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The Role of Inflammasomes in Ischemic Stroke: From Pathophysiology to Treatments

David Yang-Wei Fann

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Abstract

Stroke is the second leading cause of mortality worldwide and a major cause of long-term disability. Clinically, stroke can be classified as either ischemic or haemorrhagic. Ischemic stroke is the most common type of stroke and accounts for approximately 80% of all stroke cases. The pathophysiological processes following stroke are complex and extensive, and include bioenergetic failure, excitotoxicity, oxidative stress and inflammation, which leads to necrotic and apoptotic cell death. Recent findings have provided insight into a newly described inflammatory mechanism that may contribute to neuronal and glial cell death during cerebral ischemia known as sterile inflammation involving intracellular multi-protein complexes termed inflammasomes.

Despite neuroprotective agents decreasing neuronal cell death and infarct size under *in vitro* and *in vivo* stroke models, respectively, all such agents tested in stroke patients have failed in clinical trials. Novel potential therapies envisaged to target multiple cell injury mechanisms in the brain following cerebral ischemia include – intravenous immunoglobulin (IVIg) and intermittent fasting (IF). IVIg is a purified polyclonal immunoglobulin preparation obtained from the plasma of several thousand healthy donors. Numerous experimental studies by our laboratory demonstrated that administration of IVIg was able to significantly attenuate brain injury in mice subjected to experimental stroke. Moreover, IF is a form of dietary energy restriction and encompasses alternate periods of *ad libitum* feeding and fasting, which have been proven to decrease the development of age-related diseases. Previous experimental studies demonstrated that IF was able to significantly attenuate brain injury outcome in mice subjected to experimental stroke. However, the precise mechanism(s) in how IVIg and IF directly protect neurons and cerebral tissue from inflammasome-mediated sterile inflammation following ischemic stroke remains to be determined and is a major focus of this research thesis.

In the first study of this research thesis, we performed a comprehensive investigation into the expression patterns of NLRP1 and NLRP3 inflammasome proteins and both IL-1β and IL-18 in mouse primary cortical neurons subjected to simulated ischemia and in a model of focal ischemic stroke in C57BL/6J mice. In addition, determined whether the NLRP1 and NLRP3 inflammasome could be targeted with a Caspase-1 inhibitor and IVIg for therapeutic intervention. The study demonstrated that ischemia-like conditions increased the levels of NLRP1 and NLRP3 inflammasome proteins and both IL-1β and IL-18 in neurons and brain tissues. Moreover, Caspase-1

inhibitor and IVIg treatment protected neurons and brain tissue by a mechanism(s) involving Caspase-1 inhibition and suppression of NLRP1 and NLRP3 inflammasome activity, respectively, under *in vitro* and *in vivo* ischemic conditions.

In the second study of this research thesis, we provide evidence that the NF-κB and MAPK(s) signaling pathways are involved in regulating the expression and activation of NLRP1 and NLRP3 inflammasomes in neurons subjected to simulated ischemic conditions. This study established that activation of either the NF-κB and MAPK(s) signaling pathways are responsible for inducing the expression and activation of NLRP1 and NLRP3 inflammasomes in neurons under ischemic conditions. In addition, the present study demonstrated that pharmacological inhibition of both the NF-κB and MAPKs signaling pathways was able to directly attenuate activation of NLRP inflammasomes in neurons under ischemic conditions. Furthermore, this study provided supporting evidence that IVIg treatment was able to significantly decrease NF-κB and MAPK(s) signaling pathway activation, which decreased the expression of NLRP inflammasomes, and subsequently attenuate inflammasome activity; in addition to increasing the expression of anti-apoptotic proteins, Bcl-2 and Bcl-xL, in cortical neurons following ischemic conditions.

In the third study of this research thesis, we investigated the impact of prophylactic IF on NLRP1 and NLRP3 inflammasome activity in a model of focal ischemic stroke in C57BL/6J mice. This study demonstrated that prophylactic IF was able to significantly decrease apoptotic tissue damage by attenuating the activation of the NF-κB and MAPK(s) signaling pathways, and the expression of NLRP inflammasome proteins, and both IL-1β and IL-18; in addition to increasing the expression of anti-apoptotic proteins, Bcl-2 and Bcl-xL in ischemic brain tissues.

In summary, the findings from this research thesis provided evidence of expression and a functional role for the NLRP inflammasomes in neuronal apoptosis and cerebral tissue damage under *in vitro* and *in vivo* ischemic conditions. It was demonstrated that activation of the NF-κB and MAPK(s) signaling pathways are responsible for inducing the expression and activation of NLRP inflammasomes. Furthermore, we established that a neuroprotective effect of IVIg and IF involved suppressing NLRP inflammasome activity through a mechanism(s) associated with decreasing the NF-κB and MAPK(s) signaling pathway in ischemic conditions. Finally, it was demonstrated that another neuroprotective effect of IVIg and IF involved increasing the expression of anti-apoptotic proteins, Bcl-2 and Bcl-xL, through an unknown mechanism(s). Collectively, our findings identified

inflammasome inhibition as a novel mechanism by which IVIg and IF can protect brain cells against ischemic damage, suggesting a potential clinical benefit of therapeutic interventions that can target inflammasome activation in ischemic stroke.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Contributor	Statement of Contribution
Author – Fann D.Y (Candidate)	Wrote the Manuscript – (95%) Images Constructed – (95%) Edited Manuscript – (55%)
Author – Lee S.Y	Images Constructed – (5%)
Author – Manzanero S	Edited Manuscript – (5%)
Author – Chunduri P	Edited Manuscript – (5%)
Author – Sobey C.G	Wrote the Manuscript – (5%) Edited Manuscript – (30%)
Author – Arumugam T.V	Edited Manuscript – (5%)

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Contributor	Statement of Contribution
Author – Fann D.Y (Candidate)	Experiments Designed – (60%) Experiments Conducted – (70%) Wrote the Manuscript – (50%) Edited Manuscript – (50%)
Author – Lee S.Y	Experiments Conducted – (15%)
Author – Manzanero S	Edited Manuscript – (0.5%)
Author – Tang S.C	Edited Manuscript – (0.5%)
Author – Gelderblom M	Edited Manuscript – (0.5%)
Author – Chunduri P	Edited Manuscript – (0.5%)
Author – Bernreuther C	Edited Manuscript – (0.5%)
Author – Glatzel M	Edited Manuscript – (0.5%)
Author – Cheng Y.L	Edited Manuscript – (0.5%)
Author – Thundyil J	Edited Manuscript – (0.5%)
Author – Widiapradja A	Edited Manuscript – (0.5%)
Author – Lok K.Z	Edited Manuscript – (0.5%)
Author – Foo S.L	Experiments Conducted – (5%)
Author – Wang Y.C	Experiments Conducted – (5%)
Author – Li Y.I	Experiments Conducted – (5%)
Author – Drummond G.R	Edited Manuscript – (0.5%)
Author – Basta M	Edited Manuscript – (0.5%)
Author – Magnus T	Edited Manuscript – (0.5%)
Author – Jo D.G	Edited Manuscript – (0.5%)
Author – Mattson M.P	Wrote the Manuscript – (15%) Edited the Manuscript – (21.5%)
Author – Sobey C.G	Wrote the Manuscript – (15%) Edited the Manuscript – (21.5%)

Author – Arumugam T.V	Experiments Designed – (40%) Wrote the Manuscript – (20%)

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Contributor	**Statement of Contribution**
Fann D.Y (Candidate)	Experiments Designed – (90%) Experiments Conducted – (95%) Wrote the Manuscript – (50%) Edited the Manuscript – (50%)
Cheng Y.L	Experiments Conducted – (2.5%)
Lok K.Z	Experiments Conducted – (2.5%)
Manzanero S	Experiments Designed – (10%)
Chunduri P	Edited the Manuscript – (5%)
Arumugam T.V	Wrote the Manuscript – (50%) Edited the Manuscript – (45%)

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Contributor	**Statement of Contribution**
Fann D.Y (Candidate)	Experiments Designed – (30%) Experiments Conducted – (93.5%) Wrote the Manuscript – (50%) Edited the Manuscript – (70%)
Santro T	Experiments Conducted – (0.5%)
Manzanero S	Experiments Designed – (30%) Experiments Conducted – (0.5%)
Widiapradja A	Experiments Conducted – (5%)
Cheng Y.L	Experiments Conducted – (0.5%)
Lee S.Y	Edited the Manuscript – (0.5%)
Chunduri P	Edited the Manuscript – (0.5%)
Jo D.G	Edited the Manuscript – (0.5%)
Stranahan A.M	Edited the Manuscript – (0.5%)
Mattson M.P	Edited the Manuscript – (28%)
Arumugam T.V	Experiments Designed – (40%) Wrote the Manuscript – (50%)

Contributions by others to the thesis

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Ischemic stroke, inflammasome, intravenous immunoglobulin (IVIg), intermittent fasting, neuronal cell death

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List of Abbreviations

2-VO	2-vessel occlusion
4-VO	4-vessel occlusion
ω-3 Fas	omega-3 fatty acids
ADCC	antibody-dependent cytotoxicity
AIF	apoptosis inducing factor
AIHA	autoimmune hemolytic anemia
AL	ad libitum
AMP	adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPK	adenosine monophosphate activated protein kinase
ANOVA	analysis of variance
AP-1	activator protein-1
ASC	apoptosis-associated speck-like protein containing a caspase recruitment domain
ASICs	acid sensing ion channels
ATP	adenosine triphosphate
BBB	blood brain barrier
BCA	Bicinchoninic Acid
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BID	Bcl-2 interacting domain
BSA	bovine serum albumin
CARD	caspase recruitment domain
CaSRs	calcium-sensing receptors
CBF	cerebral blood flow
CCA	common carotid artery
CIDP	chronic inflammatory demyelinating polyneuropathy
CR	calorie reduction
CREB	cyclic AMP response element binding protein
CRIDs	cytokine release inhibitory drugs
DAG	diacylglycerol
DAMPs	damage-associated molecular patterns
DD	death domain
DEAE	diethylaminoethanol
DHA	docosahexaenoic acid
DIABLO	direct IAP-binding protein with low pl
DISC	death-inducing signalling complex
EAAT2	excitatory amino acid transporter 2
ECA	external carotid artery
eNOS	endothelial nitric oxide synthase
EPA	eicosapentaenoic acid
ERK	extracellular signal-regulated kinase
$F(ab')_2$	antigen binding fragment
FADD	Fas-associated death domain
FasL	Fas ligand
Fc	fragment crystallizable
FcγRs	Fcγ receptors
FcRn	neonatal Fc receptor

FDA	Food and Drug Administration
FGF2	fibroblast growth factor 2
FGFR1	fibroblast growth factor receptor 1
FIIND	function to find
FOXP3	forkhead box P3
GD	glucose deprivation
GPR40	G-protein-coupled receptor 40
GRP78	glucose regulated protein 78
GSTO1	glutathione-S-transferase omega 1
H_2O_2	hydrogen peroxide
HIF	hypoxia inducible factor
HIV	human immunodeficiency virus
HMGB1	high mobility group box 1
HO-1	heme oxygenase-1
Hsp70	heat shock protein 70
HTLV	human T cell lymphotropic retrovirus
ICA	internal carotid artery
ICAM-1	intercellular adhesion molecule-1
IF	intermittent fasting
IFN	interferon
IgG	immunoglobulin G
IL-1α	interleukin-1alpha
IL-1β	interleukin-1beta
IL-1R1	interleukin-1 receptor 1
IL-1ra	interleukin-1 receptor antagonist
IL-18R	interleukin-18 receptor
iNOS	inducible nitric oxide synthase
$InsP_3$	inositol triphosphate
$InsP_3$-R	inositol triphosphate receptors
IPS	ipsilateral
I/R	ischemia and reperfusion
ITAM	immunoreceptor tyrosine based activation motifs
ITIM	immunoreceptor tyrosine based inhibitory motifs
ITP	thrombocytopenic purpura
IVIg	intravenous immunoglobulin
JNK	c-Jun-N-terminal kinase
LC3B	light chain 3B
LPS	lipopolysaccharide
LRRs	leucine rich repeats
MAC	membrane attack complex
MAP2	microtubule-associated protein 2
MAPKs	mitogen activated protein kinases
MAVS	mitochondrial antiviral signalling protein
MCA	middle cerebral artery
MCAO/R	middle cerebral artery occlusion/reperfusion
MCP-1	monocyte chemoattractant protein 1
MDA	malondialdehyde
MFGE8	milk fat globule-EGF 8
MHCI	major histocompatibility class I
MMP	mitochondrial membrane potential
MMPs	matrix metalloproteinases
MPT	mitochondrial permeability transition

MSU	monosodium urate
MTPs	mitochondria transition pores
mTOR	mammalian target of rapamycin
mtTFA	mitochondrial transcription factor A
NACHT	NAIP, CIITA, HET-E and TP1
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide hydrogenated
NADPH	nicotinamide adenine dinucleotide phosphate
NBD	nucleotide-binding domain
NF-κB	nuclear factor kappa B
NK	natural killer
NLR	nod like-receptor
NLRP1	NOD (nucleotide-binding oligomerization domain)-like receptor (NLR) Pyrin domain containing 1
NLRP3	NOD (nucleotide-binding oligomerization domain)-like receptor (NLR) Pyrin domain containing 3
NMDA	N-methyl-d-aspartic acid
NO	nitric oxide
nNOS	neuronal nitric oxide synthase
NRF	nuclear respiratory factor
$O_2^{\cdot -}$	superoxide
OGD	oxygen and glucose deprivation
OH^-	hydroxyl radical
$ONOO^-$	peroxynitrite
PAMPs	pathogen-associated molecular patterns
PARP	poly (ADP-ribose) polymerase
PBS	phosphate-buffered saline
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3-kinase	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKR	protein kinase R
PLA_2	phospholipase A_2
PRRs	pattern recognition receptors
PUMA	p53-upregulated modulator of apoptosis
PYD	pyrin domain
RAGE	receptor for advanced glycation end products
r-tPA	recombinant tissue plasminogen activator
RIP1	receptor interacting protein 1
RIPA	radio-immunoprecipitation assay
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
shRNA	short hairpin RNA
SIGLEC	sialic acid-binding immunoglobulin-like lectin
SIRT1	silent information regulator-1
SLE	systemic lupus erythematosus
Smac	second mitochondria-derived activator of caspases
SOD	superoxide dismutase
SUR1	sulfonylurea receptor 1
SVZ	subventricular zone

TAK1	TGFβ-activated kinase 1
tBID	truncated Bcl-2 interacting domain
T_H1	T helper 1
TLRs	toll-like receptors
TNF-α	tumor necrosis factor-alpha
TNFR	tumor necrosis factor receptor
TPER	tissue protein extraction reagent
TRADD	tumor necrosis factor receptor associated death domain
TRAF2	tumor necrosis factor receptor-associated protein 2
T_{Reg}	regulatory T cells
TrkB	tyrosine kinase receptor B
TRP	transient receptor potential
TRPM2	transient receptor potential melastatin 2
TTC	2,3,5-triphenyltetrazolium chloride
TXNIP	thioredoxin-interacting protein
UCP	uncoupling protein
VCAMs	vascular adhesion molecules
VEGF	vascular endothelial growth factor
WHO	world health organization
XIAP	X-linked inhibitor of apoptosis

CHAPTER 1:

Pathogenesis of Acute Stroke and the Role of Inflammasomes – A Systematic Review

1.1 Introduction:

1.1.1 Definition

In accordance with the World Health Organization (WHO), stroke or cerebrovascular accidents is an acute condition characterized by a sudden decrease in blood flow to brain tissue resulting in impairment or loss of neurological function with symptoms persisting for more then 24 hours, which can either be initiated by transient or permanent loss of cerebral blood flow (World Health Organization, 2010). The condition typically involves an immediate deprivation of both glucose and oxygen, which are needed to maintain the metabolic demands of the brain as it holds no energy reserves that can be drawn upon (Ahmad & Graham, 2010).

1.1.2 Epidemiology

According to the World Health Organization (WHO), stroke is the second leading cause of mortality worldwide resulting in approximately 6.2 million deaths each year, which accounted for 9.7% of all deaths in 2004 (World Health Organization, 2011). In Australia, stroke is considered one of the leading contributors to adult-related deaths and long-term permanent disability. According to the Australian Bureau of Statistics an estimated 12,000 people are affected by stroke annually, where 73% were first-time stroke patients. Of these patients, approximately 30% died within the first year of occurrence. Since stroke is a leading cause of permanent disability in Australia, it is recognised as a major economic health burden accounting for a total healthcare expenditure cost of $2.14 billion each year (Australian Bureau of Statistics).

Numerous lifestyle risk factors such as obesity, diabetes mellitus, hypertension, hyperlipidemia, cigarette smoking, physical inactivity and excessive consumption of alcohol have been associated with increasing the likelihood of stroke. Furthermore, it is recognized that ageing, a non-modifiable risk factor, is associated with increasing the incidence of stroke each year. Accordingly, it is predicted from statistical models that the incidence of stroke will increase from 1.6-2.7 per 1000 people in the general population to 14.3 per 1000 people from 45 years of age and subsequently double with each decade to approximately 120 per 1000 people amongst individuals over 75 years of age (Mukherjee & Patil, 2012; Strong *et al*., 2007). The emergence of an ageing population in developed countries will inevitably increase the incidence of stroke annually where it

is predicted that worldwide mortality from stroke will be 12.1% by the year 2030 (Mukherjee & Patil, 2012; Strong *et al*., 2007). These alarming statistics only reinforces the notion that stroke is indeed a major public health concern with enormous financial implications to the healthcare system in developed countries in treating these patients. Hence, the need for comprehensive research in the field of stroke is warranted, which will incite an improved understanding of stroke pathophysiology and subsequently develop improved future treatments for stroke patients.

1.1.3 Classification of Stroke:

Stroke can be classified into two major subtypes such as ischemic stroke or haemorrhagic stroke. Ischemic stroke commonly accounts for approximately 80-87% of all stroke cases, and can be instigated by an embolic or thrombotic occlusion of a cerebral artery, whereas haemorrhagic stroke accounts for approximately 13-20% of all stroke cases and is initiated by the rupture of a cerebral blood vessel (Amarenco *et al*., 2009; Gilgun-Sherki *et al*., 2002). Haemorrhagic stroke can be further divided into sub-arachnoid haemorrhage and intra-cerebral haemorrhage (Strandgaard, 1996; Wang, 2010). Since ischemic stroke is the major focus of this research thesis further discussions will be in the context of ischemic stroke.

Classification of Ischemic Stroke

Depending on the involvement of both the affected brain area and pathophysiological mechanisms, ischemic stroke can be further categorized into: global and focal ischemia (Bacigaluppi *et al*., 2010; Durukan & Tatlisumak, 2007).

a. Global ischemic stroke

Global ischemic stroke occurs when blood flow to the entire brain or a majority part of the brain is stopped or severely reduced due to hemodynamic changes in the peripheral circulatory system (Bottiger *et al*., 1999; Yonekura *et al*., 2004). For example, this commonly occurs during a cardiac arrest associated with myocardial infarction (i.e. heart attack) where blood flow to the brain immediately ceases within seconds (Bottiger *et al*., 1999; Yonekura *et al*., 2004). In addition, other major causes include carotid stenosis and hypotensive shock where a decrease in mean peripheral arterial blood pressure reduces cerebral blood flow and subsequent perfusion pressure in the brain (Jovicevic *et al*., 2010). During a global ischemic stroke, the brain area commonly affected will be the regions between the major cerebral and cerebellar arteries, known as the “boundary zone” or “watershed areas”, which accounts for approximately 10% of all ischemic stroke cases (Demaerschalk *et al*., 2010).

b. Focal ischemic stroke

Focal ischemic stroke occurs when cerebral blood flow is attenuated in a specific brain region (Hata *et al*., 2000; McAuley, 1995). Dependent on the nature of the occlusion in the cerebral artery, focal ischemic stroke can be further subdivided into thrombotic or embolic stroke (Adams *et al*., 1993; Amarenco *et al*., 2009).

Thrombotic stroke occurs when a blood clot is formed within a cerebral artery, which is commonly caused by atherosclerosis where the vascular endothelium is constantly damaged resulting in the activation of numerous vasoactive enzymes that leads to the formation of an atherosclerotic plaque within the cerebral artery (Fukusumi, 2010). Furthermore, additional pathological changes in atherosclerosis such as thrombosis, ulceration and calcification increases the risk of blood clot formation (Andrade-Machado, *et al*., 2001). Other pathological conditions such as hypercoagulable states, fibromuscular dysplasia, arteritis and arterial trauma can comparably lead to thrombotic strokes (Broussalis *et al*., 2012).

Embolic strokes occurs when a blood clot or atherosclerotic plaque fragment that is formed elsewhere in the circulatory system detaches and is mobilized through the blood stream and occludes a cerebral artery (Donnan, 2009). The two major causes of embolic strokes are large arterial emboli and left cardioembolic emboli. Moreover, additional sources of emboli that may occlude the cerebral vasculature are fat, bacterial clumps, metastatic tumours and foreign bodies (Dudney & Elliot, 1994; Jovicevic *et al*., 2010). The most common artery to be occluded by an embolus are the left and right middle cerebral arteries since 80% of blood volume that travels through the arteries in the neck eventually flows through the middle cerebral artery (Demaerschalk *et al*., 2010).

1.1.4 Cerebral Blood Supply and Flow Parameters

There are 4 major types of arteries responsible for supplying blood to cerebral tissue, which include the vertebral arteries (left and right) and internal carotid arteries (left and right). The internal carotid arteries further subdivide into the anterior and middle cerebral arteries (Purves *et al*., 2001). The middle cerebral artery is anatomically the largest cerebral artery and supplies blood to the cortical surface of the brain, and is the site where most cerebrovascular accidents occur in humans (Becker, 2009). Normal physiological cerebral blood flow (mL/100g of brain tissue/minute) and cerebral perfusion pressure (mmHg) to the brain is approximately 50-60mL/100g of brain tissue/minute and 60-130mmHg, respectively (Astrup *et al*., 1981).

1.2 Pathophysiology of Focal Ischemic Stroke – An Overview

Focal ischemic stroke occurs when cerebral blood flow is transiently or permanently attenuated, which initiates ischemic changes in a specific brain region caused by an embolic or thrombotic occlusion to a major cerebral artery. It is characterized by the formation of two regions within the ischemic territory, a central ischemic core surrounded by an ischemic penumbra (or peri-infarct zone) due to focal hypoperfusion (Kumar *et al*., 2010; Lo, 2008). The size of the ischemic core and penumbra region will usually depend on the severity and duration of the cerebral artery occlusion, the affected brain region and vulnerability of certain populations of neurons and glial cells to ischemia (e.g. CA1 pyramidal neurons in the hippocampus are more susceptible to ischemic damage than dentate granule neurons) (Brouns & De Deyn, 2009; Mattson *et al*., 2001).

An important consideration to recognize in the formation of the ischemic core and ischemic penumbra region during ischemic stroke is limited by the level of cerebral blood flow that continues to perfuse the affected tissue (Mehta *et al*., 2007). Under physiological conditions, cerebral tissue requires continuous blood flow of at least 50mL/100g/min to sustain an adequate supply of both glucose and oxygen, which are utilized to maintain neurological function through energy (i.e. adenosine triphosphate; ATP) production by glycolysis and oxidative phosphorylation (Bisdas *et al*., 2004; Mehta *et al*., 2007). Conversely, if cerebral blood flow is reduced to less then 10mL/100g/min during ischemic stroke, an ischemic core region will develop (Astrup *et al*., 1981; Bisdas *et al*., 2004; Mehta *et al*., 2007). This ischemic core region will then undergo rapid, irreversible, necrotic cell death, resulting in an infarcted region of cerebral tissue that is metabolically, electrically and functionally inactive (Mehta *et al*., 2007). However, if cerebral blood flow remains between 10 and 50mL/100g/min, an ischemic penumbra may form between the ischemic core and normal healthy tissue (Astrup *et al*., 1981; Hossmann, 1994). This may generate a heterogeneous, meta-stable region of cerebral tissue that is metabolically active but electrically and functionally impaired (Astrup *et al*., 1981; Moskowitz *et al*., 2010). The availability of glucose and oxygen in the ischemic penumbra from collateral blood vessels will usually lead to a slower energy-dependent mode of cell death, known as apoptosis (**Figure 1.1**) (Broughton *et al*., 2009). If normal levels of perfusion are not restored in sufficient time, the penumbra will effectively merge with the ischemic core and increase infarct size (Baron, 1999; Weinstein *et al*., 2004). Since salvage of the ischemic penumbra may be associated with improved neurological outcome and recovery, this region is currently considered to be the most clinically relevant target for acute stroke therapy.

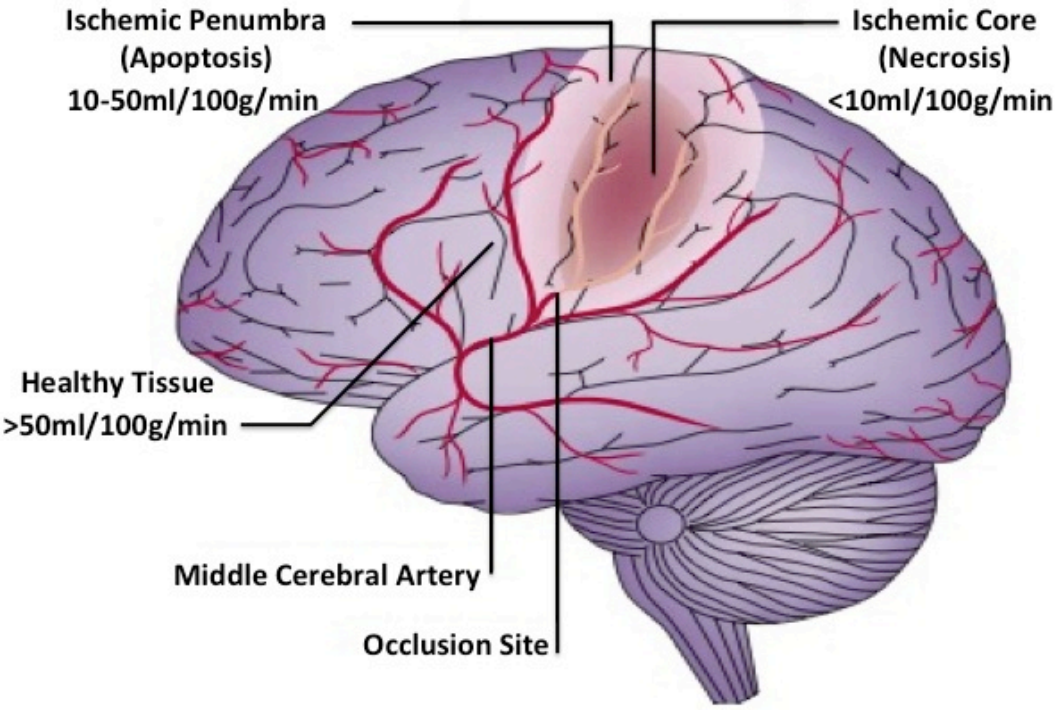

Figure 1.1: Schematic diagram of the regions defined in the ischemic territory following occlusion of the middle cerebral artery in ischemic stroke. The level of cerebral blood flow that continues to perfuse the affected tissue following an ischemic stroke determines the formation of the ischemic core and penumbra. This figure is adapted and modified from Molecular targets in cerebral ischemia for developing novel therapeutics. Mehta *et al*., (2007). *Brain Resarch Reviews*; 4: p-34-66.

1.3 The Ischemic Cascade

The ischemic cascade is a complex biochemical process of interlinked molecular and cellular reactions that are initiated in the brain following cerebral ischemia (Brouns & De Deyn, 2009). The pathological effects of the ischemic cascade are highly dependent on a number of factors such as the severity and duration of the process, which can usually last from hours to days following blood restoration, whereby blood restoration alone can contribute significantly to the propagation of the ischemic cascade known as reperfusion injury (Brouns & De Deyn, 2009; Suwanwela & Koroshetz, 2007). In addition, the amount of damage inflicted upon cerebral tissue can be dependant on the brain region and cell type affected, where neurons are the most sensitive followed by microglia and endothelial cells to ischemia during an ischemic stroke (Mattson *et al*., 2000; Mehta *et al*., 2007). In general, the ischemic cascade is characterized by the following biochemical events – bioenergetic failure, ionic imbalance, acidotoxicity, excitotoxicity, oxidative stress, inflammation and ultimately cell death via necrosis or apoptosis (**Figure 1.2**).

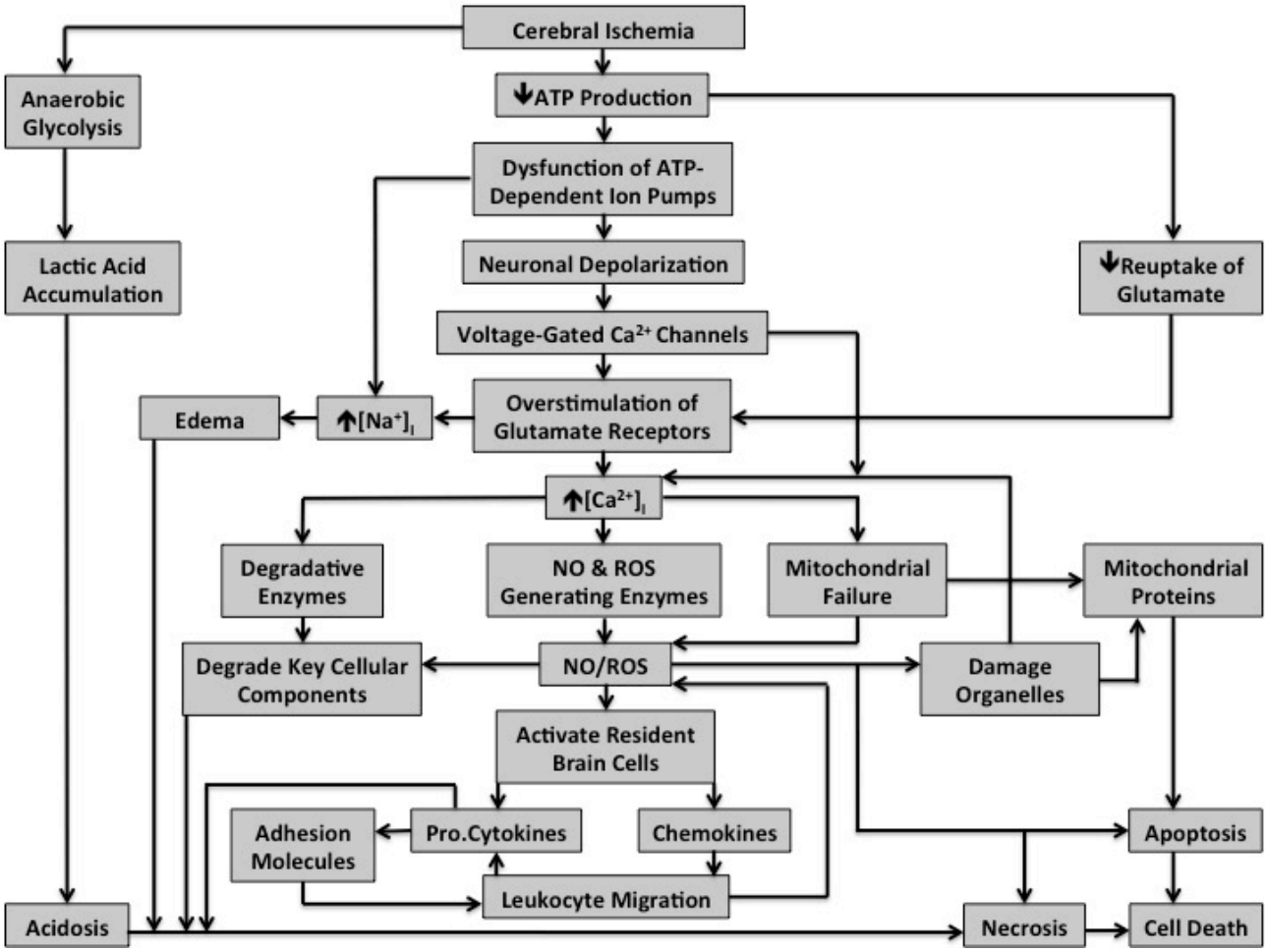

Figure 1.2: A schematic diagram of the major cell injury mechanisms involved in causing neuronal and glial cell death in cerebral ischemia. These cell injury mechanisms include bioenergetic failure, acidotoxicity, excitotoxicity, oxidative stress and inflammation. During cerebral ischemia there is decreased blood flow, and accordingly, insufficient delivery of both glucose and oxygen to the brain, which will induce bioenergetic failure by stopping or slowing ATP production via glycolysis and oxidative phosphorylation. In addition, reduced oxygen availability will initiate anaerobic glycolysis, which leads to increased production and accumulation of lactate within the ischemic tissue decreasing intracellular pH (acidosis) causing acidotoxicity and necrotic cell death in the brain. During cerebral ischemia there is a decreased production of ATP, which causes ATP-dependent ion pumps (e.g. Na^+/K^+-ATPase pumps) to fail causing widespread anoxic depolarization in neurons. This causes voltage-gated Ca^{2+} channels to open at the pre-synaptic terminals and allows an influx of Ca^{2+} ions, inducing uncontrolled release of glutamate into the synaptic cleft. In addition, the energy failure will impair the re-uptake of glutamate by glutamate transporters. The resultant build-up of glutamate at synapses will then overstimulate glutamate receptors on neighboring neurons, driving a further influx of Na^+ and Ca^{2+} ions through channels gated by these receptors. The increased influx of Na^+ ions into neurons will cause an osmotic movement of water into the cell, leading to cell swelling and brain edema. If energy supply is not restored in time, these changes will result in rapid necrotic cellular lysis of neurons. Concurrently, the increased concentration of Ca^{2+} ions within neurons can initiate a series of nuclear and cytoplasmic events that lead to lethal or non-lethal metabolic derangements known as excitotoxicity by activating catabolic enzymes, NO and ROS generating enzymes, and causing mitochondrial failure, which increases the production of ROS (oxidative stress) that degrade key cellular components inducing necrotic or apoptotic cell death depending on severity. ROS can damage organelles such as the endoplasmic reticulum and mitochondria, which can facilitate the release of additional Ca^{2+} ions and pro-apoptotic proteins into the cytosol, leading to local amplification of the initial ischemic insult by Ca^{2+} ions, and both endoplasmic reticulum stress and apoptosis through the intrinsic and extrinsic pathway. Finally, ROS can activate resident brain cells to increase the production and release of pro-inflammatory cytokines, which can cause cell damage and induce the expression of cell adhesion molecules on endothelial cells and leukocytes to facilitate leukocyte infiltration into the ischemic territory during reperfusion releasing

additional pro-inflammatory cytokines and ROS. In addition, chemokines can be released by activated brain cells and contribute to guiding leukocyte migration toward the ischemic tissue (ATP, adenosine triphosphate; I, intracellular; NO, nitric oxide; ROS, reactive oxygen species; Pro, pro-inflammatory).

