate transporters 1/2; V-SVZ, ventricular subventricular zone

MLKL inactivation and inhibition studies

As explained in Chapter Four, Section 4.4, complete silencing of MLKL expression may exert different effect on mitophagy and mitochondrial quality control than having MLKL in an inactive state. Although deficiency and inhibition of MLKL would both prevent necroptosis and necrosome translocation into mitochondrial fraction, the inhibited/inactive MLKL may still be capable of interacting with mitophagy mediators and mitophagy-related factors in the cytosol to exert an additional layer of control over mitophagy.

The MLKL-selective inhibitor necrosulfonamide (NSA) was shown prevent MLKL oligomerisation and translocation to membrane fractions by targeting the cysteine 86 residue on human MLKL, whereas the murine MLKL contains a tryptophan residue in place of cysteine to render the murine MLKL untargetable by NSA^{296, 448}. Therefore, in a parallel study, human neuroblastoma cells SY-5-SHY could be treated with MLKL inhibitor NSA and its effect on OGD/Rep-induced mitophagy can be compared with Neuro-2a cells with MLKL knockdown.

As an alternative, it is also desirable to generate Neuro-2a cell line stably expressing mutant MLKL that cannot be phosphorylated and activated by RIPK3, and therefore unable to oligomerise and translocate to lipid membranes. In this regard, endogenous MLKL expression will need to be silenced by short-hairpin RNA (shRNA-MLKL) before reconstitution of a phosphor-dead MLKL mutant, for example double point mutations at T357A/S358A³¹². Neuro-2a cells with phosphor-dead MLKL mutant will then be subjected to OGD/Rep to investigate the effect of MLKL inactivation on mitophagy.

Mechanistic study using mouse primary neurons

In view of the scarcity of mouse primary neurons and the difficulties associated with their long-term maintenance *in vitro* (>7 days) and efficient, stable transfection for gene knockdown study, our studies have primarily utilised the neuronal-like Neuro-2a cells for mechanistic studies. Notwithstanding, we acknowledge that cell lines and primary neurons are fundamentally different, exemplified by their highly variable susceptibilities towards OGD/Rep (I/R) challenge, such that findings and conclusions derived from Neuro-2a cells may not be completely applicable to primary cortical and hippocampal neurons. In future studies, it will be imperative to optimise the culture protocols for mouse primary neurons for obtainment of an ample supply of cells for an extended period of time, to allow for sufficient extraction of mitochondrial fraction as well as a suitably lengthy timeframe for transgenic manipulation. Moreover, the vulnerability of mouse primary cortical and hippocampal neurons towards OGD/Rep injury requires careful titrations of the severity of OGD and the respective durations of OGD and reperfusion, in order to avoid excessive cell death while still allowing for adequate activation of necroptosis and mitophagy for subsequent analysis. Further still, unlike Neuro-2a cells, post-mitotic, non-proliferative primary cortical and hippocampal neurons will be more difficult to transfect, and optimisation to the lipofection method (e.g. liposomes encapsulating RNA) is necessary for efficient delivery and expression of the RNAi construct(s)⁴⁹⁹. To this end, commercially available lipofection-based technologies such as Lipofectamine™ RNAiMAX reagent (13778030, Invitrogen) and FuGENE® SI Transfection Reagent (SI-1000, FuGENE; Middleton, WI, USA) may be employed with suitable modifications to transfect mouse primary neurons with small interfering RNAs (e.g. siRNAs targeting MLKL or ClpP) to study the necroptosis-mitophagy crosstalk during I/R injury.

Co-immunoprecipitation study with tagged proteins

To enhance the efficiency of protein enrichment and immunoprecipitation, and to improve detection accuracy of potential protein-protein interaction, the protein of interest can be fused with an affinity tag, which is a small, linear peptide sequence that can be attached at the N- or the C-terminal of the protein⁵⁰⁰ without affecting the folding and properties of the protein of interest. For example, FLAG (DYKDDDD, 1kDa), haemagglutinin antigen (YPYDVPDYA, HA, 1.1kDa) and Myc (EQKLISEEDL, 1.2kDa) represent the most widely used affinity tags to be incorporated into target protein for immunoprecipitation and co-immunoprecipitation applications⁵⁰⁰. The tagged protein can be recognised and enriched by antibody highly specific for the affinity tag (e.g. anti-FLAG antibody)⁵⁰¹, and the pull-down/enrichment efficiency of tagged protein by anti-tag antibody can be significantly superior than enriching the naked protein with a standard antibody. In order to generate Neuro-2a cell line that stably expresses the MLKL-FLAG fusion protein, the full length MLKL coding sequence is inserted into an expression plasmid containing the FLAG-tag sequence followed by cell transfection and selection. In this regard, the FLAG-tagged MLKL and any protein complex it forms can be immunoprecipitated with anti-FLAG antibody, and potential interaction with mitophagy mediators and mitophagy-related proteins (e.g. PGAM5) can be interrogated.

Visualisation of mitophagy and additional mitochondrial morphology and function studies

In the present study, mitophagy was visualised by co-localisation between mitochondrial proteins (TOM20, COXIV) and autophagosome marker LC3A/B *in vitro* and *in vivo*, and the co-localised signals were semi-quantified as a measure of mitophagy rate. However, immunofluorescence staining requires sample fixation for antibody binding and only captures a “snapshot” of a dynamic cellular process, and it will undoubtedly be more informative to be able to track mitophagy overtime. In this regard, we propose double labelling live cells with fluorescent chemical probes that respectively target the mitochondria and the lysosomes, for example with MitoTracker™ and LysoTracker™, and co-localisation of probes would indicate delivery of mitochondrial fraction into the lysosomal compartment for degradation, i.e. mitophagy, as described in Chapter One, Section F.3.3. In future studies, we plan to perform live cell imaging with MitoTracker and LysoTracker on Neuro-2a cells and primary neuronal cells to visualise and semi-quantify the dynamic process of mitophagy overtime following OGD/Rep and necroptosis inhibition. Moreover, cells could be examined under transmission electron microscope (TEM) to directly visualise and track the progress of mitophagy at subcellular level under different challenge/treatment conditions. Mitochondrial morphology visualised by MitoTracker dye can be further analysed to “semi-quantify” connectivity and elongation of mitochondrial networks to inform mitochondrial health and function⁵⁰².

In this study, JC-10 mitochondrial membrane potential assay was used to measure mitochondrial function and mitochondrial depolarisation seen as increased intensity of green JC-10 monomer is indicative of

mitochondrial dysfunction. Additional mitochondrial assays can be performed to complement our JC-10 data and to supplement our study with more mechanistic insights. For example, quantification of mitochondrial ROS level is commonly used by many to assess the overall mitochondrial health, and changes to mitochondrial ROS following necroptosis inhibition will enable us to better verify the role of ROS during the necroptosis-mitophagy crosstalk. With ROS quantification assay, ROS scavenger (butylated hydroxyanisole BHA)⁴⁰⁵ or mitochondrial anti-oxidant (mitoquinone, MitoQ)⁴⁰⁹ can be included to delineate whether mitochondrial ROS relays RIPK1/RIPK3 activation to indirectly stimulate mitophagy. Moreover, ATP quantification assay can also be performed to enable dual detection of cell death/injury and mitochondrial dysfunction. Fluctuations to mitochondrial ATP synthesis can be used to approximate cell viability and to reflect respiratory chain efficiency following OGD/Rep and necroptosis inhibition.

5.4.3 *In vivo* experiments

Identifying cell type and region-specific susceptibility to regulated cell death pathways

As discussed in section 5.4.1 Main limitations and corresponding to the *in vitro* aspect of the study, cell type/subtype-specific activation of necroptosis and apoptosis, amongst other modes of RCD, can be visualised within the neonatal mouse brain following cerebral H/I and reperfusion using markers specified above. Co-labelling brain sections also allows for comparison of the extent of RCD activities between the different brain regions prone to H/I challenge, including but not limited to the neocortex, hippocampus, striatum and thalamus. Moreover, from a neurodevelopment perspective, investigating RCD and related pathways in the neurogenic and gliogenic niches (V-SVZ, SGZ) will shed light on how early-life H/I challenge impact long-term neurogenesis, gliogenesis and neurofunctional development.

MLKL-knockout or MLKL-knockdown mice

To recapitulate our *in vitro* findings from MLKL-knockdown in murine model of HIE, and to complement our *in vivo* findings with the RIPK1 inhibitor Nec-1s, homozygous MLKL knockout C57BL/6 mice can be generated to establish whether MLKL silencing is neuroprotective in neonatal ischaemia-reperfusion brain injury. Using wildtype and Nec-1s treated cohorts as comparisons, survival, brain infarct size and long-term functional outcomes will be determined in the MLKL^{-/-} mice following neonatal exposure to cerebral hypoxia-ischaemia, along with biochemical analysis on mitophagy and mitochondrial function. In view of our *in vitro* findings, we hypothesise that heightened mitophagy is correlated with preserved tissue volume, restored mitochondrial function and improved functional outcome in a MLKL deficient background, and demonstration of such correlation would justify the therapeutic development of MLKL-targeted strategy as novel treatment for I/R brain injury. MLKL^{-/-} mice have been successfully generated and are viable without exhibiting immunological and developmental abnormalities compared to WT mice⁵⁰³. Animal studies have revealed that MLKL

deficiency is protective in kidney ischaemia-reperfusion injury⁵⁰⁴, liver fibrosis⁵⁰⁵, cerulean-induced acute pancreatitis⁵⁰³ and intracerebral haemorrhage⁵⁰⁶, corresponding to an increased resistance in MLKL^{-/-} cells towards cytotoxicity from necroptosis-inducing cocktail treatment (TNF- α /SMAC mimetic/pan-caspase inhibitor)⁵⁰³. Conditional knockout of MLKL within specific neuronal subtypes can also be employed, for example MLKL deletion within the neural stem cells, to assess the long-term neuroprotective and neurodevelopmental outcomes when I/R-induced necroptosis is inhibited only within the neurogenic/gliogenic niches. In this regard, MLKL-floxed mice can be crossed with tamoxifen-inducible Cre mice under a neural stem cell-specific promoter, such as a Nestin⁵⁰⁷ or a glioma-associated oncogene homolog 1 (Gli1)⁵⁰⁸ promoter, to enable neural stem cell-specific recombination and MLKL silencing.

Alternatively, *in vivo* knockdown of MLKL confined to the injured hemisphere can be achieved by short-hairpin RNA (shRNA) specific towards the mouse MLKL transcripts. shRNA contains RNA sequence that are complementary to endogenous mRNA and an internal hybridisation region for folding into a “hairpin” structure. Unlike small interfering RNA (siRNA) that is delivered as “naked” RNA double strands for cell transfection and temporary expression silencing, shRNA is packaged into a viral vector (adeno-associated virus or lentivirus), and integration of vector containing the shRNA sequence into the genomic DNA will enable long-term, stable expression of shRNA and therefore knockdown of the target mRNA⁵⁰⁹. For this reason, shRNA maybe preferred over siRNA for *in vivo* knockdown study. Once expressed in the cell, shRNA is firstly processed by the RISC into siRNA and subsequently targets the complementary mRNA for RISC-dependent degradation⁴²¹, as detailed in Chapter Two, section 2.5. As future study, it will be feasible to perform intracerebrovascular (i.c.v.) injection of MLKL-shRNA viral vector into the ipsilateral hemisphere, to selectively knockdown MLKL expression prior to cerebral H/I exposure and to determine potential neuroprotective as described above.

Additional behavioural studies for improved anatomical and functional correlations

In light of the hippocampal lesions observed in our murine model of HIE, future study will place more emphasis on hippocampus-dependent functional parameters, which maybe more sensitive to cerebral H/I insult and more amenable to neuroprotection from necroptosis inhibition/mitophagy induction. Hippocampus belongs to the limbic system and plays a crucial role in cognition, memory and learning through extensive, direct interconnections with the cortex and amygdala. As explained in Chapter One, section C.1.3.2, spatial memory and learning in rodents, commonly assessed by Morris water maze, is highly dependent on hippocampal integrity and susceptible to neonatal H/I injury, whereby rodent with hippocampal lesion will exhibit increased escape latency and difficulty in locating the escape platform^{103, 510, 511}. Fear conditioning is a behavioural paradigm that assesses the collective functioning of hippocampus and basolateral amygdala,

whereby the rodent needs to learn and associate a neutral conditioned stimulus (e.g. sound, light) with an unconditioned aversive stimulus (e.g. foot shock), such that exposure to the neutral stimulus/cue will elicit fear that can be measured as freezing behaviour in rodents^{105, 106}. Studies have shown that neonatal cerebral H/I impaired long-term memory and associative learning, seen as reduced freezing behaviour to the conditioned stimulus^{109, 111}. As reliable measures of hippocampal-dependent functions, future animal study will employ Morris water maze and fear conditioning to comprehensively evaluate long-term neuroprotection from necroptosis inhibition following neonatal H/I exposure.

Moreover, as explained in Chapter Seven, Section 7.4.2, the perceived deficits in motor movement and spontaneous locomotion following neonatal HIE injury maybe attributable to lesions in the motor cortex and striatum amongst other susceptible brain regions that may also be responsive to neuroprotection by Nec-1s. Motor coordination and exploratory activity could also be further assessed in injured and treated mice by using CatWalk gait analysis and novel object recognition test. Therefore, additional histopathological study and immunofluorescence staining of the motor-related anatomical regions (motor cortex, striatum), along with more diverse assessments of motor function, will allow us to better establish the anatomy-function correlation in the context of necroptosis and mitophagy during HIE and to better evaluate the therapeutic potential of necroptosis inhibitor against HIE.

5.5 Conclusions

In conclusion, the present study highlights necroptosis as a potential upstream regulator of mitophagy during ischaemia-reperfusion (I/R) brain injury and implicates the necroptosis-mitophagy signalling axis in hypoxic-ischaemic encephalopathy (HIE) of the immature brain. To our knowledge, our study is the first to examine the respective contribution of different necrosome components on mitophagy in neurons and neuron-like cells during I/R challenge. Our findings revealed the unexpected, opposing effects of receptor-interacting protein kinase 1 and 3 (RIPK1/RIPK3) and the pseudokinase mixed lineage kinase-like (MLKL) on mitochondrial clearance, whereby RIPK1/RIPK3 kinase activity promotes mitophagy and MLKL likely inhibits I/R-induced mitophagy. Notwithstanding their opposing effects on mitophagy, either pharmacological inhibition of RIPK1/RIPK3 or genetic silencing of MLKL improved mitochondrial function and mitochondrial morphology, and additionally RIPK1 inhibition maybe associated with long-term improvement to motor functions post-HIE. Our study underscores necroptosis and associated mitophagy as promising molecular targets for therapeutic development for HIE and I/R brain injuries alike. Mitochondrial translocation of necrosome maybe conducive for necroptosis regulation over mitophagy during I/R, and futures studies are warranted to uncover the precise mechanisms underpinning the necroptosis-mitophagy axis.