1.3.1 Bioenergetic Failure and Ionic Imbalance

The primary insult caused by cerebral ischemia is hypoperfusion, and accordingly, insufficient delivery of both glucose and oxygen to the brain, which will induce bioenergetic failure by stopping or slowing ATP production in the mitochondria (Hertz, 2008; Hertz & Dienel, 2002; Hertz *et al*., 2007; Rossi *et al*., 2007). The loss of ATP results in dysfunction of all ATP-dependent ion pumps, thus rendering neurons and glial cells highly susceptible to cerebral ischemia. A major consequence of ATP loss that occurs within minutes of ischemic insult is inhibition of both the Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase pumps, which commonly elicits rapid deterioration of ionic gradients across the plasma membrane, resulting in an abnormal influx of Ca^{2+} and Na^{+} ions, and efflux of K^{+} ions (Kaplan, 2002; Khanna *et al*., 2014; Lipton, 1999; Mongin, 2007; Song & Yu, 2014) across the plasma membrane. The increased influx of Na^{+} ions into neurons and glial cells can cause an osmotic movement of water through aquaporins into the cell, leading to cytotoxic swelling and/or cell lysis in the ischemic core (Khanna *et al*., 2014; Song & Yu, 2014). In addition, this ionic imbalance across the plasma membrane will induce widespread anoxic depolarization in neurons and glial cells (Higuchi *et al*., 2002; Jarvis *et al*., 2001; Khanna *et al*., 2014; Leichsenring *et al*., 2013; Mongin, 2007; Song & Yu, 2014; White *et al*., 2012).

1.3.2 Acidotoxicity

During an ischemic stroke, the reduced delivery and availability of oxygen within cerebral tissue will initiate anaerobic glycolysis, which will lead to an increased production of lactate within ischemic tissue (Brouns & De Deyn, 2009). Consequently, the accumulation of lactate within the ischemic tissue decreases intracellular pH (acidosis) and causes acidotoxicity, which is mediated by acid sensing ion channels (ASICs) that are abnormally more permeable to Na^{+} and Ca^{2+} ions across the plasma membrane (Brouns *et al*., 2008; Ding *et al*., 2000; Katsura *et al*., 1994; Park *et al*., 1999; Sherwood *et al*., 2011; Xiang *et al*., 2004; Xiong *et al*., 2004). The increased influx of Na^{+} and Ca^{2+} ions can induce glutamate excitotoxicity, enhance pro-oxidant production and antioxidant inactivation leading to neuronal and glial cell death by necrosis or apoptosis depending on the severity of acidosis (Lewerenz *et al*., 2010; Ying *et al*., 1999). The damage inflicted upon ischemic tissue from acidotoxicity is known as the lactate-acidosis-hypothesis, which can induce metabolic stress and secondary damage in ischemic stroke (Brouns *et al*., 2008; Ding *et al*., 2000; Sherwood *et al*., 2011).

1.3.3 Excitoxicity

Excitoxicity is a pathological process where neurons are damaged by excessive stimulation by excitatory neurotransmitters such as glutamate during an ischemic stroke (Lai *et al*., 2014). Anoxic depolarization in neurons causes opening of voltage-gated Ca^{2+} channels at the pre-synaptic terminal and allows an influx of Ca^{2+} ions, inducing uncontrolled release of glutamate into the synaptic cleft, which is the major excitatory neurotransmitter in the mammalian brain (Arundine & Tymianski, 2003; Zhang *et al*., 2006). Energy failure will also impair the re-uptake of glutamate by glutamate transporters (EAAT2; excitatory amino acid transporter 2) located on pre-synaptic neurons and surrounding astrocytes (Camacho & Massieu, 2006; Rossi *et al*., 2000). The resultant accumulation of glutamate at synapses will then overstimulate AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid), kainate and NMDA (N-methyl-d-aspartic acid)-type glutamate receptors on neighbouring neurons, driving a further influx of Na^+ ions and Ca^{2+} ions through channels gated by these receptors (Arias *et al*., 1999; Arundine & Tymianski, 2003; Lai *et al*., 2014; Li *et al*., 2007; Seo *et al*., 2001; Suzuki *et al*., 2012; Zhang *et al*., 2006). Depolarization of additional neurons causes further Ca^{2+} ion influx and glutamate release, leading to local amplification of the initial ischemic insult. In addition, the increased influx of Na^+ ions into neurons causes an osmotic movement of water through aquaporins into the cell, leading to cytotoxic swelling and brain oedema (Ayata & Ropper, 2002; Breder *et al*., 2000; Khanna *et al*., 2014; Mongin, 2007; Simard *et al*., 2007; Song & Yu, 2014). If energy supply is not restored in time, these changes will result in rapid necrotic cellular lysis, especially in the ischemic core (Khanna *et al*., 2014; Sattler & Tymianski, 2000; Song & Yu, 2014). Concurrently, the increased Ca^{2+} ion influx mediated by the combined effects of activation of voltage-gated Ca^{2+} channels, ASICs, glutamate receptors and reverse operation of the Na^+/Ca^{2+} exchanger, and the decreased Ca^{2+} ion efflux due to inhibition of the Na^+/Ca^{2+} exchanger and plasma membrane Ca^{2+}-ATPase pump, will initiate a series of nuclear and cytoplasmic events that lead to lethal or non-lethal metabolic derangements known as excitotoxicity (Bano *et al*., 2005; Jeffs *et al*., 2007; Li *et al*., 2007; Schwab *et al*., 2002).

When calcium homeostasis is disrupted during cerebral ischemia, Ca^{2+} ions can become a powerful activator of multiple damaging mechanisms, including activation of catabolic enzymes, especially endonuclease and calpain, ultimately leading to necrotic or apoptotic cell death depending on the degree of damage. The increased concentration of intracellular Ca^{2+} ions can activate nuclear and cytosolic proteases such as endonuclease and calpains, i.e. calpain I (μ-calpain) and II (m-calpain), respectively (Lee *et al*., 2005; Neumar *et al*., 2001). It has been shown that endonuclease can cleave DNA to cause apoptosis, while activated calpain can hydrolyse

cytoskeletal proteins, including spectrin, fodrin, actin and tubulin; anti-apoptotic proteins, including Bcl-2 (B-cell lymphoma 2) and Bcl-xL (B-cell lymphoma-extra large); membrane proteins, including glutamate and ryanodine receptors; and regulatory and signalling proteins, including calmodulin-binding protein, protein kinase C and G-proteins (Aki *et al*., 2002; Buddle *et al*., 2003; Ling *et al*., 2002; Liu *et al*., 2004b; Nakagawa & Yuan, 2000; Neumar *et al*., 2001; Roberts-Lewis *et al*., 1994; Xu *et al*., 2009). In addition, through an unknown mechanism(s), calpain can induce the rupture of lysosomes, releasing cathepsins (i.e. cathepsin B, D and L) into the cytosol, which can hydrolyse similar calpain targets (Yamashima *et al*., 1998; Yamashima, 2004; Yamashima & Oikawa, 2009). Such a process is known as the calpain-cathepsin hypothesis. The uncontrolled proteolysis of these cellular proteins in neurons and glial cells is an important component of neurodegeneration detected in necrosis that is observed primarily in the ischemic core (Yamashima, 2004; Yamashima & Oikawa, 2009).

1.3.4 Oxidative Stress

Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and/or a decreased ability of the cellular antioxidant defence system to neutralize these reactive intermediates, which inflicts cerebral tissue damage during an ischemic stroke. Disruption of calcium homeostasis is a major contributor towards the production of ROS. In neurons and glial cells, the primary mechanism of Ca^{2+} ion uptake into the mitochondrial matrix is through the calcium uniporter during an ischemic stroke (Kirichok *et al*., 2004; Triantafilou *et al*., 2013). Consequently, abnormal accumulation of Ca^{2+} ions within the mitochondrial matrix will decrease the mitochondrial transmembrane potential to facilitate the formation of the mitochondrial transition pore, and induce the formation of calcium precipitates (i.e. calcium phosphate and calcium hydroxyapatite) within the inner mitochondrial membrane, perturbing the electron transport chain and causing electron leakage that can react with oxygen to produce superoxide ($O_2^{\cdot -}$) (Green & Kroemer, 2004; Nieminen, 2003; Triantafilou *et al*., 2013). The increase in cytosolic Ca^{2+} can activate protein kinase C, which in turn activates NADPH (nicotinamide adenine dinucleotide phosphate) oxidase, producing $O_2^{\cdot -}$ (Brennan *et al*., 2009; Kahles *et al*., 2010; Yoshioka *et al*., 2011). Accumulation of Ca^{2+} within neurons can induce the translocation of cytosolic phospholipase A_2 (PLA_2) into the plasma membrane, catalyzing the formation of arachidonic acid, which is utilized by cyclooxygenase and lipoxygenase to produce prostaglandins and leukotrienes, respectively, with a concomitant production of $O_2^{\cdot -}$ (Kishimoto *et al*., 2010; Tomimoto *et al*., 2002). In addition, conversion of xanthine dehydrogenase to xanthine oxidase by Ca^{2+}-activated proteases can result in an increased output of $O_2^{\cdot -}$ (Abramov *et al*., 2007; Al-Gonaiah *et al*., 2009; Ono *et al*., 2009). Increased production of $O_2^{\cdot -}$ from numerous sources can lead to the formation of additional

free radicals, such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and peroxynitrite ($ONOO^-$) by reacting with nitric oxide (NO) produced from Ca^{2+}-activated neuronal and endothelial NO synthase (n/eNOS) (Chan, 2001; Heeba & El-Hanafy, 2012; Nanetti *et al*., 2007).

The increased production of reactive oxygen species (ROS) induces oxidative stress, a major cause of tissue damage that can impact multiple cellular components, including nucleic acids, proteins, carbohydrates and lipids via oxidation reactions (Allen & Bayraktutan, 2009). In addition, ROS can stimulate transcription factors such as nuclear factor kappa B (NF-κB) directly, and activator protein-1 (AP-1) indirectly by activating mitogen activated protein kinases (MAPKs) (in particular p38 MAPK and c-Jun-N-terminal kinase; JNK) to cause neuronal and glial damage by modulating caspase-mediated apoptosis (Barone *et al*., 2001; Chen *et al*., 2011; Kratsovnik *et al*., 2005; Ridder & Schwaninger, 2009; Suzuki *et al*., 1997). Furthermore, oxidative stress can damage organelles such as the endoplasmic reticulum (i.e. the major site of calcium storage) and mitochondria, which can facilitate the release of additional Ca^{2+} ions and pro-apoptotic proteins (such as cytochrome c and apoptosis inducing factor) into the cytosol, leading to local amplification of the initial ischemic insult by Ca^{2+} ions, and both endoplasmic reticulum stress and apoptosis through the intrinsic and extrinsic pathway (Cao *et al*., 2004; Hayashi *et al*., 2005; Malhotra & Kaufman, 2007; Nieminen, 2003). In general, severe oxidative stress can cause cell death through necrosis, while moderate oxidative stress can elicit apoptosis that is observed primarily in the ischemic penumbra (Chen *et al*., 2011).

1.3.5 Ischemic Inflammation

Inflammation plays a significant role in the overall pathogenesis of ischemic stroke. The inflammatory response is a double-edged sword, initially contributing to ischemic brain injury and then to tissue regeneration (Chamorro & Hallenbeck, 2006). It is characterized by the production and release of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6 and IL-18 from activated cells in the brain parenchyma including neurons, astrocytes, microglia and endothelial cells by initiating various pro-death signalling pathways, resulting in neuronal and glial cell death during cerebral ischemia (Allan & Rothwell, 2001; Vila *et al*., 2000). Pro-inflammatory cytokines can also induce the expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecules (VCAMs), selectins (e.g. E-selectin, P-selectin) and integrins (e.g. Mac-1, LFA-1) on endothelial cells, leukocytes and platelets (Arumugam *et al*., 2004a; Ehrensperger *et al*., 2005; Huang *et al*., 2000; Yilmaz & Granger, 2008; Zhang *et al*., 1998). These adhesion molecules are crucial for the infiltration of leukocytes (e.g. neutrophils and monocytes/macrophages) whereby both E and P-selectins mediate

leukocyte recruitment and rolling, and ICAM-1 and VCAM assist in leukocyte adherence to the endothelium to facilitate transmigration into the ischemic territory during reperfusion, which paradoxically, often leads to secondary damage known as ischemic reperfusion injury (Buck *et al*., 2008; Iadecola & Alexander, 2001; Tang *et al*., 2006; Wang *et al*., 2007). In addition, monocyte chemoattractant protein 1 (MCP-1/CCL2), the major chemokine in mammalian systems, and other chemokines such as macrophage inflammatory protein 1-α and fractalkine are released by activated neurons and glial cells, which is important in guiding leukocyte migration toward the damaged tissue (Dimitrijevic *et al*., 2006; Lakhan *et al*., 2009; Stamatovic *et al*., 2003). The infiltration of leukocytes usually occurs within 4-6 hours after the onset of ischemia with neutrophils being the first immune cells to infiltrate the ischemic penumbra followed by monocytes, macrophages and T-lymphocytes (Buck *et al*., 2008; Campanella *et al*., 2002; Tang *et al*., 2006; Wang *et al*., 2007). In particular, $CD4^+$ and $CD8^+$ T-lymphocytes have been shown to induce ischemic injury while regulatory T-lymphocytes demonstrate a protective role in post-ischemic inflammation (Hurn *et al*., 2007; Iadecola & Alexander, 2001; Liesz *et al*., 2009; Planas & Chamorro, 2009; Shichita *et al*., 2009; Yilmaz *et al*., 2006). However, a recent study suggested that neurovascular leukocyte accumulation showed no spatial correlation with increased vessel permeability and enhanced expression of endothelial cell adhesion molecules. These observations may indicate that the neurovascular endothelium rather than the brain parenchyma is the site of leukocyte action after stroke (Enzmann *et al*., 2013). Despite the mechanisms of ischemic reperfusion injury remaining incompletely understood, it has been shown that infiltrating leukocytes can release a variety of cytotoxic agents, including additional pro-inflammatory cytokines (i.e. TNF-α, IL-1β, IL-6, IL-12 and IL-18), NADPH oxidase-derived ROS, NO from inducible nitric oxide synthase (iNOS), and matrix metalloproteinases (MMPs, particularly MMP-2 and MMP-9). These MMPs can cause damage to the extracellular matrix and blood brain barrier (BBB), exacerbating brain oedema, haemorrhage, and ultimately, neuronal and glial cell death (Amantea *et al*., 2009; Asashi *et al*., 2001; Kriz, 2006; Yang *et al*., 2007a). In addition, the complement cascade has been shown to be involved in ischemic reperfusion injury through the production of several inflammatory mediators, including C1, C3a and C5a anaphylatoxins, that are involved in leukocyte recruitment and formation of the membrane attack complex (MAC) in neurons and glial cells, which causes cell lysis and further tissue damage (Arumugam *et al*., 2004b; Barnum *et al*., 2002; Gesuete *et al*., 2009; Leinhase *et al*., 2006; Van Beek *et al*., 2000).

1.4 Cell Death Pathways in Ischemic Stroke – Necrosis and Apoptosis

There are two main types of cell death pathways evident during an ischemic stroke - necrosis and apoptosis. Necrosis is primarily seen in the ischemic core as it receives the least

amount of blood flow where neurons and glial cells will undergo an unregulated, rapid and irreversible form of cell death that results in cellular lysis causing an inflammatory response in surrounding tissue (Astrup *et al*., 1981; Bisdas *et al*., 2004; Mehta *et al*., 2007). The cell death mechanisms responsible for inducing necrotic cell death are extensive and severe, and include biochemical events associated with the ischemic cascade such as bioenergetic failure, acidotoxicity, excitotoxicity and oxidative stress previously discussed in detail in Section 1.3.1-1.3.4. Conversely, apoptosis is observed primarily in the ischemic penumbra as it receives more blood flow in comparison to the ischemic core where neurons and glial cells will undergo a delayed programmed form of cell death that is potentially reversible following immediate treatment (Astrup *et al*., 1981; Broughton *et al*., 2009; Hossmann, 1994; Sairanen *et al*., 2006). The cell death mechanisms responsible for inducing apoptotic cell death are the same aforementioned biochemical events associated with the ischemic cascade, although less severe, and is responsible for activating the extrinsic and intrinsic apoptotic pathways.

1.4.1 Extrinsic Apoptotic Pathway

The extrinsic apoptotic pathway involves activation of death receptors on the plasma membrane of neurons and glial cells during an ischemic stroke (Broughton *et al*., 2009; Sairanen *et al*., 2006). Death receptors belong to the tumor necrosis factor receptor (TNFR) superfamily, and include death receptors 3,4,5, TNFR-1 (p55 or CD120a) and Fas receptor (CD95 or Apo1), which all possesses an intracellular death domain (DD) that is able to interact with two adaptor proteins such as the TNF receptor associated death domain (TRADD) or the Fas-associated death domain (FADD) to facilitate downstream signalling (Choi & Benveniste, 2004; Mehta *et al*., 2007; Nakka *et al*., 2008; Sessler *et al*., 2013; Wilson *et al*., 2009). The recruitment of FADD is regarded as the canonical pathway for mediating extrinsic apoptosis (Wilson *et al*., 2009). During an ischemic stroke, a member of the forkhead family of transcription factors, forkhead1, stimulates the expression of target genes such as Fas ligand (FasL), which is released into the extracellular environment (Fukunaga *et al*., 2005; Kavurma & Khachigian, 2003; Sugawara *et al*., 2004). When FasL binds onto the Fas receptor on the plasma membrane, both FADD and procaspase-8 interact and are recruited to the Fas receptor to form a FasL-Fas-receptor-FADD-procaspase-8 complex known as a death-inducing signalling complex (DISC) (Sessler *et al*., 2013). The formation of DISC catalyses the conversion of pro-caspase-8 into biologically active caspase-8, which is released into the cytoplasm (Fu *et al*., 2012; Sessler *et al*., 2013). Once activated, caspase-8 can induce apoptotic cell death through two pathways: Firstly, caspase-8 can directly cleave pro-caspase-3 into active cleaved caspase-3, which enters the nucleus, and cleaves poly (ADP-ribose) polymerase (PARP) and cytoskeletal proteins (e.g. spectrin and gesolin) causing nuclear DNA and cytoskeletal

damage, respectively, ultimately leading to apoptosis (Badiola *et al*., 2009; Lee *et al*., 2004; Pike *et al*., 2004; Sairanen *et al*., 2009). Secondly, caspase-8 can directly cleave Bcl-2 interacting domain (BID) into its truncated form (tBID), which translocates to the outer mitochondrial membrane and interacts with other pro-apoptotic proteins, such as Bad, Bax, Bak or Bcl-XS (Broughton *et al*., 2009; Ferrer & Planas, 2003; Lovell *et al*., 2008; Plesnila *et al*., 2001). Interaction of tBID with either pro-apoptotic protein is thought to induce the formation of mitochondria transition pores (MTPs) through an unknown mechanism to facilitate the release of cytochrome c from the mitochondria intermembrane space into the cytoplasm (Gillick & Crompton, 2008; Jemmerson *et al*., 2009; Kim *et al*., 2000; Wei *et al*., 2000; Zhai *et al*., 2000). Cytochrome c can bind with Apaf-1 and pro-caspase-9 to form a complex known as an apoptosome (Hu *et al*., 2014). The formation of the apoptosome will catalyse the conversion of pro-caspase-9 into active caspase-9, which subsequently converts pro-caspase-3 into active caspase-3 that causes nuclear DNA and cytoskeletal damage, ultimately leading to apoptosis (Lee *et al*., 2004; McStay & Green, 2014; Pike *et al*., 2004; Sairanen *et al*., 2009; Yuan *et al*., 2011).

1.4.2 Intrinsic Apoptotic Pathway

The intrinsic apoptotic pathway involves the release of several pro-apoptotic proteins such as cytochrome c, apoptosis inducing factor (AIF), second mitochondria-derived activator of caspases/direct IAP-binding protein with low pl (Smac/DIABLO), endonuclease G and pro-caspases 2,3,8,9 from the mitochondria (Arnoult *et al*., 2003; Broughton *et al*., 2009; Chan, 2005; Sugawara *et al*., 2004). During an ischemic stroke, the ischemic cascade induces the release of cytochrome c from the mitochondria into the cytoplasm often regarded as the most crucial pro-apoptotic protein in initiating the intrinsic apoptotic pathway (Jemmerson *et al*., 2009; Lin *et al*., 2005). Specifically, an increased concentration of Ca^{2+} ions in the cytoplasm activates calpain enzymes, which cleaves BID into tBID that translocates to the outer mitochondrial membrane and interacts with other pro-apoptotic proteins, such as Bad, Bax, Bak or Bcl-XS (D'Orsi *et al*., 2012; Krajewska *et al*., 2004; Lovell *et al*., 2008). Interaction of tBID with either pro-apoptotic protein is thought to induce the formation of MTPs through an unknown mechanism to facilitate the release of AIF and cytochrome c from the inner mitochondrial membrane space into the cytoplasm (Gillick & Crompton, 2008; Jemmerson *et al*., 2009; Kim *et al*., 2000; Wei *et al*., 2000; Zhai *et al*., 2000). AIF rapidly translocates into the nucleus to induce large-scale DNA fragmentation and apoptosis via a caspase-independent mechanism (Plesnila *et al*., 2004). Conversely, cytochrome c binds with Apaf-1 and pro-caspase-9 to form a complex known as an apoptosome (Hu *et al*., 2014). Consequently, the formation of the apoptosome will catalyse the conversion of pro-caspase-9 into active caspase-9, which subsequently converts pro-caspase-3 into active caspase-3 that causes nuclear DNA and

cytoskeletal damage, ultimately leading to apoptosis (Lee *et al*., 2004; McStay & Green, 2014; Pike *et al*., 2004; Sairanen *et al*., 2009; Yuan *et al*., 2011).

1.4.3 Caspase-Independent Apoptosis

Despite the extrinsic and intrinsic apoptotic pathways, there are a number of caspase-independent mechanisms responsible for inducing apoptosis during an ischemic stroke (Cho & Toledo-Pereyra, 2008). The release of AIF from the inner mitochondrial space into the cytoplasm mediated by the formation of MTPs is a prime example where AIF rapidly translocates into the nucleus to induce large-scale DNA fragmentation causing apoptosis (Culmsee *et al*., 2005; Galluzzi *et al*., 2009; Moroni, 2008; Plesnila *et al*., 2004; Zhu *et al*., 2003). PARP-1 is an important regulator of this particular caspase-independent pathway where it is a key regulatory protein that initiates nuclear signaling to the mitochondria to release AIF during the apoptotic process through mechanism(s) involving interactions with receptor interacting protein 1 (RIP1), TNFR-associated protein 2 (TRAF2) and c-Jun N-Terminal kinase, especially JNK1, although, it remains to be fully established (Culmsee *et al*., 2005; Gao *et al*., 2005; Komjati *et al*., 2004; Xu *et al*., 2006). Furthermore, the increased production and accumulation of ROS in the cytoplasm can directly cause irreversible nuclear DNA damage via oxidation reactions inducing apoptosis (Allen & Bayraktutan, 2009; ArunaDevi *et al*., 2010; Olmez & Ozyurt, 2012). Finally, in response to DNA damage, phosphorylation and activation of p53, a tumor-suppressor transcription factor, initiates apoptosis by promoting pro-apoptotic protein expression (i.e. Bax and Bak) and suppresses anti-apoptotic protein regulation (i.e. Bcl-2) by increasing p53-mediated expression of BH3-only proteins, such as p53-upregulated modulator of apoptosis (PUMA) and NOXA in the brain under ischemic conditions (Culmsee & Mattson, 2005; Hong *et al*., 2010; Kim *et al*., 2004; Kuroki *et al*., 2009; Luo *et al*., 2009; Niizuma *et al*., 2009; Steckley *et al*., 2007).

1.4.4 Regulators of Apoptosis

The interaction between pro-apoptotic and anti-apoptotic proteins is a constantly regulated process, which ensures apoptosis is tightly regulated. Normally, anti-apoptotic proteins, Bcl-2 and Bcl-xL, are located on the outer mitochondrial membrane, whereby neutralization of pro-apoptotic proteins (Bad, Bax, Bak and Bcl-XS) from interacting with tBID occurs (Billen *et al*., 2008; Ganesan *et al*., 2012; Garcia-Saez *et al*., 2004; Gonzalvez *et al*., 2005; Howells *et al*., 2011; Liu *et al*., 2004c; Lovell *et al*., 2008; Luo *et al*., 2014; Shamas-Din *et al*., 2014; Webster *et al*., 2006; Yao *et al*., 2009). Hence, neutralization of pro-apoptotic proteins from interacting with tBID prevents the formation of MTPs and inhibits the release of pro-apoptotic proteins, AIF and cytochrome c into the cytoplasm during cerebral ischemia (Dubal *et al*., 1999; Gal *et al*., 2008; Hata *et al*., 1999; Kilic

et al., 2002; Martinou *et al*., 1994; Shamas-Din *et al*., 2014; Webster *et al*., 2006; Wiessner *et al*., 1999; Zhao *et al*., 2003).

1.5 Experimental Animal Models in Ischemic Stroke

Research into understanding the molecular and cellular biochemical events associated with the pathophysiology of ischemic stroke has been made possible by the use of experimental animal stroke models. Since the 1970s, the development of animal models of ischemic stroke has vastly improved stroke research by providing new avenues of therapeutic targets and strategies towards the prevention, treatment and rehabilitation of stroke-induced brain injury and functional deficits in stroke patients (Canazza *et al*., 2014; Van der Worp *et al*., 2010).

An important criterion concerning the development of experimental stroke models in animals is that the underlying pathophysiology and clinical features must be accurately represented in human ischemic stroke patients. At present, most experimental stroke models are performed on mammals and include the use of mice, rats, gerbils, rabbits, pigs, cats, dogs and non-human primates such as monkeys (Jeon *et al*., 2014; Kim *et al*., 2014; Lapchak *et al*., 2015; Lee *et al*., 2015a; Liu *et al*., 2014a; Mattingly *et al*., 2015; Zhang *et al*., 2015a; Zhang *et al*., 2015b). There are a number of advantages in the use of animals in stroke research. Firstly, possessing a similar cerebrovascular anatomy and physiology between animals and humans will often produce a comparable biological response to potential pharmacological stroke treatments (Durukan & Tatlisumak, 2007; Durukan *et al*., 2008; Graham *et al*., 2004; Leker & Constantini, 2002). Secondly, the pathophysiology and severity of stroke is equivalent following experimental stroke induction between animals and humans; and finally, from an experimental outlook the ability to induce reproducible stroke infarcts with minimal invasive surgery is ideal and achievable in animals (Ahmed *et al*., 2000a; Durukan *et al*., 2008; Graham *et al*., 2004; Leker & Constantini, 2002). Despite the recognition that no single animal stroke model is able to accurately represent all the clinical heterogeneous features associated in human ischemic stroke, which still needs to be addressed, the outcome from current models are extrapolated in order to successfully translate new pathophysiological concepts and pre-clinical treatments from bench to beside.

In relation to animal size there are several advantages and disadvantages in the use of small and large animals in ischemic stroke models. The advantages of using large animals in ischemic stroke models is that the brain is anatomically and physiologically similar to humans as both brains are gyrencephalic and possess a high white matter to grey matter ratio (Dirnagl *et al*., 1999; Howells *et al*., 2010; Krafft *et al*., 2012; Macrae, 2011). In addition, the use of large animals allows

you to perform a number of experiments simultaneously and at multiple time-points such as measuring physiological, neurobehavioral and sensorimotor parameters (Traystman, 2003). Conversely, the disadvantage of using larger animals in ischemic stroke models is that it is labour intensive and financially expensive as it involves complicated invasive surgery, which often increases the risk of haemorrhage and mortality rates (Canazza *et al*., 2014).

The advantage of using small animals in ischemic stroke models is that it is financially feasible to purchase and maintain small animals for a sustained period of time, especially rodents, due to low husbandry costs (Durukan & Tatlisumak, 2007). From an experimental outlook, it is easier to achieve reproducible ischemic infarcts in small animals, which is ideal for the success of each experiment (Krafft *et al*., 2012). In addition, small animals are genetically homogenous, which allows you to generate transgenic or knockout animals (Liang *et al*., 2004). Conversely, the disadvantage of using small animals is that the brain is anatomically dissimilar to humans as small animals, such as rodents, have lissencephalic brains and have a higher grey matter to white matter ratio, and thus functionally different (Dirnagl *et al*., 1999; Liu *et al*., 2011). In addition, the use of small animals often does not allow you to perform concurrent experiments at multiple times points (Traystman, 2003).

Experimental animal models in ischemic stroke can be divided into two categories – global and focal ischemia.

1.5.1 Global Ischemic Stroke Models

The global ischemic stroke model was developed to investigate the effect of a widespread disruption of blood flow to a majority or the whole brain due to hemodynamic changes in the peripheral circulatory system following clinical events such as asphyxiation or cardiac arrest (Allen & Buckberg, 2012; Krafft *et al*., 2012; Kristian & Hu, 2013). The global ischemic stroke model is primarily conducted in small animals such as rodents (i.e. mice and rats) as larger animals require the induction of ventricular fibrillation to produce a cardiac arrest and cardio-pulmonary resuscitation, which is often labour intensive and expensive (Kristian & Hu, 2013; Traystman, 2003). The global ischemic stroke model is divided into two types: 2-vessel occlusion model (2-VO) or 4-vessel occlusion model (4-VO).

The 2-vessel occlusion model (2-VO) involves the temporary bilateral occlusion of the common carotid arteries combined with systemic hypotension to produce reversible forebrain ischemia (Atlasi *et al*., 2013; Clark *et al*., 2007; Kenny *et al*., 2013; Onken *et al*., 2012; Sanderson & Wider, 2013; Smith *et al*., 1984; Traystman, 2003). This model induces cerebral tissue damage

within two minutes of global ischemic onset and causes damage to primarily CA1 pyramidal neurons in the hippocampus, neocortex and caudoputamen that progress over the course of 6-24 hours of reperfusion (Traystman, 2003). A major limitation towards the use of the 2-VO model is inconsistency in achieving the same degree of damage between experimental animals due to variations in collateral blood flow, especially at the Circle of Willis, whilst maintaining a high survival rate (Kitagawa *et al*., 1998; Martinez *et al*., 2012; Murakami *et al*., 1998; Yang *et al*., 1997; Zhen & Dore, 2007). Subsequently, the three-vessel occlusion model was developed in an attempt to overcome this problem where the basilar artery was additionally occluded, however, similar problems were encountered due to anatomical and experimental difficulties to locate, isolate and occlude the basilar artery (Panahian *et al*., 1996; Thal *et al*., 2010; Yonekura *et al*., 2004).

The 4-vessel occlusion model is the most common method to induce global ischemia in the forebrain via a two-stage process (Atlasi *et al*., 2013; Pegorini *et al*., 2005; Yamaguchi *et al*., 2005; Yonekura *et al*., 2004). The first stage involves the location and isolation of both common carotid arteries, and an atraumatic clasp loosely attached to each common carotid artery followed by electro-cauterization of both vertebral arteries (Traystman, 2003; Yamaguchi *et al*., 2005). On the following day, the second stage involves occluding both the common carotid arteries by narrowing both atraumatic clasps to induce forebrain ischemia (Pegorini *et al*., 2005; Traystman, 2003). This model induces cerebral tissue damage within 30 minutes of ischemic onset and causes damage to primarily striatal neurons in the hippocampus and neocortex following 3-6 hours and 1-3 days after reperfusion, respectively (Yamaguchi *et al*., 2005; Yonekura *et al*., 2004). A major limitation towards the use of the 4-VO model is that it is surgically challenging in comparison to the 2-VO model to achieve global ischemia (Kristian & Hu, 2013).

1.5.2 Focal Ischemic Stroke Models

The focal ischemic stroke model was developed to investigate the effect of a local disruption of blood flow in a specific brain region due to an embolic or thrombotic occlusion in the middle cerebral artery (MCA), the most clinically relevant site where a majority of focal ischemic strokes occur in humans (Canazza *et al*., 2014; Howells *et al*., 2010). This model is primarily conducted in small mammals such as rodents but has been applied to larger mammals such as cats, dogs and non-human primates (Jeon *et al*., 2014; Kim *et al*., 2014; Zhang *et al*., 2015b). It should be recognized that there are two important pathological differences between focal and global ischemic stroke models. Firstly, the amount of blood flow will be greater in the ischemic core region in a focal ischemic stroke model in comparison to the global ischemic stroke model, and hence a longer time period will be required to induce cerebral tissue damage during focal ischemia (Bandera *et al*.,

2006). Secondly, a gradient of ischemia will be observed from the ischemic core to the surrounding ischemic penumbra in a focal ischemic stroke model but absent in comparison to a global ischemic model, and hence an increasing concentric gradient of metabolic damage will be observed from the ischemic penumbra to the ischemic core within the ischemic territory during focal ischemia (Bonova *et al*., 2013; Heiss, 2012; Iwabuchi *et al*., 2013). Currently, the focal ischemic stroke model is the most widely accepted and accurate representation of ischemic stroke in terms of occlusion site, pathophysiology and symptoms that occur in human patients making it a clinically relevant model (Braeuninger *et al*., 2012; Macrae, 2011). There are two types of focal ischemic stroke models – the transient ischemic stroke model and permanent ischemic stroke model.

The most common transient ischemic stroke model involves the occlusion of the middle cerebral artery (MCA) where a nylon suture is inserted into the common carotid artery (CCA) and advanced past the bifurcation point between the internal carotid artery (ICA) and pterygopalatine artery so that the origin of the MCA is occluded (Ansari *et al*., 2011; Chiang *et al*., 2011; Engel *et al*., 2011; Liu & McCullough, 2011; Rousselet *et al*., 2012). In detail, the procedure begins with a midline incision in the neck where the left external carotid artery (ECA) and pterygopalatine artery are isolated and ligated with silk thread (Chen *et al*., 2008a). The ICA is occluded at the bifurcation point between the ICA and pterygopalatine artery with a small clip and the CCA ligated with a silk thread (Chen *et al*., 2008a; Chu *et al*., 2008). A small incision is made into the ECA and a nylon intraluminal monofilament with a blunted tip (0.2-0.22mm) with a coagulator is inserted into ECA (Chiang *et al*., 2011; Rousselet *et al*., 2012). The ECA and inserted nylon monofilament is ligated and tightened with a silk thread to prevent bleeding from rotational displacement of the nylon monofilament during advancement into the ICA and removal at the time of reperfusion (Ansari *et al*., 2011; Engel *et al*., 2011). Following removal of the clip from the ICA, the nylon monofilament is advanced into the ICA until light resistance is felt where the origin of the MCA is occluded for 30 minutes to 2 hours depending on the severity intended (Chinag *et al*., 2011; Rousselet *et al*., 2012). Occlusion of the MCA is deemed successful when Laser Doppler Flowmetry measurements on the affected parietal bone show a 20% decrease in blood flow from baseline (Ansari *et al*., 2011; Arumugam *et al*., 2007; Taninishi *et al*., 2015). In order for reperfusion to occur, the ligation on the CCA and inserted monofilament is removed allowing blood flow through the ICA (Engel *et al*., 2011). This focal ischemic stroke model induces damage to the frontal, temporal and parietal occipital cortex and striatum including the thalamus, hypothalamus and substantia nigra (Traystman, 2003).

The permanent ischemic stroke model commonly involves occlusion of the MCA with a silk thread for 24 hours (Mdzinarishvili *et al*., 2005; Xi *et al*., 2004). Alternatively, the permanent

ischemic stroke model can be achieved by the trans-temporal approach. This involves retraction of the temporalis muscle followed by a 2-3mm burr hole made rostral to the fusion of the zygomatic and squamosal bones to locate and isolate the MCA (Taguchi *et al*., 2010). Using a steel hook and micromanipulator, the MCA is elevated and occluded by electrocoagulation for 24 hours (Taguchi *et al*., 2010).

1.6 DAMPs and Inflammasomes: An Overview in Stroke

Inflammation is an innate immune response to infection and tissue damage designed to limit harm to the host (Medzhitov, 2008). However, as mentioned, the inflammatory response in cerebral tissue damaged following ischemic stroke contributes to the progression of ischemic brain injury and exacerbation of neurological deficits (Chamorro & Hallenbeck, 2006). The inflammatory response is initiated by the detection of acute damage via extracellular and intracellular pattern recognition receptors (PRRs), which respond to conserved microbial structures, termed pathogen-associated molecular patterns (PAMPs) and/or host-derived danger signals termed damage-associated molecular patterns (DAMPs). PAMPs and DAMPs may be released from stressed or damaged cells following either microbial or non-microbial insults (Akira *et al*., 2006; Kono & Rock, 2008; Kono *et al*., 2014; Maslanik *et al*., 2013; Matzinger, 2002a; Matzinger, 2002b; Matzinger, 2012; Medzhitov, 2008; Medzhitov & Janeway, 1997; Meylan *et al*., 2006; Rock & Kono, 2008). Hence, the initiation of an inflammatory response requires sensors to detect any noxious agent or irregularity within the cellular microenvironment, and molecular platforms such as the NLRP1, NLRP2, NLRP3, NLRP6, NLRP7, NLRP12, NLRC4, AIM2 and Pyrin inflammasomes, that process this signal to trigger an appropriate effector response (Agostini *et al*., 2004; Chae *et al*., 2011; Fernandes-Alnemri *et al*., 2009; Kempster *et al*., 2011; Khare *et al*., 2012; Martinon *et al*., 2002; Miao *et al*., 2010; Minkiewicz *et al*., 2013; Vladimer *et al*., 2012).

Recent findings have provided insight into new inflammatory mechanisms that may contribute to neuronal and glial cell death during cerebral ischemia. There is emerging evidence to suggest that plasma membrane PRRs on neurons and glial cells can play an important role in activating NF-κB and MAPK(s) signalling pathways. This is in response to endogenous DAMPs released by necrotic cells in the ischemic core, leading to increased production of pro-inflammatory cytokines, and neuronal and glial cell death mediated by large intracellular multi-protein complexes (approximately 700 kDa) termed inflammasomes (Abulafia *et al*., 2009; Denes *et al*., 2015; Deroide *et al*., 2013; Ito *et al*., 2015; Iyer *et al*., 2009; Kono & Rock, 2008; Kono *et al*., 2014; Legos *et al*., 2001; Li *et al*., 2009; Martinon *et al*., 2002; Savage *et al*., 2012; Tamatani *et al*., 2000; Zhang *et al*., 2014).

At present, it is thought that the NLRP1 and NLRP3 inflammasome in neurons and glial cells may play an important role in detecting cellular damage and mediating inflammatory responses to aseptic tissue injury during ischemic stroke (Abulafia *et al*., 2009; Deroide *et al*., 2013; Ito *et al*., 2015; Savage *et al*., 2012; Zhang *et al*., 2014). In humans, the NLRP1 inflammasome is composed of four cytoplasmic components: the NLRP1 (NOD (nucleotide-binding oligomerization domain)-like receptor (NLR) Pyrin domain containing 1) receptor; ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain); precursor caspase-1 and precursor caspase-4 or 5 (Lamkanfi *et al*., 2002; Martinon *et al*., 2002). However, in mice, the NLRP1 inflammasome is composed of the NLRP1 receptor, ASC, precursor caspase-1, precursor caspase-11 (homologs to precursor caspase-4 or 5 in humans) and XIAP (X-linked inhibitor of apoptosis) (De Rivero Vaccari *et al*., 2008; De Rivero Vaccari *et al*., 2009; Mawhinney *et al*., 2011; Silverman *et al*., 2009). The NLRP3 inflammasome is composed of three cytoplasmic components: the NLRP3 (NOD (nucleotide-binding oligomerization domain)-like receptor (NLR) Pyrin domain containing 3) receptor, ASC and precursor caspase-1 in both mice and humans (Agostini *et al*., 2004; Schroder & Tschopp, 2010).

Activation and subsequent homo-oligomerization of the NLRP1 and NLRP3 receptors independently will lead to the formation of the NLRP1 and NLRP3 inflammasomes, respectively, which converts precursor caspase-1 into cleaved caspase-1 (Agostini *et al*., 2004; Martinon *et al*., 2002). Following activation, cleaved caspase-1 will cleave precursor IL-1β and precursor IL-18 into biologically active pro-inflammatory cytokines – mature IL-1β and mature IL-18, which are then released into the extracellular environment (Bauernfeind *et al*., 2011a). In addition, cleaved capase-1 may induce apoptosis and a particular type of cell death known as pyroptosis (Bergsbaken *et al*., 2009; Erener *et al*., 2012; Fink *et al*., 2008; Fink & Cookson, 2006; Lamkanfi, 2011; Sagulenko *et al*., 2013; Walsh *et al*., 2011; Zhang *et al*., 2003). The production and maturation of precursor IL-1β and precursor IL-18 is a tightly regulated process, and involves two distinct regulatory signals (Bauernfeind *et al*., 2011a; Khare *et al*., 2010; Martinon *et al*., 2009; Medzhitov, 2008; Yu & Finlay, 2008) (**Figure 1.3**). The first signal (Priming) involves the activation of plasma membrane PRRs (e.g. toll-like receptors, TLRs; TLR-2 and TLR-4), receptor for advanced glycation end products (RAGE), and IL-1 receptor 1 (IL-1R1), by DAMPs (e.g. HMGB1, High mobility group box 1; and IL-1α) released from necrotic cells in the ischemic core (Alfonso-Loeches *et al*., 2014; Burm *et al*., 2015; Caso *et al*., 2007a; Caso *et al*., 2008; Codolo *et al*., 2013; Eigenbrod *et al*., 2008; Frank *et al*., 2015; Lee *et al*., 2013; Lippai *et al*., 2013; Lok *et al*., 2015; Nagyoszi *et al*., 2015; Nystrom *et al*., 2013; Tang *et al*., 2007; Tang *et al*., 2013; Weber *et al*., 2015; Zhao *et al*., 2014; Zheng *et al*., 2013). This up-regulates gene transcription of inflammasome proteins, and both

precursor IL-1β and precursor IL-18 mediated by NF-κB and MAPK(s) signalling pathways (Bauernfeind *et al*., 2011b; Bauernfeind *et al*., 2009; Budai *et al*., 2013; Burm *et al*., 2015; Frederick Lo *et al*., 2008; Ghonime *et al*., 2014; Hara *et al*., 2013; He *et al*., 2012; Juliana *et al*., 2010; Kang *et al*., 2000; Legos *et al*., 2001; Liao *et al*., 2012; Liu *et al*., 2004a; Liu *et al*., 2013; Mariathasan & Monack, 2007; Okada *et al*., 2014; Qiao *et al*., 2012; Savage *et al*., 2012; Schroder *et al*., 2012; Tamatani *et al*., 2000; Weber *et al*., 2015; Zhao *et al*., 2013). The second signal involves activation and consequent homo-oligomerization of the NLRP1 and NLRP3 receptors individually by either DAMPs, or irregularities within the cellular microenvironment from cellular stress, resulting in the formation of the NLRP1 and NLRP3 inflammasome, which then activates precursor caspase-1 to produce cleaved caspase-1 (Faustin *et al*., 2007; Li *et al*., 2009; Martinon *et al*., 2002; Maslanik *et al*., 2013; Savage *et al*., 2012).

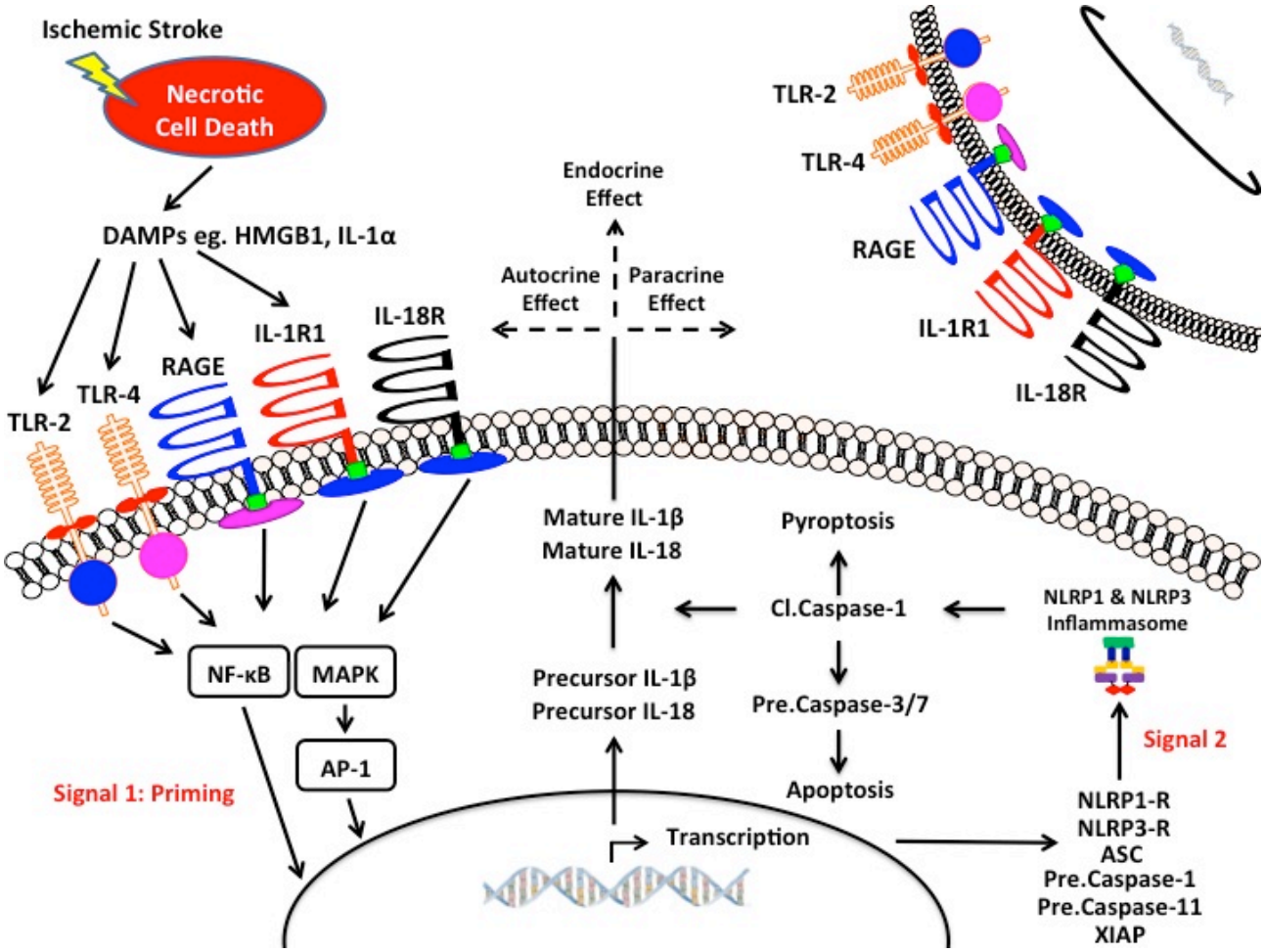

Figure 1.3: Mechanisms involved in the production and maturation of both precursor IL-1β and IL-18. DAMPs encoded by PRRs such as TLRs, RAGE and IL-1R1 allow the activation of NF-κB and MAPK(s)-dependent transcription of NLRP1 and NLRP3 inflammasome proteins, and precursor IL-1β and precursor IL-18 – known as Signal 1 (Priming). The second signal involves the activation and homo-oligomerization of the NLRP1 and NLRP3 receptors independently, resulting in the formation of the NLRP1 and NLRP3 inflammasome. Inflammasome formation is responsible for activating precursor caspase-1 into cleaved caspase-1, which then cleaves both precursor IL-1β and precursor IL-18 into mature proinflammatory cytokines that are released from the cell. These mature proinflammatory cytokines – mature

IL-1β and mature IL-18 can then initiate autocrine, paracrine and endocrine effects by binding onto their respective receptors on the same cell, neighboring neurons, astrocytes or microglia and/or peripheral leukocytes indicated by the "dashed line". In addition, cleaved caspase-1 can initiate cell death through apoptosis and pyroptosis (DAMPs, damage-associated molecular patterns; TLR, toll-like receptor; RAGE, receptor for advanced glycation end products; IL-1R1, interleukin-1 receptor 1; IL-18R, interleukin-18 receptor; NF-κB, nuclear factor kappa-B; MAPK, mitogen activated protein kinase; AP-1, activator protein-1; NLRP, (NOD (nucleotide-binding oligomerization domain)-like receptor (NLR) Pyrin domain containing 1 and 3); ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; XIAP, X-linked inhibitor of apoptosis; Cl, Cleaved; IL, interleukin; Pre, Precursor).

1.6.1 Molecular Structure of NLRP1 and NLRP3 Receptors

The NLRP1 receptor in humans is characterised by five structural domains: an N-terminal PYD (pyrin) domain, a central NACHT (NAIP, CIITA, HET-E and TP1) domain, LRRs (leucine rich repeats), FIIND (function to find) and C-terminal CARD (caspase recruitment domain) domain (Letunic *et al*., 2009; Lechtenberg *et al*., 2014; Martinon *et al*., 2009; Schultz *et al*., 1998). However, in mice the N-terminal PYD domain is absent (Faustin *et al*., 2007; Hsu *et al*., 2008; Jha & Ting, 2009). The NLRP3 receptor is characterised by three structural domains: an N-terminal PYD domain, a central NACHT domain and a C-terminal LRR domain (Bae & Park, 2011; Lechtenberg *et al*., 2014) (**Figure 1.4**). The functions of the following domains are as follows: the N-terminal PYD domain facilitates downstream homotypic PYD-PYD protein interactions with the adaptor protein ASC (Masumoto *et al*., 1999; Sahillioglu *et al*., 2014; Srinivasula *et al*., 2002). The NACHT domain is responsible for both the NLRP1 and NLRP3 receptor, once activated, to oligomerize and form the central core of the inflammasome, which is an ATP-dependent process (Duncan *et al*., 2007; Faustin *et al*., 2007; Koonin & Aravind, 2000; Levinsohn *et al*., 2012; Martinon *et al*., 2002). The LRR domain is considered to be implicated in ligand sensing and autoregulation due to reports that deletion of the LRR domain results in a constitutively active receptor by removing a possible autoinhibitory role of the LRR (Liao & Mogridge, 2009; Truhlar & Komives, 2008). The FIIND domain is autolytically cleaved upon NLRP1 receptor activation, which is necessary for NLRP1 inflammasome activity (Finger *et al*., 2012; D'Osualdo *et al*., 2011). The C-terminal CARD domain facilitates downstream homotypic CARD-CARD protein interactions with the effector protein precursor caspase-1 (Srinivasula *et al*., 2002).

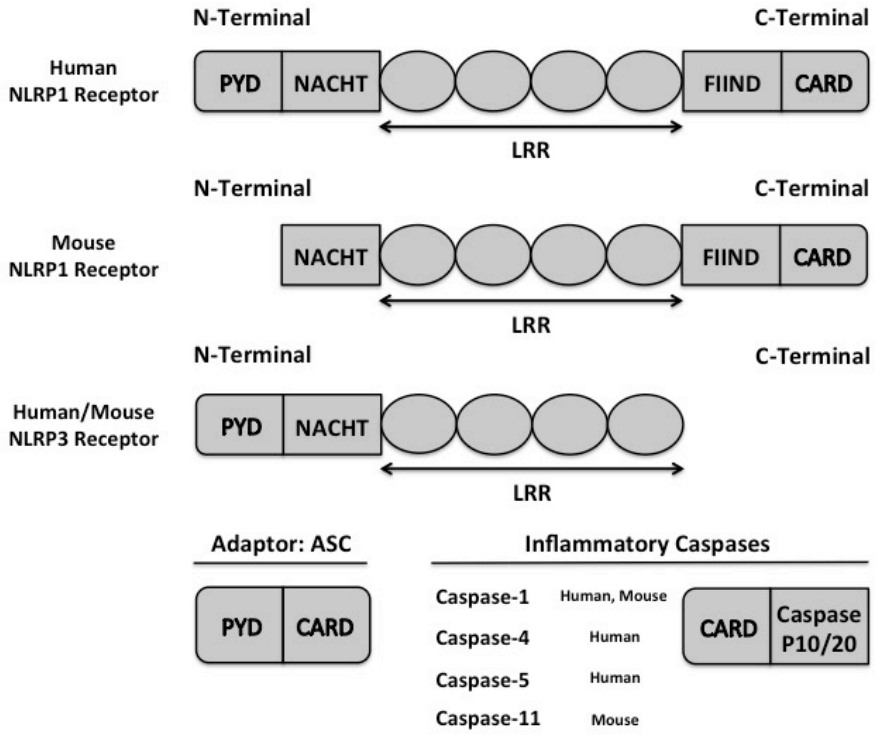

Figure 1.4: Structural domains in the NLRP1 and NLRP3 receptors and associated inflammasome proteins in human and mice. The NLRP1 receptor is characterized by five structural domains in humans – a PYD, NACHT, LRRs, FIIND and CARD domain. However, in mice, the PYD domain is absent. The NLRP3 receptor is characterized by three structural domains in humans and mice – a PYD, NACHT and LRRs. The adaptor – ASC, is characterized by two structural domains – a PYD and CARD domain. Inflammatory caspases from humans and mice are characterized by a CARD domain (NLRP, (NOD (nucleotide-binding oligomerization domain)-like receptor (NLR) Pyrin domain containing 1 and 3); ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; PYD, pyrin domain; NACHT, NAIP, CIITA, HET-E and TP1 domain; LRR, leucine rich repeats; FIIND, function to find; CARD, caspase recruitment domain).

1.6.2 Potential Stimuli(s) of NLRP1 and NLRP3 Receptor Activation in Stroke

The precise molecular and cellular stimuli(s) for NLRP1 and NLRP3 receptor activation during cerebral ischemia are unknown. Despite the extensive list of stimuli(s) described to be capable of activating the NLRP1 and NLRP3 receptor, there is no evidence of direct ligand binding (Petrilli *et al*., 2007a). Hence, it is now proposed that the NLRP1 and NLRP3 receptor is a sensor for abnormal changes in the intracellular environment in times of cellular stress (Davis *et al*., 2011; Kersse *et al*., 2011; Schroder & Tschopp, 2010). Although a fully defined mechanism leading to NLRP1 and NLRP3 receptor activation has not been elucidated during cerebral ischemia, numerous contributing cellular events are considered plausible, including energy depletion, acidosis, cathepsin release, decreased intracellular potassium (K^+) concentration, increased ROS production, oxidized mitochondrial DNA, increased intracellular calcium (Ca^{2+}) concentration, cell swelling, and protein kinase R (PKR) activation (Compan *et al*., 2012; Lee *et al*., 2012; Liao & Mogridge, 2012; Lindestam Arlehamn *et al*., 2010; Lu *et al*., 2012; Munoz-Planillo *et al*., 2013; Nakahira *et al*.,

2011; Petrilli *et al*., 2007b; Rajamaki *et al*., 2013; Rossol *et al*., 2012; Shimada *et al*., 2012; Zhou *et al*., 2010a; Zhou *et al*., 2011) (**Figure 1.5**).

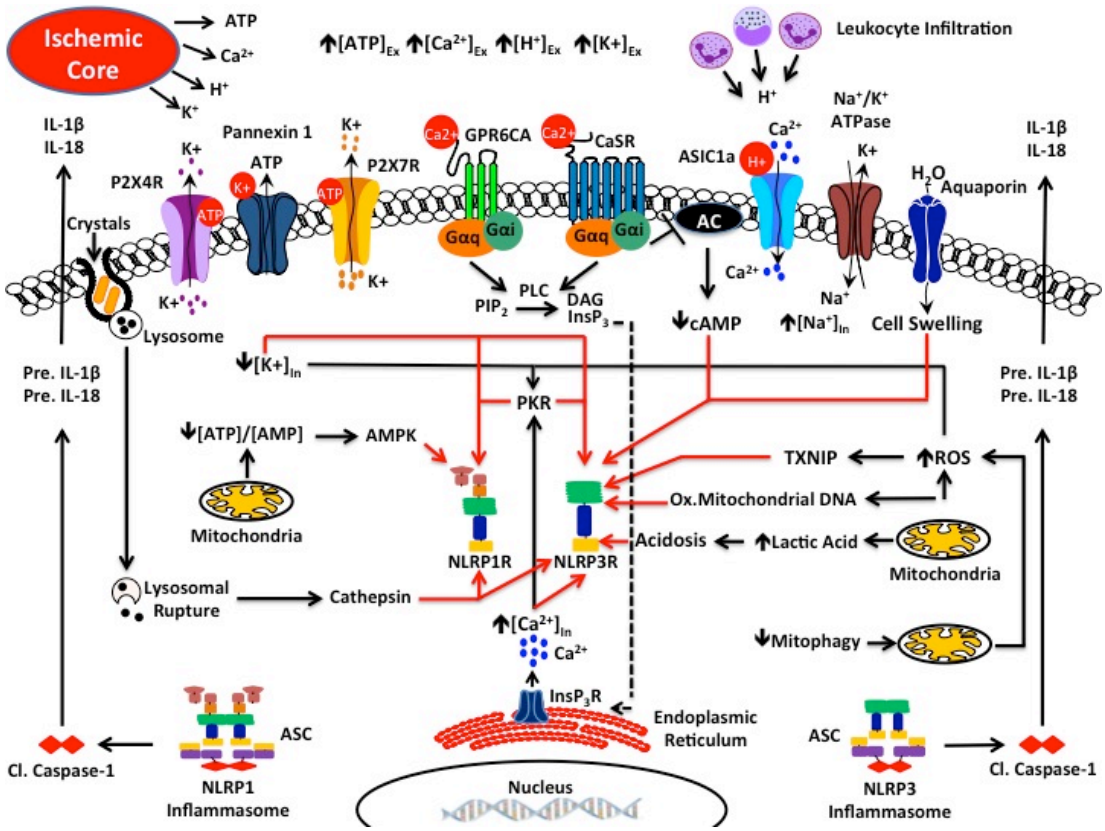

Figure 1.5: Potential stimulus involved in NLRP1 and NLRP3 receptor activation in cerebral ischemia. The precise molecular and cellular stimuli of NLRP1 and NLRP3 receptor activation during cerebral ischemia are unknown. However, several cellular events are considered plausible during cerebral ischemia including – energy depletion, acidosis, cathepsin release, decreased intracellular K^+ concentration, increased ROS production, oxidized mitochondrial DNA, increased intracellular Ca^{2+} concentration, cell swelling and PKR activation. During cerebral ischemia there is decreased levels of cytosolic ATP, thereby lowering the ratio of ATP/AMP in the cytoplasm, which activates AMPK to promote NLRP1 receptor activation. Extracellular and intracellular acidosis may activate the NLRP3 receptor. Extracellular acidosis caused by passive release of H^+ ions from necrotic cells in the ischemic core or secretion from metabolically active leukocytes may activate the NLRP3 receptor by H^+ ions binding onto ASIC1a on neurons and glial cells resulting in the influx of Ca^{2+} ions into the cytoplasm. Consequently, increasing the concentration of Ca^{2+} ions in the intracellular environment, which has recently been suggested to activate the NLRP3 receptor. However, intracellular acidosis caused by a reduction in oxygen availability under ischemic conditions, initiates anaerobic glycolysis in the mitochondria resulting in the production and accumulation of lactic acid within the cell leading to a decrease in intracellular pH (acidosis) that appears to activate the NLRP3 receptor in synergy with a decreased intracellular K^+ concentration. The NLRP1 and NLRP3 receptor can be activated by cathepsins caused by lysosomal membrane permeabilization, destabilization, and rupture releasing cathepsins into the cytoplasm induced by particulate crystals. The NLRP1 and NLRP3 receptor can be activated by a decrease in K^+ levels in the cytoplasm caused by dysfunction of the Na^+/K^+-ATPase pump due to a decreased production of ATP resulting in both an increased influx and efflux of Na^+ and K^+ ions, respectively. The increased influx of Na^+ ions will promote an osmotic movement of water through aquaporins into the cell diluting the concentration of K^+ ions in the cytoplasm; together with an increased efflux of K^+ ions into the extracellular environment by dysfunctional Na^+/K^+-ATPase pumps will both decrease the concentration of K^+ ions inside the cell. Alternatively, the passive release of ATP from necrotic cells in the ischemic core may bind onto plasma membrane P2X4 receptors on neurons, astrocytes or microglia, which can cause P2X4 receptors to open allowing an efflux of K^+ ions along its concentration gradient out of the cell decreasing the concentration of K^+ ions in the cytoplasm. In addition, necrotic cells in the ischemic core will passively release K^+ ions into the extracellular environment. Collectively these

mechanisms increase the amount of K^+ ions in the extracellular environment and activate Pannexin 1 channels on the plasma membrane. Opening of Pannexin 1 channels will lead to the release of more ATP, which can further activate more P2X4 and now P2X7 receptors on the same cell causing additional K^+ efflux creating a positive feedback loop. The NLRP3 receptor can be activated by localized increases in ROS levels in the cytoplasm. This may occur through perturbation of the electron transport chain in the mitochondria or impaired mitophagy during cerebral ischemia causing TXNIP to bind with the NLRP3 receptor leading to its activation. The NLRP3 receptor can be activated by oxidized mitochondrial DNA released by the mitochondria due to an increase in K^+ efflux and ROS. The NLRP3 receptor can be activated by an increased intracellular Ca^{2+} concentration. The passive release of Ca^{2+} ions from necrotic cells in the ischemic core can bind to and activate CaSRs and GPR6CA receptors on neighboring cells. Consequently, activation of CaSRs and GPR6CA receptors can interact with Gαq and activate PLC, which cleaves PIP2 into DAG and InsP3. InsP3 will bind onto InsP3-R on the endoplasmic reticulum to stimulate the release of Ca^{2+} ions into the cytoplasm. A reduced concentration of cAMP in the cytoplasm could promote NLRP3 receptor activation caused by passive release of Ca^{2+} ions from necrotic cells in the ischemic core binding to and activating CaSRs but interacting with Gαi to inhibit adenylate cyclase, which converts ATP to cAMP. Therefore, inhibition of adenylate cyclase will decrease the formation and concentration of cAMP in the cytoplasm, which is suggested to inhibit the NLRP3 receptor. The NLRP3 receptor is activated by cell swelling caused by an increased influx of Na^+ ions into neurons, which causes an osmotic movement of water through aquaporins into the cell. The NLRP1 and NLRP3 receptor can be activated by PKR, which is activated by cellular stress, including a decreased intracellular K^+ concentration, increased intracellular ROS production and increased intracellular Ca^{2+} concentration, which all occur during cerebral ischemia (ATP, adenosine triphosphate; AMP, adenosine monophosphate; cAMP, cyclic adenosine monophosphate; AMPK, adenosine monophosphate-activated protein kinase; CaSR, calcium-sensing receptor; AC, adenylate cyclase; ASIC, acid sensing ion channel; PIP2, phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C; DAG, diacylglycerol; InsP3, inositol triphosphate; InsP3R, inositol triphosphate receptor; ROS, reactive oxygen species; Ox, oxidized; TXNIP, thioredoxin-interacting protein; PKR, protein kinase R; NLRP, (NOD (nucleotide-binding oligomerization domain)-like receptor (NLR) Pyrin domain containing 1 and 3); ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; Cl, cleaved; Ex, extracellular; IL, interleukin; In, intracellular; Pre, precursor).

1.6.2.1 Adenosine Triphoshate (ATP)-mediated NLRP Activation

The NLRP1 receptor was recently demonstrated to be activated by energy depletion under *in vitro* conditions in human fibroblast cells subjected to oxygen and glucose deprivation (Liao & Mogridge, 2012). It was shown that ischemic conditions could decrease the levels of cytosolic ATP, thereby lowering the ratio of ATP to AMP (adenosine monophosphate) in the cytoplasm, to activate the main cellular energy sensor, AMPK (AMP-activated protein kinase), and promote NLRP1 receptor activation through an unknown mechanism. Importantly, although AMPK promotes NLRP1 receptor activation, activation of AMPK in the absence of ATP depletion in the cytoplasm was not sufficient to activate the NLRP1 receptor (Liao & Mogridge, 2012). Lastly, it was shown that mutation of the ATPase binding motif in the NLRP1 receptor caused constitutive activation, suggesting that ATP might normally inhibit the NLRP1 receptor instead of being required for assembly, which is redundant under ischemic conditions due to decreased levels of cytosolic ATP. This is in direct contrast to the abolition of activity seen when the same mutation is introduced into the NLRP3 receptor (Liao & Mogridge, 2012).

1.6.2.2 Acidosis-mediated NLRP Activation

The NLRP3 receptor was recently shown to be activated by extracellular and intracellular acidosis under *in vitro* conditions in human macrophages (Rajamaki *et al*., 2013). During cerebral ischemia, extracellular acidosis may be caused by either passive release of hydrogen (H^+) ions from necrotic cells in the ischemic core or post-ischemic inflammation. Infiltration and activation of resident inflammatory cells in damaged tissue leads to an increase in metabolic activity due to increased energy and oxygen consumption through anaerobic glycolysis, resulting in the production and secretion of lactic acid, which decreases pH in the extracellular environment (acidosis) (Krawczyk *et al*., 2010; Rajamaki *et al*., 2013; Roiniotis *et al*., 2009; Tannahill & O'Neill, 2011; Xiong *et al*., 2004). However, intracellular acidosis under ischemic conditions is usually caused by a reduction in oxygen availability, which initiates anaerobic glycolysis resulting in the production and accumulation of lactic acid within the cell (Brouns *et al*., 2008; Ding *et al*., 2000; Katsura *et al*., 1994; Park *et al*., 1999; Rossi *et al*., 2007; Xiang *et al*., 2004). An acidic extracellular environment may activate the NLRP3 receptor by H^+ ions binding to ASICs, in particular ASIC1a on neurons and glial cells resulting in Ca^{2+} influx (Li *et al*., 2010; Pignataro *et al*., 2007; Sherwood *et al*., 2011; Xiong *et al*., 2004). Increased cytosolic Ca^{2+} concentration has recently been suggested to activate the NLRP3 receptor, and is discussed in more detail in Section 1.6.2.7. Nevertheless, an acidic intracellular environment appears to activate the NLRP3 receptor in synergy with a decreased intracellular K^+ concentration seen in ischemia through a mechanism that remains to be fully determined (Rajamaki *et al*., 2013).

1.6.2.3 Edema-mediated NLRP Activation

The NLRP3 receptor was recently shown to be activated by cell swelling under *in vitro* conditions in immune cells (Compan *et al*., 2012; Rabolli *et al*., 2014; Schorn *et al*., 2011). This may occur during cerebral ischemia, as a major consequence of ATP loss is the inhibition of the Na^+/K^+-ATPase pumps, which will commonly elicit rapid deterioration of ionic gradients across the plasma membrane resulting in increased Na^+ influx and K^+ efflux (Kaplan, 2002; Khanna *et al*., 2014; Lipton, 1999; Mongin, 2007). The influx of Na^+ into neurons will result in osmotic movement of water through aquaporins into the cell so that the cell swells, causing brain edema in the ischemic penumbra (Ayata & Ropper, 2002; Breder *et al*., 2000; Khanna *et al*., 2014; Rabolli *et al*., 2014; Schorn *et al*., 2011; Simard *et al*., 2007). A recent study provided insight into the molecular events potentially driving volume-dependent NLRP3 receptor activation (Compan *et al*., 2012). It was shown that the NLRP3 receptor was oligomerized into inactive complexes in a resting state in macrophages. However, in a hypotonic environment the NLRP3 receptor was activated and underwent a conformational change dependent on decreased intracellular K^+ levels (Compan *et al*.,

2012). Hence, inhibition of K^+ efflux during hypotonic shock was sufficient to block NLRP3 receptor activation. In addition, chloride (Cl^-) ion efflux through swell-sensing Cl^- channels reduced NLRP3 receptor activation, although K^+ efflux was unaffected (Compan *et al*., 2012). Moreover, in a hypotonic environment, transient receptor potential (TRP) channels have been implicated in cell swelling and NLRP3 receptor activation as they respond to membrane stretch, especially TRPV2 in macrophages, which was demonstrated when NLRP3 receptor activation was inhibited during TRPV2 blockade. Furthermore, a hypotonic environment caused TRP channel activation, which induced changes to intracellular Ca^{2+} levels and promoted TGFβ-activated kinase 1 (TAK1) phosphorylation, which was required for NLRP3 receptor activation (Compan *et al*., 2012). Thus, it appears that cell swelling may activate the NLRP3 receptor through a pathway that involves K^+ and Cl^- ion efflux, TRP channel activation and TAK1 phosphorylation, suggesting a complicated role for cell swelling in activating the NLRP3 receptor during cerebral ischemia.