REFERENCES

- 1 Kurinczuk JJ, White-Koning M, Badawi N. Epidemiology of neonatal encephalopathy and hypoxic-ischaemic encephalopathy. *Early Hum Dev* 2010; **86**: 329-38
- 2 Douglas-Escobar M, Weiss MD. Hypoxic-ischemic encephalopathy: a review for the clinician. *JAMA Pediatr* 2015; **169**: 397-403
- 3 Ferriero DM. Neonatal brain injury. *N Engl J Med* 2004; **351**: 1985-95
- 4 Lawn JE, Cousens S, Zupan J, Lancet Neonatal Survival Steering T. 4 million neonatal deaths: when? Where? Why? *Lancet* 2005; **365**: 891-900
- 5 Black RE, Cousens S, Johnson HL, et al. Global, regional, and national causes of child mortality in 2008: a systematic analysis. *Lancet* 2010; **375**: 1969-87
- 6 Finder M, Boylan GB, Twomey D, Ahearne C, Murray DM, Hallberg B. Two-Year Neurodevelopmental Outcomes After Mild Hypoxic Ischemic Encephalopathy in the Era of Therapeutic Hypothermia. *JAMA Pediatr* 2020; **174**: 48-55
- 7 Dammann O, Ferriero D, Gressens P. Neonatal encephalopathy or hypoxic-ischemic encephalopathy? Appropriate terminology matters. *Pediatr Res* 2011; **70**: 1-2
- 8 Volpe JJ. Neonatal encephalopathy: an inadequate term for hypoxic-ischemic encephalopathy. *Ann Neurol* 2012; **72**: 156-66
- 9 Badawi N, Kurinczuk JJ, Keogh JM, et al. Antepartum risk factors for newborn encephalopathy: the Western Australian case-control study. *BMJ* 1998; **317**: 1549-53
- 10 Badawi N, Kurinczuk JJ, Keogh JM, et al. Intrapartum risk factors for newborn encephalopathy: the Western Australian case-control study. *BMJ* 1998; **317**: 1554-8
- 11 Nelson KB, Bingham P, Edwards EM, et al. Antecedents of neonatal encephalopathy in the Vermont Oxford Network Encephalopathy Registry. *Pediatrics* 2012; **130**: 878-86
- 12 Molloy EJ, Bearer C. Neonatal encephalopathy versus Hypoxic-Ischemic Encephalopathy. *Pediatr Res* 2018; **84**: 574
- 13 Nelson KB. Is it HIE? And why that matters. *Acta Paediatr* 2007; **96**: 1113-4
- 14 Ellis M, Manandhar N, Manandhar DS, Costello AM. Risk factors for neonatal encephalopathy in Kathmandu, Nepal, a developing country: unmatched case-control study. *BMJ* 2000; **320**: 1229-36
- 15 Cowan F, Rutherford M, Groenendaal F, et al. Origin and timing of brain lesions in term infants with neonatal encephalopathy. *Lancet* 2003; **361**: 736-42
- 16 Johnston MV. MRI for neonatal encephalopathy in full-term infants. *Lancet* 2003; **361**: 713-4
- 17 Huang BY, Castillo M. Hypoxic-ischemic brain injury: imaging findings from birth to adulthood. *Radiographics* 2008; **28**: 417-39; quiz 617
- 18 de Vries LS, Groenendaal F. Patterns of neonatal hypoxic-ischaemic brain injury. *Neuroradiology* 2010; **52**: 555-66
- 19 Barkovich AJ, Westmark K, Partridge C, Sola A, Ferriero DM. Perinatal asphyxia: MR findings in the first 10 days. *AJNR Am J Neuroradiol* 1995; **16**: 427-38

- 20 Heinz ER, Provenzale JM. Imaging findings in neonatal hypoxia: a practical review. *AJR Am J Roentgenol* 2009; **192**: 41-7
- 21 Guidelines QC. Queensland Clinical Guidelines: Hypoxic ischaemia encephalopathy (HIE). 2021. Available from https://www.health.qld.gov.au/_data/assets/pdf_file/0014/140162/g-hie.pdf 2022)
- 22 Blennow M, Savman K, Ilves P, Thoresen M, Rosengren L. Brain-specific proteins in the cerebrospinal fluid of severely asphyxiated newborn infants. *Acta Paediatr* 2001; **90**: 1171-5
- 23 Sun J, Li J, Cheng G, Sha B, Zhou W. Effects of hypothermia on NSE and S-100 protein levels in CSF in neonates following hypoxic/ischaemic brain damage. *Acta Paediatr* 2012; **101**: e316-20
- 24 Chalak L, Hellstrom-Westas L, Bonifacio S, et al. Bedside and laboratory neuromonitoring in neonatal encephalopathy. *Semin Fetal Neonatal Med* 2021; **26**: 101273
- 25 Toet MC, Lemmers PM, van Schelven LJ, van Bel F. Cerebral oxygenation and electrical activity after birth asphyxia: their relation to outcome. *Pediatrics* 2006; **117**: 333-9
- 26 Wintermark P, Hansen A, Warfield SK, Dukhovny D, Soul JS. Near-infrared spectroscopy versus magnetic resonance imaging to study brain perfusion in newborns with hypoxic-ischemic encephalopathy treated with hypothermia. *Neuroimage* 2014; **85 Pt 1**: 287-93
- 27 Lemmers PM, Zwanenburg RJ, Benders MJ, et al. Cerebral oxygenation and brain activity after perinatal asphyxia: does hypothermia change their prognostic value? *Pediatr Res* 2013; **74**: 180-5
- 28 Ancora G, Maranella E, Grandi S, et al. Early predictors of short term neurodevelopmental outcome in asphyxiated cooled infants. A combined brain amplitude integrated electroencephalography and near infrared spectroscopy study. *Brain Dev* 2013; **35**: 26-31
- 29 Giesinger RE, Levy PT, Ruoss JL, et al. Cardiovascular management following hypoxic-ischemic encephalopathy in North America: need for physiologic consideration. *Pediatr Res* 2021; **90**: 600-7
- 30 Pazandak C, McPherson C, Abubakar M, Zanelli S, Fairchild K, Vesoulis Z. Blood Pressure Profiles in Infants With Hypoxic Ischemic Encephalopathy (HIE), Response to Dopamine, and Association With Brain Injury. *Front Pediatr* 2020; **8**: 512
- 31 Basu P, Som S, Choudhuri N, Das H. Contribution of the blood glucose level in perinatal asphyxia. *Eur J Pediatr* 2009; **168**: 833-8
- 32 Salhab WA, Wyckoff MH, Laptook AR, Perlman JM. Initial hypoglycemia and neonatal brain injury in term infants with severe fetal acidemia. *Pediatrics* 2004; **114**: 361-6
- 33 Hagberg H, David Edwards A, Groenendaal F. Perinatal brain damage: The term infant. *Neurobiol Dis* 2016; **92**: 102-12
- 34 Azzopardi DV, Strohm B, Edwards AD, et al. Moderate hypothermia to treat perinatal asphyxial encephalopathy. *N Engl J Med* 2009; **361**: 1349-58
- 35 Gluckman PD, Wyatt JS, Azzopardi D, et al. Selective head cooling with mild systemic hypothermia after neonatal encephalopathy: multicentre randomised trial. *Lancet* 2005; **365**: 663-70
- 36 Jacobs SE. Selective head cooling with mild systemic hypothermia after neonatal encephalopathy: Multicentre randomised trial. *J Pediatr* 2005; **147**: 122-3

- 37 Tagin MA, Woolcott CG, Vincer MJ, Whyte RK, Stinson DA. Hypothermia for neonatal hypoxic ischemic encephalopathy: an updated systematic review and meta-analysis. *Arch Pediatr Adolesc Med* 2012; **166**: 558-66
- 38 Jacobs SE, Berg M, Hunt R, Tarnow-Mordi WO, Inder TE, Davis PG. Cooling for newborns with hypoxic ischaemic encephalopathy. *Cochrane Database Syst Rev* 2013: CD003311
- 39 Rutherford M, Ramenghi LA, Edwards AD, et al. Assessment of brain tissue injury after moderate hypothermia in neonates with hypoxic-ischaemic encephalopathy: a nested substudy of a randomised controlled trial. *Lancet Neurol* 2010; **9**: 39-45
- 40 Shankaran S, Pappas A, McDonald SA, et al. Childhood outcomes after hypothermia for neonatal encephalopathy. *N Engl J Med* 2012; **366**: 2085-92
- 41 Guillet R, Edwards AD, Thoresen M, et al. Seven- to eight-year follow-up of the CoolCap trial of head cooling for neonatal encephalopathy. *Pediatr Res* 2012; **71**: 205-9
- 42 Laptook AR, Shankaran S, Tyson JE, et al. Effect of Therapeutic Hypothermia Initiated After 6 Hours of Age on Death or Disability Among Newborns With Hypoxic-Ischemic Encephalopathy: A Randomized Clinical Trial. *JAMA* 2017; **318**: 1550-60
- 43 Shankaran S, Laptook AR, Pappas A, et al. Effect of Depth and Duration of Cooling on Death or Disability at Age 18 Months Among Neonates With Hypoxic-Ischemic Encephalopathy: A Randomized Clinical Trial. *JAMA* 2017; **318**: 57-67
- 44 Sanders RD, Franks NP, Maze M. Xenon: no stranger to anaesthesia. *Br J Anaesth* 2003; **91**: 709-17
- 45 Dingley J, Tooley J, Porter H, Thoresen M. Xenon provides short-term neuroprotection in neonatal rats when administered after hypoxia-ischemia. *Stroke* 2006; **37**: 501-6
- 46 Hobbs C, Thoresen M, Tucker A, Aquilina K, Chakkarapani E, Dingley J. Xenon and hypothermia combine additively, offering long-term functional and histopathologic neuroprotection after neonatal hypoxia/ischemia. *Stroke* 2008; **39**: 1307-13
- 47 Chakkarapani E, Dingley J, Liu X, et al. Xenon enhances hypothermic neuroprotection in asphyxiated newborn pigs. *Ann Neurol* 2010; **68**: 330-41
- 48 Ma D, Hossain M, Chow A, et al. Xenon and hypothermia combine to provide neuroprotection from neonatal asphyxia. *Ann Neurol* 2005; **58**: 182-93
- 49 Thoresen M, Hobbs CE, Wood T, Chakkarapani E, Dingley J. Cooling combined with immediate or delayed xenon inhalation provides equivalent long-term neuroprotection after neonatal hypoxia-ischemia. *J Cereb Blood Flow Metab* 2009; **29**: 707-14
- 50 Martin JL, Ma D, Hossain M, et al. Asynchronous administration of xenon and hypothermia significantly reduces brain infarction in the neonatal rat. *Br J Anaesth* 2007; **98**: 236-40
- 51 Faulkner S, Bainbridge A, Kato T, et al. Xenon augmented hypothermia reduces early lactate/N-acetylaspartate and cell death in perinatal asphyxia. *Ann Neurol* 2011; **70**: 133-50
- 52 Azzopardi D, Robertson NJ, Bainbridge A, et al. Moderate hypothermia within 6 h of birth plus inhaled xenon versus moderate hypothermia alone after birth asphyxia (TOBY-Xe): a proof-of-concept, open-label, randomised controlled trial. *Lancet Neurol* 2016; **15**: 145-53

- 53 Morishita E, Masuda S, Nagao M, Yasuda Y, Sasaki R. Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents in vitro glutamate-induced neuronal death. *Neuroscience* 1997; **76**: 105-16
- 54 Nagai A, Nakagawa E, Choi HB, Hatori K, Kobayashi S, Kim SU. Erythropoietin and erythropoietin receptors in human CNS neurons, astrocytes, microglia, and oligodendrocytes grown in culture. *J Neuropathol Exp Neurol* 2001; **60**: 386-92
- 55 Siren AL, Fratelli M, Brines M, et al. Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. *Proc Natl Acad Sci U S A* 2001; **98**: 4044-9
- 56 Sun Y, Calvert JW, Zhang JH. Neonatal hypoxia/ischemia is associated with decreased inflammatory mediators after erythropoietin administration. *Stroke* 2005; **36**: 1672-8
- 57 Shingo T, Sorokan ST, Shimazaki T, Weiss S. Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neural stem cells. *J Neurosci* 2001; **21**: 9733-43
- 58 Wang L, Zhang Z, Wang Y, Zhang R, Chopp M. Treatment of stroke with erythropoietin enhances neurogenesis and angiogenesis and improves neurological function in rats. *Stroke* 2004; **35**: 1732-7
- 59 Fan X, Heijnen CJ, van der KM, Groenendaal F, van Bel F. Beneficial effect of erythropoietin on sensorimotor function and white matter after hypoxia-ischemia in neonatal mice. *Pediatr Res* 2011; **69**: 56-61
- 60 Gonzalez FF, Abel R, Almli CR, Mu D, Wendland M, Ferriero DM. Erythropoietin sustains cognitive function and brain volume after neonatal stroke. *Dev Neurosci* 2009; **31**: 403-11
- 61 Wu YW, Mathur AM, Chang T, et al. High-Dose Erythropoietin and Hypothermia for Hypoxic-Ischemic Encephalopathy: A Phase II Trial. *Pediatrics* 2016; **137**
- 62 Juul SE, Comstock BA, Heagerty PJ, et al. High-Dose Erythropoietin for Asphyxia and Encephalopathy (HEAL): A Randomized Controlled Trial - Background, Aims, and Study Protocol. *Neonatology* 2018; **113**: 331-8
- 63 Dubocovich ML, Delagrange P, Krause DN, Sugden D, Cardinali DP, Olcese J. International Union of Basic and Clinical Pharmacology. LXXV. Nomenclature, classification, and pharmacology of G protein-coupled melatonin receptors. *Pharmacol Rev* 2010; **62**: 343-80
- 64 Tan DX, Manchester LC, Terron MP, Flores LJ, Reiter RJ. One molecule, many derivatives: a never-ending interaction of melatonin with reactive oxygen and nitrogen species? *J Pineal Res* 2007; **42**: 28-42
- 65 Carloni S, Perrone S, Buonocore G, Longini M, Proietti F, Balduini W. Melatonin protects from the long-term consequences of a neonatal hypoxic-ischemic brain injury in rats. *J Pineal Res* 2008; **44**: 157-64
- 66 Welin AK, Svedin P, Lapatto R, et al. Melatonin reduces inflammation and cell death in white matter in the mid-gestation fetal sheep following umbilical cord occlusion. *Pediatr Res* 2007; **61**: 153-8
- 67 Robertson NJ, Faulkner S, Fleiss B, et al. Melatonin augments hypothermic neuroprotection in a perinatal asphyxia model. *Brain* 2013; **136**: 90-105
- 68 Pang R, Avdic-Belltheus A, Meehan C, et al. Melatonin and/or erythropoietin combined with hypothermia in a piglet model of perinatal asphyxia. *Brain Commun* 2021; **3**: fcaa211
- 69 Hoehn M, Kustermann E, Blunk J, et al. Monitoring of implanted stem cell migration in vivo: a highly resolved in vivo magnetic resonance imaging investigation of experimental stroke in rat. *Proc Natl Acad Sci U S A* 2002; **99**: 16267-72

- 70 Hicks AU, Hewlett K, Windle V, et al. Enriched environment enhances transplanted subventricular zone stem cell migration and functional recovery after stroke. *Neuroscience* 2007; **146**: 31-40
- 71 Park KI, Hack MA, Ourednik J, et al. Acute injury directs the migration, proliferation, and differentiation of solid organ stem cells: evidence from the effect of hypoxia-ischemia in the CNS on clonal "reporter" neural stem cells. *Exp Neurol* 2006; **199**: 156-78
- 72 Park KI, Himes BT, Stieg PE, Tessler A, Fischer I, Snyder EY. Neural stem cells may be uniquely suited for combined gene therapy and cell replacement: Evidence from engraftment of Neurotrophin-3-expressing stem cells in hypoxic-ischemic brain injury. *Exp Neurol* 2006; **199**: 179-90
- 73 Obenaus A, Dilmac N, Tone B, et al. Long-term magnetic resonance imaging of stem cells in neonatal ischemic injury. *Ann Neurol* 2011; **69**: 282-91
- 74 Nabetani M, Shintaku H, Hamazaki T. Future perspectives of cell therapy for neonatal hypoxic-ischemic encephalopathy. *Pediatr Res* 2018; **83**: 356-63
- 75 Pimentel-Coelho PM, Rosado-de-Castro PH, da Fonseca LM, Mendez-Otero R. Umbilical cord blood mononuclear cell transplantation for neonatal hypoxic-ischemic encephalopathy. *Pediatr Res* 2012; **71**: 464-73
- 76 Pimentel-Coelho PM, Magalhaes ES, Lopes LM, deAzevedo LC, Santiago MF, Mendez-Otero R. Human cord blood transplantation in a neonatal rat model of hypoxic-ischemic brain damage: functional outcome related to neuroprotection in the striatum. *Stem Cells Dev* 2010; **19**: 351-8
- 77 Meier C, Middelani J, Wasielewski B, et al. Spastic paresis after perinatal brain damage in rats is reduced by human cord blood mononuclear cells. *Pediatr Res* 2006; **59**: 244-9
- 78 Lee OK, Kuo TK, Chen WM, Lee KD, Hsieh SL, Chen TH. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood* 2004; **103**: 1669-75
- 79 Cox CS, Jr., Baumgartner JE, Harting MT, et al. Autologous bone marrow mononuclear cell therapy for severe traumatic brain injury in children. *Neurosurgery* 2011; **68**: 588-600
- 80 Cotten CM, Murtha AP, Goldberg RN, et al. Feasibility of autologous cord blood cells for infants with hypoxic-ischemic encephalopathy. *J Pediatr* 2014; **164**: 973-9 e1
- 81 Wang X, Cheng H, Hua R, et al. Effects of bone marrow mesenchymal stromal cells on gross motor function measure scores of children with cerebral palsy: a preliminary clinical study. *Cytotherapy* 2013; **15**: 1549-62
- 82 Northington FJ. Brief update on animal models of hypoxic-ischemic encephalopathy and neonatal stroke. *ILAR J* 2006; **47**: 32-8
- 83 Semple BD, Blomgren K, Gimlin K, Ferriero DM, Noble-Haeusslein LJ. Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. *Prog Neurobiol* 2013; **106-107**: 1-16
- 84 Clancy B, Darlington RB, Finlay BL. Translating developmental time across mammalian species. *Neuroscience* 2001; **105**: 7-17
- 85 Clancy B, Kersh B, Hyde J, Darlington RB, Anand KJ, Finlay BL. Web-based method for translating neurodevelopment from laboratory species to humans. *Neuroinformatics* 2007; **5**: 79-94
- 86 Roohey T, Raju TN, Moustogiannis AN. Animal models for the study of perinatal hypoxic-ischemic encephalopathy: a critical analysis. *Early Hum Dev* 1997; **47**: 115-46