1.6.2.4 Cathepsin-mediated NLRP Activation

The NLRP1 and NLRP3 receptor may be activated by cathepsins released into the cytoplasm due to lysosomal membrane permeabilization, destabilization and rupture induced by particulate crystals (Averette *et al*., 2009; Hari *et al*., 2014; Hornung *et al*., 2008; Hoegen *et al*., 2011; Newman *et al*., 2009; Newman *et al*., 2010; Savage *et al*., 2012; Shi *et al*., 2013; Terada *et al*., 2010). During cerebral ischemia, this could be caused by the passive release of cholesterol crystals from atherosclerotic plaques at the site of occlusion, or the release of soluble uric acid and Ca^{2+} ions from necrotic cells in the ischemic core undergoing crystallization to produce monosodium urate (MSU) and calcium phosphate (i.e. calcium pyrophosphate dihydrate and octacalcium phosphate) crystals, respectively, in the extracellular environment. These particulate crystals may then be taken up by resident cells such as astrocytes, microglia and infiltrating leukocytes via endocytosis, phagocytosis or membrane-bound scavenger receptors (i.e. CD36) to be degraded by lysosomes in the cell (Duewell *et al*., 2010; Denoble *et al*., 2011; Ea *et al*., 2011; Freigang *et al*., 2011; Freigang *et al*., 2013; Gasse *et al*., 2009; Ghaemi-Oskouie & Shi, 2011; Grebe & Latz, 2013; Hari *et al*., 2014; Hoffman *et al*., 2010; Jin *et al*., 2011; Martinon *et al*., 2006; Narayan *et al*., 2011; Pazar *et al*., 2011; Peng *et al*., 2015; Rajamaki *et al*., 2010; Rock *et al*., 2013; Sheedy *et al*., 2013; Zhang *et al*., 2015c). Consequently, the uptake of certain particulate crystals by endosomes that fuse with acidic lysosomes downstream induces lysosomal membrane permeabilization, destabilization and rupture through an unknown mechanism. This releases proteases such as cathepsins (e.g. cathepsin B & L) into the cytoplasm, which are proposed to either stimulate the receptor itself, its receptor activators, or cleave either inhibitory domains within the receptor or inhibitory proteins associated with the receptor. Such a sequence is thought to release

the NLRP1 and NLRP3 receptor from an inactive conformation to an activated state during cerebral ischemia (Benchoua *et al*., 2004; Fukuda *et al*., 2004; Kilinc *et al*., 2010; Qin *et al*., 2008; Seyfried *et al*., 2001; Wen *et al*., 2008). Despite a recent study demonstrating liposomes as a new type of particulate matter that can activate the NLRP3 receptor, an alternative mechanism was observed whereby liposomes induced the production of ROS from the mitochondria, which subsequently activated transient receptor potential melastatin 2 (i.e. TRPM2) channels to induce calcium influx in neurons and glial cells, to activate the NLRP3 receptor (Zhong *et al*., 2013).

1.6.2.5 Potassium (K^+)-mediated NLRP Activation

The NLRP1 and NLRP3 receptor can be activated by a decrease in K^+ levels (<90 mM) in the cytoplasm under *in vitro* conditions in immune cells (Franchi *et al*., 2014; Katsnelson *et al*., 2015; Lindestam Arlehamn *et al*., 2010; Munoz-Planillo *et al*., 2013; Petrilli *et al*., 2007b). During cerebral ischemia, this may result from a number of mechanisms including dysfunction of the Na^+/K^+-ATPase pump due to a decreased production of ATP (Kaplan, 2002; Lipton, 1999; Mongin, 2007). Consequently, the increased influx of Na^+ ions will promote an osmotic movement of water through aquaporins into the intracellular environment diluting the concentration of K^+ ions in the cytoplasm (Schorn *et al*., 2011), together with an increased efflux of K^+ ions (Kaplan, 2002; Lipton, 1999; Mongin, 2007). Alternatively, the passive release of ATP from cell stress and/or necrotic cells in the ischemic core may bind to plasma membrane P2X4 receptors on neighbouring neurons and glial cells to cause receptor opening and K^+ efflux (Carta *et al*., 2015; De Rivero Vaccari *et al*., 2012; Iyer *et al*., 2009; Mariathasan *et al*., 2006; Schwab *et al*., 2005; Wilhelm *et al*., 2010). In addition, necrotic cells in the ischemic core will passively release K^+ ions into the extracellular environment. Therefore, these mechanisms will collectively increase K^+ ions in the extracellular environment and activate Pannexin 1 channels on the plasma membrane (Silverman *et al*., 2009). Opening of Pannexin 1 channels will lead to further release of ATP and activation of P2X4 and P2X7 receptors, creating a positive feedback loop by leading to additional K^+ efflux (De Rivero Vaccari *et al*., 2012; Ferrari *et al*., 2006; Franchi *et al*., 2007; Hung *et al*., 2013; Kahlenberg *et al*., 2005; Le Feuvre *et al*., 2003; Locovei *et al*., 2007; Pelegrin & Surprenant, 2006). The later activation of P2X7 receptors is due to P2X4 receptors being more sensitive (approximately 100 times) to ATP than P2X7 receptors in the CNS (North & Surprenant, 2000; Raouf *et al*., 2007). In addition, the Pannexin 1 channel can be activated by other stimuli, including hypoxia, mechanical stress, increased cytosolic Ca^{2+} and increased extracellular concentrations of ATP and glutamate that occur in cerebral ischemia (Bao *et al*., 2004; Locovei *et al*., 2006; Thompson *et al*., 2006; Thompson *et al*., 2008). Consequently, the decreased concentration of K^+ ions in the cytoplasm will create an environment that is favourable for activating the NLRP1 and NLRP3 receptor

(Kahlenberg & Dubyak, 2004; Lindestam Arlehamn *et al*., 2010; Munoz-Planillo *et al*., 2013; Petrilli *et al*., 2007b; Yu, 2003).

1.6.2.6 Reactive Oxygen Species (ROS)-mediated NLRP Activation

The NLRP3 receptor may be activated by localised increases in ROS levels in the cytoplasm (Meissner *et al*., 2008; Nakahira *et al*., 2011; Zhou *et al*., 2010a; Zhou *et al*., 2011) although this remains controversial (Meissner *et al*., 2010). During cerebral ischemia, ROS elevation may occur through perturbation of the electron transport chain in the mitochondria or by an increased activation of NADPH oxidase, phospholipase A_2, xanthine dehydrogenase and/or nitric oxide synthase, all of which are driven by an increased cytosolic Ca^{2+} level (Abramov *et al*., 2007; Al-Gonaiah *et al*., 2009; Brennan *et al*., 2009; Green & Kroemer, 2004; Heeba & El-Hanafy, 2012; Kahles *et al*., 2010; Kishimoto *et al*., 2010; Nanetti *et al*., 2007; Nieminen, 2003; Ono *et al*., 2009; Tomimoto *et al*., 2002; Yoshioka *et al*., 2011). All known activators of the NLRP3 receptor can trigger the production of ROS, and furthermore, treatment with various ROS inhibitors and scavengers can block NLRP3 receptor activation (Bauernfeind *et al*., 2011b; Cassel *et al*., 2008; Cruz *et al*., 2007; Dostert *et al*., 2008; Gross *et al*., 2009; Meissner *et al*., 2008; Petrilli *et al*., 2007b; Shio *et al*., 2009).

Recent evidence suggests that the mitochondria could be a central source of ROS involved in NLRP3 receptor activation (Nakahira *et al*., 2011; Zhou *et al*., 2010a; Zhou *et al*., 2011). Using various experimental techniques to manipulate mitochondrial function and uncouple the respiratory chain, it has been demonstrated that mitochondrial dysfunction increases ROS production and leads to NLRP3 receptor activation, as would be expected to occur following cerebral ischemia (Nakahira *et al*., 2011; Zhou *et al*., 2010a; Zhou *et al*., 2011). Upon NLRP3 receptor activation and oligomerization on the endoplasmic reticulum, ASC on mitochondria is moved into the perinuclear space towards the endoplasmic reticulum via the motor protein dynein, which binds to polymerized microtubules through acetylated α-tubulin (induced by inflammasome activators). This brings ASC on the mitochondria into close proximity to the NLRP3 receptor on the endoplasmic reticulum via its N-terminal PYD binding with a mitochondria-associated adaptor protein, MAVS (mitochondrial antiviral signaling protein) on the mitochondrial outer membrane. This places the NLRP3 receptor in a position to receive mitochondria-derived signals such as ROS, which may cause continued receptor activation (Park *et al*., 2013; Misawa *et al*., 2013; Subramanian *et al*., 2013; Zhou *et al*., 2011).

Mitochondrial function is equally sensitive to elevated ROS levels due to disturbances to the respiratory chain. This results in a decrease in the mitochondrial membrane potential (MMP) during

apoptosis, which may further enhance ROS production and create a chain reaction with neighbouring mitochondria, ultimately augmenting total ROS levels (Zhou *et al*., 2010b). In order to protect the cell, ROS-generating mitochondria are removed by autophagy (i.e. mitophagy) but this may not occur efficiently due to depletion of autophagic proteins such as microtubule-associated protein 1 light chain 3B (LC3B) and beclin 1 in the presence of cellular stress and damage during cerebral ischemia (Nakahira *et al*., 2011). Hence, impaired autophagy will promote the accumulation of damaged mitochondria in the cytoplasm and thus enhance the levels of ROS produced activating the NLRP3 receptor (Nakahira *et al*., 2011; Yang *et al*., 2014; Zhou *et al*., 2011). Nevertheless, although dysfunctional mitochondria and autophagy may provide the source of ROS for NLRP3 receptor activation, it remains unclear as to how ROS activates the NLRP3 receptor. However, a recent study provided insight into the molecular events potentially driving ROS-dependent NLRP3 receptor activation (Zhou *et al*., 2010a). In unstimulated cells, thioredoxin-interacting protein (TXNIP) is constitutively bound to and inhibited by oxidoreductase thioredoxin. Following an increase in cytoplasmic ROS, this complex dissociates and allows TXNIP to bind with the NLRP3 receptor (mainly in the LRR), leading to NLRP3 receptor activation during cerebral ischemia (Ishrat *et al*., 2015; Lane *et al*., 2013; Zhou *et al*., 2010a).

Recent studies elegantly connected both an increase in K^+ efflux and generation of ROS with the production of oxidized mitochondrial DNA, and demonstrated that once released into the cytosol, oxidized mitochondrial DNA acts as a danger signal and activates the NLRP3 receptor (Mathew *et al*., 2012; Shimada *et al*., 2012). The study showed that K^+ efflux-induced mitochondrial dysfunction, demonstrated by a decreased MMP (a marker of mitochondrial damage during apoptosis), which released oxidized mitochondrial DNA into the cytosol through the mitochondrial permeability transition (MPT) pore that forms across the inner mitochondrial membrane during ischemic conditions. This occurs because mitochondrial DNA that is normally attached to the inner mitochondrial membrane will be prone to oxidation due to its close proximity to a major source of ROS during cerebral ischemia (Shimada *et al*., 2012). Consequently, this report demonstrated that oxidized mitochondrial DNA can bind to and activate the NLRP3 receptor, consistent with the mitochondria playing a key role in NLRP3 inflammasome signaling (Shimada *et al*., 2012). Overall, the study has provided evidence for a potentially unified mechanism by which K^+ efflux and ROS may activate the NLRP3 receptor during cerebral ischemia.

1.6.2.7 Calcium (Ca^{2+})-mediated NLRP Activation

The NLRP3 receptor was recently shown to be activated by an increased intracellular Ca^{2+} concentration under *in vitro* and *in vivo* conditions (Chae *et al*., 2015; Lee *et al*., 2012; Murakami *et*

al., 2012; Rada *et al*., 2014; Rossol *et al*., 2012; Triantafilou *et al*., 2013). As mentioned, during cerebral ischemia this may occur by an increased calcium influx, a decreased calcium efflux and/or an increased release of calcium from intracellular stores (mediated primarily by oxidative damage and formation of MAC on Ca^{2+} storing organelles such as the endoplasmic reticulum) in neurons and glial cells (Bano *et al*., 2005; Jeffs *et al*., 2007; Li *et al*., 2007; Murakami *et al*., 2012; Schwab *et al*., 2002; Triantafilou *et al*., 2013). In addition, recent studies have shown that an increased extracellular concentration of Ca^{2+} ions can indirectly mediate NLRP3 receptor activation through both plasma membrane calcium-sensing receptors (CaSRs) and GPR6CA receptors, together with a decreased concentration of intracellular cAMP (Lee *et al*., 2012; Rossol *et al*., 2012). During cerebral ischemia, this may be achieved by passive release of Ca^{2+} from necrotic cells in the ischemic core binding to and activating CaSRs and GPR6CA receptors on neighbouring neurons and glial cells (Korff *et al*., 2006; Lee *et al*., 2012; Rossol *et al*., 2012; Tzimas *et al*., 2004). CaSRs and GPR6CA receptors are both G-protein coupled receptors that can interact with Gαq and Gαi proteins in the plasma membrane (Christiansen *et al*., 2007; Faure *et al*., 2009; Hofer & Brown, 2003; Khan & Conigrave, 2010; Pi *et al*., 2005; Riccardi & Kemp, 2012). Consequently, Ca^{2+}-mediated activation of CaSRs and GPR6CA receptors can interact with Gαq and activate membrane-bound phospholipase C, which cleaves phosphatidylinositol-4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate ($InsP_3$) (Chae *et al*., 2015; Hofer & Brown, 2003; Khan & Conigrave, 2010; Lee *et al*., 2012; Rossol *et al*., 2012). The main effect of DAG is to activate PKC, which catalyses the phosphorylation of a variety of intracellular proteins. Whether PKC has any effect on the activation of the NLRP3 receptor remains to be determined. Moreover, $InsP_3$ that is released into the cytoplasm can bind to $InsP_3$ receptors ($InsP_3$-R) on the endoplasmic reticulum to stimulate the release of Ca^{2+} into the cytoplasm (Lee *et al*., 2012; Hofer & Brown, 2003; Khan & Conigrave, 2010; Rossol *et al*., 2012).

Lastly, it was shown that a reduced concentration of cAMP in the cytoplasm could promote NLRP3 receptor activation (Bos, 2003; Kim *et al*., 2007; Lee *et al*., 2012; Peters-Golden, 2009; Trophy, 1998). During cerebral ischemia, this may be caused by passive release of Ca^{2+} from necrotic cells in the ischemic core, which then binds to and activates CaSRs on neighbouring neurons and glial cells (Korff *et al*., 2006; Lee *et al*., 2012; Tzimas *et al*., 2004). Consequently, Ca^{2+}-mediated activation of CaSRs can similarly interact with Gαi and inhibit the membrane-bound enzyme adenylate cyclase, which converts ATP to cAMP (Lee *et al*., 2012). Therefore, inhibition of adenylate cyclase will tend to decrease the formation and concentration of cAMP in the cytoplasm, which is thought to inhibit the NLRP3 receptor by interfering with the NACHT domain without preventing ATP from binding onto the NLRP3 receptor. In contrast, Rossol and colleagues (2012)

detected no influence of cAMP on NLRP3 receptor activation. Hence, the mechanism(s) by which increased concentrations of Ca^{2+} in conjunction with a decreased concentration of cAMP in the cytoplasm promotes NLRP3 receptor activation in cerebral ischemia remains to be clarified.

1.6.2.8 Protein Kinase R (PKR)-mediated NLRP Activation

The NLRP1 and NLRP3 receptors were recently shown to be activated by protein kinase R (PKR) in the cytoplasm under *in vitro* conditions in lipopolysaccharide (LPS) primed immune cells during apoptosis (Lu *et al*., 2012). PKR is a ubiquitously expressed serine/threonine protein kinase activated by double-stranded RNA that was primarily identified as an innate immune anti-viral protein induced by interferon (IFN) (Garcia *et al*., 2006; Nakamura *et al*., 2010). In addition, PKR is involved in inflammation and appears to be activated by cellular stress, including a decreased intracellular K^+ concentration, increased intracellular ROS production, increased intracellular Ca^{2+} concentration and pro-inflammatory cytokines (TNFα and IFN), all of which occur during cerebral ischemia (Lu *et al*., 2012; Nakamura *et al*., 2010). However, the ability of PKR to act as a danger-sensing molecule to detect these stimuli remains to be determined. Nevertheless, upon activation by a stimulus, PKR will undergo dimerization and auto-phosphorylation reactions in order to phosphorylate the target protein – in this case NLRP1 or NLRP3 receptors – to induce activation (Dey *et al*., 2005; Garcia *et al*., 2006; Lu *et al*., 2012; Peng *et al*., 2015). The 2012 study by Lu *et al*. provides evidence for a broader role for PKR as a danger-sensing molecule that is integral to inflammasome assembly and activation. Major findings were that overexpression of PKR substantially enhanced caspase-1 activation and IL-1β cleavage, whereas knockdown of PKR by short hairpin RNA (shRNA) inhibited caspase-1 activation and IL-1β cleavage in different cell types including macrophages, dendritic cells and embryonic kidney cells (Lu *et al*., 2012). In addition, the study demonstrated that PKR physically interacted with the NLRP1, NLRP3, NLRC4 and AIM2 receptors, which was mediated by auto-phosphorylation of PKR, while a kinase-defective PKR protein failed to bind to or activate the NLRP3 receptor.

A recent study demonstrated that PKR kinase activity is not needed for ASC oligomerization and caspase-1 activation in the NLRP1 and NLRP3 inflammasome in non-primed anthrax lethal toxin infected macrophages undergoing pyroptosis (Hett *et al*., 2013). This demonstrates that PKR has an uncharacterized role in caspase-1 activation and pyroptosis that is distinct from its kinase-dependent role in inflammasome formation during apoptosis in LPS-primed cells. This might possibly occur through PKR protein interactions with the IκK complex, which causes IκB phosphorylation and proteasomal degradation activating the NF-κB signaling pathway (Hett *et al*., 2013). In other words, PKR kinase activity is present in a primed apoptotic and

pyroptotic cell death model, while PKR kinase activity is absent (i.e. PKR protein interaction is present) in a non-primed apoptotic and pyroptotic cell death model. Therefore, this study establishes a different role for PKR in two distinct cell death pathways during apoptosis and pyroptosis (Hett *et al*., 2013). Moreover, PKR failed to interact with other cytosolic receptors or inflammasome family members, including NOD2, NLRP12 and NLRX1 (Lu *et al*., 2012). Hence, the authors concluded that PKR selectively and directly interacted with the NLRP1, NLRP3, NLRC4 and AIM2 receptors to induce their activation. This proposal was recently challenged, however, as stimulus known to activate the NLRP3, NLRC4 and AIM2 receptors were also able to activate precursor caspase-1, and process both precursor IL-1β and IL-18 into their mature forms in PKR deficient macrophages, demonstrating that PKR is not required for inflammasome activation in macrophages (He *et al*., 2013). Hence, additional studies are needed to clarify the precise role of PKR in inflammasome activation.

1.6.3 Mechanism(s) of Nod Like-Receptor (NLR) Activation: NLRP1 and NLRP3

There are two proposed models of NLR activation suggested in the literature (Kadota *et al*., 2009; Mayor *et al*., 2007; Shirasu, 2009). The principal difference between the two models is the implementation of the activation signal. The first hypothetical mechanism is based upon the assumption that the NLR is present in the cell in a closed inactive form (i.e. an 'off' state), whereby the regulatory LRR domain is folded onto the NACHT domain and thus preventing ATP from binding and initiating a structural rearrangement that would promote an 'open' active state (Jha & Ting, 2009; Riedl *et al*., 2005; Yuan *et al*., 2010). However, direct binding of a PAMP/DAMP individually, or their associated complex with adaptor molecules, to the regulatory LRR domain on the NLR, would cause the regulatory LRR domain to be released from the NACHT domain, leading to the formation of an 'open' active NLR that is able to oliogomerize upon activation (Faustin *et al*., 2007; Kadota *et al*., 2009). The second hypothetical mechanism is based on the assumption that the NLR is present in a 'off' state bound to a host guard complex, which protects the NLR from proteasomal degradation and keeps the NLR in an inactive conformation (Boyer *et al*., 2011; Dangl & Jones, 2001; Fontana *et al*., 2011; Kadota *et al*., 2009; Mayor *et al*., 2007). However, direct or indirect activation of the guard complex by a PAMP/DAMP would lead to complete or partial dissociation from the NLR, producing an 'open' and active NLR that is able to oligomerize upon activation (Boyer *et al*., 2011; Dangl & Jones, 2001; Fontana *et al*., 2011; Kadota *et al*., 2009; Mayor *et al*., 2007). Clearly, more experimental evidence is needed before either model of NLR activation can be confirmed.

1.6.4 Formation of the NLRP1 and NLRP3 Inflammasome Complex

At present, there are three proposed models of NLRP1 inflammasome assembly in humans. The first model suggests that the C-terminal CARD domains of NLRP1 receptors are able to directly interact with the CARD domains in pro-caspase-1 in the absence of the adaptor protein ASC (Martinon *et al*., 2002) (**Figure 1.6A**). However, the second model suggests that the N-terminal PYD domains of NLRP1 receptors facilitate downstream homotypic protein-protein interactions with the adaptor protein ASC, as it contains two sub-domains: a PYD and CARD domain (Bauernfeind *et al*., 2011a; Faustin *et al*., 2007; Martinon *et al*., 2009; Srinivasula *et al*., 2002). Effectively, this would allow the PYD and CARD domain of ASC to bind with the PYD domain and CARD domain in the NLRP1 receptor and precursor caspase-1, respectively, through PYD-PYD or CARD-CARD homotypic protein interactions (Bauernfeind *et al*., 2011; Faustin *et al*., 2007; Martinon *et al*., 2009; Srinivasula *et al*., 2002) (**Figure 1.6B**). However, it was recently proven that the N-terminal PYD domain of the NLRP1 receptor is not required for NLRP1 inflammasome activity but the dependence upon ASC and the requirement of the C-terminal CARD domain of the NLRP1 receptor suggested an alternative model (Finger *et al*., 2012). The third model suggests that ASC dimers and/or ASC polymers in the form of filamentous structures known as ASC specks are arranged via PYD-PYD association leaving two "free" CARD domains at either end, which can bind with the C-terminal CARD domain on the NLRP1 receptor and the CARD domain of precursor caspase-1 in order to form the NLRP1 inflammasome (Cai *et al*., 2014; Finger *et al*., 2012; Franklin *et al*., 2014) (**Figure 1.6C**). In addition, it was recently confirmed that the FIIND domain on the NLRP1 receptor is autolytically cleaved, demonstrating that NLRP1 inflammasome activity is strictly dependent upon this cleavage following NLRP1 receptor activation (Finger *et al*., 2012; D'Osualdo *et al*., 2011).

Regarding NLRP3 inflammasome assembly – the N-terminal PYD domains of the NLRP3 receptor facilitates downstream homotypic PYD-PYD protein interactions with the PYD domains of ASC polymers in the form of filamentous structures known as ASC specks (Bauernfeind *et al*., 2011; Cai *et al*., 2014; Franklin *et al*., 2014; Martinon *et al*., 2009; Srinivasula *et al*., 2002). Effectively, this allows the CARD domains of ASC polymers to bind with the CARD domains in precursor caspase-1 through homotypic CARD-CARD protein interactions (Bauernfeind *et al*., Cai *et al*., 2014; Franklin *et al*., 2014; 2011; Martinon *et al*., 2009; Srinivasula *et al*., 2002) (**Figure 1.7**).

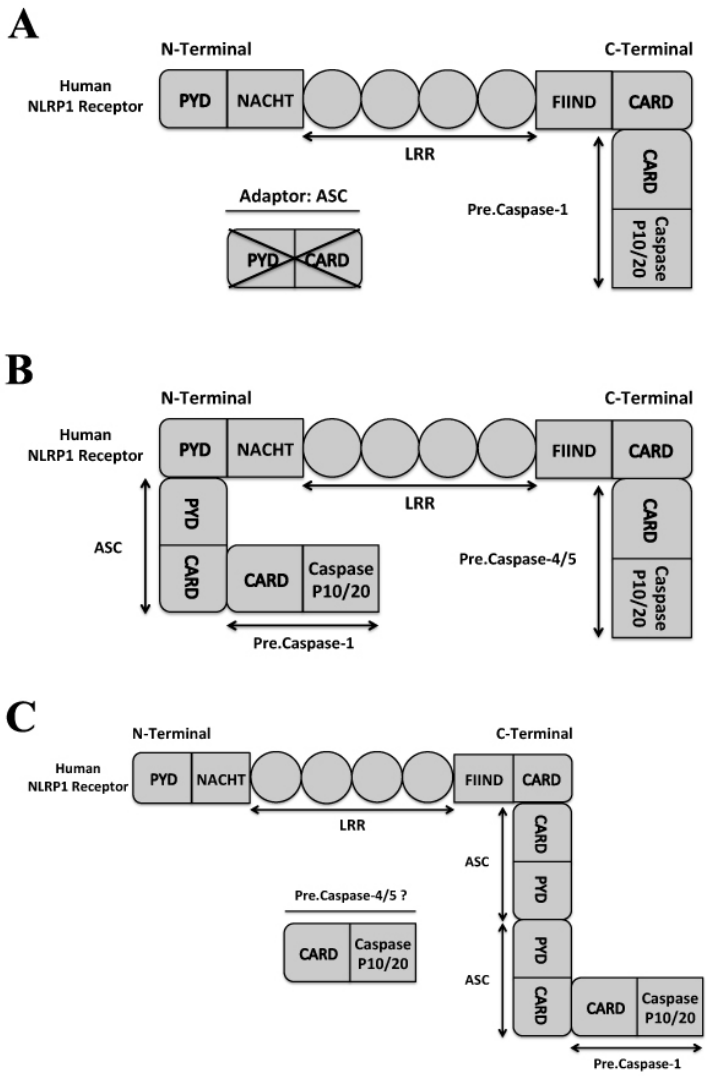

Figure 1.6: Three proposed models for NLRP1 inflammasome assembly in humans. (A). The first model suggest that the C-terminal CARD domain of the NLRP1 receptor is able to directly interect with the CARD domain in precursor caspase-1 through CARD-CARD homotypic protein interactions in the absence of the adaptor protein ASC. However, the second model (B) suggests that the N-terminal PYD domain of the NLRP1 receptor is able to facilitate downstream homotypic protein interactions with the adaptor protein ASC as it contains two sub-domains: a PYD and CARD domain. This allows the PYD and CARD domain of ASC to bind with the PYD domain and CARD domain in the NLRP1 receptor and precursor caspase-1, respectively, through PYD-PYD or CARD-CARD homotypic protein interactions. Recently, it was proven that the N-terminal PYD domain of the NLRP1 receptor is not required for NLRP1 inflammasome activity but the dependence upon ASC and the requirement of the C-terminal CARD domain of the NLRP1 receptor suggests an alternative model. (C). The third model suggests that ASC dimers form via PYD-PYD association leaving two "free" CARD domains at either end, which can bind with the C-terminal CARD domain on the NLRP1 receptor and the CARD domain of precursor caspase-1 in order to form the NLRP1 inflammasome (NLRP1, (NOD (nucleotide-binding oligomerization domain)-like receptor (NLR) Pyrin domain contain 1); ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; PYD, pyrin domain; NACHT, NAIP, CIITA, HET-E and TP1 domain; LRR, leucine rich repeats; FIIND, function to find; CARD, caspase recruitment domain; Pre, Precursor).

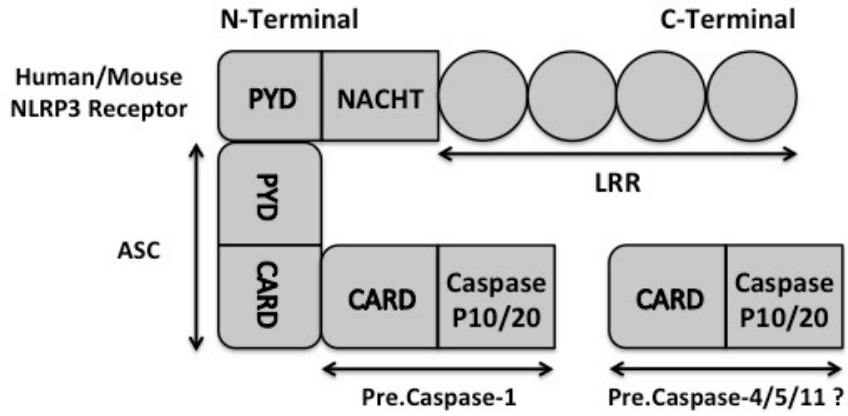

Figure 1.7: NLRP3 inflammasome assembly in humans and mice. The N-terminal PYD domain of the NLRP3 receptor facilitates downstream homotypic PYD-PYD protein interactions with the PYD domain of ASC. This effectively allows the CARD domain of ASC to bind with the CARD domain in precursor caspase-1 through homotypic CARD-CARD protein interactions (NLRP3, NOD (nucleotide-binding oligomerization domain)-like receptor (NLR) Pyrin domain containing 3); ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; PYD, pyrin domain; NACHT, NAIP, CIITA, HET-E, and TP1 domain; LRR, leucine rich repeat; CARD, caspase recruitment domain; Pre, Precursor).

The activation and subsequent oligomerization of the NLRP1 and NLRP3 receptors individually via their NACHT domain will subsequently recruit ASC polymers and precursor caspase-1 molecules, leading to the formation of the NLRP1 and NLRP3 inflammasome. Consequently, this will activate precursor caspase-1 into cleaved caspase-1 in an "all-or-none fashion" via proximity-induced auto-activation, which is a process where two or more precursor caspase-1 proteins are brought sufficiently close together to induce their autocatalytic activation (Boatright *et al*., 2003; Liu *et al*., 2014b; Salvesen & Dixit, 1999).

It should be recognised that the interaction between human precursor caspase-4/5 or murine precursor caspase-11 to precursor caspase-1 is essential for caspase-1 activation in both NLRP1 and NLRP3 inflammasomes (Kang *et al*., 2000; Kang *et al*., 2002; Kang *et al*., 2003; Martinon *et al*., 2002; Rathinam *et al*., 2012; Salskov-Iversen *et al*., 2011; Sollberger *et al*., 2012; Wang *et al*., 1998). However, the timing when human precursor caspase-4/5 or murine precursor caspase-11 binds to the inflammasome remains to be clarified. In addition, the precise location, time of binding and role of XIAP in murine NLRP1 and NLRP3 inflammasome remains to be established. However, it is suggested that full-length XIAP may serve to inhibit the NLRP1 and NLRP3 inflammasome by inhibiting precursor caspase-1 activation, although once XIAP becomes cleaved it is unable to inhibit precursor caspase-1 effectively due to the production of an XIAP fragment (BIR1-2) with an attenuated capacity to inhibit precursor caspase-1 (Katz *et al*., 2001; Keane *et al*., 2001; Lotocki & Keane, 2002; Mawhinney *et al*., 2011; Vince *et al*., 2012). Therefore, stroke-induced XIAP cleavage may reduce the threshold for activation of precursor caspase-1, allowing

unrestrained maturation of both precursor IL-1β and IL-18 (De Rivero Vaccari *et al*., 2008). Additional studies with XIAP-deficient animals are needed to determine the consequences of XIAP cleavage in stroke-induced inflammasome signaling.

1.6.5 NLRP1 and NLRP3 Inflammasome-mediated Cell Death in Stroke

The NLRP1 and NLRP3 inflammasome can mediate neuronal and glial cell death in ischemic stroke through a number of mechanisms by increasing the production and secretion of pro-inflammatory cytokines IL-1β and IL-18, and via pleiotropic effects of cleaved caspase-1 in mediating apoptosis and pyroptosis. While most studies suggest that IL-1β binding to the IL-1 receptor 1 (IL-1R1) on neurons and glial cells is harmful to the injured cerebral tissue during ischemic stroke, some studies report neuroprotective effects that seem to be dependent on the concentration of IL-1β, and on the timing of the response relative to the ischemic stroke insult (Bernardino *et al*., 2005; Jones *et al*., 2005; Lu *et al*., 2005; Shaftel *et al*., 2007a). Importantly, IL-1β alone, in the absence of cerebral tissue injury, is not neurotoxic (Lawrence *et al*., 1998, Rothwell, 1999; Shaftel *et al*., 2007b). It is thus proposed that the increase in IL-1β production after ischemic stroke is part of a protective response that goes wrong. A number of neurological disorders share common cell injury mechanisms and could provide indications to the mechanisms underlying the harmful effects of IL-1β. For example, evidence has emerged on the relationship between glutamate excitotoxicity and oxidative stress with IL-1β. Hence, it is proposed that glutamate excitotoxicity and oxidative stress with IL-1β are linked in causing neuronal and glial cell death during ischemic stroke.

1.6.5.1 IL-1β and Glutamate Excitotoxicity

There is evidence to suggest that glutamate excitotoxicity and IL-1β actions are not mutually exclusive. This was demonstrated in an experimental study that intracerebroventricular injection of an NMDA agonist (i.e. cis-2,4-methanoglutamate) increased protein expression of IL-1β in neurons, astrocytes and microglia in the parietal cortex and striatum of rats following 30 minutes to 7 days of NMDA-induced excitotoxicity (Pearson *et al*., 1999). In addition, the study revealed an early temporal expression of IL-1β in microglia localised to the site of cerebral tissue damage and a delayed, widespread expression of IL-1β in astrocytes suggesting a diverse role for IL-1β following NMDA-induced excitotoxicity. Similiarly, an experimental study demonstrated that pre-treatment with MK-801, a non-competitive NMDA receptor antagonist decreased gene expression of TNF-α and IL-1β in the parietal cortex following 4, 16 and 24 hours of ischemia in a photothrombosis model of focal ischemic stroke (Jander *et al*., 2000). This study was the first to

suggest that NMDA-induced excitotoxicity can activate inflammatory gene expression independently from neuronal and glial cell death induced by cerebral ischemia and may provide a mechanistic link as to how IL-1β mediates cell injury by regulating excitoxicity. This concept was supported by a number of experimental studies. A study demonstrated that intracerebroventricular injection of a recombinant IL-1 receptor antagonist decreased neuronal cell death and infarct size following 24 hours of focal ischemia and NMDA-induced excitotoxicity suggesting that IL-1β is a mediator for ischemic and excitotoxic damage (Relton and Rothwell, 1992). In addition, a number of experimental studies demonstrated that intracerebroventricular injection of IL-1β into the cortex failed to increase infarct volume in either the striatum or cortex, but increased excitotoxic damage in the striatum and cortex suggesting a link between excitotoxicity and IL-1β, although the mechanism(s) by which they converge may be diverse (Lawrence *et al*., 1998; Stroemer and Rothwell, 1998; Allan *et al*., 2000). This was elegantly demonstrated in an experimental study where administration of IL-1β was able to increase activation of Src tyrosine kinase, which bound and phosphorylated the NMDA receptor subunits (i.e. NR2A/B) to increase Ca^{2+} influx through NMDA receptors inducing excitotoxic cell death in primary hippocampal neurons in rats (Viviani *et al*., 2003). Moreover, an experimental study demonstrated that administration of IL-1β decreased gene expression of glutamate transporter subtype-1, which decreased the re-uptake of glutamate and increased excitotoxicity in primary human astroyctes in a dose-dependent manner after 24 hours (Hu *et al*., 2000). Finally, an experimental study demonstrated that administration of IL-1β activated the cystine/glutamate antiporter (i.e. System x(c)-) to increase intracellular cystine levels and extracellular glutamate levels inducing glutamate excitoxicity in mixed neuron-astrocyte co-cultures under ischemic conditions (Fogal *et al*., 2007). Hence, it appears that IL-1β stimulates a variety of pathways to induce glutamate excitotoxicity.

1.6.5.2 IL-1β and Oxidative Stress

There is evidence to suggest that oxidative stress and IL-1β are not mutually exclusive. A number of studies have demonstrated that ROS can induce the expression of precursor IL-1β in mixed hippocampal cultures and attenuated by antioxidants such as N-acetyl-cysteine (Brabers & Nottet, 2006; Min *et al*., 2003). In addition, an experimental study demonstrated that administration of NMDA increased intracellular Ca^{2+} concentrations, which uncoupled the mitochondrial electron transport chain increasing the production of ROS and inducing oxidative stress in mouse cortical neurons under glutamate excitotoxic conditions (Dugan *et al*., 1995). Similiarly, an experimental study demonstrated that intraperitoneal administration of a lipid peroxidation inhibitor (IRFI 042) decreased malondialdehyde (MDA) levels, prevented loss of glutathione-reduced (GSH) levels and

gene expression of IL-1β in the cortex and hippocampus of kainic-acid induced brain injury in mice (Marini *et al*., 2004; Reynolds & Hastings, 1995). Hence, these studies propose that excitotoxicity can cause oxidative stress, and that oxidative stress can induce precursor IL-1β expression suggesting a possible mechanistic link.

1.6.5.3 IL-1β, IL-18 and IL-12

Although IL-18 is structurally homologous to IL-1β, and its receptor (IL-18R) belongs to the same IL-1R/TLR superfamily, its function is quite different from IL-1β (Boraschi & Dinarello, 2006; Felderhoff-Mueser *et al*., 2005). In synergy with IL-12, IL-18 promotes T helper 1 (T_H1)-mediated immune responses, which play a critical role in the host defence against infection by inducing the production of interferon-γ (IFN-γ) from activated T_H1, natural killer (NK) and B cells (Nakahira *et al*., 2002; Yoshimoto *et al*., 1998). However, the overproduction of IL-12 from infiltrating macrophages and IL-18 from neuronal and glial cells in ischemic cerebral tissue induces a pro-inflammatory state via an increased production of IFN-γ. Consequently, this stimulates parenchymal macrophages to produce additional pro-inflammatory cytokines such as TNFα and IL-6, and neurotoxic mediators such as ROS and NO, leading to severe cerebral tissue damage (Monteforte *et al*., 2000; Nakanishi *et al*., 2001; Ohkusu *et al*., 2000; Wei *et al*., 1999). In addition, IL-18 stimulates NK cells and cytotoxic T cells ($CD8^+$) to show cytotoxic activity by utilizing perforin, which is a potent pore-forming molecule that can lyse target neurons and glial cells, and FasL, which can induce neuronal and glial apoptosis (Dao *et al*., 1998; Tsutsui *et al*., 1996; Yilmaz *et al*., 2006). In addition, IL-18 up-regulates perforin-dependent cytotoxic activity and FasL expression (Nakanishi *et al*., 2001). This suggests that IL-18 is a potent pro-inflammatory cytokine that may have pathophysiological roles in inflammatory conditions such as ischemic stroke.

Both IL-1β and IL-18 released from neurons and glial cells can have an autocrine, paracrine and endocrine effect by binding to their respective receptors on the same cell, neighbouring neurons, astrocytes, microglia or endothelial cells, and/or peripheral leukocytes triggering a complex series of signaling events in the target cell that can result in the activation of NF-κB and MAPK(s) signaling pathways (Dinarello, 1998; Dinarello, 2002; Dinarello, 2009; Gracie *et al*., 2003; Sedimbi *et al*., 2013). Consequently, this will lead to secondary transcription of multiple inflammation-associated genes, including: pro-inflammatory cytokines (e.g. TNFα, IL-1β, IL-6 and IL-18); chemokines (e.g. CXC-chemokine ligand 8, CXCL8 aka IL-8, CX_3C-chemokine ligand 1, CX_3CL1 aka fractalkine); and adhesion molecules (e.g. E-selectin and ICAM-1), all contributing to ischemic reperfusion injury resulting in neuronal and glial cell death (Allan *et al*., 2005; Allan &

Rothwell, 2001; Arumugam *et al*., 2004a; Ehrensperger *et al*., 2005; Huang *et al*., 2000; Vila *et al*., 2000; Yilmaz & Granger, 2008; Zhang *et al*., 1998).

1.6.5.4 Pleiotropic Effects of Cleaved Caspase-1

A major pleiotropic effect of cleaved caspase-1 is that it is able to induce pyroptosis. Pyroptosis is morphologically and mechanistically distinct from other forms of cell death such as necrosis and apoptosis. It is a programmed form of cell death that is highly inflammatory and exclusively mediated by cleaved caspase-1 (Bergsbaken *et al*., 2009). At present, pyroptosis has only been described in neurons, astrocytes, endothelial cells, muller cells, monocytes, macrophages and dendritic cells in experimental models of infection, traumatic brain injury, diabetic retinopathy, epilepsy, Alzheimer's disease, hypercholesterolemia, hyperlipidemia and alcohol intoxication (Adamczak *et al*., 2014; Alfonso-Loeches *et al*., 2014; Edgeworth *et al*., 2002; Feenstra *et al*., 2013; Fink *et al*., 2008; Lamkanfi, 2011; Tan *et al*., 2014; Tan *et al*., 2015; Yin *et al*., 2015, Zhang *et al*., 2015c). Whether neurons and glial cells undergo pyroptosis during cerebral ischemia remains to be determined. Pyroptosis is characterised by rapid plasma membrane rupture and release of pro-inflammatory contents into the extracellular environment due to the development of pores on the plasma membrane (diameter of 1.1-2.4 nm) mediated by cleaved capase-1 through an unknown mechanism(s) (Fink *et al*., 2008; Fink & Cookson, 2006). Consequently, these pores will dissipate cellular ionic gradients (such as Na^+ and K^+), allowing an osmotic movement of water through aquaporins into the cell causing swelling and lysis (Bergsbaken *et al*., 2009; Fink & Cookson, 2006; Fink *et al*., 2008). In addition, DNA damage can occur during pyroptosis, where cleaved capase-1 can mediate cleavage of chromosomal DNA by an unidentified endonuclease that does not produce the oligonucleosomal DNA fragmentation observed in apoptosis (Brennan & Cookson, 2000; Fink & Cookson, 2006). Hence, cell lysis and DNA cleavage are cleaved caspase-1-dependent features of pyroptosis that remain to be established in ischemic stroke.

Despite cleaved caspase-1 being responsible for inducing pyroptosis, additional pleiotropic effects of cleaved caspase-1 were shown to cause cell death through a number of alternative mechanisms by cleaving and inactivating a number of enzymes involved in glycolysis such as fructose-bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, α-enolase and pyruvate kinase, linking inactivation of bioenergetic pathways to cell death (Shao *et al*., 2007). In addition, cleaved caspase-1 was shown to initiate rapid mitochondrial disassembly and subsequent irreversible mitochondrial damage demonstrated by an increased production of mitochondrial ROS, mitochondrial swelling, dissipation of mitochondrial membrane potential, increased mitochondrial permeabilization and fragmentation of the mitochondrial network in immune cells (Yu *et al*., 2014).

Moreover, cleaved caspase-1 was shown to inhibit the clearance of dysfunctional mitochondria through a process known as mitophagy, which subsequently accumulates mitochondrial-derived DAMPs (e.g. mitochondrial DNA and ROS) that amplifies mitochondrial and cellular damage mediated in part by cleaving a key mitophagy pro-regulator – Parkin (Kahns *et al*., 2003; Yu *et al*., 2014). Furthermore, cleaved caspase-1 was shown to directly cleave and activate both executioner caspase-3 and 7, and Bid into its truncated form, inducing intrinsic and extrinsic apoptotic cell death, respectively (Erener *et al*., 2012; Frederick Lo *et al*., 2008; Guegan *et al*., 2002; Liu *et al*., 2004a; Walsh *et al*., 2011; Zhang *et al*., 2003). In addition, a pleiotropic effect of caspase-11 alone was shown to cleave and activate executioner caspase-3 inducing apoptotic cell death (Kang *et al*., 2000; Kang *et al*., 2002; Kang *et al*., 2003). Hence, these pleiotropic effects of cleaved caspase-1 and caspase-11 may contribute to neuronal and glial cell death in ischemic stroke.

1.6.6 Evidence of Inflammasome Activity in Cerebral Ischemia

An increase in inflammasome activity is associated with neuronal and glial cell death in cerebral ischemia. The following section will describe evidence for the role of the inflammasome in such pathology by highlighting the relationship between an increase in inflammasome activity with an increased production of IL-1β and IL-18 in cerebral ischemia.

IL-1β and IL-18 mRNA and protein expression is increased in the brains of rodents following cerebral ischemia (Pearson *et al*., 1999; Sairanen *et al*., 1997; Skifter *et al*., 2002). In addition, upregulation of both IL-1R1 and IL-18R can be observed in the cortex, hippocampus and striatum following cerebral ischemia in rats (Sairanen *et al*., 1997; Wang *et al*., 1997). Importantly, the ischemic injury induced elevations in IL-1β and IL-18, and both IL-1R1 and IL-18R levels contribute to neuronal and glial cell death that occurs subsequent to cerebral ischemia (Boutin *et al*., 2001; Loddick *et al*., 1997; Mizushima *et al*., 2002). The administration of either an IL-1β neutralizing antibody or pharmacological IL-1 receptor antagonist (IL-1ra) markedly reduced infract volume, blood brain barrier disruption, microglial activation, neutrophil infiltration and cytokines in the brain, in addition to reversing peripheral immune suppression following cerebral ischemia (Mulcahy *et al*., 2003; Pradillo *et al*., 2012; Smith *et al*., 2012; Yamasaki *et al*., 1995; Yang *et al*., 1999). Rodents deficient in caspase-1 or caspase-1 inhibition showed a reduction in IL-1β and IL-18 levels associated with diminished infarct volumes (Fann *et al*., 2013; Hara *et al*., 1997; Liu *et al*., 1999; Rabuffetti *et al*., 2000; Ross *et al*., 2007; Schielke *et al*., 1998). Immuno-neutralization of endogenous IL-1ra markedly enhanced ischemic damage, indicating that IL-1ra plays an important role in controlling endogenous IL-1β levels (Loddick *et al*., 1997). A study demonstrated that loss of IL-1R1 signaling was neuroprotective in a hypoxic-ischemic model with

an associated decrease in cytotoxic edema (Basu *et al*., 2005; Lazovic *et al*., 2005). In agreement, IL-1R1 null mice are less susceptible than wild-type control mice to focal cerebral ischemic damage induced by reversible middle cerebral artery occlusion (Fogal *et al*., 2007).

The important discovery of the NLRP1 inflammasome finally conveyed a mechanism as to how precursor caspase-1 was activated, by providing a molecular platform for activation (Martinon *et al*., 2002). In addition, a study stipulated evidence for the first time that stroke could induce the formation of the NLRP1 inflammasome in neurons and glial cells, and activate precursor caspase-1 to produce both mature IL-1β and IL-18 to mediate neuronal and glial cell death (Abulafia *et al*., 2009). Similiarly, another recent study showed that milk fat globule-EGF 8 (MFGE8) inhibited necrotic cell-induced and ATP-dependent IL-1β production in macrophages (Deroide *et al*., 2013). MFGE8 deficiency was associated with enhanced IL-1β production and larger infarct size following cerebral ischemia, whereas MFGE8 supplementation significantly dampened caspase-1 activation and IL-1β production, and reduced infarct size, in wild-type mice, indicating that MFGE8 can inhibit NLRP3 inflammasome-induced IL-1β production and attenuate post-ischemic cerebral injury (Deroide *et al*., 2013). Furthermore, a recent experimental study demonstrated that intravenous administration of a caspase-1 inhibitor and intravenous immunoglobulin significantly decreased caspase-1 activation, maturation of IL-1β and IL-18, and infarct size by suppressing NLRP1 and NLRP3 inflammasome activity following 24 hrs of reperfusion in a transient mouse model of focal ischemic stroke (Fann *et al*., 2013). In addition, another recent experimental study elegantly demonstrated that the AIM2 and NLRC4 inflammasomes contribute with ASC to acute brain injury by increasing infarct size and neurological deficits in a mouse model of focal ischemic stroke (Denes *et al*., 2015). Moreover, the concentration of IL-1β and IL-18 was increased in the cerebrospinal fluid of stroke patients and significantly attenuated following intravenous administration of recombinant human IL-1ra in stroke patients in a Phase II placebo-controlled study (Emsley *et al*., 2005; Tarkowski *et al*., 1999). Thus, the totality of experimental and now human data provide compelling evidence that in brain cells the AIM2, NLRP1, NLRP3 and NLRC4 inflammasome may be responsible for activating precursor caspase-1 to produce mature IL-1β and IL-18, which are contributing factors in brain injury following cerebral ischemia.

1.6.7 Current Treatments in Stroke

Current therapeutic approaches for ischemic stroke can be categorised into two major strategies – vessel recanalization and neuroprotection. Vessel recanalization can be achieved surgically by mechanical removal of the blood clot using intracranial clot removers (MERCI device and Penumbra system), or pharmacologically by thrombolysis using recombinant tissue

plasminogen activator (r-tPA) (NINDS, 1995; Smith *et al*., 2008; Taschner *et al*., 2011). At present, the only pharmacological treatment for acute ischemic stroke approved by the US Food and Drug Administration (FDA) continues to be intravenous r-tPA (alteplase) (NINDS, 1995). However, there are limitations towards the use of r-tPA in the treatment of acute ischemic stroke, such as a narrow therapeutic window of 3-4.5 hours due to an increased risk of intracerebral hemorrhage (ascribed to r-tPA increasing the activation and expression levels of MMP-9), neuronal excitotoxicity (attributed to r-tPA increasing NMDA receptor-evoked calcium influx through cleavage of the NR1 subunit), and an inability to rescue dying neurons, thus precluding the use of r-tPA beyond this time frame (Hacke *et al*., 2008; Kelly *et al*., 2006; Nicole *et al*., 2001; Ning *et al*., 2006). Hence, due to safety concerns and the restrictive timeframe, only a small percentage (5-10%) of eligible patients are treated with r-tPA (Kleindorfer *et al*., 2008).

An alternative approach for treating acute ischemic stroke is neuroprotection. The basic concept underlying the use of neuroprotective agents evolved in response to the idea that pharmaceutical drugs could interfere with the ischemic cascade in an attempt to save neurons in the ischemic penumbra from irreversible injury. In the past decade, a number of neuroprotective agents have undergone clinical trials including ion channel modulators such as Na^+ channel (Fosphenytoin), Ca^{2+} channel blockers (Nimodipine), glutamate receptor modulators (NMDA-glutamate receptor antagonists, e.g. Selfotel), free radical scavengers (Trilizad) and anti-inflammatory therapies (Enlimomab) (Ahmed *et al*., 2000b; Chan *et al*., 1998; Davis *et al*., 2000; Fosphenytoin - Internet Stroke Centre, 2007; Furuya *et al*., 2001; Van der Worp *et al*., 2002). Despite neuroprotective agents decreasing neuronal cell death and infarct size in animal stroke models, each of these agents have failed in clinical trials due to deleterious side effects and/or low efficacy (Cheng *et al*., 2004; Green, 2002). The discrepancy between outcomes of such therapies in animal stroke studies and clinical trials may be due to several reasons. Firstly, anatomical and physiological differences in the brains of animals and humans may be an issue as animal brains are smaller and less gyrated (Dirnagl *et al*., 1999). Hence, neuronal and glial densities will be smaller in rodents in comparison to humans. Cerebral energy metabolism and blood flow is inversely related to body mass (Dirnagl *et al*., 1999). Hence, glucose and oxygen metabolism, in addition to blood flow, will generally be higher in animals in comparison to humans. Accordingly, the size and development of the ischemic core will vary between species while characterization of the ischemic penumbra in rodents is well established in comparison to humans (Tagaya *et al*., 1997). Therefore, greater emphasis should be placed on conducting experimental stroke and neuroprotection in species that are related closer to humans. Secondly, age and associated illness or comorbidities may be an issue as most experimental studies have been conducted on relatively young and healthy

animals (Howells *et al*., 2010; O'Collins *et al*., 2011; Schaller, 2007; Wang *et al*., 2003). However, stroke patients are typically elderly and afflicted with numerous risk factors and complicating diseases such as hypertension and diabetes. Therefore, developing animal stroke models with appropriate comorbidities should better reflect human stroke pathology. Thirdly, the administration of neuroprotective agents has often occurred beyond the period of efficacy for the drug being tested (De Keyser, *et al*., 1999; Dirnagl *et al*., 1999; Ginsberg, 2008; O'Collins *et al*., 2011). In animal stroke models, the onset of ischemia and reperfusion, and the administration of treatment are precisely defined: generally at the onset of ischemia, immediately after reperfusion or at various times after reperfusion. However, in human stroke patients this is not always possible, as the onset of symptoms does not always coincide with the onset of ischemia, and neuroprotective agents were thus likely often administered many hours after the stroke began (Ginsberg, 2008). Finally, neuroprotective agents only target a particular cell injury mechanism in the ischemic cascade, and in either single or multiple cell types (Woodruff *et al*., 2011). Hence, development of neuroprotective agents that can target multiple cell injury mechanisms in different cell types is warranted. Thus, intracellular complexes known as inflammasomes that can target diverse pathogenic events in multiple cell types could provide an attractive target for superior approaches in the treatment of stroke and should be further investigated.

1.6.8 Future Treatments in Stroke – Targeting Inflammasome Signalling

In recent years the inflammasome has emerged as a key mediator in inflammation, via activation of precursor caspase-1 into cleaved caspase-1, which is responsible in initiating and amplifying the production of pro-inflammatory cytokines IL-1β and IL-18, and ultimately causing apoptotic neuronal and glial cell death following cerebral ischemia (Abulafia *et al*., 2009; Denes *et al*., 2015; Deroide *et al*., 2013; Fann *et al*., 2013; Savage *et al*., 2012; Zhang *et al*., 2014). Hence, targeting pathways upstream and downstream of inflammasome signaling, in particular to its expression, assembly, activity and products, may offer substantial promise in developing new therapeutics for stroke. These potential targets include signaling pathways (i.e. NF-κB and MAPKs), inflammasome components (i.e. NLRPs, ASC and Caspase-1), plasma membrane receptors/channels (i.e. P2X7 receptors, Pannexin 1 and K^+ channels), secondary messengers (i.e. ROS, PKR and β-arrestin-2), cytokines (i.e. IL-1β and IL-18) and cytokine receptors (i.e. IL-1R1 and IL-18R) involved in inflammasome signaling.

1.6.8.1 Targeting Signalling Pathways – NF-κB and MAPK(s) Pathway

The rationale behind targeting the NF-κB and MAPK(s) signaling pathways have emerged from observations that both pathways are involved in increasing the expression of inflammasome proteins and both precursor IL-1β and precursor IL-18 in the cytoplasm following cerebral ischemia (Bauernfeind *et al*., 2011b; Bauernfeind *et al*., 2009; Budai *et al*., 2013; Burm *et al*., 2015; Frederick Lo *et al*., 2008; Ghonime *et al*., 2014; Hara *et al*., 2013; He *et al*., 2012; Juliana *et al*., 2010; Kang *et al*., 2000; Legos *et al*., 2001; Liao *et al*., 2012; Liu *et al*., 2004a; Liu *et al*., 2013; Mariathasan & Monack, 2007; Okada *et al*., 2014; Qiao *et al*., 2012; Savage *et al*., 2012; Schroder *et al*., 2012; Tamatani *et al*., 2000; Weber *et al*., 2015; Zhao *et al*., 2013). Accordingly, this would "prime" cells to be able to form more inflammasome complexes and activate precursor caspase-1 to cleave precursor IL-1β and precursor IL-18 into their active forms – mature IL-1β and mature IL-18. Recently, a number of experimental studies have demonstrated that administration of intravenous immunoglobulin (IVIg); a highly purified blood preparation containing immunoglobulin G (IgG) was able to decrease the expression of NLRP1 and NLRP3 inflammasome proteins, and both precursor IL-1β and precursor IL-18, and thus inflammasome activity by conceivably attenuating the activation of the NF-κB (i.e. p-p65) and MAPK(s) (i.e. p-P38 and p-JNK) pathway via an unknown mechanism(s) in mouse primary cortical neurons and brain tissue under *in vitro* and *in vivo* ischemic conditions (Fann *et al*., 2013; Lok *et al*., 2015; Widiapradja *et al*., 2012). In addition, IVIg was shown to increase the expression levels of anti-apoptotic protein Bcl-2 in primary cortical neurons and brain tissue following ischemia, which have been shown to bind and inhibit the NLRP1 and NLRP3 receptor in macrophages by preventing ATP from binding onto the NACHT domain in the NLRP1 and NLRP3 receptor (Bruey *et al*., 2007; Fann *et al*., 2013; Faustin *et al*., 2009; Lok *et al*., 2015; Shimada *et al*., 2012; Widiapradja *et al*., 2012). Therefore, inhibiting the oligomerization of the NLRP1 and NLRP3 receptors is expected to attenuate caspase-1 activation and maturation of IL-1β and IL-18. Similiarly, a recent experimental study demonstrated that thymoquinone, a major ingredient in the seed of the *Nigella sativa* plant revealed an ability to inhibit the NF-κB pathway decreasing expression of the NLRP3 receptor, maturation of precursor caspase-1 and secretion of mature IL-1β and IL-18 into the extracellular environment in human (A375) and mouse (B16F10) melanoma cell lines (Ahmad *et al*., 2013). Moreover, another recent study demonstrated that administration of *Aloe vera*, an immunomodulatory agent, was able to decrease expression of the P2X7 receptor, NLRP3 receptor, precursor caspase-1 and precursor IL-1β, and thus attenuate secretion of IL-1β, TNF-α, IL-6 and IL-8 in a dose dependent manner by inhibiting the NF-κB, p38, JNK and ERK signaling pathways in LPS-activated primary macrophages and human THP-1 cells (Budai *et al*., 2013). This suggests that targeting the NF-κB

and MAPK(s) pathway may provide a clinical benefit of therapeutic interventions that target inflammasome expression and activity during cerebral ischemia. At present, NF-κB and MAPK(s) inhibition is not used in clinical trials to treat stroke patients.

1.6.8.2 Targeting Inflammasome Components: NLRPs, ASC and Caspase-1

During cerebral tissue injury there is an increased expression of inflammasome components such as NLRP1, NLRP3, ASC, and precursor caspase-1 and 11 (Bauernfeind *et al*., 2011b; Bauernfeind *et al*., 2009; Budai *et al*., 2013; Burm *et al*., 2015; Frederick Lo *et al*., 2008; Ghonime *et al*., 2014; Hara *et al*., 2013; He *et al*., 2012; Juliana *et al*., 2010; Kang *et al*., 2000; Legos *et al*., 2001; Liao *et al*., 2012; Liu *et al*., 2004a; Liu *et al*., 2013; Mariathasan & Monack, 2007; Okada *et al*., 2014; Qiao *et al*., 2012; Savage *et al*., 2012; Schroder *et al*., 2012; Tamatani *et al*., 2000; Weber *et al*., 2015; Zhao *et al*., 2013). Therefore, targeting these inflammasome components is predicted to attenuate the formation of the inflammasome complex and activation of caspase-1 following cerebral ischemia. This concept was demonstrated in a number of experimental studies using antibodies (e.g. NLRP1 and ASC antibody), inhibitors (e.g. Bay-11-7082, Parthenolide, Ac-YVAD.cmk and VX-765/VRT-018858) and other blockers (e.g. CRID3) that target components of the inflammasome complex (Abulafia *et al*., 2009; Coll & O'Neill, 2011; De Rivero Vaccari *et al*., 2009; Fann *et al*., 2013; Juliana *et al*., 2010; Laliberte *et al*., 2003; Perregaux *et al*., 2001; Rabuffetti *et al*., 2000; Ray *et al*., 2000; Ross *et al*., 2007). A recent study showed that intracerebroventricular injection of a neutralizing antibody against the NLRP1 receptor was able to cross the BBB and interfere with the assembly of the NLRP1 inflammasome complex in neuronal and glial cells, producing a decreased activation of caspase-1, maturation of IL-1β and IL-18, and reduced infarct size after 24 hours in a thromboembolic mouse model of ischemic stroke (Abulafia *et al*., 2009). Similiarly, another study showed that intracerebroventricular and intraperitoneal injection of a neutralizing antibody against the adaptor protein ASC was able to cross the BBB and interfere with the assembly of the NLRP1 inflammasome complex in cortical neurons producing a decreased activation of caspase-1 and XIAP, maturation of IL-1β and IL-18, and contusion volume after 3 days in a fluid-percussion injury rat model of traumatic brain injury (De Rivero Vaccari *et al*., 2009). Currently, antibodies against inflammasome components have not been used in clinical trials to treat cerebral ischemia. Besides using antibodies against inflammasome components a recent study showed that Bay-11-7082 and Parthenolide, both NF-κB pathway inhibitors were able to inhibit NLRP3 receptor ATPase activity, which is required to recruit and oligomerize ASC in order to form the NLRP3 inflammasome and activate precursor caspase-1 to cleave precursor IL-1β and precursor IL-18 in mouse NG5 macrophages independent of NF-κB inhibition (Juliana *et al*., 2010). Furthermore, an experimental study demonstrated that pre-treatment with a selective

precursor caspase-1 inhibitior (e.g. Ac-YVAD.cmk) was able to decrease DNA fragmentation, which attenuated apoptotic neuronal cell death and preserved synaptic function in organotypic hippocampal slices from rat pups after 24 hours of oxygen and glucose deprivation *in vitro* (Ray *et al*., 2000). Similiarly, a number of experimental studies have established that intracerebroventricular injection of a selective precursor caspase-1 inhibitor (e.g. Ac-YVAD.cmk & VX-765/VRT-018858) can inhibit the activation of caspase-1 and 3, and decrease the production of IL-1β and TNF-α, and neuronal apoptotic cell death, respectively, and also infarct size after 24 hours and 7 days in two rat models of ischemic stroke, demonstrating long-term neuroprotection from ischemic insult (Fann *et al*., 2013; Rabuffetti *et al*., 2000; Ross *et al*., 2007).

Other reasons for caspase-1 inhibition may also arise from pleiotropic effects of cleaved capase-1 in potentially stimulating pyroptotic cell death; mitochondrial dysfunction; direct cleavage and activation of both executioner caspase-3 and 7; and pro-apoptotic Bid into its truncated form can mediate intrinsic and extrinsic apoptotic cell death, respectively, contributing to the progression of ischemic brain injury and to the exacerbation of focal neurological deficits (Fink & Cookson, 2006; Guegan *et al*., 2002; Walsh *et al*., 2011; Yu *et al*., 2014; Zhang *et al*., 2003). Despite precursor caspase-1 inhibition by conventional caspase-1 inhibitors, alternative therapeutic drugs indicated for other targets and disorders have been shown to inhibit precursor caspase-1. This was previously demonstrated from a number of experimental studies that ritonavir, an orally active HIV protease inhibitor used to treat HIV infection; disulfiram, an orally active acetalaldehyde dehydrogenase inhibitor used to treat recovering alcoholics abstain from alcohol consumption; and captopril, an angiotensin converting enzyme inhibitor used to treat high blood pressure have all shown to inhibit precursor caspase-1 and therefore potentially decrease maturation of IL-1β and IL-18 (Nobel *et al*., 1997; Sloand *et al*., 2000; Uhal *et al*., 1998). Similiarly, it was recently demonstrated that thalidomide, an anti-inflammatory and anti-angiogenic drug used to treat inflammatory skin diseases and certain types of cancers at pharmacological doses can decrease precursor caspase-1 activation and subsequently decrease maturation and secretion of IL-1β and fibroblast growth factor 2 (FGF2) without affecting the expression of inflammasome proteins, mediated by a metabolite of the drug in human primary keratinocytes and fibroblast cells (Keller *et al*., 2009). In addition, it was shown that parthenolide, a herbal NF-κB inhibitor was able to directly inhibit precursor caspase-1 activity by alkylating the active site of the enzyme following ASC oligomerization at low concentrations (μM) and subsequently decrease maturation of IL-1β in human THP-1 macrophages (Juliana *et al*., 2010). At present, caspase-1 inhibitors are not approved for clinical use, but they have been used in clinical trials (i.e. VX-765/VRT-043198; Vertex Pharmaceuticals) for treating seizures in epileptic patients (Bialer *et al*., 2013; Maroso *et al*., 2011).

Moreover, a number of experimental studies have identified that cytokine release inhibitory drugs (CRIDs) are able to inhibit glutathione-S-transferase omega 1 (GSTO1), which was found to associate with ASC and inhibit ASC oligomerization, and consequently caspase-1 activation in NLRP3 and AIM2 inflammasomes, suggesting that GSTO1 might play a role in inflammasome formation in murine bone marrow derived macrophages (Coll & O'Neill, 2011; Laliberte *et al*., 2003; Perregaux *et al*., 2001). To date, antibodies, inhibitors or blockers of inflammasome components have not been tested in clinical trials to treat cerebral ischemia.

1.6.8.3 Targeting Receptors and Ion Channels: P2X7 Receptor, Pannexin 1 and Potassium (K^+) Channels

The rationale behind targeting P2X7 receptors and Pannexin 1 channels has emerged from observations that during cerebral ischemia both are involved in decreasing the intracellular concentration of K^+, which is responsible in activating the NLRP1 and NLRP3 receptors in neurons and glial cells. Numerous experimental studies have shown that both P2X7 receptor antagonism (e.g. using Brilliant Blue G) and Pannexin 1 inhibition (e.g. using Carbenoxolone & Brilliant Blue FCF) can decrease the production and secretion of proinflammatory cytokines such as IL-1β, TNF-α and IL-6, and attenuate neuronal and glial apoptotic cell death, infarct size, neurological impairment and improve survival rate in *in vitro* and *in vivo* models of cerebral ischemia (Arbeloa *et al*., 2012; Chu *et al*., 2012; Eyo *et al*., 2013; Iglesias *et al*., 2008; Poornima *et al*., 2012; Thompson *et al*., 2006; Thompson *et al*., 2008; Wang *et al*., 2013). Currently, P2X7 receptor antagonists (i.e. AZD9056, AstraZeneca; CE-224,535, Pfizer; EVT-401, Evotec; GSK1482160, GlaxoSmithKline) are not approved for clinical use, but have been used in clinical trials for treating chronic inflammatory diseases such as rheumatoid arthritis (Ali *et al*., 2013; Arulkumaran *et al*., 2011; Keystone *et al*., 2012; Stock *et al*., 2012). To date, P2X7 receptor antagonists have not been used in clinical trials to treat cerebral ischemia. A Pannexin 1 channel inhibitor (Probenecid) has long been used to treat hyperuricemia in gout by decreasing urate levels through increased urine excretion in pateints with normal renal function (Reinders *et al*., 2009; Stocker *et al*., 2011). However, its rationale in targeting inflammasome signaling has come under question due to a recent study demonstrating that Pannexin 1 channels could be dispensable for P2X7 receptor-induced inflammasome activation in murine macrophages, and furthermore the lack of selective Pannexin 1 channel inhibitors available for clinical use have made Pannexin 1 an unfavourable therapeutic target (Qu *et al*., 2011). Downstream from P2X7 receptor and Pannexin 1 activation, K^+ efflux is a powerful activator of NLRP1 and NLRP3 receptors. Therefore, inhibiting K^+ efflux or increasing K^+ concentrations in the extracellular environment may provide a strategy to inhibit NLRP1 and NLRP3 receptor activation. An experimental study provided evidence that inhibiting voltage-gated

K^+ channels (using Idebenone) prevented NLRP1 receptor activation following anthrax lethal toxin treatment in mouse macrophages (Newman *et al*., 2011). In addition, an experimental study showed that glibenclamide, an orally active sulfonylurea receptor 1 (SUR1) inhibitor towards the regulatory subunit of ATP-sensitive K^+ channels (K^+_{ATP}) used to treat Type 2 diabetes have shown a remarkable ability to inhibit caspase-1 activation, and processing and secretion of IL-1β from murine and human macrophages through an unknown mechanism independent of SUR1 inhibition (Lamkanfi *et al*., 2009). Hence, more work is needed to elucidate glibenclamide's unique ability to inhibit NLRP3 inflammasome activity.

1.6.8.4 Targeting Secondary Messengers: ROS, PKR and β-arrestin-2

Production of ROS and activation of PKR are increased in the cytoplasm during cerebral ischemia, which may be responsible for activating the NLRP1 and/or NLRP3 receptor in neurons and glial cells. A number of experimental studies have shown a more general therapeutic approach by neutralizing ROS via the use of antioxidants (e.g. N-acetyl-L-cysteine, diphenyleneiodonium chloride, epigallocatechin-3-gallate) and by eliminating ROS via the use of free radical scavengers (e.g. Ebselen), have shown a decrease in caspase-1 activation and production and secretion of IL-1β and IL-18 during mitochondrial dysfunction and apoptosis (Dostert *et al*., 2008; Jabaut *et al*., 2013; Shimada *et al*., 2012; Tassi *et al*., 2010; Tsai *et al*., 2011). However, the use of antioxidants must be used with caution as inhibiting the production of ROS may instead stimulate inflammasome activity (Van de Veerdonk *et al*., 2010). Antioxidants (e.g. Vitamin C) and free radical scavengers (e.g. NXY-059; AstraZeneca) have not been approved for clinical use due to poor efficacy in clinical trials in treating cerebral ischemia (Diener *et al*., 2008; Lagowska-Lenard *et al*., 2010).

A new free radical scavenger (Edaravone; Mitsubishi Pharma) has recently shown promise by enhancing early recanalization during t-PA infusion, suppressing serum MMP-9 levels, alleviating BBB disruption, decreasing infarct size and improving neurological deficits in stroke patients during the subacute period of stroke (Isahaya *et al*., 2012; Kimura *et al*., 2012; Nakase *et al*., 2011). Alternative approaches to using antioxidants and free radical scavengers may involve decreasing the expression of TXNIP, an NLRP3 receptor activator, by inducing the production of TXNIP-destabilizing miRNA (miRNA-17) to downregulate TXNIP activity (Lerner *et al*., 2012). Despite avenues to regulate TXNIP expression and function, attempts to inhibit inflammasome signaling by this approach are still preliminary (Watanabe *et al*., 2010). Moreover, a recent experimental study demonstrated that PKR inhibition through its kinase activity (using 2-Aminopurine and C16) was able to decrease caspase-1 activation and cleavage of precursor IL-1β by inhibiting auto-phosphorylation interactions of PKR with the NLRP1 and NLRP3 receptor,

hence preventing its activation (Lu *et al*., 2012). However, PKR inhibition through its kinase-independent activity (using 7DG) was able to decrease caspase-1 activation by reducing precursor caspase-1 expression via inhibiting protein interactions with the IκK complex in NF-κB signaling during pyroptosis (Hett *et al*., 2013). To date, PKR inhibitors have not been used in clinical trials to treat cerebral ischemia. In addition, the rationale behind targeting β-arrestin-2 was demonstrated in a recent experimental study that Omega-3 fatty acids (ω-3 FAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) could activate G-protein-coupled receptor 40 (GPR40) and GPR120, which caused the downstream scaffold protein, β-arrestin-2, to specifically bind and inhibit NLRP1 and NLRP3 receptor activation (Yan *et al*., 2013). Therefore, inhibiting the oligomerization of the NLRP1 and NLRP3 receptor is expected to attenuate NLRP1 and NLRP3 inflammasome formation, caspase-1 activation, and maturation and secretion of IL-1β and IL-18 in LPS-primed mouse macrophages (Yan *et al*., 2013). At present, activation of β-arrestin-2 has not been used in clinical trials to treat cerebral ischemia.