87 Mallard C, Vexler ZS. Modeling Ischemia in the Immature Brain: How Translational Are Animal Models? *Stroke* 2015; **46**: 3006-11

88 Rice JE, 3rd, Vannucci RC, Brierley JB. The influence of immaturity on hypoxic-ischemic brain damage in the rat. *Ann Neurol* 1981; **9**: 131-41

89 Vannucci SJ, Back SA. The Vannucci Model of Hypoxic-Ischemic Injury in the Neonatal Rodent: 40 years later. *Dev Neurosci* 2022

90 Vannucci RC, Lyons DT, Vasta F. Regional cerebral blood flow during hypoxia-ischemia in immature rats. *Stroke* 1988; **19**: 245-50

91 Palmer C, Brucklacher RM, Christensen MA, Vannucci RC. Carbohydrate and energy metabolism during the evolution of hypoxic-ischemic brain damage in the immature rat. *J Cereb Blood Flow Metab* 1990; **10**: 227-35

92 Ferriero DM, Holtzman DM, Black SM, Sheldon RA. Neonatal mice lacking neuronal nitric oxide synthase are less vulnerable to hypoxic-ischemic injury. *Neurobiol Dis* 1996; **3**: 64-71

93 Graham EM, Sheldon RA, Flock DL, et al. Neonatal mice lacking functional Fas death receptors are resistant to hypoxic-ischemic brain injury. *Neurobiol Dis* 2004; **17**: 89-98

94 Hagberg H, Wilson MA, Matsushita H, et al. PARP-1 gene disruption in mice preferentially protects males from perinatal brain injury. *J Neurochem* 2004; **90**: 1068-75

95 Knox R, Zhao C, Miguel-Perez D, et al. Enhanced NMDA receptor tyrosine phosphorylation and increased brain injury following neonatal hypoxia-ischemia in mice with neuronal Fyn overexpression. *Neurobiol Dis* 2013; **51**: 113-9

96 Sheldon RA, Sedik C, Ferriero DM. Strain-related brain injury in neonatal mice subjected to hypoxia-ischemia. *Brain Res* 1998; **810**: 114-22

97 Barone FC, Knudsen DJ, Nelson AH, Feuerstein GZ, Willette RN. Mouse strain differences in susceptibility to cerebral ischemia are related to cerebral vascular anatomy. *J Cereb Blood Flow Metab* 1993; **13**: 683-92

98 Rumajogee P, Bregman T, Miller SP, Yager JY, Fehlings MG. Rodent Hypoxia-Ischemia Models for Cerebral Palsy Research: A Systematic Review. *Front Neurol* 2016; **7**: 57

99 Diaz J, Abiola S, Kim N, et al. Therapeutic Hypothermia Provides Variable Protection against Behavioral Deficits after Neonatal Hypoxia-Ischemia: A Potential Role for Brain-Derived Neurotrophic Factor. *Dev Neurosci* 2017; **39**: 257-72

100 Borjini N, Sivilia S, Giuliani A, et al. Potential biomarkers for neuroinflammation and neurodegeneration at short and long term after neonatal hypoxic-ischemic insult in rat. *J Neuroinflammation* 2019; **16**: 194

101 Moran J, Stokowska A, Walker FR, Mallard C, Hagberg H, Pekna M. Intranasal C3a treatment ameliorates cognitive impairment in a mouse model of neonatal hypoxic-ischemic brain injury. *Exp Neurol* 2017; **290**: 74-84

102 Vorhees CV, Williams MT. Morris water maze: procedures for assessing spatial and related forms of learning and memory. *Nat Protoc* 2006; **1**: 848-58

103 Huang Z, Liu J, Cheung PY, Chen C. Long-term cognitive impairment and myelination deficiency in a rat model of perinatal hypoxic-ischemic brain injury. *Brain Res* 2009; **1301**: 100-9

- 104 Smith AL, Alexander M, Rosenkrantz TS, Sadek ML, Fitch RH. Sex differences in behavioral outcome following neonatal hypoxia ischemia: insights from a clinical meta-analysis and a rodent model of induced hypoxic ischemic brain injury. *Exp Neurol* 2014; **254**: 54-67
- 105 Curzon P, Rustay NR, Browman KE. Cued and Contextual Fear Conditioning for Rodents. In: nd, Buccafusco JJ, eds. *Methods of Behavior Analysis in Neuroscience*. Boca Raton (FL), 2009
- 106 Lugo JN, Smith GD, Holley AJ. Trace fear conditioning in mice. *J Vis Exp* 2014
- 107 Sun Y, Gooch H, Sah P. Fear conditioning and the basolateral amygdala. *F1000Res* 2020; **9**
- 108 Chowdhury N, Quinn JJ, Fanselow MS. Dorsal hippocampus involvement in trace fear conditioning with long, but not short, trace intervals in mice. *Behav Neurosci* 2005; **119**: 1396-402
- 109 Wang X, Han W, Du X, et al. Neuroprotective effect of Bax-inhibiting peptide on neonatal brain injury. *Stroke* 2010; **41**: 2050-5
- 110 Jarlestedt K, Atkins AL, Hagberg H, Pekna M, Mallard C. Trace fear conditioning detects hypoxic-ischemic brain injury in neonatal mice. *Dev Neurosci* 2011; **33**: 222-30
- 111 Jarlestedt K, Rousset CI, Stahlberg A, et al. Receptor for complement peptide C3a: a therapeutic target for neonatal hypoxic-ischemic brain injury. *FASEB J* 2013; **27**: 3797-804
- 112 Reinboth BS, Koster C, Abberger H, et al. Endogenous hypothermic response to hypoxia reduces brain injury: Implications for modeling hypoxic-ischemic encephalopathy and therapeutic hypothermia in neonatal mice. *Exp Neurol* 2016; **283**: 264-75
- 113 Ten VS, Wu EX, Tang H, et al. Late measures of brain injury after neonatal hypoxia-ischemia in mice. *Stroke* 2004; **35**: 2183-8
- 114 Cengiz P, Uluc K, Kendigelen P, et al. Chronic neurological deficits in mice after perinatal hypoxia and ischemia correlate with hemispheric tissue loss and white matter injury detected by MRI. *Dev Neurosci* 2011; **33**: 270-9
- 115 Alexander M, Garbus H, Smith AL, Rosenkrantz TS, Fitch RH. Behavioral and histological outcomes following neonatal HI injury in a preterm (P3) and term (P7) rodent model. *Behav Brain Res* 2014; **259**: 85-96
- 116 Ten VS, Bradley-Moore M, Gingrich JA, Stark RI, Pinsky DJ. Brain injury and neurofunctional deficit in neonatal mice with hypoxic-ischemic encephalopathy. *Behav Brain Res* 2003; **145**: 209-19
- 117 Feather-Schussler DN, Ferguson TS. A Battery of Motor Tests in a Neonatal Mouse Model of Cerebral Palsy. *J Vis Exp* 2016
- 118 Lubics A, Reglodi D, Tamas A, et al. Neurological reflexes and early motor behavior in rats subjected to neonatal hypoxic-ischemic injury. *Behav Brain Res* 2005; **157**: 157-65
- 119 Lotgering FK, Bishai JM, Struijk PC, et al. Ten-minute umbilical cord occlusion markedly reduces cerebral blood flow and heat production in fetal sheep. *Am J Obstet Gynecol* 2003; **189**: 233-8
- 120 Gonzalez H, Hunter CJ, Bennet L, Power GG, Gunn AJ. Cerebral oxygenation during postasphyxial seizures in near-term fetal sheep. *J Cereb Blood Flow Metab* 2005; **25**: 911-8
- 121 Reddy K, Mallard C, Guan J, et al. Maturation change in the cortical response to hypoperfusion injury in the fetal sheep. *Pediatr Res* 1998; **43**: 674-82

- 122 Koehler RC, Yang ZJ, Lee JK, Martin LJ. Perinatal hypoxic-ischemic brain injury in large animal models: Relevance to human neonatal encephalopathy. *J Cereb Blood Flow Metab* 2018; **38**: 2092-111
- 123 Thoresen M, Haaland K, Loberg EM, et al. A piglet survival model of posthypoxic encephalopathy. *Pediatr Res* 1996; **40**: 738-48
- 124 Foster KA, Colditz PB, Lingwood BE, Burke C, Dunster KR, Roberts MS. An improved survival model of hypoxia/ischaemia in the piglet suitable for neuroprotection studies. *Brain Res* 2001; **919**: 122-31
- 125 Thoresen M, Penrice J, Lorek A, et al. Mild hypothermia after severe transient hypoxia-ischemia ameliorates delayed cerebral energy failure in the newborn piglet. *Pediatr Res* 1995; **37**: 667-70
- 126 Munkeby BH, De Lange C, Emblem KE, et al. A piglet model for detection of hypoxic-ischemic brain injury with magnetic resonance imaging. *Acta Radiol* 2008; **49**: 1049-57
- 127 Derrick M, Luo NL, Bregman JC, et al. Preterm fetal hypoxia-ischemia causes hypertonia and motor deficits in the neonatal rabbit: a model for human cerebral palsy? *J Neurosci* 2004; **24**: 24-34
- 128 Derrick M, He J, Brady E, Tan S. The in vitro fate of rabbit fetal brain cells after acute in vivo hypoxia. *J Neurosci* 2001; **21**: RC138
- 129 Derrick M, Drobyshesky A, Ji X, Tan S. A model of cerebral palsy from fetal hypoxia-ischemia. *Stroke* 2007; **38**: 731-5
- 130 Drobyshesky A, Derrick M, Luo K, et al. Near-term fetal hypoxia-ischemia in rabbits: MRI can predict muscle tone abnormalities and deep brain injury. *Stroke* 2012; **43**: 2757-63
- 131 Gluckman PD, Pinal CS, Gunn AJ. Hypoxic-ischemic brain injury in the newborn: pathophysiology and potential strategies for intervention. *Semin Neonatol* 2001; **6**: 109-20
- 132 Hagberg H, Mallard C, Rousset CI, Thornton C. Mitochondria: hub of injury responses in the developing brain. *Lancet Neurol* 2014; **13**: 217-32
- 133 Thornton C, Leaw B, Mallard C, Nair S, Jinnai M, Hagberg H. Cell Death in the Developing Brain after Hypoxia-Ischemia. *Front Cell Neurosci* 2017; **11**: 248
- 134 Tan WK, Williams CE, Gunn AJ, Mallard CE, Gluckman PD. Suppression of postischemic epileptiform activity with MK-801 improves neural outcome in fetal sheep. *Ann Neurol* 1992; **32**: 677-82
- 135 McDonald JW, Silverstein FS, Johnston MV. MK-801 protects the neonatal brain from hypoxic-ischemic damage. *Eur J Pharmacol* 1987; **140**: 359-61
- 136 Hagberg H, Gilland E, Diemer NH, Andine P. Hypoxia-ischemia in the neonatal rat brain: histopathology after post-treatment with NMDA and non-NMDA receptor antagonists. *Biol Neonate* 1994; **66**: 205-13
- 137 Follett PL, Rosenberg PA, Volpe JJ, Jensen FE. NBQX attenuates excitotoxic injury in developing white matter. *J Neurosci* 2000; **20**: 9235-41
- 138 Koh S, Jensen FE. Topiramate blocks perinatal hypoxia-induced seizures in rat pups. *Ann Neurol* 2001; **50**: 366-72
- 139 Follett PL, Deng W, Dai W, et al. Glutamate receptor-mediated oligodendrocyte toxicity in periventricular leukomalacia: a protective role for topiramate. *J Neurosci* 2004; **24**: 4412-20
- 140 Noh MR, Kim SK, Sun W, et al. Neuroprotective effect of topiramate on hypoxic ischemic brain injury in neonatal rats. *Exp Neurol* 2006; **201**: 470-8

- 141 Liu Y, Barks JD, Xu G, Silverstein FS. Topiramate extends the therapeutic window for hypothermia-mediated neuroprotection after stroke in neonatal rats. *Stroke* 2004; **35**: 1460-5
- 142 Hardingham GE, Bading H. Synaptic versus extrasynaptic NMDA receptor signalling: implications for neurodegenerative disorders. *Nat Rev Neurosci* 2010; **11**: 682-96
- 143 Pirianov G, Brywe KG, Mallard C, et al. Deletion of the c-Jun N-terminal kinase 3 gene protects neonatal mice against cerebral hypoxic-ischaemic injury. *J Cereb Blood Flow Metab* 2007; **27**: 1022-32
- 144 Nijboer CH, Bonestroo HJ, Zijlstra J, Kavelaars A, Heijnen CJ. Mitochondrial JNK phosphorylation as a novel therapeutic target to inhibit neuroinflammation and apoptosis after neonatal ischemic brain damage. *Neurobiol Dis* 2013; **54**: 432-44
- 145 Cook DJ, Teves L, Tymianski M. Treatment of stroke with a PSD-95 inhibitor in the gyrencephalic primate brain. *Nature* 2012; **483**: 213-7
- 146 Tu W, Xu X, Peng L, et al. DAPK1 interaction with NMDA receptor NR2B subunits mediates brain damage in stroke. *Cell* 2010; **140**: 222-34
- 147 Vannucci RC, Brucklacher RM, Vannucci SJ. Intracellular calcium accumulation during the evolution of hypoxic-ischemic brain damage in the immature rat. *Brain Res Dev Brain Res* 2001; **126**: 117-20
- 148 Peng TI, Greenamyre JT. Privileged access to mitochondria of calcium influx through N-methyl-D-aspartate receptors. *Mol Pharmacol* 1998; **53**: 974-80
- 149 Puka-Sundvall M, Gajkowska B, Cholewinski M, Blomgren K, Lazarewicz JW, Hagberg H. Subcellular distribution of calcium and ultrastructural changes after cerebral hypoxia-ischemia in immature rats. *Brain Res Dev Brain Res* 2000; **125**: 31-41
- 150 Sanderson TH, Reynolds CA, Kumar R, Przyklenk K, Huttemann M. Molecular mechanisms of ischemia-reperfusion injury in brain: pivotal role of the mitochondrial membrane potential in reactive oxygen species generation. *Mol Neurobiol* 2013; **47**: 9-23
- 151 Hopper RK, Carroll S, Aponte AM, et al. Mitochondrial matrix phosphoproteome: effect of extra mitochondrial calcium. *Biochemistry* 2006; **45**: 2524-36
- 152 Lee I, Salomon AR, Ficarro S, et al. cAMP-dependent tyrosine phosphorylation of subunit I inhibits cytochrome c oxidase activity. *J Biol Chem* 2005; **280**: 6094-100
- 153 Huttemann M, Lee I, Pecinova A, Pecina P, Przyklenk K, Doan JW. Regulation of oxidative phosphorylation, the mitochondrial membrane potential, and their role in human disease. *J Bioenerg Biomembr* 2008; **40**: 445-56
- 154 Iijima T, Mishima T, Akagawa K, Iwao Y. Mitochondrial hyperpolarization after transient oxygen-glucose deprivation and subsequent apoptosis in cultured rat hippocampal neurons. *Brain Res* 2003; **993**: 140-5
- 155 Starkov AA, Fiskum G. Regulation of brain mitochondrial H₂O₂ production by membrane potential and NAD(P)H redox state. *J Neurochem* 2003; **86**: 1101-7
- 156 Piantadosi CA, Zhang J. Mitochondrial generation of reactive oxygen species after brain ischemia in the rat. *Stroke* 1996; **27**: 327-31; discussion 32
- 157 Starkov AA, Chinopoulos C, Fiskum G. Mitochondrial calcium and oxidative stress as mediators of ischemic brain injury. *Cell Calcium* 2004; **36**: 257-64