1.6.8.5 Targeting Cytokines and Cytokine Receptors: IL-1β, IL-18, IL-1R1 and IL-18R.

An increased expression and secretion of IL-1β and IL-18 into the extracellular environment (i.e. cerebrospinal fluid and blood plasma), which bind to elevated numbers of IL-1R1 and IL-18Rs, respectively, on neurons and glial cells contributes to cerebral tissue damage and neurological impairment (Abulafia *et al*., 2009; Denes *et al*., 2015; Deroide *et al*., 2013; Fann *et al*., 2013; Mallat *et al*., 2001; Wang *et al*., 1997; Yuen *et al*., 2007). Therefore, targeting IL-1β and IL-18 and their corresponding receptors (IL-1R1 and IL-18R) may attenuate receptor activation following cerebral ischemia. This concept was demonstrated in a number of experimental or clinical studies utilizing antibodies, antagonists or soluble decoy receptors that target the downstream pathway of inflammasome signaling. Experimental studies have shown that intracerebroventricular injection of an anti-mouse IL-1β neutralizing polyclonal antibody decreased infiltration of leukocytes (i.e. neutrophils, monocytes and lymphocytes) into the perivascular and middle cerebral artery areas, oedema, infarct volume and neurological and behavioural deficits in a dose-dependent manner 6 hours before or 24 hours after reperfusion in rat models of focal ischemic stroke (Caso *et al*., 2007b; Yamasaki *et al*., 1995). Currently, a human IL-1β monoclonal antibody (i.e. Canakinumab; Novartis) that selectively neutralizes IL-1β activity with high affinity over a long half-life (21-28 days) has been approved for clinical use to treat inherited chronic inflammatory diseases such as cryopyrin-associated periodic syndrome in particular Muckle-Wells syndrome (Chakraborty *et al*., 2012; Lachmann *et al*., 2009). Canakinumab has not been used in clinical trials for the treatment of cerebral ischemia. However, it is being used in an ongoing clinical trial (CANTOS) to determine

whether IL-1β inhibition can decrease the risk of recurrent myocardial infarction, stroke, and cardiovascular death among high risk pateints who persistently have high levels of C-reactive protein, an inflammatory biomarker, despite secondary treatment (Ridker *et al*., 2011).

A number of experimental studies have shown that intravenous injection of an anti-mouse IL-18 neutralizing antibody 30 to 60 min prior to ischemia decreased NF-κB and AP-1 activation, serum levels of pro-inflammatory TNF-α, suppression of anti-inflammatory IL-4 and IL-10, CXC chemokine expression, neutrophil infiltration, pulmonary extravasation of Evans Blue dye, apoptosis, and hepatic, pulmonary and myocardial infarct size at 3-24 hours following reperfusion in mouse models of ischemia/reperfusion injury (Takeuchi *et al*., 2004; Venkatachalam *et al*., 2009; Yang *et al*., 2007b). Similarly experimental studies have shown that intravenous or intramyocardial injection of exogenous IL-18 binding protein or mesenchymal stem cells overexpressing IL-18 binding protein, a naturally occurring inhibitor, selectively neutralized IL-18, and decreased expression of proinflammatory mediators (TNF-α, IL-1β, IL-6, IL-18, MCP-1 and ICAM-1), macrophage infiltration, renal tubule epithelium apoptosis, infarct size and increased vascular endothelial growth factor (VEGF) expression, proliferation of renal tubule epithelium and left-ventricular ejection fraction at 6-72 hours after reperfusion in rat models of renal ischemia/reperfusion injury and myocardial infarction (Wang *et al*., 2009; Wang *et al*., 2012a). Furthermore, a clinical study showed that subcutaneous injections of recombinant human IL-18 binding protein selectively neutralized IL-18 activity with high affinity and proved to be safe in patients with rheumatoid arthritis and psoriasis (Tak *et al*., 2006). However, the use and efficacy of anti-IL-18 neutralizing antibodies and IL-18 binding proteins remains to be tested in experimental and clinical studies following cerebral ischemia.

Numerous experimental studies have also shown that subcutaneous injection (25-100 mg/kg) or overexpression of human IL-1 receptor antagonist was able to cross the BBB and decrease BBB disruption, infiltration of neutrophils, proinflammatory mediators (i.e. IL-6 and CXCL1), microglial activation, infarct volume, neurological (i.e. sensory and motor function) and behavioural deficits in a dose-dependent manner following experimental stroke (Banwell *et al*., 2009; Greenhalgh *et al*., 2010; Pradillo *et al*., 2012; Yang *et al*., 1999). At present, a human recombinant IL-1 receptor antagonist (Anakinra; BioVitrum) that selectively blocks IL-1 (i.e. IL-1α and IL-1β) from binding to the IL-1Rs (i.e. IL-1R1 and IL-1R2) with high affinity over a short half-life (4 hours) has been approved for clinical use to treat rheumatoid arthritis (Dinarello, 2011; Cunnane *et al*., 2001). Anakinra is not used in clinical practice to treat cerebral ischemia but was tested in a randomised, double blind, placebo-controlled, Phase II clinical trial in patients with acute

ischemic stroke (Emsley *et al*., 2005). Numerous clinical studies have demonstrated that intravenous administration of human recombinant IL-1 receptor antagonist is able to cross the BBB and achieve therapeutic concentrations in the cerebrospinal fluid to decrease serum levels of IL-6, C-reactive protein, neutrophilia, infarct volume, and improve cognitive function within 4-6 hours of stroke onset following 7 days to 3 months of treatment suggesting that human recombinant IL-1 receptor antagonist is efficacious, safe and well tolerated in subarachnoid haemorrhage and acute ischemic stroke patients (Emsley *et al*., 2005; Galea *et al*., 2011). The efficacy of an IL-18 receptor antagonist is yet to be determined in experimental and clinical stroke studies. Currently, a human recombinant dimeric protein containing the extracellular component of IL-1R1 and the IL-1R assessory protein (Rilonacept; Regeneron) that acts as a soluble IL-1 "decoy" receptor that selectively binds IL-1α and IL-1β with high affinity over a moderate half-life (67 hours) has been approved for clinical use to treat cryopyrin-associated periodic syndromes in particular familial cold autoinflammatory and Muckle-Wells syndrome (Goldbach-Mansky *et al*., 2008; Hoffman *et al*., 2008; Moll & Kuemmerie-Deschner, 2013). Rilonacept has not been used in experimental or clinical studies for the treatment of cerebral ischemia.

1.7 Novel Treatments in Stroke: Intravenous Immunoglobulin (IVIg) and Intermittent Fasting (IF) – An Overview

Development of novel neuroprotective agents and treatment strategies that can target a number of cell injury mechanisms and cell types is warranted in the prospective treatment of cerebral ischemia. Innovative potential therapies envisaged to target multiple cell injury mechanisms in multiple cell types in the brain during an ischemic stroke includes - intravenous immunoglobulin (IVIg) and intermittent fasting (IF).

IVIg is a sterile blood preparation of natural antibodies that was initially indicated as a replacement therapy to treat immunocompromised individuals, such as those with primary immunodeficiency diseases (Rezaei *et al*., 2011; Wasserman *et al*., 2012). Since the 1950s, the improved clinical outcome evident in the treatment of primary immunodeficiency diseases with IVIg inspired experimental and clinical research into understanding the molecular and cellular mechanism(s) of action of IVIg and other potential clinical indications of IVIg for decades (Gelfand, 2012; Rezaei *et al*., 2011). Currently, IVIg is a therapeutic modality that is approved by the US Food and Drug Administration (FDA) to treat a number of autoimmune and inflammatory conditions such as primary immune deficiency diseases, immune (idiopathic) thrombocytopenic purpura (ITP) and Kawasaki syndrome, and neurological conditions such as Guillain-Barre syndrome, chronic inflammatory demyelinating polyneuropathy (CIDP) and multifocal motor

neuropathy (Arumugam *et al*., 2008; Dash *et al*., 2014; Hahn *et al*., 2013; Kuitwaard *et al*., 2009; Leger *et al*., 2013; Rezaei *et al*., 2011; Sakata *et al*., 2007; Wasserman *et al*., 2012). In addition, off-label use of IVIg treatment following randomized controlled trials of efficacy included dermatomyositis, Lambert-Eaton syndrome, Myasthenia Gravis and Stiff-Person syndrome (Dalakas, 2005; Katz *et al*., 2011; Miyasaka *et al*., 2012; Rezaei *et al*., 2011; Rich *et al*., 1997; Zinman *et al*., 2007).

Commercial IVIg is a purified polyclonal preparation of natural antibodies that is extracted from the plasma of several thousand (3000-10,000) healthy human donors in order to ensure the preparation is consistent and functionally heterogeneous (Arumugam *et al*., 2008; Saeedian & Randhawa, 2014; Simon & Spath, 2003). The primary component of IVIg preparations is immunoglobulin G (IgG; >95%), with low amounts of IgA, and minor traces of IgM (Lemieux *et al*., 2005; Negi *et al*., 2007; Prins *et al*., 2007; Rezaei *et al*., 2011, Schwab & Nimmerjahn, 2013). Despite the normal physiological functions of IgG antibodies directed against a broad range of pathogens, as well as a number of foreign and self antigens, IgG autoantibodies have been found to be responsible for inducing a number of autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus (SLE) and autoimmune hemolytic anemia (AIHA) (Hogarth & Pietersz, 2012; Takai, 2002). This unique and contradictory phenomenon is referred to as the IgG paradox, whereby the same class of IgG is able to induce both pathological symptoms and possess anti-inflammatory properties to the same disease as indicated by the successful treatment of ITP and CIPD with IVIg (Nimmerjahn & Ravetch, 2007). A major limitation towards the use of IVIg in clinical practice is the shortage in supply of IVIg due to a number of factors such as the high dose (1-2g/kg) required over 2-5 days on a monthly basis to promote an anti-inflammatory effect combined with an increased demand for IVIg in treating additional pathological disorders have made IVIg an expensive (US$100/g) therapeutic agent (Gelfand, 2005; Saeedian & Randhawa, 2014; Stiehm, 2013). Hence, a clearer understanding of the molecular structure and mechanism(s) of IgG will develop cheaper substitutes with equal efficacy to fulfill the clinical demand and reduce the cost of IVIg, and understanding the ability of IgG to attain its anti-inflammatory properties at higher doses will be important in order to maximize its full potential perhaps in the future treatment of ischemic stroke.

In conjunction to using pharmacological interventions, an alternative approach is to perhaps implement lifestyle modification regimens as a prophylactic treatment to improve an individual's health benefits ideally demonstrated by intermittent fasting (IF). IF is a form of dietary energy restriction and involves alternate periods of *ad libitum* feeding and fasting, which have been proven to extend lifespan and decrease the development and severity of age-related diseases such as

cardiovascular (e.g. Type 2 diabetes mellitus, myocardial infarction and stroke) and neurodegenerative diseases (e.g. Alzheimer's disease, Parkinson's disease and Huntington's disease) demonstrated in a number of animal models (Belkacemi *et al*., 2011; Halagappa *et al*., 2007; Katare *et al*., 2009; Longo & Mattson, 2014; Manzanero *et al*., 2011; Manzanero *et al*., 2014; Mattson *et al*., 2003; Mattson, 2005; Mattson, 2014; Mattson & Wan, 2005; Pedersen *et al*., 1999; Wan *et al*., 2010). The efficacy of prophylactic IF treatment appears to precondition and protect neurons and glial cells against brain injury by increasing their cellular resistance against excitotoxicity, oxidative stress and inflammation via coordinating an upregulation of multiple neuroprotective proteins such as neurotrophic factors, protein chaperones and antioxidant enzymes, and down regulation of pro-inflammatory cytokines at the site of injury (Arumugam *et al*., 2010; Duan *et al*., 2001a; Duan *et al*., 2001b; Faris *et al*., 2012; Guo *et al*., 2000; Liu *et al*., 2006; Sanz *et al*., 2005; Sohal *et al*., 1994; Weindruch *et al*., 2001). Despite numerous experimental studies suggesting that prophylactic IF treatment may be beneficial for overall health, a major limitation towards the practice of IF is due to the shortage of clinical studies to formulate evidence-based practice recommendations (Skaznik-Wikiel & Polotsky, 2014). Hence, more clinical research into understanding the molecular mechanism(s) of prophylactic IF treatment increasing cellular resistance to excitotoxicity, oxidative stress and inflammation in the brain may provide new opportunities in the future treatment of ischemic stroke.

1.7.1 Intravenous Immunoglobulin (IVIg): Preparation and Composition

a). Preparation of Intravenous Immunoglobulin

IVIg is extracted from healthy human plasma by using a precipitation process such as cold ethanol fractionation (Dichtelmuller *et al*., 2012; Radosevich & Burnouf, 2010). Despite the process extracting immunoglobulins (i.e. IgG, IgA and IgM), highly reactive aggregates and contaminants (prekallikrein activator, prekallikrein, activated coagulation factors) often remain in the preparation, which can activate the immune system such as the complement system causing a significant allergic reaction (Nesterova *et al*., 2009; Radosevich & Burnouf, 2010). Hence, IVIg preparations undergo a second processing step such as anion exchange diethylaminoethanol (DEAE)-sepharose chromatography to separate IgG from contaminants to ensure the preparation primarily contains immunoglobulins (Laursen *et al*., 2014; Martin, 2006).

Since IVIg is a blood-derived product several steps are undertaken to ensure the preparation is safe for commercial use. Firstly, the plasma is bacterial and viral tested for all known human bacterial (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bordetella pertussis*, *Klebsiella pneumoniae*, Group B streptococcus, diphtheria and tetanus toxin) and viral (Epstein-

Barr, measles, mumps, myxovirus influenza, adenovirus, herpes simplex, human immunodeficiency virus (HIV), hepatitis B and C, human T cell lymphotropic retrovirus (HTLV), varicella-zona, coxsackie and rubella) diseases (Kaveri, 2013; Kempf *et al*., 2007). Secondly, the preparation is made safe by removing bacterial toxins and viral particles through several sophisticated treatment strategies such as detergent treatment, trypsinization, pasteurization, nano-filtration and low pH treatment to eliminate all known and unknown human transmissible bacterial and viral pathogens (Boros *et al*., 2005; Bridonneau *et al*., 1996; Caballero *et al*., 2010; Dichtelmuller *et al*., 2009; Dichtelmuller *et al*., 2012; Kempf *et al*., 2007; Radosevich & Burnouf, 2010; Soluk *et al*., 2008).

An important consideration that needs to be understood is that the different processing modifications conducted on IVIg preparations can affect the integrity and activity of the final product by changing the chemical structure, antibody content, electrophoretic profile and subclass distribution of IgG, which can potentially lead to biological variations amongst IVIg batches in relation to both Fc receptor and opsonization activity, and complement fixation (Bridonneau *et al*., 1996; Radosevich & Burnouf, 2010). Hence, clinical physicians need to be vigilant that these changes can negatively or positively influence the anti-inflammatory properties of IVIg and affect patient outcome.

b). Composition of Intravenous Immunoglobulin

IVIg preparations primarily contain IgG (>95%) with low amounts of IgA, and minor traces of IgM, in addition to stabilizers such as sucrose, maltose, mannitol and sorbitol (Lemieux *et al*., 2005; Negi *et al*., 2007; Prins *et al*., 2007; Rezaei *et al*., 2011; Stein, 2010). Commercial IVIg preparations come in two forms: a liquid and lypophilized form, whereby the latter is reconstituted with sterile water into a liquid at time of infusion. Two important product parameters of IVIg are important at time of infusion – osmolality and pH (Shah, 2005; Stangel & Pul, 2006; Stein, 2010).

The final osmolality of IVIg at time of infusion ranges between physiological values of 280-296 mOsm to values greater then 1 Osm, which is primarily determined by the sugar (sucrose, maltose, mannitol and sorbitol) and sodium content of the preparation (Dantal, 2013; Hooper, 2008; Radosevich & Burnouf, 2010; Shah, 2005; Stein, 2010). This is an important consideration as hyperosmotic preparations of IVIg can increase osmotic pressure resulting in adverse compartmental fluid-shifts in the body (Ahsan *et al*., 1994). The sugar content contained in IVIg preparations is designed to prevent IgG dimer (1-10%) aggregate formation within individual IVIg preparations, although complications may occur such as acute renal failure where the patient has to undergo emergency renal haemodialysis with mortality occurring at 10-15% of cases (Dantal, 2013; Graumann & Zawada, 2010; Itkin & Trujillo, 2005; Renjen *et al*., 2004; Stein, 2010). The sodium

content contained in IVIg preparations is approximately 0.9%, which similarly contributes to the final osmolality, tolerability and adverse effects of IVIg (Lemm, 2002; Stein, 2010; Vo *et al*., 2006). The final pH of IVIg at time of infusion is approximately neutral between 6-7, which is ultimately determined by the physiological buffering capacity of the plasma in the recipient (Roberts *et al*., 2014; Szenczi *et al*., 2006). However, this is problematic as a low pH is often required to prevent IVIg aggregate formation and hence additional medical agents to lower pH are often needed to maintain the products stability and prevent aggregate formation (Solano *et al*., 2012; Stein, 2010; Szenczi *et al*., 2006).

In general, the adverse effects associated with IVIg administration ranges in both severity (mild to severe) and on-set of symptoms (immediate to late). Examples of some of the most common and immediate symptoms experienced by patients include headache, fever, fatigue, nausea and tachycardia in approximately 5-10% of recipients primarily due to high osmolality of IVIg preparations, which can be diluted by sterile water at time of infusion, while some of the less common and delayed symptoms encountered by patients include persistent headache, aseptic meningitis, hemolytic anemia and dermatological complications (Hamrock, 2006; Katz *et al*., 2007; Stiehm, 2013; Vo *et al*., 2006). An immediate and severe complication that can occur following the administration of IVIg is often observed in IgA deficient patients where recipients have developed immunity against IgA, which can cause anaphylactic reactions due to the presence of IgA3 in some batches of IVIg preparations (Rachid & Bonilla, 2012). Hence, the administration of IVIg is often contraindicated in IgA deficient patients, despite several strategies implemented to prevent this occurrence such as pre-treating IVIg preparations with autologous plasma or subcutaneously injecting IVIg preparations in order to limit the risk of an allergic reaction in high anti-IgA patients (Rachid & Bonilla, 2012; Salama *et al*., 2004).

1.7.2 Immunoglobulin G (IgG): Subclass, Structure and Half-Life

The IgG family is composed of four different subclasses such as IgG_1-IgG_4 in humans and IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 in rodents, with IgG_1 being the most abundant and primarily responsible for most of the immunomodulatory effects observed due to its high binding affinity and efficacy on immune receptors in both species (Kapur *et al*., 2014; Nimmerjahn & Ravetch, 2011; Vidarsson *et al*., 2014). However, the remaining subclasses of IgG all vary in their ability to activate downstream effector pathways due to different levels in abundance and binding affinity to their respective receptors (Saeedian & Randhawa, 2014).

Monomeric IgG is a protein complex composed of four peptide chains with two identical light chains and two identical heavy chains arranged in a Y-shaped configuration that forms two important structural domains, which are functionally distinct – the constant or fragment crystallizable (Fc) region, and the variable antigen binding fragment $F(ab')_2$ region (Liu & May, 2012; Vidarsson *et al*., 2014). The $F(ab')_2$ region is the amino terminal end of the IgG structure and contains two identical light and heavy chains, whereas the Fc region is the carboxy-terminal end of the IgG structure and contains only two identical heavy chains (**Figure 1.8**) (Lunemann *et al*., 2015; Vidarsson *et al*., 2014).

The average serum half-life of IgG is approximately 2-3 weeks where monthly administration of IVIg is required to maintain its therapeutic effect (Lunemann *et al*., 2015). The long serum half-life of IgG is dependent on neonatal Fc receptor (FcRn), which is responsible for binding to serum IgG in endosomes following endocytosis and protects it from catabolism by lysosomes in endothelial cells and macrophages under low pH conditions and recycles it back to the cell surface (Schwab & Nimmerjahn, 2013). Hence, in the absence of FcRn, the half-life of IgG is significantly attenuated (Garg & Balthasar, 2007; Tam *et al*., 2013; Xiao, 2012).

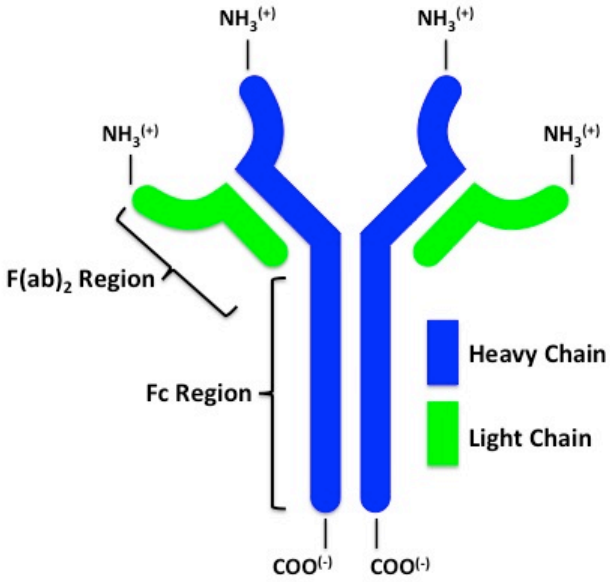

Figure 1.8. A schematic structure of monomeric immunoglobulin G (IgG). IgG is composed of four peptide chains with two identical light and heavy chains arranged in a Y-shaped configuration that forms two structural domains – the antigen binding fragment $F(ab)_2$ region and the fragment crystallisable (Fc) region. Each IgG is composed of two $F(ab)_2$ regions and one Fc region at the amino terminal and carboxy terminal end of the IgG structure, respectively.

1.7.3 Mechanisms of Action of Intravenous Immunoglobulin (IVIg) Preparations

Despite the widespread use and therapeutic success of IVIg preparations in the treatment of autoimmune and anti-inflammatory diseases for over half a century, the therapeutic mode of action of IVIg remains to be fully understood, although it appears to involve numerous immunomodulatory processes (Nagelkerke & Kuijpers, 2015). Intravenous administration of IVIg preparations can exert both pro-inflammatory and anti-inflammatory properties depending on the concentration administered (Schwab & Nimmerjahn, 2013). At low concentrations, IVIg is pro-inflammatory where activation of the complement system and innate immune cells is induced, whilst a high concentration of IVIg exerts an anti-inflammatory response (Nimmerjahn & Ravetch, 2007). Although the precise anti-inflammatory mechanisms of IVIg remain to be fully elucidated, a number of models have been proposed that are based on two general types of mechanisms mediated independently by either the $F(ab')_2$ region, which is responsible for antigen recognition; and the Fc region, which is critical for modulating the activity of the innate immune system (Lunemann *et al*., 2015; Schwab & Nimmerjahn, 2013). In essence, both regions are suggested to be responsible for the anti-inflammatory and immunomodulatory properties of IVIg at high concentrations.

1.7.3.1 $F(ab)_2$ site mediated mechanisms

The anti-inflammatory $F(ab)_2$-dependent mechanisms of IVIg is determined by the ability of autoreactive antibodies contained within IVIg preparations to be directed against a number of self-antigens such as sialic acid-binding immunoglobulin-like lectin (SIGLEC), FasL (CD95L) or Fas (CD95), the variable domains of IgG, pro-inflammatory cytokines and anaphylatoxins (Arumugam *et al*., 2007; Basta *et al*., 2003; Kalay *et al*., 2014; Murakami *et al*., 2014; Prasad *et al*., 1998; Schaub *et al*., 2011; Seite *et al*., 2014; Tawfik *et al*., 2012; Viard et al., 1998; Von Gunten *et al*., 2006; Von Gunten *et al*., 2007). The natural antibodies that are directed against each self antigen mentioned above represents a potential model of a different $F(ab)_2$ site mediated mechanism of IVIg including - cell depletion, cellular signaling blockade, pro-inflammatory cytokine neutralization, and anaphylatoxin scavenging (**Figure 1.9**).

IVIg preparations contain autoreactive antibodies directed against SIGLEC, especially SIGLEC8 and SIGLEC9 expressed on eosinophils and neutrophils, respectively (Schaub *et al*., 2011; Von Gunten *et al*., 2006; Von Gunten *et al*., 2007). As both immune cells are responsible for driving inflammation, it is suggested that eliminating these cells by SIGLEC8 and SIGLEC9-specific antibodies will decrease inflammation. Despite promising *in vitro* data, experimental and clinical studies have established that IVIg did not attenuate these cell types in mice and humans possibly indicating that the amount of SIGLEC8 and SIGLEC9-specific antibodies in IVIg

preparations was not sufficient enough to deplete these cell types at therapeutic doses (Schaub *et al*., 2011; Von Gunten & Simon, 2008). Hence, the following example is a potetial model whereby IVIg eliminates and depletes target cells by antibody-dependent cytotoxicity (ADCC) (Schwab & Nimmerjahn, 2013).

Despite containing SIGLEC antibodies, IVIg preparations contain autoreactive antibodies against self-antigens such as FasL (CD95L) expressed on cytotoxic T lymphocytes and the FasL receptor (CD95) that is ubiquitously expressed on all cell types, which blocks FasL from binding onto the FasL receptor preventing signal transduction and apoptosis (Prasad *et al*., 1998; Reipert *et al*., 2008; Viard *et al*., 1998). However, it was demonstrated from a number of experimental studies that IVIg was able to induce apoptosis in leukemic lymphocytes and monocytes mediated in part via anti-FasL receptor antibodies present in IVIg preparations supporting the notion that IVIg possesses anti-inflammatory properties by inducing apoptosis in activated leukocytes (Prasad *et al*., 1998; Viard *et al*., 1998). In addition, IVIg preparations contain autoreactive antibodies directed against self-antigens such as the variable domains of IgG - including the hinge region and the constant light or heavy chains (Spath & Lutz, 2012). This unique natural antibody is known as an anti-idiotype antibody, which can bind to either the antigen specific binding region of the immunoglobulin antibody (i.e. autoantibody) or the T cell receptor, and subsequently compete with the antigen for binding (Lemieux & Bazin, 2006). Observations from numerous experimental studies have demonstrated that anti-idiotype antibodies found in IVIg preparations include autoantibodies against the variable region of the T cell receptors and antigen receptors, which prevents autoantigen-mediated T cell activation, resulting in long-term T cell downregulation and prevention of autoantigen-mediated B and T cell activation, respectively (Macias *et al*., 1999; Seite *et al*., 2014; Tawfik *et al*., 2012). Hence, both examples above is a potential model whereby IVIg is able to block cellular signaling and communication by antagonizing either the ligand or receptor (Schwab & Nimmerjahn, 2013).

IVIg preparations contain autoreactive antibodies directed against pro-inflammatory cytokines, in particular, interleukin-1β (IL-1β), IL-6 and TNF-α, which bind and inactivates circulating pro-inflammatory cytokines with a high degree of affinity and efficacy, subsequently decreasing the concentration of circulating pro-inflammatory cytokines in the plasma (Kalay *et al*., 2014; Murakami *et al*., 2014; Panacek *et al*., 2004; Terenghi *et al*., 2006). Hence, the following example is a potential model whereby IVIg is able to prevent pro-inflammatory cytokines from binding to their respective receptors by neutralization (Ballow, 2011).

Numerous experimental studies have shown that the $F(ab)_2$ region of IgG was able to bind and sequester active complement components including anaphylatoxins such as C3a and C5a from binding onto the C3a and C5a receptors, respectively (Arumugam *et al*., 2007; Basta, 2008; Basta *et al*., 2003; Lutz *et al*., 2004; Vivanco *et al*., 1999). This interaction prevents binding of complement fragments to their receptors on target cells, which inhibits downstream effector functions such as enhanced phagocytosis of antigens, leukocyte recruitment and formation of the membrane attack complex reducing complement-mediated tissue damage (Arumugam *et al*., 2009). The following example is a potential model whereby IVIg is able to scavenge activated complement components from binding onto their respective receptors on target cells (Schwab & Nimmerjahn, 2013).

In summary, some of the potential $F(ab)_2$ site mediated mechanisms of IVIg include eliminating target cells by antibody-dependent cytotoxicity (ADCC), blocking cellular signalling by ligand or receptor antagonism, pro-inflammatory cytokine neutralization and complement scavenging.

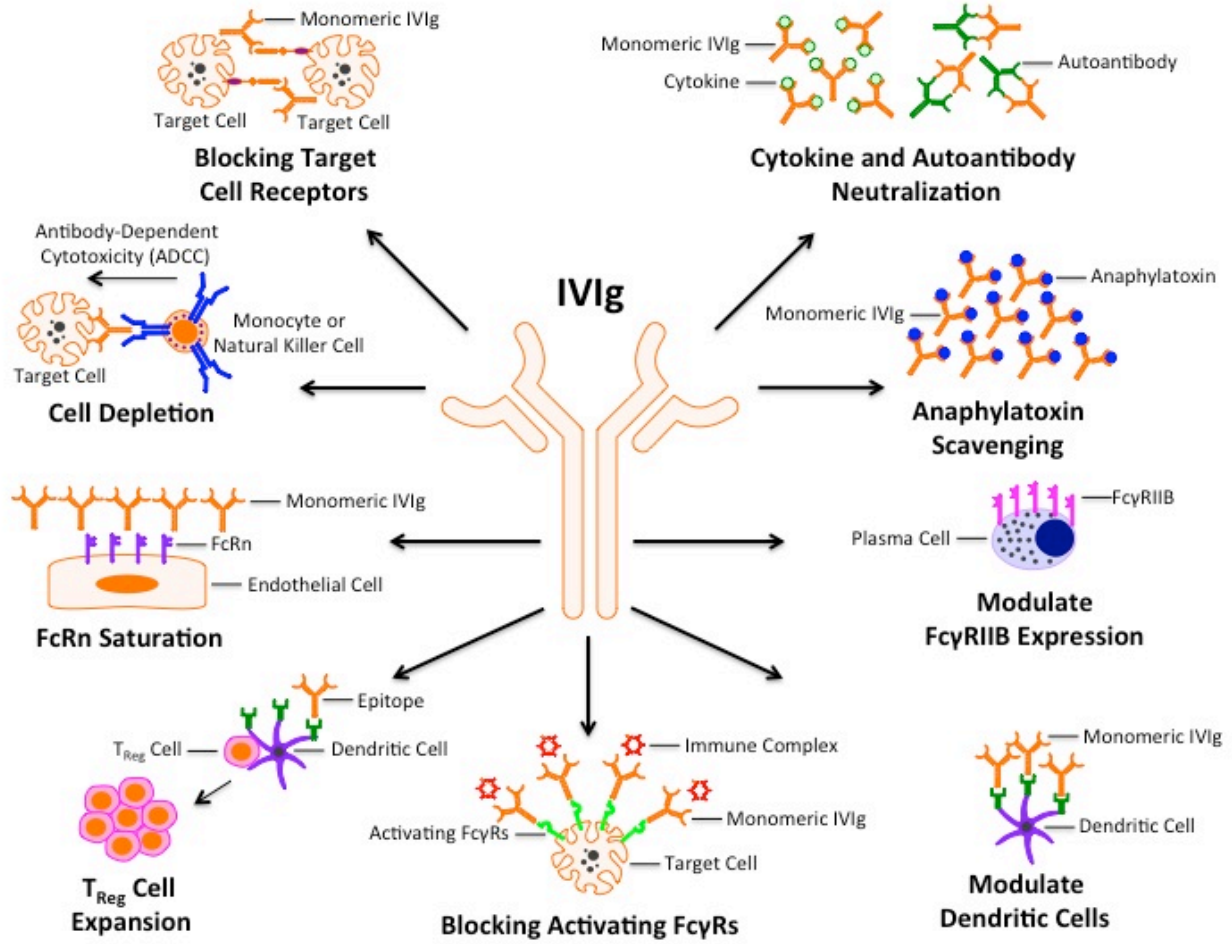

Figure 1.9: Mechanisms of action of intravenous immunoglobulin (IVIg) preparations. An overview of different pathways implicated in the anti-inflammatory and immunomodulatory properties of IVIg. The $F(ab)_2$-dependent mechanisms include - eliminating target cells by antibody-dependent cytotoxicity (ADCC), blocking cellular signalling by ligand or receptor antagonism, pro-inflammatory cytokine and autoantibody

neutralization, and complement scavenging. The Fc-dependent mechanisms include - blocking activating FcγRs, increasing the expression of inhibitory FcγRIIB, saturating FcRn, and modulating the expression and activity of immune cells such as dendritic and T cells. This figure is adapted from Intravenous immunoglobulin therapy: how does IgG modulate the immune system? Schwab and Nimmerjahn, (2013). *Nature Reviews*; **13**: p.176-189.

1.7.3.2 Fc site mediated mechanisms

The anti-inflammatory Fc-dependent mechanisms of IVIg is determined by the Fc region of IgG from IVIg preparations binding onto and affecting three different types of cognate immune receptors such as the activating family of Fcγ receptors (FcγRs), the inhibitory FcγR (FcγRIIB), and the neonatal Fc receptor (FcRn); in addition to modulating the expression and activity of immune cells (**Figure 1.9**) (Nimmerjahn & Ravetch, 2007).

The activating FcγRs are a conserved family of glycoproteins that initiates activating signaling pathways via adaptor proteins containing immunoreceptor tyrosine based activation motifs (ITAM) (Nimmerjahn & Ravetch, 2011). There are five activating FcγRs in humans including - FcγRIA, FcγRIIA, FcγRIIC, FcγRIIIA and FcγRIIIB whereas three receptors are found in rodents including - FcγRI, FcγRIII and FcγRIV, which are all widely expressed on the surface of innate immune cells such as monocytes, macrophages, basophils, neutrophils, eosinophils, mast cells, natural killer cells, and platelets (Hogarth & Pietersz, 2012; Nimmerjahn & Ravetch, 2008; Nimmerjahn & Ravetch, 2011). In both humans and mice, the FcγRI class has the highest binding affinity towards the Fc region to different IgG subtypes such as IgG1, IgG3 and IgG4 in humans and IgG2a in rodents while the binding affinity of other classes is considered low to medium as their binding affinity to monomeric IgG is poor and can only be activated by multimeric IgG molecules, especially present in immune complexes (Nimmerjahn & Ravetch, 2011). Hence, it is suggested that the FcγRI class will become saturated as IVIg preparations primarily contain monomeric IgGs, which will competitively prevent pathological autoantibodies from binding onto activating FcγRs on immune effector cells, thereby blocking cell activation and their pathogenic potential (Nimmerjahn & Ravetch, 2007). The first evidence of this mechanism was provided by a clinical trial in patients with ITP where opsonized platelets remained in the peripheral circulation due to Fc-mediated inhibition of the phagocytic system in the liver and spleen (Bussel, 2000; Debre *et al*., 1993; Ibanez *et al*., 2003). Furthermore, infusion of monoclonal antibodies against the Fc fragment of IgG or purified IVIg preparations without the Fc fragment demonstrated no immunomodulatory effect on ITP (Anthony *et al*., 2008; Erickson *et al*., 1996; Kaneko *et al*., 2006a). Other autoimmune diseases demonstrating this mechanism of action were observed in Guillain-Barre syndrome, Myastenia Gravis and multiple sclerosis (Fokkink *et al*., 2014; Roades *et*

al., 2000; Thiruppathi *et al*., 2014; Vedeler *et al*., 2001). Despite monomeric IgG primarily binding onto the FcγRI class, this model fails to take into account the limited ability of low or medium binding FcγRs to bind to monomeric IgG contained in IVIg preparations and suggests direct blockade of IVIg to activating FcγRs is only one component of its anti-inflammatory properties mediated by the Fc region of IgG (Lunemann *et al*., 2015).

The inhibitory FcγR (FcγRIIB) is a low affinity binding glycoprotein that initiates inhibitory signaling pathways via adaptor proteins containing immunoreceptor tyrosine based inhibitory motifs (ITIM) (Nimmerjahn & Ravetch, 2011). The inhibitory FcγR (FcγRIIB) is found in both humans and rodents, which is often co-expressed with activating FcγRs on the surface of innate immune cells such as monocytes, macrophages, basophils, neutrophils, eosinophils, mast cells, natural killer cells, and platelets in order to establish a threshold level for the initiation of activating FcγR-dependent effector responses (Hogarth & Pietersz, 2012; Nimmerjahn & Ravetch, 2008; Nimmerjahn & Ravetch, 2011). Hence, it is suggested that IVIg preparations are able to upregulate the surface expression of inhibitory FcγRIIB on immune effector cells, which increases the threshold level required to initiate the activating FcγRs by pathogenic immune complexes and subsequently inhibits the release of destructive and cytotoxic mediators from effector immune cells (Nimmerjahn & Ravetch, 2007). This mechanism was elegantly demonstrated in experimental studies where FcγRIIB expression on human and mouse myeloid cells and B lymphocytes were increased following IVIg administration and the therapeutic effects of IVIg was attenuated via the disruption of FcγRIIB by monoclonal antibody blockade and genetic deletion in a number of autoimmune animal models of ITP, lupus erythematosus, rheumatoid arthritis and nephrotoxic nephritis (Brownlie *et al*., 2008; Bruhns *et al*., 2003; Kaneko *et al*., 2006b; Leontyev *et al*., 2012; Mackay *et al*., 2006; McGaha *et al*., 2005; Samuelsson *et al*., 2001; Siragam *et al*., 2006; Tackenberg *et al*., 2009). However, the mechanism(s) by which IVIg preparations are able to increase the surface expression of FcγRIIB on immune effector cells remains to be determined.

The neonatal Fc receptor (FcRn) is a member of the major histocompatibility class I molecule (MHCI) located in the endosomal compartment of intestinal epithelial and vascular endothelial cells, and immune cells such as macrophages in humans and rodents (Abdiche *et al*., 2015; Nimmerjahn & Ravetch, 2011; Sockolosky & Szoka, 2015). The FcRn is responsible for binding to serum IgG in endosomes following endocytosis and protects it from catabolism by lysosomes in endothelial cells and macrophages under low pH conditions and recycles it back to the cell surface (Borrok *et al*., 2015; Lunemann *et al*., 2015; Schwab & Nimmerjahn, 2013). Hence, it is suggested that the administration of a therapeutic high dose of IVIg will increase the

concentration of exogenous IgG in the plasma and saturate FcRn, which can no longer protect serum IgG and pathological autoantibodies from catabolism causing both serum IgG and pathological autoantibodies to be degraded and cleared more rapidly due to saturation and shortage of available FcRn (Abdiche *et al*., 2015; Nimmerjahn & Ravetch, 2011). This mechanism was demonstrated in experimental studies where administration of a high dose of IVIg decreased autoantibody half-life by approximately 50% in a rat model of ITP and neonatal mouse model of bullous pemphigoid (Hansen & Balthasar, 2002ab; Li *et al*., 2005). However, a recent study has argued against a role of FcRn in contributing to the anti-inflammatory properties of IVIg where administration of IVIg did not demonstrate any amelioration of ITP in FcRn-deficient mice (Crow *et al*., 2011).

Further Fc-mediated mechanisms of IgG include increasing the expression and activation of forkhead box P3 (FOXP3), which is an important transcription factor responsible for increasing the development and suppressive properties of regulatory T (T_{Reg}) cells through mechanism(s) that remains to be fully established (Kessel *et al*., 2007; Olivito *et al*., 2010; Tjon *et al*., 2013). Hence, by increasing the number of T_{Reg} cells the ratio between T helper cells and T suppressor cells will shift in favor of the suppressor phenotype where cytotoxic T cell-mediated immunity is suppressed in autoimmune diseases demonstrated by experimental and clinical studies in rheumatoid arthritis, Kawasaki disease, EAE, SLE, eosinophilic granulomatosis and Gullain-Barre syndrome following the administration of IVIg (**Figure 1.9**) (Costa *et al*., 2013; Ephrem *et al*., 2008; Guo *et al*., 2015; Jia *et al*., 2010; Lee *et al*., 2014; Maddur *et al*., 2014; Okuda *et al*., 2012; Olivito *et al*., 2010; Tselios *et al*., 2015; Tsurikisawa *et al*., 2012). It is interesting to indicate that IVIg can bind to both $CD4^+CD25^+$ T_{Reg} cells and conventional $CD4^+CD25^+$ T cells, however, preferentially binds T_{Reg} cells suggesting that most of the direct effects of IVIg on T cells is mediated by the activation of T_{Reg} cells despite not identifying the T_{Reg} cell surface molecule(s) responsible for binding to IVIg (Ephrem *et al*., 2008).

1.7.3.3 Both $F(ab')_2$ and Fc-mediated mechanisms

Other anti-inflammatory mechanisms of IgG can sometimes involve both the $F(ab')_2$ and Fc regions of IgG, whereby the differentiation, maturation and activation of dendritic cells are inhibited possibly due to suppression in the upregulation of co-stimulatory molecules such as CD80 and CD86, which is important in mediating dendritic cell and T cell communications; in addition to decreasing the production and secretion of pro-inflammatory cytokines such as IL-12 associated with mature dendritic cell differentiation, while simultaneously increasing the production and secretion of anti-inflammatory cytokines such as IL-10 from dendritic cells through mechanism(s)

that remain to be fully established following IVIg administration (**Figure 1.9**) (Aubin *et al*., 2010; Bayry *et al*., 2003; Bayry *et al*., 2005; Press *et al*., 2005; Qian *et al*., 2014). This mechanism was demonstrated in clinical studies where the number of dendritic cells was decreased and both pro- and anti-inflammatory cytokine profile modulated in the cerebrospinal fluid in patients with CIDP and Guillain-Barre syndrome (Press *et al*., 2005). However, a recent study controversially argued against the aforementioned anti-inflammatory effects of IVIg on dendritic cells, in fact, suggesting that IVIg stimulated the differentiation and maturation of human dendritic cells while leaving both pro- and anti-inflammatory cytokine production unaffected (Tjon *et al*., 2014). Hence, more research is warranted in order to confirm the precise immunomodulatory effects of IVIg on dendritic cells.

In summary, some of the potential Fc site-mediated mechanisms of IVIg include blocking activating FcγRs, increasing the expression of inhibitory FcγRIIB, saturating FcRn, and modulating the expression and activity of immune cells such as T cells and dendritic cells.

1.7.4 Intravenous Immunoglobulin (IVIg) Treatment in Stroke

As recombinant tissue plasminogen activator (r-tPA) is recognized as the only pharmacological agent approved for the treatment of ischemic stroke, there remains major limitations towards its use such as its narrow therapeutic window (3-4.5 hours) and increased risk of intracerebral hemorrhage (NINDS, 1995). An alternative approach for treating acute ischemic stroke is neuroprotection. Despite neuroprotective agents decreasing neuronal cell death and infarct size in cell culture and animal stroke models, respectively, all such agents tested in stroke patients have failed in clinical trials (Cheng *et al*., 2004; Green, 2002). Although there a number of reasons contributing to the failure, a common underlying feature is that neuroprotective agents only target a particular cell injury mechanism in the ischemic cascade, and in either single or multiple cell types (Woodruff *et al*., 2011). Hence, development and application of neuroprotective agents that can target multiple cell injury mechanisms in multiple cell types is warranted in the future treatment of ischemic stroke.

A novel potential candidate envisaged to target multiple cell injury mechanisms in multiple cell types in the brain following cerebral ischemia is intravenous immunoglobulin (IVIg). Recent experimental studies by our laboratory were able to demonstrate that administration of IVIg was able to significantly attenuate brain infarct size (50-60%) and mortality, and improve functional outcome in mice subjected to experimental ischemic stroke (Arumugam *et al*., 2007). The efficacy of IVIg is attributed to a number of mechanisms including its ability to neutralise active

complement fragments (C3b) in ischemic brain tissue, which accordingly reduced endothelial cell adhesion molecule (i.e. ICAM-1) production, and activation (i.e. microglia) and infiltration of inflammatory cells (i.e. neurtrophils), subsequently reducing inflammation and caspase-mediated neuronal apoptosis at the site of injury (Arumugam *et al*., 2007). In addition, IVIg was demonstrated to decrease NF-κB and MAPK(s) signalling pathway activity and increase anti-apoptotic proteins (i.e. Bcl-2) in primary cortical neurons under ischemic conditions, which reduced neuronal apoptosis through unknown mechanism(s) (Widiapradja *et al*., 2012). Finally, IVIg was demonstrated to protect the endothelium in the brain, a key component of the neurovascular unit and blood brain barrier (BBB) by preventing the down-regulation of tight junctions (i.e. claudin 5 and occludin) and anti-apoptotic proteins (i.e. Bcl-2 and Bcl-xL) in endothelial cells under simulated ischemic conditions (Widiapradja *et al*., 2014). However, the precise mechanism(s) in how IVIg directly protect neurons and cerebral tissue from inflammasome-mediated sterile inflammation following ischemic stroke remains to be determined and is a major focus of this PhD Thesis.

1.7.5 Intermittent Fasting (IF): Definition

Intermittent fasting (IF) is a dietary protocol where energy restriction is induced by alternate periods of *ad libitum* feeding and fasting, which have been proven to extend lifespan and decrease the development and severity of age-related diseases such as cardiovascular (e.g. Type 2 diabetes mellitus, myocardial infarction and stroke) and neurodegenerative diseases (e.g. Alzheimer's disease, Parkinson's disease and Huntington's disease) demonstrated in a number of animal models (Belkacemi *et al*., 2011; Bruce-Keller *et al*., 1999; Duan *et al*., 2003; Halagappa *et al*., 2007; Katare *et al*., 2009; Longo & Mattson, 2014; Manzanero *et al*., 2011; Manzanero *et al*., 2014; Mattson *et al*., 2003; Mattson, 2005; Mattson, 2014; Mattson & Wan, 2005; Patterson *et al*., 2015; Pedersen *et al*., 1999; Wan *et al*., 2010).

1.7.6 Protective Mechanisms of Intermittent Fasting (IF) in the Brain

The protective effects of prophylactic intermittent fasting (IF) treatment have been shown to prevent and attenuate cellular dysfunction and degeneration in the brain by preconditioning neurons and glial cells with energy restriction, which acts as a mild metabolic stressor that effectively upregulates the expression of several key neuroprotective proteins including - neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and basic fibroblast growth factor (bFGF); stress response proteins including, protein chaperones, such as heat shock protein 70 (Hsp70) and glucose regulated protein 78 (GRP78); regulatory proteins, such as peroxisome proliferator-activated

receptor gamma coactivator 1-alpha (PGC-1α); antioxidant enzymes, such as heme oxygenase-1 (HO-1); and uncoupling proteins, such as UCP2 and UCP4; in addition to down regulation of mammalian target of rapamycin (mTOR) activity (Akerfelt *et al*., 2010; Arumugam *et al*., 2010; Chu *et al*., 2009; Fontana & Partridge, 2015; Kouda & Iki, 2010; Liu *et al*., 2006; Mattson & Wan, 2005; Tajes *et al*., 2010; Vasconcelos *et al*., 2014). However, the precise mechanism(s) by which prophylactic IF treatment induces the expression of these neuroprotective proteins remains to be fully established. Nevertheless, it is known that energy depletion in cells will activate energy sensor proteins such as adenosine monophosphate (AMP)-activated protein kinase (AMPK) and silent information regulator-1 (SIRT1) through their respective phosphorylation and deacetylation reactions in response to increases in the AMP/ATP, and nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide hydrogenated (NAD^+/NADH) ratio, respectively (**Figure 1.10**) (Braidy *et al*., 2014; Burkewitz *et al*., 2014; Canto & Auwerx, 2011; Chen *et al*., 2008b; Fontana & Partridge, 2015; Graff *et al*., 2013; Mouchiroud *et al*., 2013; Tajes *et al*., 2010; Yuen & Sander, 2014; Zhang *et al*., 2011). Hence, it is suggested that the protective effects of prophylactic IF treatment are primarily mediated by the activation of AMPK and SIRT1, and their downstream upregulation of several key neuroprotective protein targets that synergistically interact to increase cellular resistance against a number of molecular and cellular pathological processes that occur during brain injury, especially in ischemic stroke such as excitotoxicity, oxidative stress and inflammation, in addition to regulating neurogenesis and angiogenesis.

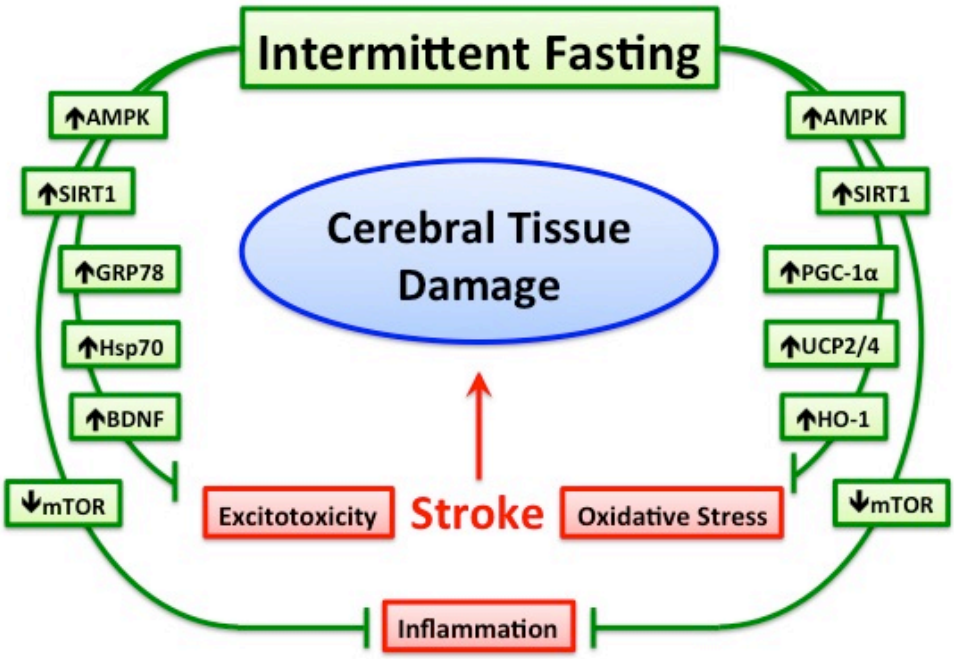

Figure 1.10: Protective mechanisms of prophylactic intermittent fasting (IF) treatment against cerebral tissue damage in stroke. Stroke induces cerebral tissue damage through different mechanisms including excitotoxicity, oxidative stress and inflammation. The efficacy of prophylatic IF treatment appears

to precondition and protect neurons and glial cells against brain injury by increasing their cellular resistance against excitotoxicity, oxidative stress and inflammation via coordinating an upregulation of multiple neuroprotective proteins including - neurotrophic factors, such as brain-derived neurotrohic factor (BDNF); protein chaperones such as heat shock protein 70 (Hsp70) and glucose regulated protein 78 (GRP78); regulatory proteins, such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α); antioxidant enzymes, such as heme oxygenase-1 (HO-1); and uncoupling proteins (UCPs), such as UCP2 and UCP4; in addition to down regulation of mammalian target of rapamycin (mTOR) activity at the site of injury following stroke. The precise mechanism(s) by which prophylatic IF treatment induces expression of these neuroprotective proteins remains to be fully established. Nevertheless, it is known that energy depletion in cells will activate energy sensor proteins such as adenosine monophosphate (AMP)-activated protein kinase (AMPK) and silent information regulator-1 (SIRT1) through their respective phosphorylation and deacetylation reactions, respectively. This figure is adapted from Calorie restriction and stroke. Manzanero *et al* (2011). *Experimental & Translational Stroke Medicine*; **3**: p.8.

1.7.6.1 Neuroprotective Effects of Intermittent Fasting (IF) on Excitotoxicity

Numerous lines of evidence have shown that prophylatic IF treatment is able to protect and improve neuronal survival from glutamate excitotoxicity in rodent models of epilepsy and focal cerebral ischemia through a number of mechanisms by increasing neuroprotective proteins; in particular, neurotrophic factors such as BDNF and bFGF; and protein chaperones, including Hsp70 and GRP78 in the brain (Brandoli *et al*., 1998; Mokrushin *et al*., 2005; Ribeiro *et al*., 2009; Sharma & Kaur, 2005; Sommer *et al*., 2003; Yu & Mattson, 1999; Yu *et al*., 1999).

Neurotrophic factors such as BDNF is both widely expressed and is responsible for a number of physiological functions in the brain by promoting the survival of existing neurons, the growth and development of dendrites and synapses (synaptic plasticity), and differentiation of new neurons from neural stem cells (neurogenesis), whereas bFGF is expressed in blood vessels and is responsible for promoting the formation of new blood vessels (angiogenesis) (Abe & Saito, 2001; Adachi *et al*., 2014; Chen *et al*., 2013; Rose *et al*., 2007). Both BDNF and bFGF mediate its neuroprotective effects by binding onto membrane bound tyrosine kinase receptor B (TrkB) and fibroblast growth factor receptor 1 (FGFR1), respectively, which activate the same phosphoinositide 3-kinase (PI3-kinase)/Akt (protein kinase B) and mitogen activated protein kinase (MAPK), in particular, the extracellular signal-regulated kinase (ERK) signaling pathway resulting in the activation of transcription factor cyclic AMP response element binding protein (CREB) (Almeida *et al*., 2005; Longo & Mattson, 2014; Nguyen *et al*., 2010; Wang *et al*., 2012b; Zheng & Quirion, 2004). The genes induced by CREB include the DNA repair enzyme, APE1; the master regulator of mitochondrial biogenesis, PGC-1α and the anti-apoptotic protein, Bcl-2, which can all provide neuroprotective functions during an ischemic stroke (Longo & Mattson, 2014).

Moreover, BDNF was demonstrated to possess neuroprotective pleiotropic effects where the administration of exogenous BDNF to an experimental focal ischemic rodent model was shown to

reduce infarct size in the penumbra by modulating the expression and function of neurotransmitter receptors in the brain; mediated in part by reducing both the binding affinity of glutamate to NMDA and AMPA receptors, the expression of NMDA and AMPA receptors, and by preventing the decrease in number of GABA receptors in the penumbra so that the inhibitory function of GABA receptors are maintained in order to inhibit excitatory NMDA and AMPA receptors to reduce glutamate excitotoxicity under ischemic conditions (Brandoli *et al*., 1998; Sommer *et al*., 2003). In addition, another mechanism(s) prophylactic IF treatment could protect cerebral tissue from glutamate excitotoxicity is by modulating the function of astrocytes in the brain; mediated firstly by increasing glutamate uptake into astrocytes, and secondly by increasing glutamine synthetase activity in astrocytes, which is responsible for catalyzing the reaction between glutamate and ammonia to produce glutamine, therefore decreasing the concentration of glutamate in the extracellular environment; suggesting prophylactic IF treatment might exert its neuroprotective effects by modulating the function of astrocytes (Ribeiro *et al*., 2009). However, the precise mechanism(s) involved in modulating the aforementioned functions induced by prophylactic IF treatment in astrocytes remains to be determined.

Protein chaperones such as Hsp70 is ubiquitously expressed and is responsible for a number of physiological functions in the brain by folding, stabilizing and transporting newly synthesized proteins in the cytosol, protecting cells by binding onto damaged and defective proteins from aggregation induced by oxidative stress and subsequently eliminating them through ubiquitination and proteolysis pathways, and inhibiting apoptosis by blocking the interaction of pro-caspase-9 with Apaf-1 and cytochrome c to form the apoptosome complex; whereas GRP78 is abundantly expressed in the endoplasmic reticulum and is primarily responsible for binding onto newly synthesized proteins and maintains them in a state that allows them to be correctly folded and assembled in the endoplasmic reticulum, especially under pathological conditions where the accumulation of misfolded and unfolded proteins occur; known as endoplasmic reticulum stress that commonly develops during an ischemic stroke (Franklin *et al*., 2005; Giffard & Yenari, 2004; Giffard *et al*., 2004; Gonzalez-Gronow et al., 2009; Kim *et al*. 2012; Luo *et al*., 2013; Ni *et al*., 2011; Niforou *et al*., 2014; Quinones *et al*., 2008; Sharp *et al*., 2013; Yenari *et al*., 2005). Both Hsp70 and GRP78 mediate its neuroprotective effects through the same ATP-dependent mechanism whereby ATP is used to bind onto the nucleotide binding domain on Hsp70 and GRP78, which subsequently allows the substrate binding domain of Hsp70 and GRP78 to interact with unfolded or misfolded proteins in order to maintain the structural integrity and function of the protein (Gonzalez-Gronow et al., 2009; Luo *et al*., 2013; Ni *et al*., 2011; Sharp *et al*., 2013).

Moreover, Hsp70 was demonstrated to possess neuroprotective pleiotropic effects where

pre-incubation of exogenous Hsp70 was able to increase neuronal resistance to excitotoxic damage by protecting the conformational structure of both AMPA and NMDA-glutamate receptors and pre-synaptic ion channels in order to maintain presynaptic and postsynaptic functions of glutamate transmission in cultured rat brain slices of the cortex in an *in vitro* model of glutamate excitotoxicity (Mokrushin *et al*., 2005; Sharma & Kaur, 2005). In addition, GRP78 was also demonstrated to possess neuroprotective pleiotropic effects where siRNA knockdown of GRP78 was seen to increase the concentration of Ca^{2+} ions in cultured hippocampal neurons and subsequently induce apoptotic cell death in comparison to untreated hippocampal neurons following glutamate treatment; indicating that GRP78 is responsible for maintaining low intracellular Ca^{2+} ion concentrations (Yu *et al*., 1999). Furthermore, the administration of a neuroprotective agent such as 2-deoxy-d-glucose, a potent inducer of GRP78 expression with similar effects to IF was shown to protect hippocampal neurons against glutamate excitotoxicity suggesting that GRP78 serves a neuroprotective function (Yu & Mattson, 1999).

1.7.6.2 Neuroprotective Effects of Intermittent Fasting (IF) on Oxidative Stress

Numerous lines of evidence have demonstrated that prophylatic IF treatment is able to protect and improve neuronal survival from oxidative stress in rodent models of focal cerebral ischemia through a number of potential mechanisms by either decreasing the production and release of reactive oxygen species (ROS) or increasing antioxidant defenses in the brain (Amigo & Kowaltowski, 2014; Bevilacqua *et al*., 2005; Chu *et al*., 2009; Goffart & Wiesner, 2003; Gouspillou & Hepple, 2013; Haines *et al*., 2010; Hancock *et al*., 2011; Liu *et al*., 2006; Mattiasson *et al*., 2003; Wareski *et al*., 2009; Wu *et al*., 1999).

Recent experimental studies have shown that prophylactic IF treatment is able to decrease the production and release of ROS by counter intuitively increasing the metabolic respiratory rate of the mitochondria, which is achieved by a combination of two mechanisms in terms of increasing both the expression of uncoupling proteins (UCP) such as UCP2 and UCP4 in the mitochondria, and the number and activity of the mitochondria in the brain (Amigo & Kowaltowski, 2014; Caldeira da Silva *et al*., 2008; Chu *et al*., 2009; Haines *et al*., 2010; Hancock *et al*., 2011; Liu *et al*., 2006; Mattiasson *et al*., 2003; Nakase *et al*., 2007; Sanz *et al*., 2005; Wareski *et al*., 2009; Wu *et al*., 1999). A central mediator of these effects appear to be driven by PGC-1α, which is activated by increased levels and activity of AMPK and SIRT1 induced by prophylactic IF treatment through phosphorylation and deacetylation reactions, respectively (Canto & Auwerx, 2009). The activation of PGC-1α increases the expression of electron transport chain proteins such as UCP2 and UCP4 through an undefined mechanism(s) that mildly uncouples the passage of protons through the inner

mitochondrial membrane during oxidative phosphorylation resulting in increased electron transport and oxygen consumption in the mitochondria (Bevilacqua *et al*., 2005; Chu *et al*., 2009; Haines *et al*., 2010; Liu *et al*., 2006; Mattiasson *et al*., 2003). Currently, there are many proposed mechanisms behind mild uncoupling that decreases the production and release of ROS. Firstly, an increase in respiratory rate will increase the consumption of oxygen, which is suggested to lower oxygen tension and decrease the probability of oxygen being chemically reduced into superoxide in the mitochondria (Balaban *et al*., 2005). Secondly, an increase in respiratory rate will cause protein complexes I and III in the electron transport chain to be maintained in an oxidized state, which subsequently prevents electron transfer to chemically reduce oxygen into superoxide in the mitochondria (Sanz *et al*., 2005; Turrens, 2003). Finally, an increase in respiratory rate will increase the availability of NAD^+, which will in turn decrease the production of ROS by pyruvate and α-ketoglutarate in the mitochondria (Starkov *et al*., 2004; Tahara *et al*., 2007). Hence, it appears that IF will induce mild chronic uncoupling in the mitochondria in order to decrease the production of ROS through a number of mechanisms in the brain (Caldeira da Silva *et al*., 2008; Chu *et al*., 2009; Kwok *et al*., 2010; Liu *et al*., 2006; Mattiasson *et al*., 2003). In addition, PGC-1α have been shown to activate nuclear respiratory factor 1 and 2 (NRF-1 and NRF-2), which are transcription factors responsible for activating nuclear genes involved in stimulating mitochondrial biogenesis, in addition to NRF-2 independently activating mitochondrial transcription factor A (mtTFA) that is responsible for inducing the replication and transcription of the mitochondrial genome required in mitochondrial biogenesis (Goffart & Wiesner, 2003; Gouspillou & Hepple, 2013; Hancock *et al*., 2011; Wareski *et al*., 2009; Wu *et al*., 1999) In general, the aforementioned changes will ultimately increase the metabolic respiratory activity of the mitochondria, which in contradiction increases its oxidative buffering capacity and cellular resistance through mechanism(s) that remains to be fully determined highlighting the complex neuroprotective effects of prophylactic IF treatment towards oxidative stress.

Numerous experimental studies have shown that prophylactic IF treatment is unable to consistently increase the expression or activity of commonly measured antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase or catalase, but is able to increase the expression of HO-1 in the brain that is activated by hypoxia and oxidative stress following cerebral ischemia (Walsh *et al*., 2014). The physiological effects of HO-1 is that it is a rate-limiting enzyme responsible for catalyzing the degradation of heme into diverse neuroprotective by products such as carbon monoxide and biliverdin, whereby the latter is further processed into bilirubin by biliverdin reductase, which can be converted back into biliverdin when oxidized by ROS demonstrating that HO-1 activity is able to be regulated by oxidative stress levels (Idriss *et al*., 2008; Kim *et al*., 2011).

The neuroprotective property of carbon monoxide is that it is able to activate cGMP in vascular smooth muscle cells in the vasculature causing vasodilatation, which increases blood flow to ischemic tissues, in addition to biliverdin and bilirubin possessing antioxidant properties by potently scavenging and neutralizing ROS in the brain to decrease oxidative damage and improve functional outcome in rodent models of ischemic stroke (Beschorner *et al*., 2000; Chao *et al*., 2013; Deguchi *et al*., 2008; Hanafy *et al*., 2013; Leffler *et al*., 2011; Namiranian *et al*., 2005).

Moreover, a number of experimental studies demonstrated HO-1 to possess neuroprotective pleiotropic effects where overexpression of HO-1 was able to protect neurons from apoptosis by either decreasing nuclear localization of p53 and/or increase the expression of Bcl-2, an anti-apoptotic protein or BDNF, which mediates its protective effects by activating the TrkB-PI3K/Akt pathway, in addition to decreasing infarct volume and neurological deficits by preserving NO bioavailability mediated by increasing eNOS phosphorylation and activity in rodent models of ischemic stroke (Chao *et al*., 2013; Panahian *et al*., 1999; Qi *et al*., 2014). However, the precise mechanism(s) behind HO-1 overexpression decreasing nuclear localization of p53 and increasing the expression of Bcl-2, BDNF, and eNOS phosphorylation and activity in the brain following ischemic stroke remains to be fully established.

1.7.6.3 Neuroprotective Effects of Intermittent Fasting (IF) on Inflammation

Numerous lines of evidence have demonstrated that prophylactic IF treatment is able to protect and improve neuronal survival from inflammation in rodent models of focal cerebral ischemia through a number of mechanisms by either decreasing the expression of pro-inflammatory genes or eliminating inflammatory causing stimuli in the brain (Desai *et al*., 2010; Nijboer *et al*., 2008; Yeung *et al*., 2004; Zhang *et al*., 2005).

Experimental studies have shown that prophylactic IF treatment is able to decrease the NF-κB signaling pathway by attenuating the activity of NF-κB, which have been implicated in inducing the expression of pro-inflammatory genes such as pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), chemokines (CCL2/MCP-1 and CXCL2/MIP2) and endothelial cell adhesion molecules (E-selectin, ICAM-1 and VCAM-1) in the brain following cerebral ischemia (Aljada *et al*., 2006; Harari & Liao, 2010; Howard *et al*., 1998; Lee *et al*., 2015b; Nam *et al*., 2009; Sanacora *et al*., 2014; Schwaninger *et al*., 2006; Son *et al*., 2008; Supanc *et al*., 2011; Xing & Remick, 2007; Yilmaz & Granger, 2010; Zampetaki *et al*., 2004). The mechanism(s) behind prophylactic IF treatment decreasing the activity of the NF-κB signaling pathway is mediated by an increase in SIRT1 activity induced by IF through an undefined mechanism(s), which physically deacetylates

the RelA/p65 subunit of NF-κB inhibiting its transactivation potential and rendering it inactive (Yeung *et al*., 2004). Hence, inhibiting the ability of NF-κB to initiate transcription of pro-inflammatory genes and subsequently the inflammatory response will improve infarct size and neurological deficits from brain injury during cerebral ischemia (Desai *et al*., 2010; Nijboer *et al*., 2008; Zhang *et al*., 2005). Moreover, experimental studies have shown that prophylactic IF treatment is able to increase autophagy, an indispensable cellular process where unnecessary or dysfunctional cellular components that are capable in causing an inflammatory response are degraded by lysosomes in the brain following cerebral ischemia (Alirezaei *et al*., 2010; Chen *et al*., 2014; Michalsen & Li, 2013). The mechanism(s) behind prophylactic IF increasing autophagy is mediated by the diet itself where cellular components are broken down to maintain cellular energy levels in order to promote cell survival during dietary restriction and/or by inhibiting the activity of mammalian target of rapamycin (mTOR) (Alirezaei *et al*., 2010). mTOR is an endogenous protein kinase activated by oxidative stress via the PI3K/Akt pathway, which is responsible for promoting cell growth and proliferation, and pro-inflammatory cytokine production in order to initiate an immune response (Chong *et al*., 2013; Maiese, 2014; Xie *et al*., 2014). Hence, the neuroprotective rationale behind prophylactic IF increasing autophagy by inhibiting mTOR activity will function to remove inflammatory causing stimuli such as toxins or damaged organelles, in particular, mitochondria (i.e. mitophagy) in order to suppress an inflammatory response induced from brain injury following cerebral ischemia (Baek et al., 2014; Li *et al*., 2014; Viscomi *et al*., 2012).

1.7.6.4 Other Neuroprotective Effects of Intermittent Fasting (IF) in Stroke

Other beneficial effects of prophylactic IF treatment include increasing neurogenesis and angiogenesis mediated by BDNF and vascular endothelial growth factor (VEGF), respectively, in an attempt to reconstruct brain tissue following brain injury, especially during an ischemic stroke (Arumugam *et al*., 2010; Kernie and Parent, 2010; Marti *et al*., 2000; Mattson and Wan, 2005; Rothman *et al*., 2012; Sonanez-Organis *et al*., 2013). Numerous experimental studies have shown that prophylactic IF treatment was able to increase BDNF levels and activate the TrkB-PI3K/Akt pathway, which subsequently enhanced the production rate of new neurons from neural progenitor cells contained within the subventricular zone (SVZ)-olfactory bulb pathway in the brain following cerebral ischemia (Arumugam *et al*., 2010; Lee *et al*., 2002; Longo and Mattson, 2014; Pikula *et al*., 2013; Schabitz *et al*., 2007; Tajes *et al*., 2010; Vasconcelos *et al*., 2014). In addition, experimental studies have shown that prophylactic IF treatment was able to increase the hypoxia inducible factor (HIF) signaling pathway by increasing the activity of HIF-1α, in particular, which is a transcription factor responsible for increasing gene expression of VEGF-A under hypoxic conditions in the brain following cerebral ischemia (Harms *et al*., 2010; Reischi *et al*., 2014; Sonanez-Organis *et al*., 2013;

Yan *et al*., 2011). Evidence demonstrates that VEGF-A was able to decrease infarct size and improve neurological function through a number of mechanisms such as stimulating angiogenesis mediated by VEGF receptor 2 in the striatum; but interestingly was also able to enhance neurogenesis in the dentate gyrus and SVZ mediated possibly via the PI3-AkT pathway in the ischemic penumbra, however, more experimental studies are needed to confirm the precise mechanism(s) (Chiba *et al*., 2008; Harms *et al*., 2010; Kaya *et al*., 2005; Marti *et al*., 2000; Stowe *et al*., 2008; Sun *et al*., 2003; Zhang *et al*., 2000).