- 158 Zhang Y, Marcillat O, Giulivi C, Ernster L, Davies KJ. The oxidative inactivation of mitochondrial electron transport chain components and ATPase. *J Biol Chem* 1990; **265**: 16330-6
- 159 Kagan VE, Tyurin VA, Jiang J, et al. Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. *Nat Chem Biol* 2005; **1**: 223-32
- 160 Ramonet D, Perier C, Recasens A, et al. Optic atrophy 1 mediates mitochondria remodeling and dopaminergic neurodegeneration linked to complex I deficiency. *Cell Death Differ* 2013; **20**: 77-85
- 161 Shalbuyeva N, Brustovetsky T, Bolshakov A, Brustovetsky N. Calcium-dependent spontaneously reversible remodeling of brain mitochondria. *J Biol Chem* 2006; **281**: 37547-58
- 162 Pivovarova NB, Andrews SB. Calcium-dependent mitochondrial function and dysfunction in neurons. *FEBS J* 2010; **277**: 3622-36
- 163 Li V, Brustovetsky T, Brustovetsky N. Role of cyclophilin D-dependent mitochondrial permeability transition in glutamate-induced calcium deregulation and excitotoxic neuronal death. *Exp Neurol* 2009; **218**: 171-82
- 164 Khaspekov L, Friberg H, Halestrap A, Viktorov I, Wieloch T. Cyclosporin A and its nonimmunosuppressive analogue N-Me-Val-4-cyclosporin A mitigate glucose/oxygen deprivation-induced damage to rat cultured hippocampal neurons. *Eur J Neurosci* 1999; **11**: 3194-8
- 165 Schinzel AC, Takeuchi O, Huang Z, et al. Cyclophilin D is a component of mitochondrial permeability transition and mediates neuronal cell death after focal cerebral ischemia. *Proc Natl Acad Sci U S A* 2005; **102**: 12005-10
- 166 Wang X, Carlsson Y, Basso E, et al. Developmental shift of cyclophilin D contribution to hypoxic-ischemic brain injury. *J Neurosci* 2009; **29**: 2588-96
- 167 Kale J, Osterlund EJ, Andrews DW. BCL-2 family proteins: changing partners in the dance towards death. *Cell Death Differ* 2018; **25**: 65-80
- 168 Gibson ME, Han BH, Choi J, et al. BAX contributes to apoptotic-like death following neonatal hypoxia-ischemia: evidence for distinct apoptosis pathways. *Mol Med* 2001; **7**: 644-55
- 169 Ness JM, Harvey CA, Strasser A, Bouillet P, Klocke BJ, Roth KA. Selective involvement of BH3-only Bcl-2 family members Bim and Bad in neonatal hypoxia-ischemia. *Brain Res* 2006; **1099**: 150-9
- 170 Parsadanian AS, Cheng Y, Keller-Peck CR, Holtzman DM, Snider WD. Bcl-xL is an antiapoptotic regulator for postnatal CNS neurons. *J Neurosci* 1998; **18**: 1009-19
- 171 Yin W, Cao G, Johnnides MJ, et al. TAT-mediated delivery of Bcl-xL protein is neuroprotective against neonatal hypoxic-ischemic brain injury via inhibition of caspases and AIF. *Neurobiol Dis* 2006; **21**: 358-71
- 172 Nijboer CH, Heijnen CJ, van der Kooij MA, et al. Targeting the p53 pathway to protect the neonatal ischemic brain. *Ann Neurol* 2011; **70**: 255-64
- 173 Green DR, Kroemer G. Cytoplasmic functions of the tumour suppressor p53. *Nature* 2009; **458**: 1127-30
- 174 Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 2000; **102**: 33-42
- 175 Verhagen AM, Ekert PG, Pakusch M, et al. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 2000; **102**: 43-53
- 176 Porter AG, Janicke RU. Emerging roles of caspase-3 in apoptosis. *Cell Death Differ* 1999; **6**: 99-104

- 177 Cheng Y, Deshmukh M, D'Costa A, et al. Caspase inhibitor affords neuroprotection with delayed administration in a rat model of neonatal hypoxic-ischemic brain injury. *J Clin Invest* 1998; **101**: 1992-9
- 178 Renolleau S, Fau S, Goyenvallé C, et al. Specific caspase inhibitor Q-VD-OPh prevents neonatal stroke in P7 rat: a role for gender. *J Neurochem* 2007; **100**: 1062-71
- 179 Zhu C, Wang X, Huang Z, et al. Apoptosis-inducing factor is a major contributor to neuronal loss induced by neonatal cerebral hypoxia-ischemia. *Cell Death Differ* 2007; **14**: 775-84
- 180 Holcik M, Korneluk RG. XIAP, the guardian angel. *Nat Rev Mol Cell Biol* 2001; **2**: 550-6
- 181 Saito A, Hayashi T, Okuno S, Ferrand-Drake M, Chan PH. Interaction between XIAP and Smac/DIABLO in the mouse brain after transient focal cerebral ischemia. *J Cereb Blood Flow Metab* 2003; **23**: 1010-9
- 182 Wang X, Zhu C, Wang X, et al. X-linked inhibitor of apoptosis (XIAP) protein protects against caspase activation and tissue loss after neonatal hypoxia-ischemia. *Neurobiol Dis* 2004; **16**: 179-89
- 183 Russell JC, Whiting H, Szuflita N, Hossain MA. Nuclear translocation of X-linked inhibitor of apoptosis (XIAP) determines cell fate after hypoxia ischemia in neonatal brain. *J Neurochem* 2008; **106**: 1357-70
- 184 Modjtahedi N, Giordanetto F, Madeo F, Kroemer G. Apoptosis-inducing factor: vital and lethal. *Trends Cell Biol* 2006; **16**: 264-72
- 185 Wang Y, Dawson VL, Dawson TM. Poly(ADP-ribose) signals to mitochondrial AIF: a key event in parthanatos. *Exp Neurol* 2009; **218**: 193-202
- 186 Zhu C, Qiu L, Wang X, et al. Involvement of apoptosis-inducing factor in neuronal death after hypoxia-ischemia in the neonatal rat brain. *J Neurochem* 2003; **86**: 306-17
- 187 Matsumori Y, Hong SM, Aoyama K, et al. Hsp70 overexpression sequesters AIF and reduces neonatal hypoxic/ischemic brain injury. *J Cereb Blood Flow Metab* 2005; **25**: 899-910
- 188 Du L, Bayir H, Lai Y, et al. Innate gender-based proclivity in response to cytotoxicity and programmed cell death pathway. *J Biol Chem* 2004; **279**: 38563-70
- 189 Yuan M, Siegel C, Zeng Z, Li J, Liu F, McCullough LD. Sex differences in the response to activation of the poly (ADP-ribose) polymerase pathway after experimental stroke. *Exp Neurol* 2009; **217**: 210-8
- 190 McCullough LD, Zeng Z, Blizzard KK, Debchoudhury I, Hurn PD. Ischemic nitric oxide and poly (ADP-ribose) polymerase-1 in cerebral ischemia: male toxicity, female protection. *J Cereb Blood Flow Metab* 2005; **25**: 502-12
- 191 Zhu C, Xu F, Wang X, et al. Different apoptotic mechanisms are activated in male and female brains after neonatal hypoxia-ischaemia. *J Neurochem* 2006; **96**: 1016-27
- 192 Hill CA, Fitch RH. Sex differences in mechanisms and outcome of neonatal hypoxia-ischemia in rodent models: implications for sex-specific neuroprotection in clinical neonatal practice. *Neurol Res Int* 2012; **2012**: 867531
- 193 Hagberg H, Mallard C, Ferriero DM, et al. The role of inflammation in perinatal brain injury. *Nat Rev Neurol* 2015; **11**: 192-208
- 194 Wu YW, Escobar GJ, Grether JK, Croen LA, Greene JD, Newman TB. Chorioamnionitis and cerebral palsy in term and near-term infants. *JAMA* 2003; **290**: 2677-84
- 195 Jenster M, Bonifacio SL, Ruel T, et al. Maternal or neonatal infection: association with neonatal encephalopathy outcomes. *Pediatr Res* 2014; **76**: 93-9

- 196 Eklind S, Mallard C, Leverin AL, et al. Bacterial endotoxin sensitizes the immature brain to hypoxic--ischemic injury. *Eur J Neurosci* 2001; **13**: 1101-6
- 197 Wang X, Stridh L, Li W, et al. Lipopolysaccharide sensitizes neonatal hypoxic-ischemic brain injury in a MyD88-dependent manner. *J Immunol* 2009; **183**: 7471-7
- 198 Wang LW, Chang YC, Chen SJ, et al. TNFR1-JNK signaling is the shared pathway of neuroinflammation and neurovascular damage after LPS-sensitized hypoxic-ischemic injury in the immature brain. *J Neuroinflammation* 2014; **11**: 215
- 199 Stridh L, Mottahedin A, Johansson ME, et al. Toll-like receptor-3 activation increases the vulnerability of the neonatal brain to hypoxia-ischemia. *J Neurosci* 2013; **33**: 12041-51
- 200 Ni J, Zhao J, Zhang X, Reinheckel T, Turk V, Nakanishi H. Cathepsin H deficiency decreases hypoxia-ischemia-induced hippocampal atrophy in neonatal mice through attenuated TLR3/IFN-beta signaling. *J Neuroinflammation* 2021; **18**: 176
- 201 Fleiss B, Gressens P. Tertiary mechanisms of brain damage: a new hope for treatment of cerebral palsy? *Lancet Neurol* 2012; **11**: 556-66
- 202 Stridh L, Smith PL, Naylor AS, Wang X, Mallard C. Regulation of toll-like receptor 1 and -2 in neonatal mice brains after hypoxia-ischemia. *J Neuroinflammation* 2011; **8**: 45
- 203 Du X, Fleiss B, Li H, et al. Systemic stimulation of TLR2 impairs neonatal mouse brain development. *PLoS One* 2011; **6**: e19583
- 204 Hagberg H, Gilland E, Bona E, et al. Enhanced expression of interleukin (IL)-1 and IL-6 messenger RNA and bioactive protein after hypoxia-ischemia in neonatal rats. *Pediatr Res* 1996; **40**: 603-9
- 205 Girard S, Sebire H, Brochu ME, Briota S, Sarret P, Sebire G. Postnatal administration of IL-1Ra exerts neuroprotective effects following perinatal inflammation and/or hypoxic-ischemic injuries. *Brain Behav Immun* 2012; **26**: 1331-9
- 206 Liu XH, Kwon D, Schielke GP, Yang GY, Silverstein FS, Barks JD. Mice deficient in interleukin-1 converting enzyme are resistant to neonatal hypoxic-ischemic brain damage. *J Cereb Blood Flow Metab* 1999; **19**: 1099-108
- 207 Hedtjarn M, Leverin AL, Eriksson K, Blomgren K, Mallard C, Hagberg H. Interleukin-18 involvement in hypoxic-ischemic brain injury. *J Neurosci* 2002; **22**: 5910-9
- 208 Hu X, Nesic-Taylor O, Qiu J, et al. Activation of nuclear factor-kappaB signaling pathway by interleukin-1 after hypoxia/ischemia in neonatal rat hippocampus and cortex. *J Neurochem* 2005; **93**: 26-37
- 209 Hu X, Leak RK, Shi Y, et al. Microglial and macrophage polarization-new prospects for brain repair. *Nat Rev Neurol* 2015; **11**: 56-64
- 210 Donat CK, Scott G, Gentleman SM, Sastre M. Microglial Activation in Traumatic Brain Injury. *Front Aging Neurosci* 2017; **9**: 208
- 211 Takaki J, Fujimori K, Miura M, Suzuki T, Sekino Y, Sato K. L-glutamate released from activated microglia downregulates astrocytic L-glutamate transporter expression in neuroinflammation: the 'collusion' hypothesis for increased extracellular L-glutamate concentration in neuroinflammation. *J Neuroinflammation* 2012; **9**: 275
- 212 Bezzi P, Domercq M, Brambilla L, et al. CXCR4-activated astrocyte glutamate release via TNFalpha: amplification by microglia triggers neurotoxicity. *Nat Neurosci* 2001; **4**: 702-10

213 Paolicelli RC, Sierra A, Stevens B, et al. Microglia states and nomenclature: A field at its crossroads. *Neuron* 2022; **110**: 3458-83

214 Faustino JV, Wang X, Johnson CE, et al. Microglial cells contribute to endogenous brain defenses after acute neonatal focal stroke. *J Neurosci* 2011; **31**: 12992-3001

215 Kobayashi K, Imagama S, Ohgomori T, et al. Minocycline selectively inhibits M1 polarization of microglia. *Cell Death Dis* 2013; **4**: e525

216 Arvin KL, Han BH, Du Y, Lin SZ, Paul SM, Holtzman DM. Minocycline markedly protects the neonatal brain against hypoxic-ischemic injury. *Ann Neurol* 2002; **52**: 54-61

217 Escartin C, Galea E, Lakatos A, et al. Reactive astrocyte nomenclature, definitions, and future directions. *Nat Neurosci* 2021; **24**: 312-25

218 Lindsberg PJ, Strbian D, Karjalainen-Lindsberg ML. Mast cells as early responders in the regulation of acute blood-brain barrier changes after cerebral ischemia and hemorrhage. *J Cereb Blood Flow Metab* 2010; **30**: 689-702

219 Jin Y, Silverman AJ, Vannucci SJ. Mast cells are early responders after hypoxia-ischemia in immature rat brain. *Stroke* 2009; **40**: 3107-12

220 Biran V, Cochois V, Karroubi A, Arrang JM, Charriaut-Marlangue C, Heron A. Stroke induces histamine accumulation and mast cell degranulation in the neonatal rat brain. *Brain Pathol* 2008; **18**: 1-9

221 Jin Y, Silverman AJ, Vannucci SJ. Mast cell stabilization limits hypoxic-ischemic brain damage in the immature rat. *Dev Neurosci* 2007; **29**: 373-84

222 Mesples B, Fontaine RH, Lelievre V, Launay JM, Gressens P. Neuronal TGF-beta1 mediates IL-9/mast cell interaction and exacerbates excitotoxicity in newborn mice. *Neurobiol Dis* 2005; **18**: 193-205

223 Ek CJ, D'Angelo B, Baburamani AA, et al. Brain barrier properties and cerebral blood flow in neonatal mice exposed to cerebral hypoxia-ischemia. *J Cereb Blood Flow Metab* 2015; **35**: 818-27

224 Winerdal M, Winerdal ME, Kinn J, Urmaliya V, Winqvist O, Aden U. Long lasting local and systemic inflammation after cerebral hypoxic ischemia in newborn mice. *PLoS One* 2012; **7**: e36422

225 Jellema RK, Lima Passos V, Zwanenburg A, et al. Cerebral inflammation and mobilization of the peripheral immune system following global hypoxia-ischemia in preterm sheep. *J Neuroinflammation* 2013; **10**: 13

226 Yao HW, Kuan CY. Early neutrophil infiltration is critical for inflammation-sensitized hypoxic-ischemic brain injury in newborns. *J Cereb Blood Flow Metab* 2020; **40**: 2188-200

227 Mirza MA, Ritzel R, Xu Y, McCullough LD, Liu F. Sexually dimorphic outcomes and inflammatory responses in hypoxic-ischemic encephalopathy. *J Neuroinflammation* 2015; **12**: 32

228 Shechter R, London A, Schwartz M. Orchestrated leukocyte recruitment to immune-privileged sites: absolute barriers versus educational gates. *Nat Rev Immunol* 2013; **13**: 206-18

229 Hurn PD, Subramanian S, Parker SM, et al. T- and B-cell-deficient mice with experimental stroke have reduced lesion size and inflammation. *J Cereb Blood Flow Metab* 2007; **27**: 1798-805

230 Iadecola C, Anrather J. The immunology of stroke: from mechanisms to translation. *Nat Med* 2011; **17**: 796-808

- 231 Lin CY, Chang YC, Wang ST, Lee TY, Lin CF, Huang CC. Altered inflammatory responses in preterm children with cerebral palsy. *Ann Neurol* 2010; **68**: 204-12
- 232 Kitase Y, Chin EM, Ramachandra S, et al. Sustained peripheral immune hyper-reactivity (SPIHR): an enduring biomarker of altered inflammatory responses in adult rats after perinatal brain injury. *J Neuroinflammation* 2021; **18**: 242
- 233 Ramlackhansingh AF, Brooks DJ, Greenwood RJ, et al. Inflammation after trauma: microglial activation and traumatic brain injury. *Ann Neurol* 2011; **70**: 374-83
- 234 Plane JM, Liu R, Wang TW, Silverstein FS, Parent JM. Neonatal hypoxic-ischemic injury increases forebrain subventricular zone neurogenesis in the mouse. *Neurobiol Dis* 2004; **16**: 585-95
- 235 Covey MV, Loporchio D, Buono KD, Levison SW. Opposite effect of inflammation on subventricular zone versus hippocampal precursors in brain injury. *Ann Neurol* 2011; **70**: 616-26
- 236 Buono KD, Goodus MT, Guardia Clausi M, Jiang Y, Loporchio D, Levison SW. Mechanisms of mouse neural precursor expansion after neonatal hypoxia-ischemia. *J Neurosci* 2015; **35**: 8855-65
- 237 Yang Z, Covey MV, Bitel CL, Ni L, Jonakait GM, Levison SW. Sustained neocortical neurogenesis after neonatal hypoxic/ischemic injury. *Ann Neurol* 2007; **61**: 199-208
- 238 Monje ML, Toda H, Palmer TD. Inflammatory blockade restores adult hippocampal neurogenesis. *Science* 2003; **302**: 1760-5
- 239 Bauer S. Cytokine control of adult neural stem cells. *Ann N Y Acad Sci* 2009; **1153**: 48-56
- 240 Li J, Ramenaden ER, Peng J, Koito H, Volpe JJ, Rosenberg PA. Tumor necrosis factor alpha mediates lipopolysaccharide-induced microglial toxicity to developing oligodendrocytes when astrocytes are present. *J Neurosci* 2008; **28**: 5321-30
- 241 Peferoen L, Kipp M, van der Valk P, van Noort JM, Amor S. Oligodendrocyte-microglia cross-talk in the central nervous system. *Immunology* 2014; **141**: 302-13
- 242 Sofroniew MV. Molecular dissection of reactive astrogliosis and glial scar formation. *Trends Neurosci* 2009; **32**: 638-47
- 243 Colombo E, Farina C. Astrocytes: Key Regulators of Neuroinflammation. *Trends Immunol* 2016; **37**: 608-20
- 244 Ma Q, Zhang L. Epigenetic programming of hypoxic-ischemic encephalopathy in response to fetal hypoxia. *Prog Neurobiol* 2015; **124**: 28-48
- 245 Faraco G, Pancani T, Formentini L, et al. Pharmacological inhibition of histone deacetylases by suberoylanilide hydroxamic acid specifically alters gene expression and reduces ischemic injury in the mouse brain. *Mol Pharmacol* 2006; **70**: 1876-84
- 246 Fleiss B, Nilsson MK, Blomgren K, Mallard C. Neuroprotection by the histone deacetylase inhibitor trichostatin A in a model of lipopolysaccharide-sensitised neonatal hypoxic-ischaemic brain injury. *J Neuroinflammation* 2012; **9**: 70
- 247 Shen S, Li J, Casaccia-Bonnel P. Histone modifications affect timing of oligodendrocyte progenitor differentiation in the developing rat brain. *J Cell Biol* 2005; **169**: 577-89

248 Montgomery RL, Hsieh J, Barbosa AC, Richardson JA, Olson EN. Histone deacetylases 1 and 2 control the progression of neural precursors to neurons during brain development. *Proc Natl Acad Sci U S A* 2009; **106**: 7876-81

249 Hsieh J, Nakashima K, Kuwabara T, Mejia E, Gage FH. Histone deacetylase inhibition-mediated neuronal differentiation of multipotent adult neural progenitor cells. *Proc Natl Acad Sci U S A* 2004; **101**: 16659-64

250 Nunez JL, McCarthy MM. Androgens predispose males to GABAA-mediated excitotoxicity in the developing hippocampus. *Exp Neurol* 2008; **210**: 699-708

251 Hill CA, Threlkeld SW, Fitch RH. Early testosterone modulated sex differences in behavioral outcome following neonatal hypoxia ischemia in rats. *Int J Dev Neurosci* 2011; **29**: 381-8

252 Rosen GD, Herman AE, Galaburda AM. Sex differences in the effects of early neocortical injury on neuronal size distribution of the medial geniculate nucleus in the rat are mediated by perinatal gonadal steroids. *Cereb Cortex* 1999; **9**: 27-34

253 Ota A, Ikeda T, Ikenoue T, Toshimori K. Sequence of neuronal responses assessed by immunohistochemistry in the newborn rat brain after hypoxia-ischemia. *Am J Obstet Gynecol* 1997; **177**: 519-26

254 Silverstein FS, Torke L, Barks J, Johnston MV. Hypoxia-ischemia produces focal disruption of glutamate receptors in developing brain. *Brain Res* 1987; **431**: 33-9

255 Johnston MV. Neurotransmitter alterations in a model of perinatal hypoxic-ischemic brain injury. *Ann Neurol* 1983; **13**: 511-8

256 Northington FJ, Kratimenos P, Turnbull V, et al. Basal forebrain magnocellular cholinergic systems are damaged in mice following neonatal hypoxia-ischemia. *J Comp Neurol* 2022; **530**: 1148-63

257 Romanko MJ, Rothstein RP, Levison SW. Neural stem cells in the subventricular zone are resilient to hypoxia/ischemia whereas progenitors are vulnerable. *J Cereb Blood Flow Metab* 2004; **24**: 814-25

258 Pantoni L, Garcia JH, Gutierrez JA. Cerebral white matter is highly vulnerable to ischemia. *Stroke* 1996; **27**: 1641-6; discussion 7

259 Uehara H, Yoshioka H, Kawase S, et al. A new model of white matter injury in neonatal rats with bilateral carotid artery occlusion. *Brain Res* 1999; **837**: 213-20

260 Wang F, Yang YJ, Yang N, et al. Enhancing Oligodendrocyte Myelination Rescues Synaptic Loss and Improves Functional Recovery after Chronic Hypoxia. *Neuron* 2018; **99**: 689-701 e5

261 Tekkok SB, Goldberg MP. Ampa/kainate receptor activation mediates hypoxic oligodendrocyte death and axonal injury in cerebral white matter. *J Neurosci* 2001; **21**: 4237-48

262 Husain J, Juurlink BH. Oligodendroglial precursor cell susceptibility to hypoxia is related to poor ability to cope with reactive oxygen species. *Brain Res* 1995; **698**: 86-94

263 Back SA, Han BH, Luo NL, et al. Selective vulnerability of late oligodendrocyte progenitors to hypoxia-ischemia. *J Neurosci* 2002; **22**: 455-63

264 Engelhardt S, Huang SF, Patkar S, Gassmann M, Ogunshola OO. Differential responses of blood-brain barrier associated cells to hypoxia and ischemia: a comparative study. *Fluids Barriers CNS* 2015; **12**: 4

265 Xu J, He L, Ahmed SH, et al. Oxygen-glucose deprivation induces inducible nitric oxide synthase and nitrotyrosine expression in cerebral endothelial cells. *Stroke* 2000; **31**: 1744-51

- 266 Naito MG, Xu D, Amin P, et al. Sequential activation of necroptosis and apoptosis cooperates to mediate vascular and neural pathology in stroke. *Proc Natl Acad Sci U S A* 2020; **117**: 4959-70
- 267 Martin LJ, Brambrink AM, Lehmann C, et al. Hypoxia-ischemia causes abnormalities in glutamate transporters and death of astroglia and neurons in newborn striatum. *Ann Neurol* 1997; **42**: 335-48
- 268 Northington FJ, Chavez-Valdez R, Martin LJ. Neuronal cell death in neonatal hypoxia-ischemia. *Ann Neurol* 2011; **69**: 743-58
- 269 Ofengeim D, Yuan J. Regulation of RIP1 kinase signalling at the crossroads of inflammation and cell death. *Nat Rev Mol Cell Biol* 2013; **14**: 727-36
- 270 Linkermann A, Green DR. Necroptosis. *N Engl J Med* 2014; **370**: 455-65
- 271 Sarhan M, Land WG, Tonnus W, Hugo CP, Linkermann A. Origin and Consequences of Necroinflammation. *Physiol Rev* 2018; **98**: 727-80
- 272 Dostert C, Grusdat M, Letellier E, Brenner D. The TNF Family of Ligands and Receptors: Communication Modules in the Immune System and Beyond. *Physiol Rev* 2019; **99**: 115-60
- 273 Hsu H, Xiong J, Goeddel DV. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell* 1995; **81**: 495-504
- 274 Hsu H, Shu HB, Pan MG, Goeddel DV. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell* 1996; **84**: 299-308
- 275 Wang L, Du F, Wang X. TNF-alpha induces two distinct caspase-8 activation pathways. *Cell* 2008; **133**: 693-703
- 276 Degterev A, Ofengeim D, Yuan J. Targeting RIPK1 for the treatment of human diseases. *Proc Natl Acad Sci U S A* 2019; **116**: 9714-22
- 277 Geng J, Ito Y, Shi L, et al. Regulation of RIPK1 activation by TAK1-mediated phosphorylation dictates apoptosis and necroptosis. *Nat Commun* 2017; **8**: 359
- 278 Wilson NS, Dixit V, Ashkenazi A. Death receptor signal transducers: nodes of coordination in immune signaling networks. *Nat Immunol* 2009; **10**: 348-55
- 279 Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 1998; **94**: 491-501
- 280 Lin Y, Devin A, Rodriguez Y, Liu ZG. Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. *Genes Dev* 1999; **13**: 2514-26
- 281 Feng S, Yang Y, Mei Y, et al. Cleavage of RIP3 inactivates its caspase-independent apoptosis pathway by removal of kinase domain. *Cell Signal* 2007; **19**: 2056-67
- 282 O'Donnell MA, Perez-Jimenez E, Oberst A, et al. Caspase 8 inhibits programmed necrosis by processing CYLD. *Nat Cell Biol* 2011; **13**: 1437-42
- 283 Vanden Berghe T, Linkermann A, Jouan-Lanhuet S, Walczak H, Vandenabeele P. Regulated necrosis: the expanding network of non-apoptotic cell death pathways. *Nat Rev Mol Cell Biol* 2014; **15**: 135-47
- 284 Krueger A, Schmitz I, Baumann S, Krammer PH, Kirchhoff S. Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. *J Biol Chem* 2001; **276**: 20633-40

- 285 Micheau O, Thome M, Schneider P, et al. The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. *J Biol Chem* 2002; **277**: 45162-71
- 286 Oberst A, Dillon CP, Weinlich R, et al. Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. *Nature* 2011; **471**: 363-7
- 287 Kaiser WJ, Upton JW, Long AB, et al. RIP3 mediates the embryonic lethality of caspase-8-deficient mice. *Nature* 2011; **471**: 368-72
- 288 Gunther C, Martini E, Wittkopf N, et al. Caspase-8 regulates TNF-alpha-induced epithelial necroptosis and terminal ileitis. *Nature* 2011; **477**: 335-9
- 289 Bonnet MC, Preukschat D, Welz PS, et al. The adaptor protein FADD protects epidermal keratinocytes from necroptosis in vivo and prevents skin inflammation. *Immunity* 2011; **35**: 572-82
- 290 Zhang DW, Shao J, Lin J, et al. RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. *Science* 2009; **325**: 332-6
- 291 He S, Wang L, Miao L, et al. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. *Cell* 2009; **137**: 1100-11
- 292 Cho YS, Challa S, Moquin D, et al. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell* 2009; **137**: 1112-23
- 293 Li J, McQuade T, Siemer AB, et al. The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. *Cell* 2012; **150**: 339-50
- 294 Wu XN, Yang ZH, Wang XK, et al. Distinct roles of RIP1-RIP3 hetero- and RIP3-RIP3 homo-interaction in mediating necroptosis. *Cell Death Differ* 2014; **21**: 1709-20
- 295 McQuade T, Cho Y, Chan FK. Positive and negative phosphorylation regulates RIP1- and RIP3-induced programmed necrosis. *Biochem J* 2013; **456**: 409-15
- 296 Sun L, Wang H, Wang Z, et al. Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. *Cell* 2012; **148**: 213-27
- 297 Kaiser WJ, Upton JW, Mocarski ES. Viral modulation of programmed necrosis. *Curr Opin Virol* 2013; **3**: 296-306
- 298 Lawlor KE, Khan N, Mildenhall A, et al. RIPK3 promotes cell death and NLRP3 inflammasome activation in the absence of MLKL. *Nat Commun* 2015; **6**: 6282
- 299 Conos SA, Chen KW, De Nardo D, et al. Active MLKL triggers the NLRP3 inflammasome in a cell-intrinsic manner. *Proc Natl Acad Sci U S A* 2017; **114**: E961-E9
- 300 Pasparakis M, Vandenabeele P. Necroptosis and its role in inflammation. *Nature* 2015; **517**: 311-20
- 301 He S, Liang Y, Shao F, Wang X. Toll-like receptors activate programmed necrosis in macrophages through a receptor-interacting kinase-3-mediated pathway. *Proc Natl Acad Sci U S A* 2011; **108**: 20054-9
- 302 Kaiser WJ, Sridharan H, Huang C, et al. Toll-like receptor 3-mediated necrosis via TRIF, RIP3, and MLKL. *J Biol Chem* 2013; **288**: 31268-79
- 303 Takaoka A, Wang Z, Choi MK, et al. DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature* 2007; **448**: 501-5

- 304 Kaiser WJ, Upton JW, Mocarski ES. Receptor-interacting protein homotypic interaction motif-dependent control of NF-kappa B activation via the DNA-dependent activator of IFN regulatory factors. *J Immunol* 2008; **181**: 6427-34
- 305 Upton JW, Kaiser WJ, Mocarski ES. DAI/ZBP1/DLM-1 complexes with RIP3 to mediate virus-induced programmed necrosis that is targeted by murine cytomegalovirus vIRA. *Cell Host Microbe* 2012; **11**: 290-7
- 306 Upton JW, Kaiser WJ, Mocarski ES. Virus inhibition of RIP3-dependent necrosis. *Cell Host Microbe* 2010; **7**: 302-13
- 307 Xie T, Peng W, Yan C, Wu J, Gong X, Shi Y. Structural insights into RIP3-mediated necroptotic signaling. *Cell Rep* 2013; **5**: 70-8
- 308 Rodriguez DA, Weinlich R, Brown S, et al. Characterization of RIPK3-mediated phosphorylation of the activation loop of MLKL during necroptosis. *Cell Death Differ* 2016; **23**: 76-88
- 309 Hildebrand JM, Tanzer MC, Lucet IS, et al. Activation of the pseudokinase MLKL unleashes the four-helix bundle domain to induce membrane localization and necroptotic cell death. *Proc Natl Acad Sci U S A* 2014; **111**: 15072-7
- 310 Murphy JM, Czabotar PE, Hildebrand JM, et al. The pseudokinase MLKL mediates necroptosis via a molecular switch mechanism. *Immunity* 2013; **39**: 443-53
- 311 Dondelinger Y, Declercq W, Montessuit S, et al. MLKL compromises plasma membrane integrity by binding to phosphatidylinositol phosphates. *Cell Rep* 2014; **7**: 971-81
- 312 Wang H, Sun L, Su L, et al. Mixed lineage kinase domain-like protein MLKL causes necrotic membrane disruption upon phosphorylation by RIP3. *Mol Cell* 2014; **54**: 133-46
- 313 Gong YN, Guy C, Olauson H, et al. ESCRT-III Acts Downstream of MLKL to Regulate Necroptotic Cell Death and Its Consequences. *Cell* 2017; **169**: 286-300 e16
- 314 Yuan J, Amin P, Ofengeim D. Necroptosis and RIPK1-mediated neuroinflammation in CNS diseases. *Nat Rev Neurosci* 2019; **20**: 19-33
- 315 Ito Y, Ofengeim D, Najafov A, et al. RIPK1 mediates axonal degeneration by promoting inflammation and necroptosis in ALS. *Science* 2016; **353**: 603-8
- 316 Ofengeim D, Mazzitelli S, Ito Y, et al. RIPK1 mediates a disease-associated microglial response in Alzheimer's disease. *Proc Natl Acad Sci U S A* 2017; **114**: E8788-E97
- 317 Degterev A, Huang Z, Boyce M, et al. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nat Chem Biol* 2005; **1**: 112-9
- 318 Degterev A, Hitomi J, Gernscheid M, et al. Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nat Chem Biol* 2008; **4**: 313-21
- 319 King MD, Whitaker-Lea WA, Campbell JM, Alleyne CH, Jr., Dhandapani KM. Necrostatin-1 reduces neurovascular injury after intracerebral hemorrhage. *Int J Cell Biol* 2014; **2014**: 495817
- 320 You Z, Savitz SI, Yang J, et al. Necrostatin-1 reduces histopathology and improves functional outcome after controlled cortical impact in mice. *J Cereb Blood Flow Metab* 2008; **28**: 1564-73
- 321 Askalan R, Gabarin N, Armstrong EA, Fang Liu Y, Couchman D, Yager JY. Mechanisms of neurodegeneration after severe hypoxic-ischemic injury in the neonatal rat brain. *Brain Res* 2015; **1629**: 94-103

- 322 Vieira M, Fernandes J, Carreto L, et al. Ischemic insults induce necroptotic cell death in hippocampal neurons through the up-regulation of endogenous RIP3. *Neurobiol Dis* 2014; **68**: 26-36
- 323 Northington FJ, Chavez-Valdez R, Graham EM, Razdan S, Gauda EB, Martin LJ. Necrostatin decreases oxidative damage, inflammation, and injury after neonatal HI. *J Cereb Blood Flow Metab* 2011; **31**: 178-89
- 324 Chavez-Valdez R, Martin LJ, Flock DL, Northington FJ. Necrostatin-1 attenuates mitochondrial dysfunction in neurons and astrocytes following neonatal hypoxia-ischemia. *Neuroscience* 2012; **219**: 192-203
- 325 Northington FJ, Ferriero DM, Flock DL, Martin LJ. Delayed neurodegeneration in neonatal rat thalamus after hypoxia-ischemia is apoptosis. *J Neurosci* 2001; **21**: 1931-8
- 326 Hallin U, Kondo E, Ozaki Y, Hagberg H, Shibasaki F, Blomgren K. Bcl-2 phosphorylation in the BH4 domain precedes caspase-3 activation and cell death after neonatal cerebral hypoxic-ischemic injury. *Neurobiol Dis* 2006; **21**: 478-86
- 327 Karbowski M, Norris KL, Cleland MM, Jeong SY, Youle RJ. Role of Bax and Bak in mitochondrial morphogenesis. *Nature* 2006; **443**: 658-62
- 328 Karbowski M, Arnoult D, Chen H, Chan DC, Smith CL, Youle RJ. Quantitation of mitochondrial dynamics by photolabeling of individual organelles shows that mitochondrial fusion is blocked during the Bax activation phase of apoptosis. *J Cell Biol* 2004; **164**: 493-9
- 329 Neuspiel M, Zunino R, Gangaraju S, Rippstein P, McBride H. Activated mitofusin 2 signals mitochondrial fusion, interferes with Bax activation, and reduces susceptibility to radical induced depolarization. *J Biol Chem* 2005; **280**: 25060-70
- 330 Sugioka R, Shimizu S, Tsujimoto Y. Fzo1, a protein involved in mitochondrial fusion, inhibits apoptosis. *J Biol Chem* 2004; **279**: 52726-34
- 331 Frank S, Gaume B, Bergmann-Leitner ES, et al. The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. *Dev Cell* 2001; **1**: 515-25
- 332 Cassidy-Stone A, Chipuk JE, Ingberman E, et al. Chemical inhibition of the mitochondrial division dynamin reveals its role in Bax/Bak-dependent mitochondrial outer membrane permeabilization. *Dev Cell* 2008; **14**: 193-204
- 333 Thornton C, Jones A, Nair S, Aabdien A, Mallard C, Hagberg H. Mitochondrial dynamics, mitophagy and biogenesis in neonatal hypoxic-ischaemic brain injury. *FEBS Lett* 2018; **592**: 812-30
- 334 Pickles S, Vigie P, Youle RJ. Mitophagy and Quality Control Mechanisms in Mitochondrial Maintenance. *Curr Biol* 2018; **28**: R170-R85
- 335 Wu Z, Puigserver P, Andersson U, et al. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* 1999; **98**: 115-24
- 336 Lehman JJ, Barger PM, Kovacs A, Saffitz JE, Medeiros DM, Kelly DP. Peroxisome proliferator-activated receptor gamma coactivator-1 promotes cardiac mitochondrial biogenesis. *J Clin Invest* 2000; **106**: 847-56
- 337 Vega RB, Huss JM, Kelly DP. The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. *Mol Cell Biol* 2000; **20**: 1868-76

- 338 Huss JM, Kopp RP, Kelly DP. Peroxisome proliferator-activated receptor coactivator-1 α (PGC-1 α) coactivates the cardiac-enriched nuclear receptors estrogen-related receptor- α and - γ . Identification of novel leucine-rich interaction motif within PGC-1 α . *J Biol Chem* 2002; **277**: 40265-74
- 339 Yoon JC, Puigserver P, Chen G, et al. Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. *Nature* 2001; **413**: 131-8
- 340 Yin W, Signore AP, Iwai M, Cao G, Gao Y, Chen J. Rapidly increased neuronal mitochondrial biogenesis after hypoxic-ischemic brain injury. *Stroke* 2008; **39**: 3057-63
- 341 Youle RJ, Narendra DP. Mechanisms of mitophagy. *Nat Rev Mol Cell Biol* 2011; **12**: 9-14
- 342 Ashrafi G, Schwarz TL. The pathways of mitophagy for quality control and clearance of mitochondria. *Cell Death Differ* 2013; **20**: 31-42
- 343 Onishi M, Yamano K, Sato M, Matsuda N, Okamoto K. Molecular mechanisms and physiological functions of mitophagy. *EMBO J* 2021; **40**: e104705
- 344 Nakatogawa H. Mechanisms governing autophagosome biogenesis. *Nat Rev Mol Cell Biol* 2020; **21**: 439-58
- 345 Farre JC, Subramani S. Mechanistic insights into selective autophagy pathways: lessons from yeast. *Nat Rev Mol Cell Biol* 2016; **17**: 537-52
- 346 Zhu Y, Massen S, Terenzio M, et al. Modulation of serines 17 and 24 in the LC3-interacting region of Bnip3 determines pro-survival mitophagy versus apoptosis. *J Biol Chem* 2013; **288**: 1099-113
- 347 Kubli DA, Ycaza JE, Gustafsson AB. Bnip3 mediates mitochondrial dysfunction and cell death through Bax and Bak. *Biochem J* 2007; **405**: 407-15
- 348 Chen G, Ray R, Dubik D, et al. The E1B 19K/Bcl-2-binding protein Nip3 is a dimeric mitochondrial protein that activates apoptosis. *J Exp Med* 1997; **186**: 1975-83
- 349 Chen G, Cizeau J, Vande Velde C, et al. Nix and Nip3 form a subfamily of pro-apoptotic mitochondrial proteins. *J Biol Chem* 1999; **274**: 7-10
- 350 Ray R, Chen G, Vande Velde C, et al. BNIP3 heterodimerizes with Bcl-2/Bcl-X(L) and induces cell death independent of a Bcl-2 homology 3 (BH3) domain at both mitochondrial and nonmitochondrial sites. *J Biol Chem* 2000; **275**: 1439-48
- 351 Zhang J, Loyd MR, Randall MS, Waddell MB, Kriwacki RW, Ney PA. A short linear motif in BNIP3L (NIX) mediates mitochondrial clearance in reticulocytes. *Autophagy* 2012; **8**: 1325-32
- 352 Novak I, Kirkin V, McEwan DG, et al. Nix is a selective autophagy receptor for mitochondrial clearance. *EMBO Rep* 2010; **11**: 45-51
- 353 Marinkovic M, Sprung M, Novak I. Dimerization of mitophagy receptor BNIP3L/NIX is essential for recruitment of autophagic machinery. *Autophagy* 2021; **17**: 1232-43
- 354 Rogov VV, Suzuki H, Marinkovic M, et al. Phosphorylation of the mitochondrial autophagy receptor Nix enhances its interaction with LC3 proteins. *Sci Rep* 2017; **7**: 1131
- 355 Liu L, Feng D, Chen G, et al. Mitochondrial outer-membrane protein FUNDC1 mediates hypoxia-induced mitophagy in mammalian cells. *Nat Cell Biol* 2012; **14**: 177-85
- 356 Chen G, Han Z, Feng D, et al. A regulatory signaling loop comprising the PGAM5 phosphatase and CK2 controls receptor-mediated mitophagy. *Mol Cell* 2014; **54**: 362-77

- 357 Murakawa T, Yamaguchi O, Hashimoto A, et al. Bcl-2-like protein 13 is a mammalian Atg32 homologue that mediates mitophagy and mitochondrial fragmentation. *Nat Commun* 2015; **6**: 7527
- 358 Schapira AH. Mitochondria in the aetiology and pathogenesis of Parkinson's disease. *Lancet Neurol* 2008; **7**: 97-109
- 359 Valente EM, Abou-Sleiman PM, Caputo V, et al. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science* 2004; **304**: 1158-60
- 360 Kitada T, Asakawa S, Hattori N, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* 1998; **392**: 605-8
- 361 Park J, Lee SB, Lee S, et al. Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. *Nature* 2006; **441**: 1157-61
- 362 Clark IE, Dodson MW, Jiang C, et al. Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. *Nature* 2006; **441**: 1162-6
- 363 Deas E, Plun-Favreau H, Gandhi S, et al. PINK1 cleavage at position A103 by the mitochondrial protease PARL. *Hum Mol Genet* 2011; **20**: 867-79
- 364 Jin SM, Lazarou M, Wang C, Kane LA, Narendra DP, Youle RJ. Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. *J Cell Biol* 2010; **191**: 933-42
- 365 Lin W, Kang UJ. Characterization of PINK1 processing, stability, and subcellular localization. *J Neurochem* 2008; **106**: 464-74
- 366 Yamano K, Youle RJ. PINK1 is degraded through the N-end rule pathway. *Autophagy* 2013; **9**: 1758-69
- 367 Okatsu K, Uno M, Koyano F, et al. A dimeric PINK1-containing complex on depolarized mitochondria stimulates Parkin recruitment. *J Biol Chem* 2013; **288**: 36372-84
- 368 Narendra D, Tanaka A, Suen DF, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J Cell Biol* 2008; **183**: 795-803
- 369 Matsuda N, Sato S, Shiba K, et al. PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. *J Cell Biol* 2010; **189**: 211-21
- 370 Okatsu K, Koyano F, Kimura M, et al. Phosphorylated ubiquitin chain is the genuine Parkin receptor. *J Cell Biol* 2015; **209**: 111-28
- 371 Shiba-Fukushima K, Arano T, Matsumoto G, et al. Phosphorylation of mitochondrial polyubiquitin by PINK1 promotes Parkin mitochondrial tethering. *PLoS Genet* 2014; **10**: e1004861
- 372 Koyano F, Okatsu K, Kosako H, et al. Ubiquitin is phosphorylated by PINK1 to activate parkin. *Nature* 2014; **510**: 162-6
- 373 Kane LA, Lazarou M, Fogel AI, et al. PINK1 phosphorylates ubiquitin to activate Parkin E3 ubiquitin ligase activity. *J Cell Biol* 2014; **205**: 143-53
- 374 Geisler S, Holmstrom KM, Skujat D, et al. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat Cell Biol* 2010; **12**: 119-31
- 375 Sarraf SA, Raman M, Guarani-Pereira V, et al. Landscape of the PARKIN-dependent ubiquitylome in response to mitochondrial depolarization. *Nature* 2013; **496**: 372-6

- 376 Lee JY, Nagano Y, Taylor JP, Lim KL, Yao TP. Disease-causing mutations in parkin impair mitochondrial ubiquitination, aggregation, and HDAC6-dependent mitophagy. *J Cell Biol* 2010; **189**: 671-9
- 377 Narendra D, Kane LA, Hauser DN, Fearnley IM, Youle RJ. p62/SQSTM1 is required for Parkin-induced mitochondrial clustering but not mitophagy; VDAC1 is dispensable for both. *Autophagy* 2010; **6**: 1090-106
- 378 Lazarou M, Sliter DA, Kane LA, et al. The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy. *Nature* 2015; **524**: 309-14
- 379 Heo JM, Ordureau A, Paulo JA, Rinehart J, Harper JW. The PINK1-PARKIN Mitochondrial Ubiquitylation Pathway Drives a Program of OPTN/NDP52 Recruitment and TBK1 Activation to Promote Mitophagy. *Mol Cell* 2015; **60**: 7-20
- 380 Richter B, Sliter DA, Herhaus L, et al. Phosphorylation of OPTN by TBK1 enhances its binding to Ub chains and promotes selective autophagy of damaged mitochondria. *Proc Natl Acad Sci U S A* 2016; **113**: 4039-44
- 381 Dolman NJ, Chambers KM, Mandavilli B, Batchelor RH, Janes MS. Tools and techniques to measure mitophagy using fluorescence microscopy. *Autophagy* 2013; **9**: 1653-62
- 382 Klionsky DJ, Abdel-Aziz AK, Abdelfatah S, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (4th edition)(1). *Autophagy* 2021; **17**: 1-382
- 383 Katayama H, Kogure T, Mizushima N, Yoshimori T, Miyawaki A. A sensitive and quantitative technique for detecting autophagic events based on lysosomal delivery. *Chem Biol* 2011; **18**: 1042-52
- 384 Yoshii SR, Mizushima N. Monitoring and Measuring Autophagy. *Int J Mol Sci* 2017; **18**
- 385 Zhang X, Yan H, Yuan Y, et al. Cerebral ischemia-reperfusion-induced autophagy protects against neuronal injury by mitochondrial clearance. *Autophagy* 2013; **9**: 1321-33
- 386 Yuan Y, Zheng Y, Zhang X, et al. BNIP3L/NIX-mediated mitophagy protects against ischemic brain injury independent of PARK2. *Autophagy* 2017; **13**: 1754-66
- 387 Zhang X, Yuan Y, Jiang L, et al. Endoplasmic reticulum stress induced by tunicamycin and thapsigargin protects against transient ischemic brain injury: Involvement of PARK2-dependent mitophagy. *Autophagy* 2014; **10**: 1801-13
- 388 Shen Z, Zheng Y, Wu J, et al. PARK2-dependent mitophagy induced by acidic postconditioning protects against focal cerebral ischemia and extends the reperfusion window. *Autophagy* 2017; **13**: 473-85
- 389 Carloni S, Buonocore G, Balduini W. Protective role of autophagy in neonatal hypoxia-ischemia induced brain injury. *Neurobiol Dis* 2008; **32**: 329-39
- 390 Ginet V, Puyal J, Clarke PG, Truttmann AC. Enhancement of autophagic flux after neonatal cerebral hypoxia-ischemia and its region-specific relationship to apoptotic mechanisms. *Am J Pathol* 2009; **175**: 1962-74
- 391 Koike M, Shibata M, Tadakoshi M, et al. Inhibition of autophagy prevents hippocampal pyramidal neuron death after hypoxic-ischemic injury. *Am J Pathol* 2008; **172**: 454-69
- 392 Demarest TG, Waite EL, Kristian T, et al. Sex-dependent mitophagy and neuronal death following rat neonatal hypoxia-ischemia. *Neuroscience* 2016; **335**: 103-13
- 393 Nair S, Leverin AL, Rocha-Ferreira E, et al. Induction of Mitochondrial Fragmentation and Mitophagy after Neonatal Hypoxia-Ischemia. *Cells* 2022; **11**

- 394 Quiros PM, Langer T, Lopez-Otin C. New roles for mitochondrial proteases in health, ageing and disease. *Nat Rev Mol Cell Biol* 2015; **16**: 345-59
- 395 Nouri K, Feng Y, Schimmer AD. Mitochondrial ClpP serine protease-biological function and emerging target for cancer therapy. *Cell Death Dis* 2020; **11**: 841
- 396 Cole A, Wang Z, Coyaude E, et al. Inhibition of the Mitochondrial Protease ClpP as a Therapeutic Strategy for Human Acute Myeloid Leukemia. *Cancer Cell* 2015; **27**: 864-76
- 397 Larkin K, Byrd JC. Antagonizing ClpP: A New Power Play in Targeted Therapy for AML. *Cancer Cell* 2015; **27**: 747-9
- 398 Ishizawa J, Zarabi SF, Davis RE, et al. Mitochondrial ClpP-Mediated Proteolysis Induces Selective Cancer Cell Lethality. *Cancer Cell* 2019; **35**: 721-37 e9
- 399 Jenkinson EM, Rehman AU, Walsh T, et al. Perrault syndrome is caused by recessive mutations in CLPP, encoding a mitochondrial ATP-dependent chambered protease. *Am J Hum Genet* 2013; **92**: 605-13
- 400 Greene AW, Grenier K, Aguilera MA, et al. Mitochondrial processing peptidase regulates PINK1 processing, import and Parkin recruitment. *EMBO Rep* 2012; **13**: 378-85
- 401 Zhang S, Che L, He C, et al. Drp1 and RB interaction to mediate mitochondria-dependent necroptosis induced by cadmium in hepatocytes. *Cell Death Dis* 2019; **10**: 523
- 402 Chen W, Zhou Z, Li L, et al. Diverse sequence determinants control human and mouse receptor interacting protein 3 (RIP3) and mixed lineage kinase domain-like (MLKL) interaction in necroptotic signaling. *J Biol Chem* 2013; **288**: 16247-61
- 403 Wang Z, Jiang H, Chen S, Du F, Wang X. The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. *Cell* 2012; **148**: 228-43
- 404 Yang Z, Wang Y, Zhang Y, et al. RIP3 targets pyruvate dehydrogenase complex to increase aerobic respiration in TNF-induced necroptosis. *Nat Cell Biol* 2018; **20**: 186-97
- 405 Lin Y, Choksi S, Shen HM, et al. Tumor necrosis factor-induced nonapoptotic cell death requires receptor-interacting protein-mediated cellular reactive oxygen species accumulation. *J Biol Chem* 2004; **279**: 10822-8
- 406 Vanlangenakker N, Vanden Berghe T, Bogaert P, et al. cIAP1 and TAK1 protect cells from TNF-induced necrosis by preventing RIP1/RIP3-dependent reactive oxygen species production. *Cell Death Differ* 2011; **18**: 656-65
- 407 Tait SW, Oberst A, Quarato G, et al. Widespread mitochondrial depletion via mitophagy does not compromise necroptosis. *Cell Rep* 2013; **5**: 878-85
- 408 Basit F, van Oppen LM, Schockel L, et al. Mitochondrial complex I inhibition triggers a mitophagy-dependent ROS increase leading to necroptosis and ferroptosis in melanoma cells. *Cell Death Dis* 2017; **8**: e2716
- 409 Mizumura K, Cloonan SM, Nakahira K, et al. Mitophagy-dependent necroptosis contributes to the pathogenesis of COPD. *J Clin Invest* 2014; **124**: 3987-4003
- 410 Wang Y, Nartiss Y, Steipe B, McQuibban GA, Kim PK. ROS-induced mitochondrial depolarization initiates PARK2/PARKIN-dependent mitochondrial degradation by autophagy. *Autophagy* 2012; **8**: 1462-76

- 411 Xiao B, Goh JY, Xiao L, Xian H, Lim KL, Liou YC. Reactive oxygen species trigger Parkin/PINK1 pathway-dependent mitophagy by inducing mitochondrial recruitment of Parkin. *J Biol Chem* 2017; **292**: 16697-708
- 412 Tremblay RG, Sikorska M, Sandhu JK, Lanthier P, Ribocco-Lutkiewicz M, Bani-Yaghoub M. Differentiation of mouse Neuro 2A cells into dopamine neurons. *J Neurosci Methods* 2010; **186**: 60-7
- 413 De Girolamo LA, Hargreaves AJ, Billett EE. Protection from MPTP-induced neurotoxicity in differentiating mouse N2a neuroblastoma cells. *J Neurochem* 2001; **76**: 650-60
- 414 Tasca CI, Dal-Cim T, Cimarosti H. In vitro oxygen-glucose deprivation to study ischemic cell death. *Methods Mol Biol* 2015; **1254**: 197-210
- 415 Yu Z, Liu N, Li Y, Xu J, Wang X. Neuroglobin overexpression inhibits oxygen-glucose deprivation-induced mitochondrial permeability transition pore opening in primary cultured mouse cortical neurons. *Neurobiol Dis* 2013; **56**: 95-103
- 416 Zhao H, Mitchell S, Koumpa S, et al. Heme Oxygenase-1 Mediates Neuroprotection Conferred by Argon in Combination with Hypothermia in Neonatal Hypoxia-Ischemia Brain Injury. *Anesthesiology* 2016; **125**: 180-92
- 417 Takahashi N, Duprez L, Grootjans S, et al. Necrostatin-1 analogues: critical issues on the specificity, activity and in vivo use in experimental disease models. *Cell Death Dis* 2012; **3**: e437
- 418 Christofferson DE, Li Y, Hitomi J, et al. A novel role for RIP1 kinase in mediating TNFalpha production. *Cell Death Dis* 2012; **3**: e320
- 419 Mandal P, Berger SB, Pillay S, et al. RIP3 induces apoptosis independent of pronecrotic kinase activity. *Mol Cell* 2014; **56**: 481-95
- 420 Mizushima N, Yoshimori T. How to interpret LC3 immunoblotting. *Autophagy* 2007; **3**: 542-5
- 421 O'Keefe EP. siRNAs and shRNAs: Tools for Protein Knockdown by Gene Silencing. . *MATER METHODS* 2013; **3**: 197
- 422 Pratt AJ, MacRae IJ. The RNA-induced silencing complex: a versatile gene-silencing machine. *J Biol Chem* 2009; **284**: 17897-901
- 423 Liao PC, Bergamini C, Fato R, Pon LA, Pallotti F. Isolation of mitochondria from cells and tissues. *Methods Cell Biol* 2020; **155**: 3-31
- 424 Frezza C, Cipolat S, Scorrano L. Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts. *Nat Protoc* 2007; **2**: 287-95
- 425 Clayton DA, Shadel GS. Isolation of mitochondria from tissue culture cells. *Cold Spring Harb Protoc* 2014; **2014**: pdb prot080002
- 426 Becton DaC. Introduction to Flow Cytometry: A Learning Guide. . In: Biosciences B, ed, 2002
- 427 Adan A, Alizada G, Kiraz Y, Baran Y, Nalbant A. Flow cytometry: basic principles and applications. *Crit Rev Biotechnol* 2017; **37**: 163-76
- 428 Manohar SM, Shah P, Nair A. Flow cytometry: principles, applications and recent advances. *Bioanalysis* 2021; **13**: 181-98
- 429 Solaini G, Sgarbi G, Lenaz G, Baracca A. Evaluating mitochondrial membrane potential in cells. *Biosci Rep* 2007; **27**: 11-21

430 Crowley LC, Christensen ME, Waterhouse NJ. Measuring Mitochondrial Transmembrane Potential by TMRE Staining. *Cold Spring Harb Protoc* 2016; **2016**

431 Andree HA, Reutelingsperger CP, Hauptmann R, Hemker HC, Hermens WT, Willems GM. Binding of vascular anticoagulant alpha (VAC alpha) to planar phospholipid bilayers. *J Biol Chem* 1990; **265**: 4923-8

432 Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* 1994; **84**: 1415-20

433 Tominaga HI, M.; Ohseto, F.; Sasamoto, K.; Hamamoto, T.; Suzuki, K.; Watanabe, M. A water-soluble tetrazolium salt useful for colorimetric cell viability assay. *Anal Commun* 1999: 47-50

434 Kim JY, Grunke SD, Levites Y, Golde TE, Jankowsky JL. Intracerebroventricular viral injection of the neonatal mouse brain for persistent and widespread neuronal transduction. *J Vis Exp* 2014: 51863

435 Seibenhener ML, Wooten MC. Use of the Open Field Maze to measure locomotor and anxiety-like behavior in mice. *J Vis Exp* 2015: e52434

436 Re DB, Le Verche V, Yu C, et al. Necroptosis drives motor neuron death in models of both sporadic and familial ALS. *Neuron* 2014; **81**: 1001-8

437 Ofengeim D, Ito Y, Najafov A, et al. Activation of necroptosis in multiple sclerosis. *Cell Rep* 2015; **10**: 1836-49

438 Iannielli A, Bido S, Folladori L, et al. Pharmacological Inhibition of Necroptosis Protects from Dopaminergic Neuronal Cell Death in Parkinson's Disease Models. *Cell Rep* 2018; **22**: 2066-79

439 Zhao H, Jaffer T, Eguchi S, Wang Z, Linkermann A, Ma D. Role of necroptosis in the pathogenesis of solid organ injury. *Cell Death Dis* 2015; **6**: e1975

440 Degterev A, Maki JL, Yuan J. Activity and specificity of necrostatin-1, small-molecule inhibitor of RIP1 kinase. *Cell Death Differ* 2013; **20**: 366

441 Teng X, Degterev A, Jagtap P, et al. Structure-activity relationship study of novel necroptosis inhibitors. *Bioorg Med Chem Lett* 2005; **15**: 5039-44

442 Murphy JM, Zhang Q, Young SN, et al. A robust methodology to subclassify pseudokinases based on their nucleotide-binding properties. *Biochem J* 2014; **457**: 323-34

443 Garnish SE, Meng Y, Koide A, et al. Conformational interconversion of MLKL and disengagement from RIPK3 precede cell death by necroptosis. *Nat Commun* 2021; **12**: 2211

444 Zhang Y, Liu J, Yu D, et al. The MLKL kinase-like domain dimerization is an indispensable step of mammalian MLKL activation in necroptosis signaling. *Cell Death Dis* 2021; **12**: 638

445 Murai S, Yamaguchi Y, Shirasaki Y, et al. A FRET biosensor for necroptosis uncovers two different modes of the release of DAMPs. *Nat Commun* 2018; **9**: 4457

446 Samson AL, Zhang Y, Geoghegan ND, et al. MLKL trafficking and accumulation at the plasma membrane control the kinetics and threshold for necroptosis. *Nat Commun* 2020; **11**: 3151

447 Liu S, Liu H, Johnston A, et al. MLKL forms disulfide bond-dependent amyloid-like polymers to induce necroptosis. *Proc Natl Acad Sci U S A* 2017; **114**: E7450-E9

448 Liao DS, L.; Liu, W.; He, S.; Wang, X.; Lei, X. Necrosulfonamide inhibits necroptosis by selectively targeting the mixed lineage kinase domain-like protein. *Med Chem Commun* 2014; **5**: 333-7

449 Alberts BJ, A.; Lewis, J. et al. . The Mitochondrion. *Molecular Biology of the Cell*. New York: Garland Science, 2002

450 Yoo SM, Jung YK. A Molecular Approach to Mitophagy and Mitochondrial Dynamics. *Mol Cells* 2018; **41**: 18-26

451 Chen M, Chen Z, Wang Y, et al. Mitophagy receptor FUNDC1 regulates mitochondrial dynamics and mitophagy. *Autophagy* 2016; **12**: 689-702

452 Hu W, Wu X, Yu D, et al. Regulation of JNK signaling pathway and RIPK3/AIF in necroptosis-mediated global cerebral ischemia/reperfusion injury in rats. *Exp Neurol* 2020; **331**: 113374

453 Liu T, Zhao DX, Cui H, et al. Therapeutic hypothermia attenuates tissue damage and cytokine expression after traumatic brain injury by inhibiting necroptosis in the rat. *Sci Rep* 2016; **6**: 24547

454 Sureshbabu A, Patino E, Ma KC, et al. RIPK3 promotes sepsis-induced acute kidney injury via mitochondrial dysfunction. *JCI Insight* 2018; **3**

455 Remijnsen Q, Goossens V, Grootjans S, et al. Depletion of RIPK3 or MLKL blocks TNF-driven necroptosis and switches towards a delayed RIPK1 kinase-dependent apoptosis. *Cell Death Dis* 2014; **5**: e1004

456 Jiang X, Mu D, Biran V, et al. Activated Src kinases interact with the N-methyl-D-aspartate receptor after neonatal brain ischemia. *Ann Neurol* 2008; **63**: 632-41

457 Ni Y, Gu WW, Liu ZH, et al. RIP1K Contributes to Neuronal and Astrocytic Cell Death in Ischemic Stroke via Activating Autophagic-lysosomal Pathway. *Neuroscience* 2018; **371**: 60-74

458 Wang X, Karlsson JO, Zhu C, Bahr BA, Hagberg H, Blomgren K. Caspase-3 activation after neonatal rat cerebral hypoxia-ischemia. *Biol Neonate* 2001; **79**: 172-9

459 Hu BR, Liu CL, Ouyang Y, Blomgren K, Siesjo BK. Involvement of caspase-3 in cell death after hypoxia-ischemia declines during brain maturation. *J Cereb Blood Flow Metab* 2000; **20**: 1294-300

460 Yang T, Zhuang L, Rei Fidalgo AM, et al. Xenon and sevoflurane provide analgesia during labor and fetal brain protection in a perinatal rat model of hypoxia-ischemia. *PLoS One* 2012; **7**: e37020

461 Joly LM, Mucignat V, Mariani J, Plotkine M, Charriaut-Marlangue C. Caspase inhibition after neonatal ischemia in the rat brain. *J Cereb Blood Flow Metab* 2004; **24**: 124-31

462 Zhu C, Wang X, Xu F, et al. The influence of age on apoptotic and other mechanisms of cell death after cerebral hypoxia-ischemia. *Cell Death Differ* 2005; **12**: 162-76

463 Hu C, Huang Y, Wu L, et al. Apoptosis and necroptosis occur in the different brain regions of hippocampus in a rat model of hypoxia asphyxia. *Int J Neurosci* 2021; **131**: 843-53

464 Sanderson TH, Raghunayakula S, Kumar R. Neuronal hypoxia disrupts mitochondrial fusion. *Neuroscience* 2015; **301**: 71-8

465 Baburamani AA, Hurling C, Stolp H, et al. Mitochondrial Optic Atrophy (OPA) 1 Processing Is Altered in Response to Neonatal Hypoxic-Ischemic Brain Injury. *Int J Mol Sci* 2015; **16**: 22509-26

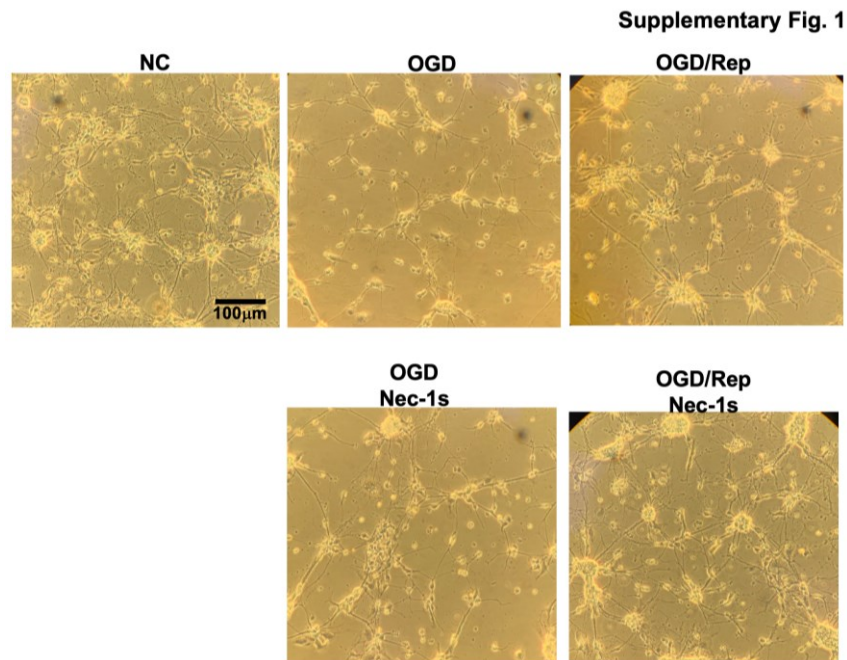
466 Grohm J, Kim SW, Mamrak U, et al. Inhibition of Drp1 provides neuroprotection in vitro and in vivo. *Cell Death Differ* 2012; **19**: 1446-58

467 Lu Q, Harris VA, Kumar S, Mansour HM, Black SM. Autophagy in neonatal hypoxia ischemic brain is associated with oxidative stress. *Redox Biol* 2015; **6**: 516-23

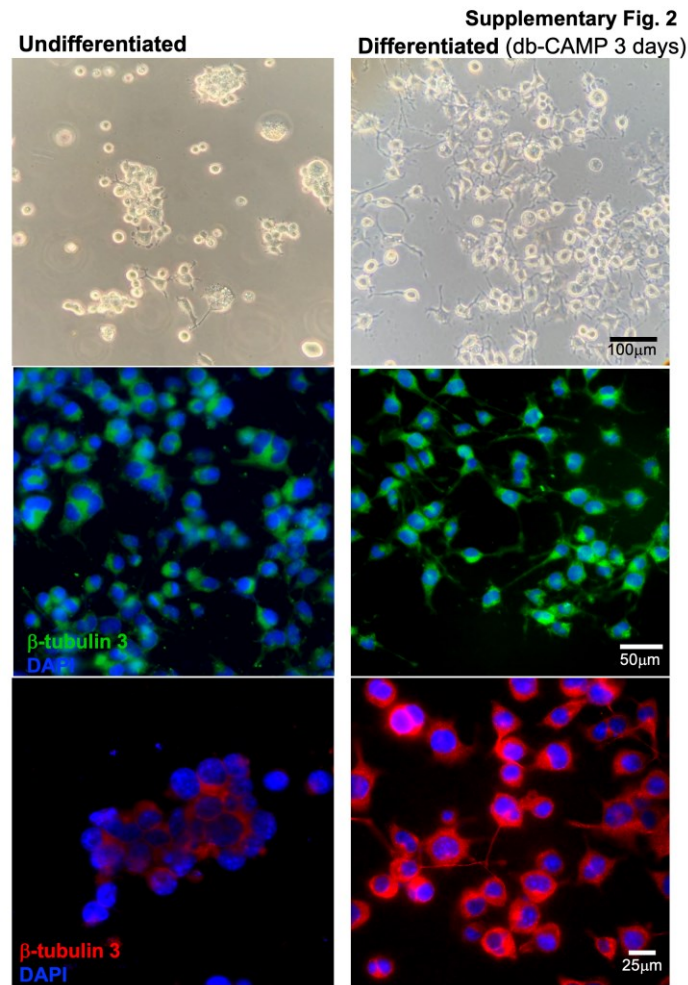
- 468 Xu Y, Tian Y, Tian Y, Li X, Zhao P. Autophagy activation involved in hypoxic-ischemic brain injury induces cognitive and memory impairment in neonatal rats. *J Neurochem* 2016; **139**: 795-805
- 469 Shi RY, Zhu SH, Li V, Gibson SB, Xu XS, Kong JM. BNIP3 interacting with LC3 triggers excessive mitophagy in delayed neuronal death in stroke. *CNS Neurosci Ther* 2014; **20**: 1045-55
- 470 Liu M, Li H, Yang R, Ji D, Xia X. GSK872 and necrostatin-1 protect retinal ganglion cells against necroptosis through inhibition of RIP1/RIP3/MLKL pathway in glutamate-induced retinal excitotoxic model of glaucoma. *J Neuroinflammation* 2022; **19**: 262
- 471 Chen Y, Zhang L, Yu H, et al. Necrostatin-1 Improves Long-term Functional Recovery Through Protecting Oligodendrocyte Precursor Cells After Transient Focal Cerebral Ischemia in Mice. *Neuroscience* 2018; **371**: 229-41
- 472 Lu W, Sun J, Yoon JS, et al. Mitochondrial Protein PGAM5 Regulates Mitophagic Protection against Cell Necroptosis. *PLoS One* 2016; **11**: e0147792
- 473 Scholz J, Niibori Y, P WF, J PL. Rotarod training in mice is associated with changes in brain structure observable with multimodal MRI. *Neuroimage* 2015; **107**: 182-9
- 474 Bureau G, Carrier M, Lebel M, Cyr M. Intrastriatal inhibition of extracellular signal-regulated kinases impaired the consolidation phase of motor skill learning. *Neurobiol Learn Mem* 2010; **94**: 107-15
- 475 Yang G, Pan F, Gan WB. Stably maintained dendritic spines are associated with lifelong memories. *Nature* 2009; **462**: 920-4
- 476 Knierim J. Chapter 4: Basal Ganglia *Neuroscience Online*: Department of Neurobiology and Anatomy, McGovern Medical School at UTHealth, 2020
- 477 Calabresi P, Picconi B, Tozzi A, Ghiglieri V, Di Filippo M. Direct and indirect pathways of basal ganglia: a critical reappraisal. *Nat Neurosci* 2014; **17**: 1022-30
- 478 Rutherford MA, Pennock JM, Counsell SJ, et al. Abnormal magnetic resonance signal in the internal capsule predicts poor neurodevelopmental outcome in infants with hypoxic-ischemic encephalopathy. *Pediatrics* 1998; **102**: 323-8
- 479 Martinez-Biarge M, Diez-Sebastian J, Kapellou O, et al. Predicting motor outcome and death in term hypoxic-ischemic encephalopathy. *Neurology* 2011; **76**: 2055-61
- 480 Calhoon GG, Tye KM. Resolving the neural circuits of anxiety. *Nat Neurosci* 2015; **18**: 1394-404
- 481 Hale MW, Hay-Schmidt A, Mikkelsen JD, Poulsen B, Shekhar A, Lowry CA. Exposure to an open-field arena increases c-Fos expression in a distributed anxiety-related system projecting to the basolateral amygdaloid complex. *Neuroscience* 2008; **155**: 659-72
- 482 Zhang Y, Su SS, Zhao S, et al. RIP1 autophosphorylation is promoted by mitochondrial ROS and is essential for RIP3 recruitment into necrosome. *Nat Commun* 2017; **8**: 14329
- 483 Takeda K, Komuro Y, Hayakawa T, et al. Mitochondrial phosphoglycerate mutase 5 uses alternate catalytic activity as a protein serine/threonine phosphatase to activate ASK1. *Proc Natl Acad Sci U S A* 2009; **106**: 12301-5
- 484 Liang MZ, Ke TL, Chen L. Mitochondrial Protein PGAM5 Emerges as a New Regulator in Neurological Diseases. *Front Mol Neurosci* 2021; **14**: 730604

- 485 Wu H, Xue D, Chen G, et al. The BCL2L1 and PGAM5 axis defines hypoxia-induced receptor-mediated mitophagy. *Autophagy* 2014; **10**: 1712-25
- 486 Sekine S, Kanamaru Y, Koike M, et al. Rhomboid protease PARL mediates the mitochondrial membrane potential loss-induced cleavage of PGAM5. *J Biol Chem* 2012; **287**: 34635-45
- 487 Yan C, Gong L, Chen L, et al. PHB2 (prohibitin 2) promotes PINK1-PRKN/Parkin-dependent mitophagy by the PARL-PGAM5-PINK1 axis. *Autophagy* 2020; **16**: 419-34
- 488 Lu W, Karuppagounder SS, Springer DA, et al. Genetic deficiency of the mitochondrial protein PGAM5 causes a Parkinson's-like movement disorder. *Nat Commun* 2014; **5**: 4930
- 489 Rambold AS, Kostecky B, Elia N, Lippincott-Schwartz J. Tubular network formation protects mitochondria from autophagosomal degradation during nutrient starvation. *Proc Natl Acad Sci U S A* 2011; **108**: 10190-5
- 490 Burman JL, Pickles S, Wang C, et al. Mitochondrial fission facilitates the selective mitophagy of protein aggregates. *J Cell Biol* 2017; **216**: 3231-47
- 491 Hamacher-Brady A, Brady NR, Logue SE, et al. Response to myocardial ischemia/reperfusion injury involves Bnip3 and autophagy. *Cell Death Differ* 2007; **14**: 146-57
- 492 Zhang H, Bosch-Marce M, Shimoda LA, et al. Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. *J Biol Chem* 2008; **283**: 10892-903
- 493 Quinn PMJ, Moreira PI, Ambrosio AF, Alves CH. PINK1/PARKIN signalling in neurodegeneration and neuroinflammation. *Acta Neuropathol Commun* 2020; **8**: 189
- 494 Lucking CB, Abbas N, Durr A, et al. Homozygous deletions in parkin gene in European and North African families with autosomal recessive juvenile parkinsonism. The European Consortium on Genetic Susceptibility in Parkinson's Disease and the French Parkinson's Disease Genetics Study Group. *Lancet* 1998; **352**: 1355-6
- 495 Kumazawa R, Tomiyama H, Li Y, et al. Mutation analysis of the PINK1 gene in 391 patients with Parkinson disease. *Arch Neurol* 2008; **65**: 802-8
- 496 Ye X, Sun X, Starovoytov V, Cai Q. Parkin-mediated mitophagy in mutant hAPP neurons and Alzheimer's disease patient brains. *Hum Mol Genet* 2015; **24**: 2938-51
- 497 Frank D, Vaux DL, Murphy JM, Vince JE, Lindqvist LM. Activated MLKL attenuates autophagy following its translocation to intracellular membranes. *J Cell Sci* 2019; **132**
- 498 Chen Y, Balasubramaniyan V, Peng J, et al. Isolation and culture of rat and mouse oligodendrocyte precursor cells. *Nat Protoc* 2007; **2**: 1044-51
- 499 Sariyer IK. Transfection of Neuronal Cultures. *Methods Mol Biol* 2021; **2311**: 147-53
- 500 Perkins EJ. Protein Tags. In: Addgene, ed. *Plasmids 101 A Desktop Resource*: Addgene 2014; 121-5
- 501 Kimple ME, Brill AL, Pasker RL. Overview of affinity tags for protein purification. *Curr Protoc Protein Sci* 2013; **73**: 9 1-9 23
- 502 Dagda RK, Cherra SJ, 3rd, Kulich SM, Tandon A, Park D, Chu CT. Loss of PINK1 function promotes mitophagy through effects on oxidative stress and mitochondrial fission. *J Biol Chem* 2009; **284**: 13843-55
- 503 Wu J, Huang Z, Ren J, et al. Mkl1 knockout mice demonstrate the indispensable role of Mkl1 in necroptosis. *Cell Res* 2013; **23**: 994-1006

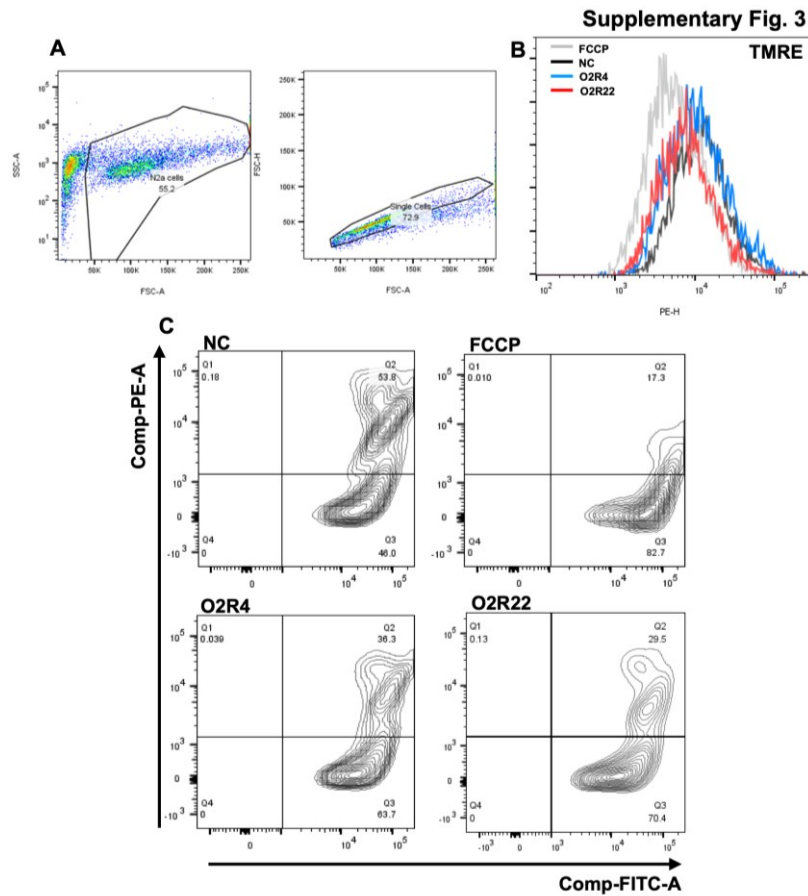
- 504 Chen H, Fang Y, Wu J, et al. RIPK3-MLKL-mediated necroinflammation contributes to AKI progression to CKD. *Cell Death Dis* 2018; **9**: 878
- 505 Guo R, Jia X, Ding Z, et al. Loss of MLKL ameliorates liver fibrosis by inhibiting hepatocyte necroptosis and hepatic stellate cell activation. *Theranostics* 2022; **12**: 5220-36
- 506 Lule S, Wu L, Sarro-Schwartz A, et al. Cell-specific activation of RIPK1 and MLKL after intracerebral hemorrhage in mice. *J Cereb Blood Flow Metab* 2021; **41**: 1623-33
- 507 Morshead CM, Reynolds BA, Craig CG, et al. Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. *Neuron* 1994; **13**: 1071-82
- 508 Samanta J, Grund EM, Silva HM, Lafaille JJ, Fishell G, Salzer JL. Inhibition of Gli1 mobilizes endogenous neural stem cells for remyelination. *Nature* 2015; **526**: 448-52
- 509 Moore CB, Guthrie EH, Huang MT, Taxman DJ. Short hairpin RNA (shRNA): design, delivery, and assessment of gene knockdown. *Methods Mol Biol* 2010; **629**: 141-58
- 510 Savage S, Ma D. III. Animal behaviour testing: memory. *Br J Anaesth* 2014; **113**: 6-9
- 511 Zhao P, Peng L, Li L, Xu X, Zuo Z. Isoflurane preconditioning improves long-term neurologic outcome after hypoxic-ischemic brain injury in neonatal rats. *Anesthesiology* 2007; **107**: 963-70

Supplementary Figures

Supplementary Figure 1. Bright field microscope images of primary neurons from embryonic day17 mouse foetus. The primary neurons were cultured in complete neurobasal medium for five days prior to 1 hr oxygen-glucose deprivation (OGD) or 1hr OGD plus 1hr reperfusion (OGD/Rep), with or without 1hr treatment of necrostatin-1s (Nec-1s, 10µM). OGD or OGD/Rep reduced the number of viable neurons when compared to naïve control (NC) and Nec-1s ameliorated the neuronal loss. Magnification x20, scale bar 100µm.



Supplementary Figure 2. Differentiation of mouse neuroblastoma Neuro-2a cells. Light microscopy and fluorescence microscopy showing Neuro-2a cells in undifferentiated state and after 3-day differentiation with dibutyl cyclc-AMP (db-cAMP, 1mM). Neuro-2a cells were labelled with the neuron-specific cytoskeletal protein β -tubulin III (or Tuj1). Upper, magnification x20, scale bar 100µm; middle, magnification x40, scale bar 50µm; lower, magnification x63, scale bar 25µm.



Supplementary Fig.3. Flow cytometric analysis of mitochondrial membrane potential in Neuro-2a cells following oxygen-glucose deprivation and reperfusion (OGD/Rep). (A) Gating strategies used to identify singlet Neuro-2a cells for subsequent analysis. **(B)** TMRE staining of Neuro-2a cells after OGD/Rep or FCCP treatment and detection of fluorescence intensity with PE channel. **(C)** JC-10 analysis of Neuro-2a cells after OGD/Rep or FCCP treatment and detection of fluorescence intensity with compensated FITC and PE channels.

Publications during PhD study

Lasting effects of general anesthetics on the brain in the young and elderly: “mixed picture” of neurotoxicity, neuroprotection and cognitive impairments. **Wu L**, Zhao H, Weng H, Ma D. Invited Review. *J Anesth*. 2019 Apr;33(2):321-335.

Dexmedetomidine inhibits astrocyte pyroptosis and subsequently protects the brain in in vitro and in vivo models of sepsis. Sun YB, Zhao H, Mu DL, Zhang W, Cui J, **Wu L**, Alam A, Wang DX, Ma D. *Cell Death Dis*. 2019 Feb 18;10(3):167.

The Role of Neutrophil NETosis in Organ Injury: Novel Inflammatory Cell Death Mechanisms. Zhen C, Zhao H, Wu L, Alam A, Eguchi S, Hao W, Ma D. *Inflammation*. 2020 Dec;43(6):2021-32.

Sevoflurane ameliorates schizophrenia in a mouse model and patients: pre-clinical and clinical feasibility study. Zhao T, Shi Z, Ling N, Qin J, Zhou Q, **Wu L**, Wang Y, Lin C, Ma D, Song X. *Curr Neuropharmacol*. 2022 Mar. doi: 10.2174/1570159X20666220310115846.

General anesthetic action profile on the human prefrontal cortex cells through comprehensive single-cell RNA-seq analysis. Chang E*, Wang Y*, Zhu R*, **Wu L***, Yang Y, Zeng S, Li N, Ruan X, Sun M, Zhang W et al. *iScience*. 2023;26(4):106534. * authors with equal contributions.

Regulated cell death and inflammasome activation in gut injury following traumatic surgery in vitro and in vivo: implication for postoperative death due to multiorgan dysfunction. **Wu L**, Zhao H, Zhang M, Sun Q, Chang E, Li X, Ouyang W, Le Y, Ma D. *Cell Death Discovery*. 2023. Accepted.

Permissions to re-use copyrighted works from the publishers

Sarnat and Sarnat staging of HIE, from Appendix D of Queensland Clinical Guidelines: Hypoxic ischaemia encephalopathy (HIE). *Guideline No. MN21.11-V11-R26. Queensland Health. 2021. Reference number 33.*

Recommended citation: Queensland Clinical Guidelines. Hypoxic ischaemic encephalopathy (HIE). Guideline No. MN21.11-V11-R26. Queensland Health. 2021 Available from:
<http://www.health.qld.gov.au/qcg>

© State of Queensland (Queensland Health) 2022.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives V4.0 International licence. In essence, you are free to copy and communicate the work in its current form for non-commercial purposes, as long as you attribute Queensland Clinical Guidelines, Queensland Health and abide by the licence terms. You may not alter or adapt the work in any way. To view a copy of this licence, visit <https://creativecommons.org/licenses/by-nc-nd/4.0/deed.en>

For further information, contact Queensland Clinical Guidelines, RBWH Post Office, Herston Qld 4029, email Guidelines@health.qld.gov.au. For permissions beyond the scope of this licence, contact: Intellectual Property Officer, Queensland Health, GPO Box 48, Brisbane Qld 4001, email ip_officer@health.qld.gov.au