1.7.7 Intermittent Fasting (IF) Treatment in Stroke

In conjunction to using pharmacological interventions, an alternative approach is to implement prophylactic lifestyle modification regimens such as dietary energy restriction in the form of intermittent fasting (IF) to target multiple cell injury mechanisms in multiple cell types in the brain following cerebral ischemia. Recent experimental studies by our laboratory were able to demonstrate that prophylactic IF treatment significantly attenuated brain infarct size and mortality, and improved functional outcome in young (3 months) and middle-aged (9 months) male mice subjected to experimental focal ischemic stroke (Arumugam *et al*., 2010; Manzanero *et al*., 2014). The efficacy of prophylactic IF to protect brain tissue against ischemic injury involved the coordinate upregulation of multiple neuroprotective proteins including neurotrophic factors, such as BDNF and bFGF; protein chaperones, including Hsp70 and GRP78; antioxidant enzymes, such as SOD and HO-1; and downregulation of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) at the site of injury (Arumugam *et al*., 2010). However, the precise mechanism(s) in how prophylactic IF treatment directly protect neurons and cerebral tissue from inflammasome-mediated sterile inflammation following ischemic stroke remains to be determined and is a major focus of this PhD Thesis.

1.8 Summary

Recent findings have provided insight into a new inflammatory mechanism in the innate immune system that may contribute to neuronal and glial cell death during cerebral ischemia. There is emerging evidence to suggest that endogenous DAMPs (e.g. IL-1α and HMGB1) released by necrotic cells in the ischemic core will bind to plasma membrane PRRs (e.g. TLR-2, TLR-4, IL-1R1 and RAGE), activating the NF-κB and MAPK(s) signaling pathways to increase expression levels of inflammasome proteins, precursor IL-1β and precursor IL-18, in the cytoplasm of surrounding neurons and glial cells in the ischemic penumbra. This is followed by the activation and homo-oligomerization of NLRP1 and NLRP3 receptors by either DAMPs or irregularities

within the cellular microenvironment, such as energy depletion, acidosis, cathepsin release, decreased intracellular K^+ concentration, increased ROS production, oxidized mitochondrial DNA, increased intracellular Ca^{2+} concentrations, cell swelling, and protein kinase R (PKR) activation. These changes induce the formation of the NLRP1 and NLRP3 inflamamsome complex, which then activates precursor caspase-1 to produce cleaved capase-1 in the cytoplasm of neurons and glial cells during cerebral ischemia. Following activation, cleaved caspase-1 cleaves precursor IL-1β and precursor IL-18 into biologically active pro-inflammatory cytokines – mature IL-1β and mature IL-18, which are then released into the extracellular environment, and induce cell death through apoptosis and/or pyroptosis. Multiple potential targets upstream and downstream of inflammasome signaling, targeting its expression, assembly, activity and products, may therefore offer substantial promise in developing and incorporating novel treatments such as IVIg and IF that may salvage penumbral tissue and attenuate neurological deficits following cerebral ischemia. However, its important to note that while certain aspects of the inflammatory response will not only exacerbate brain injury, it is also likely that other components provide a beneficial contribution to brain recovery, and it is the task of future research to distinguish these components. Unquestionably, there is still a great deal to be done to clarify the role of inflammasome signalling during the recovery phase following ischemic stroke.

1.9: Rationale and Objectives of the Project

Recent findings have provided insight into a newly described inflammatory mechanism(s) that may contribute to neuronal and glial cell death during cerebral ischemia known as sterile inflammation involving intracellular multi-protein complexes termed inflammasomes. There is emerging evidence to suggest that both NF-κB and MAPK(s) signalling pathways are able to modulate the expression and activation of NLRP inflammasomes in peripheral immune cells under inflammatory conditions. However, the connection between both the NF-κB and MAPK(s) signalling pathways with inflammasome protein expression and activation in neurons and cerebral tissue under ischemic conditions remains unclear. This may occur in response to endogenous danger signals initiated by substances released from necrotic cells at the site of injury, leading to an increased production of pro-inflammatory cytokines and to neuronal and glial cell death mediated by NLRP inflammasomes. Overall, this research thesis will investigate the pathogenic role of inflammasomes and therapeutic efficacy of a caspase-1 inhibitor (Ac-YVAD.cmk), intravenous immunoglobulin (IVIg) and intermittent fasting (IF) on neuronal cell death and cerebral tissue damage under *in vitro* and *in vivo* models of ischemic stroke.

The specific aims of the project are:

Specific Aim 1 - To determine the cellular location and temporal expression levels of the NLRP1 and NLRP3 inflammasome proteins and both IL-1β and IL-18 in neurons and cerebral tissue under *in vitro* and *in vivo* ischemic conditions.

Specific Aim 2 - To determine the effect of a caspase-1 inhibitor (Ac-YVAD.cmk), IVIg and IF on the cellular location and expression levels of the NLRP1 and NLRP3 inflammasome proteins and both IL-1β and IL-18 in neurons and cerebral tissue under *in vitro* and/or *in vivo* ischemic conditions.

Specific Aim 3 - To determine whether a caspase-1 inhibitor (Ac-YVAD.cmk), IVIg and IF will prevent or attenuate neuronal cell death and cerebral tissue damage under *in vitro* and/or *in vivo* ischemic conditions.

Specific Aim 4 - To determine whether a caspase-1 inhibitor (Ac-YVAD.cmk), IVIg and IF will prevent or attenuate inflammasome activity and its mechanism(s) of action in neurons and cerebral tissue under *in vitro* and/or *in vivo* ischemic conditions.

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CHAPTER 2:

Intravenous Immunoglobulin (IVIg) Suppresses NLRP1 and NLRP3 Inflammasome-Mediated Neuronal Death in Ischemic Stroke

2.1 Introduction:

Stroke is the second leading cause of death worldwide and a major cause of permanent disability. The molecular and cellular mechanisms responsible for the degeneration of neurons affected by stroke are complex and poorly understood, but involve bioenergetic failure, ionic imbalance, acidosis, excitotoxicity, oxidative stress and inflammation, resulting in necrotic or apoptotic cell death (Broughton *et al*., 2009; Dirnagl, 2012; Hou & MacManus, 2002; Sims & Muyderman, 2010). Post-stroke inflammation is a complex process involving activation of innate local immune responses in glial cells and recruitment of circulating leukocytes into the affected brain tissue (Gelderblom *et al*., 2009; Iadecola & Anrather, 2011). Activated glia and leukocytes produce multiple pro-inflammatory mediators including complement anaphylatoxins, cytokines, chemokines and prostaglandins (Gelderblom *et al*., 2009; Iadecola & Anrather, 2011). Recent findings have provided insight into a newly discovered inflammatory mechanism that contributes to neuronal and glial cell death in cerebral ischemia mediated by multi-protein complexes called inflammasomes. Studies of the inflammasome complex in peripheral tissues have shown that it amplifies the production and secretion of pro-inflammatory cytokines, and apoptotic and pyroptotic cell death (Lamkanfi & Dixit, 2012). It was recently reported that the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) pyrin domain-containing (NLRP) inflammasomes play a role in the inflammatory response during ischemic stroke (Abulafia *et al*., 2009; Deroide *et al*., 2013; Savage *et al*., 2012; Zhang *et al*., 2014).

The NLRP1 and NLRP3 inflammasomes are cytosolic macromolecular complexes composed of the NLRP1/3 receptor, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), precursor caspase-1, precursor caspase-11 (homologous to precursor caspase-4 or 5 in humans) and/or XIAP (X-linked inhibitor of apoptosis) (Agostini *et al*., 2004; Boyden & Dietrich, 2006; De Rivero Vaccari *et al*., 2009; Martinon *et al*., 2002). Activation and homo-oligomerization of NLRP1 and NLRP3 receptors induces formation of the NLRP1 and NLRP3 inflammasomes, respectively, which convert precursor caspase-1 into cleaved caspase-1 via proximity-induced auto-activation (Lamkanfi & Dixit, 2012; Lu *et al*., 2014; Martinon *et al*., 2002; Salvesen & Dixit, 1999). Cleaved caspase-1 converts precursors of both IL-1β and IL-18 into biologically active mature pro-inflammatory cytokines that are then released into the extracellular environment (Abulafia *et al*., 2009; Andrei *et al*., 2004; Brough & Rothwell, 2007). Moreover,

increased cleaved caspase-1 can initiate cell death directly via apoptosis or pyroptosis (Fink *et al*., 2008; Sagulenko *et al*., 2013). In stroke-related studies, reduced brain expression of mature IL-1β and IL-18 was shown in mice following cerebral ischemia, using an anti-NLRP1 antibody (Abulafia *et al*., 2009). Moreover, in caspase-1 knockout mice there was a reduction in mature IL-1β and IL-18 levels in association with a smaller infarct size (Mastronardi *et al*., 2007). Furthermore, administration of an IL-1β neutralizing antibody or IL-1 receptor antagonist reduced subarachnoid hemorrhagic injury (Jedrzejowska-Szypułka *et al*., 2009). However, the specific pathophysiologic role of the NLRP1 and NLRP3 inflammasome in neuronal cell death following ischemic stroke remains to be established.

Intravenous immunoglobulin (IVIg) is an FDA-approved therapeutic modality used for various inflammatory and autoimmune diseases such as Kawasaki's disease, immune thrombocytopenia (ITP) and humoral immunodeficiency (Gelfand, 2012; Schwab & Nimmerjahn, 2013). Thus, IVIg has potential for diminishing inappropriate inflammatory and immune activation that may offer neuroprotection (Arumugam *et al*., 2008; Arumugam *et al*., 2009). IVIg can inhibit complement activation and infiltration of leukocytes, modulate the cytokine network and inhibit endothelial dysfunction and neuronal apoptosis under *in vitr*o and *in vivo* models of ischemic stroke (Arumugam *et al*., 2007; Widiapradja *et al*., 2014). The pleiotropic effects of IVIg in inhibiting multiple components of inflammation in different cell types make it an attractive candidate for use in stroke therapy (Arumugam *et al*., 2007; Lux *et al*., 2010; Walberer *et al*., 2010; Widiapradja *et al*., 2012; Widiapradja *et al*., 2014). Potential effects and underlying mechanism(s) of IVIg on inflammasome activation in ischemic stroke-induced neuronal cell death have not been reported. In the present study, we performed a comprehensive investigation into the dynamic expression patterns of the NLRP1 and NLRP3 inflammasome in primary cortical neurons subjected to simulated ischemia, in a mouse model of focal ischemic stroke, and in brain tissue samples from stroke patients. In addition, we demonstrate expression and a functional role for the NLRP1 and NLRP3 inflammasome in neuronal cell death, and show that the neuroprotective effect of IVIg in experimental stroke involves suppression of inflammasome activity. Collectively, our findings reveal IVIg as a potential therapeutic modality for targeting ischemic stroke-induced inflammasome expression and activity.

2.2 Material & Methods:

Focal Cerebral Ischemia/Reperfusion (I/R) Stroke Model

Three-month-old C57BL6/J male mice were subjected to transient middle cerebral artery ischemia and reperfusion (I/R) injury, as described previously (Arumugam *et al*., 2004). Briefly,

after making a midline incision in the neck, the left external carotid and pterygopalatine arteries were isolated and ligated with 6-0 silk thread. The internal carotid artery (ICA) was occluded at the peripheral site of the bifurcation with a small clip and the common carotid artery (CCA) was ligated with 5-0 silk thread. The external carotid artery (ECA) was cut, and a 6-0 nylon monofilament with a tip that was blunted (0.20-0.22 mm) with a coagulator was inserted into the ECA. After the clip at the ICA was removed, the nylon thread was advanced to the origin of the middle cerebral artery (MCA) until light resistance was evident. The nylon thread and the CCA ligature were removed after 1hr to initiate reperfusion. In the Sham group, surgery was performed until the arteries were visualized but not disturbed for a period of 1hr under isofluorane-induced anaesthesia. Mice were administered with either 10 or 20mg/kg of a Caspase-1 inhibitor (20µl; Ac-YVAD-CMK, Cayman Chemical, Ann Arbor, MI, USA), 1g/kg of IVIg (250µl; Privigen, CSL Behring, King of Prussia, PA, USA, CSL) or vehicle by infusion into the femoral vein 3hr after the start of reperfusion. In a separate set of experiments, anesthetized animals from all groups (5-6 mice per group) underwent cerebral blood flow (CBF) measurements using a laser Doppler perfusion monitor (Moor Lab, Moor Instruments, Axminster, UK).

The functional consequences of I/R injury were evaluated using a five-point neurological deficit score (0, no deficit; 1, failure to extend right paw; 2, circling to the right; 3, falling to the right; and 4, unable to walk spontaneously) and were assessed in a blinded fashion (Bederson *et al*., 1986). At 72hr of reperfusion, the mice were euthanized with a lethal dose of isoflurane. Brains were immediately removed and placed into phosphate-buffered saline (PBS; Sigma-Aldrich, Castle Hill, NSW, Australia) at 4°C for 15 min, and four 2-mm coronal sections were made from the olfactory bulb to the cerebellum using an Acrylic Mouse Brain Slicer Matrix (Zivic Instruments, Pittsburgh, PA, USA). The brain sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, St. Louis, MO, USA) in PBS at 37°C for 15 min. The stained sections were photographed and the digitized images used for analysis. Borders of the infarct in the image of each brain slice were outlined and the area quantified using ImageJ v1.46 software (National Institute of Health, Bethesda, MD, USA). To correct for brain swelling, the infarct area was determined by subtracting the area of undamaged tissue in the left hemisphere from that of the intact contralateral hemisphere. The infarct volume was determined by calculating the percentage of infarct area in each brain slice, and then integrating the infarct area for all slices of each brain. All *in vivo* experimental procedures were approved by The University of Queensland Animal Care and Use Committee.

Primary Cortical Neuronal Cultures

Dissociated neuron-enriched cell cultures of cerebral cortex were established from Day 16 C57BL6/J mouse embryos, as described (Okun *et al*., 2007). Experiments were performed in 7 to 9 day-old cultures. Approximately 95% of the cells in such cultures were neurons, and the remaining cells were astrocytes. For glucose-deprivation studies, glucose-free Locke's buffer containing: 154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 5 mM HEPES, pH 7.2, supplemented with gentamicin (5 mg/L) was used. The cultured neurons were incubated in glucose-free Locke's buffer for 1-24hr. Controls were incubated in Neurobasal medium. For combined oxygen and glucose deprivation (OGD), neurons were incubated in glucose-free Locke's buffer in an oxygen-free chamber for 3, 6 or 12 hr. For simulated I/R experiments, neurons were incubated in glucose-free Locke's medium in an oxygen-free chamber for 3hr and then the medium replaced with Neurobasal medium for 3, 6, 12 or 24 hr. To observe the effect of a caspase-1 inhibitor (Ac-YVAD-CMK) or IVIg, either drug were added to cultures during and after GD, OGD or simulated I/R. Control conditions included exposure to vehicle or a negative control protein (bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA).

Cell Viability

Neuronal cell viability was determined by trypan blue exclusion assay. The assay is based on the principle that live cells possess intact cell membranes, which will exclude the dye trypan blue, while the membrane of injured or dead cells is permeable to trypan blue. Hence, injured or dead cells are stained blue whereas live cells will show no staining. Following incubation with trypan blue, the plates were emptied and the cells fixed with 4% paraformaldehyde for 20 min at room temperature. The cells were then washed with PBS three times and stored in PBS for latter observation under a light microscope to quantify the percentage of cells that were trypan-blue positive in each culture.

Cell/Tissue Lysis and Protein Quantitation

In order to extract protein, primary cortical neurons and ipsilateral (damaged) brain tissues were homogenized separately in either cell lysis buffer (Radio-Immunoprecipitation Assay (RIPA)) or tissue lysis buffer (Tissue Protein Extraction Reagent (TPER)) containing protease and phosphatase inhibitor (Thermo Scientific, Rockford, IL, USA) in 1:100 ratio, respectively, using a cell disruptor or a Tissue-Tearer (Biospec Products, Inc., Bartlesville, OK, USA). Samples were centrifuged at 15,000 rpm at 4°C for 15 minutes and the supernatant collected. Total protein concentration of each sample was measured in a microplate using the Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Bovine serum albumin (BSA) standards (20-2,000µg/mL) were prepared as per the manufacturer's instructions to generate a

standard curve with known concentrations. Absorbance was measured at 562nm using the Tecan 26 Sunrise Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland) and data was analyzed using Graphpad Prism 5 software (Graphpad Software, San Diego, CA, USA) by comparing samples to the standard curve to determine the concentration and volume of protein required to be loaded for separation by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Western Blot Analysis

Protein samples were subjected to sodium dodecyl sulfate–polyacrylamide (10%) gel electrophoresis using a Tris-glycine running buffer. Gels were then electro-blotted using a transfer apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in transfer buffer containing 0.025 mol/L Tris base, 0.15 mol/L glycine, and 10% (v/v) methanol for 2 hr at 80V onto a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membrane was then incubated in blocking buffer (5% non-fat milk in 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.2 % Tween-20) for 1hr at 23°C. The membrane was then incubated overnight at 4°C with primary antibodies including those that selectively bind NLRP1 (Novus Biologicals, Littleton, CO, USA), NLRP3 (Novus Biologicals), ASC (Abcam, Cambridge, UK), Caspase-1 (Abcam), Caspase-11 (Abcam), XIAP (Novus Biologicals), IL-1β (Abcam), IL-18 (Abcam), Bcl-2 (Cell Signaling Technology, Danvers, MA, USA), cleaved Caspase-3 (Cell Signaling) and β-actin (Sigma-Aldrich, St. Louis, MO, USA). After washing three times (10 min per wash) with Tris-buffered saline-T (20 mM Tris-HCL, pH 7.5, 137 mM NaCl, 0.2 % Tween-20), the membrane was incubated with secondary antibodies against the primary antibody and β-actin for 1hr at room temperature. The membrane was washed with Tris-Buffered saline-T and scanned using the Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Quantification of protein levels was achieved by densitometry analysis using Image J v1.46 software (National Institute of Health, Bethesda, MD, USA).

Immunocytochemistry and Immunohistochemistry

Coverslips containing primary cortical neurons subjected to either control Neurobasal medium or GD medium were fixed in 4% buffered paraformaldehyde in PBS. Fixed cells were permeabilized and incubated in blocking solution (1% BSA and 0.1% Triton-X in PBS) at room temperature for 1hr before overnight incubation at 4°C with microtubule-associated protein 2 antibody (MAP2, mouse monoclonal, Millipore, Temecula, CA, USA) along with primary antibodies that selectively bind NLRP1 (Novus Biologicals), ASC (Abcam), Caspase-1 (Abcam), Caspase-11 (Abcam), IL-1β (Abcam) or IL-18 (Abcam) diluted in blocking solution. Following incubation with primary antibodies, the cells were incubated with the appropriate Alexa Fluor-Conjugated secondary antibodies (Invitrogen) for 1hr at room temperature. The nuclei were counterstained with DAPI (AbD Setotec,

Oxford, UK) for 10 min at room temperature. Following secondary antibody incubation, coverslips were sealed with Vectashield Fluorescent Mounting Medium (Vector Laboratories, Burlingame, CA, USA) on glass slides. For immunohistochemistry, frozen cryostat brain sections were obtained from Sham and focal ischemic stroke mice following trans-cardiac perfusion with 4% paraformaldehyde and immunostained with primary antibodies against NLRP1 (Novus Biologicals), ASC (Abcam), Caspase-1 (Abcam), Caspase-11 (Abcam), IL-1β (Abcam) or MAP2 (Abcam). Images were acquired using an Olympus BX61 confocal laser-scanning microscope (Olympus, Tokyo, Japan) with a X100 oil immersion objective. Single confocal images were converted to 512 x 512 pixel 12 bit TIFF images.

Patient Brain Tissue Sample

Human brain tissues were obtained from autopsy patients from files of the Institute of Neuropathology at the University Medical Centre Hamburg-Eppendorf and National Taiwan University Hospital, as approved by the University Medical Centre Hamburg-Eppendorf and National Taiwan University Hospital ethics committees, respectively. Brain specimens had been fixed in 4% buffered formalin for at least 3 weeks before paraffin-embedding. Brain sections (3μm) were stained according to standard immunohistochemistry procedures with primary antibodies against NLRP1 (Novus Biologicals), NLRP3 (Abcam), ASC (Abcam), IL-1β (Abcam) and IL-18 (Novus Biologicals).

Statistical Analysis

All experimental data obtained are expressed as mean ± standard error of the mean (SEM). Statistical analysis of all data except the behavioural score data were performed using one-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc analysis to determine between-group differences. Statistical difference was taken as $p<0.05$. Neurological behaviour scores were analyzed using a non-parametric Kruskal-Wallis Test and Dunn's Multiple Comparison Test. Statistical analyses were performed using GraphPad Prism 5.02 software.

2.3 Results:

Ischemia induces increased expression of inflammasome proteins, and both IL-1β and IL-18, in primary cortical neurons in simulated ischemia

To determine whether ischemia-like conditions activate the inflammasome in primary cortical neurons, we evaluated the temporal expression of all NLRP1 and NLRP3 inflammasome components in neurons subjected to simulated ischemia-reperfusion (I/R). The levels of all major inflammasome components and effectors were increased in primary cortical neurons in response to glucose deprivation (GD), oxygen-glucose deprivation (OGD) and simulated I/R conditions

including NLRP1, NLRP3, ASC, XIAP, and precursor caspase-1 and -11 (**Figure 2.1A-F, Supplementary Figure 2.1-2.3**). Levels of the latter proteins increased within 1 hour of exposure to simulated ischemia and remained elevated for 12–24 hours. Activation and homo-oligomerization of the NLRP1 and NLRP3 receptors individually induced the formation of the NLRP1 and NLRP3 inflammasome, respectively, which then activated both precursor caspase-1 and -11 into biologically active cleaved caspase-1 and -11 (Martinon *et al*., 2002; Wang *et al*., 1998). Following activation, caspase-1 cleaves both precursors IL-1β and IL-18 into biologically active mature pro-inflammatory cytokines, which are released into the extracellular environment (Bauernfeind *et al*., 2011). Consistent with the notion that ischemic conditions increase NLRP1 and NLRP3 inflammasome activation, we observed significantly increased levels of both cleaved caspase-1 and -11 and both mature IL-1β and IL-18 in primary cortical neurons following GD, OGD or simulated I/R conditions over 24 hours in comparison to control (**Figure 2.1A-F; Supplementary Figure 2.1-2.3**).

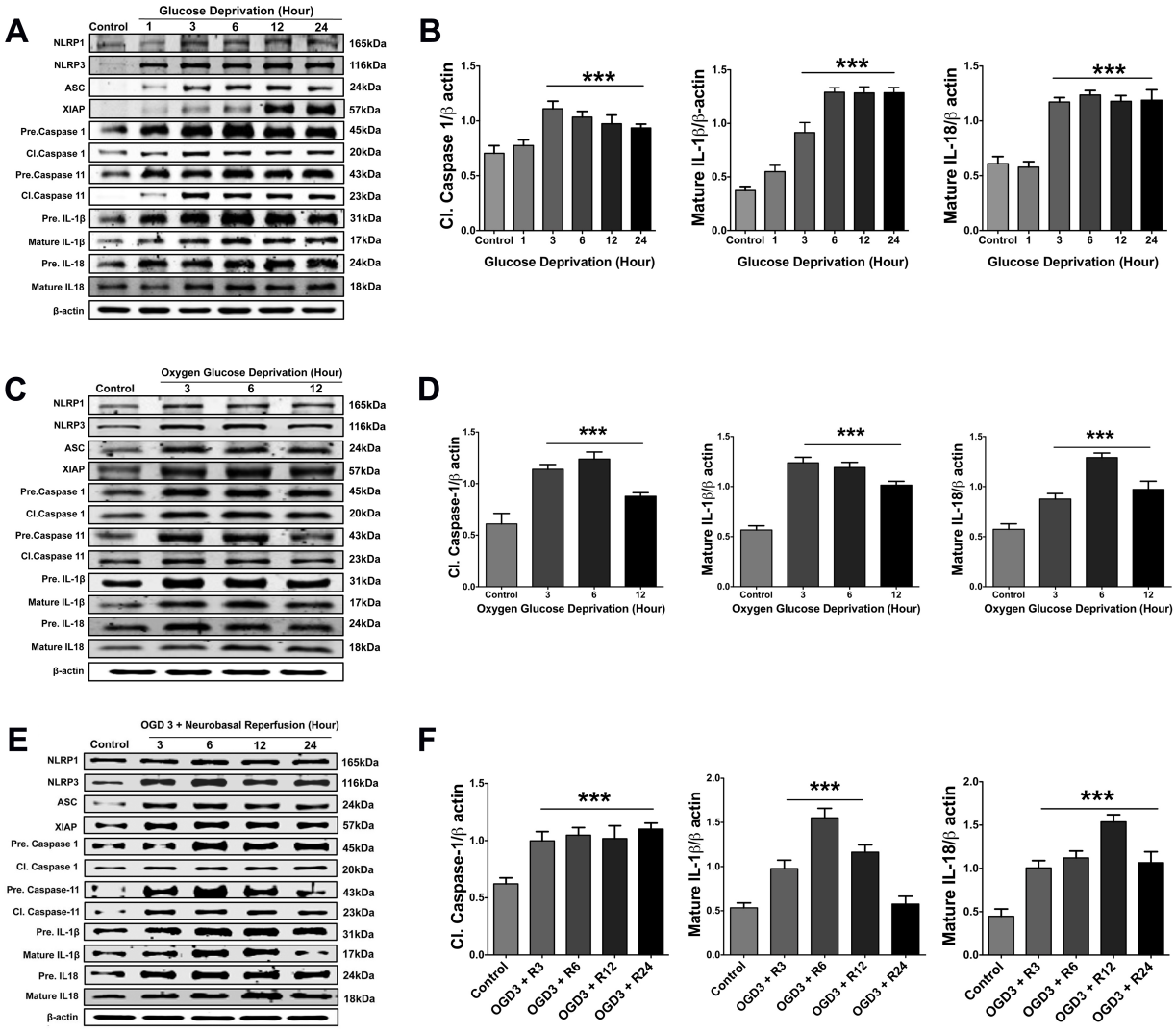


Figure 2.1: **Simulated ischemia increases the levels of multiple inflammasome proteins and both IL-1β**

and IL-18 in primary cortical neurons. (A and B). Representative immunoblots and quantification of inflammasome proteins and both IL-1β and IL-18 in lysates of cortical neurons at the indicated time points during GD. (C and D). Representative immunoblots and quantification of inflammasome proteins and both IL-1β and IL-18 in lysates of cortical neurons at the indicated time points during OGD. (E and F). Representative immunoblots and quantification of inflammasome proteins and both IL-1β and IL-18 in lysates of cortical neurons after simulated I/R. β-actin was used as a loading control. Data are represented as mean ± S.E.M. n=5 cultures. ***P<0.001 compared with controls.

Ischemia/reperfusion (I/R) induces increased expression of NLRP1 and NLRP3 inflammasome proteins and both IL-1β and IL-18 in ipsilateral brain tissues of cerebral I/R mice and stroke patients

The role of the NLRP1 and NLRP3 inflammasomes in ischemic stroke was further investigated by measuring the expression levels of NLRP1 and NLRP3 inflammasome proteins in ipsilateral (i.e. ischemic) brain tissues of cerebral I/R injured mice. It was shown that I/R significantly increased the expression of NLRP1 and NLRP3 inflammasome proteins, including NLRP1, NLRP3, ASC, XIAP, precursor caspases-1 and -11 in ipsilateral brain tissues as early as 1hr, and it remained higher at 12, 24 and 72hr following I/R in comparison to Sham controls (**Figure 2.2A-C; Supplementary Figure 2.4**). An indication of NLRP1 and NLRP3 inflammasome activation was demonstrated by increased levels of cleaved caspases 1 and 11, and both mature IL-1β and IL-18, at all time points following I/R in comparison to Sham controls (**Figure 2.2A and B**). Furthermore, to determine whether increased NLRP1 and NLRP3 inflammasome protein expression might occur in the human brain following ischemic stroke, we analysed brain tissues obtained from stroke patients at the University Medical Centre Hamburg-Eppendorf and National Taiwan University Hospital (**Figure 2.3A and B**). We found evidence that ischemic stroke increased NLRP1 and NLRP3 inflammasome proteins and both IL-1β and IL-18 in comparison to control patients **(Figure 2.3A and B)**.

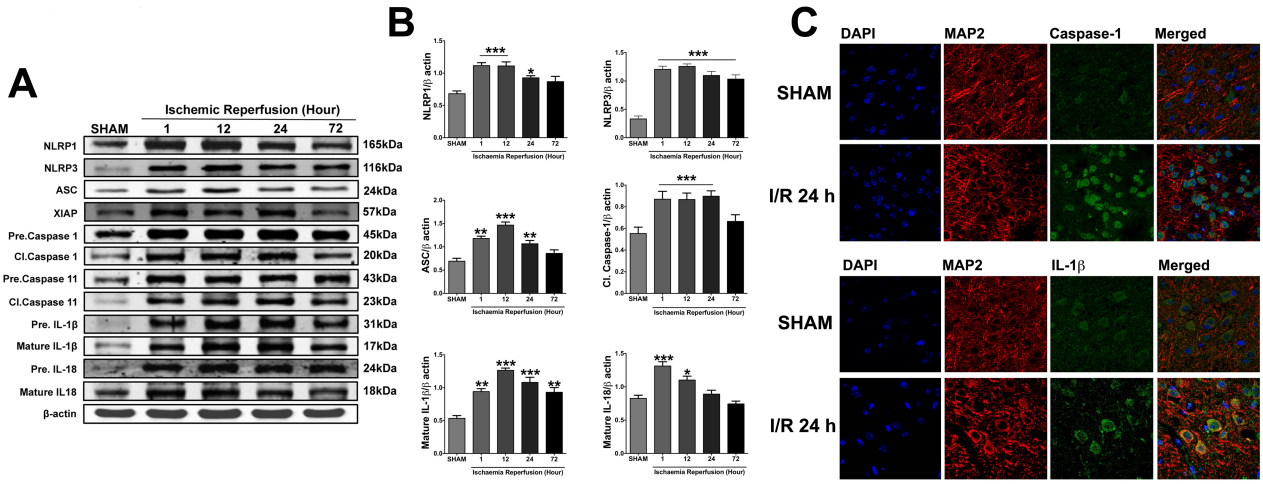


Figure 2.2: **Evidence that focal ischemic stroke activates the inflammasome in cerebral cortical cells.** (A and B). Representative immunoblots and quantification of inflammasome proteins and both IL-1β and IL-18 in ipsilateral brain lysates at indicated post-stroke time points. Data are represented as mean ± S.E.M. n=3-6. *P<0.05; **P<0.01; ***P<0.001 compared with SHAM (control). (C). Immunohistochemical analysis on caspase-1 and IL-1β show localization within the cytoplasm of cortical neurons. The levels of inflammasome proteins and both IL-1β and IL-18 are upregulated in I/R in comparison with SHAM (control). Magnification x 1000. Scale bar, 10μm. Images were taken under identical conditions and exposures.

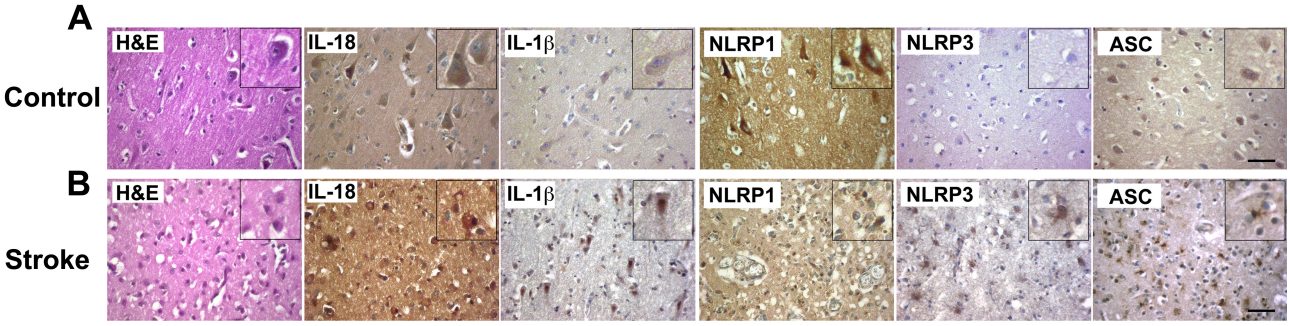


Figure 2.3: **Evidence for inflammasome expression and activation in brain tissues affected by stroke in human patients.** (A and B). Immunohistochemical analysis of NLRP1, NLRP3, ASC, IL-1β, IL-18 show localization within the cytoplasm of cortical neurons. The levels of inflammasome proteins and both IL-1β and IL-18 are elevated in brain tissues from a stroke patient in comparison with neurologically normal control patient. n=3 for each group. H&E stain was used to distinguish cell types. Images were taken under identical conditions and exposures.

Caspase-1 inhibitor (Ac-YVAD-CMK) treatment protects primary cortical neurons and cerebral tissue under simulated *in vitro* and *in vivo* models of ischemic stroke

In light of the increased expression of NLRP1 and NLRP3 inflammasome proteins and both IL-1β and IL-18 in primary cortical neurons, we next determined the functional role of inflammasomes in the degeneration of neurons subjected to ischemia-like conditions. We tested the efficacy of a caspase-1 inhibitor in primary cortical neurons under ischemic conditions. Increasing concentrations of a caspase-1 inhibitor (Ac-YVAD-CMK) (1-100 μM) were applied and neurons were then analysed for cleaved caspase-1, an indicator of inflammasome activation. Caspase-1 inhibitor (Ac-YVAD-CMK) concentrations above 30 μM were effective in reducing levels of cleaved caspase-1, in addition to cleaved caspase-3, a marker of apoptosis (**Figure 2.4A and B**). Mouse primary cortical neurons treated with a caspase-1 inhibitor (30 μM and 100 μM) were less vulnerable to apoptotic cell death under GD and OGD conditions **(Figure 2.4A and B; Figure 2.4D and E)**. The results of a cell viability assay showed that caspase-1 inhibitor (30-100 μM) treatment reduced neuronal cell death under GD conditions (**Figure 2.4A and C**). In addition, we investigated the effect of the caspase-1 inhibitor Ac-YVAD-CMK (30 μM and 100 μM) on the levels of the NLRP1 and NLRP3 inflammasome proteins and both precursor and mature forms of IL-1β and IL-18 during a 6hr period of OGD. Caspase-1 inhibition downstream had no effect on the expression levels of upstream inflammasome proteins such as NLRP1, NLRP3, ASC, XIAP, cleaved XIAP,

precursor caspase-1 and caspase-11, and both precursors IL-1β and IL-18 in comparison to vehicle controls under OGD conditions (**Figure 2.4D**). However, caspase-1 inhibitor (30 μM and 100 μM) treatment reduced the levels of cleaved caspase-1 and both mature IL-1β and IL-18 (**Figure 2.4D and E**). Furthermore, the levels of cleaved caspase-3 were lower in caspase-1 inhibitor (30 μM and 100 μM)-treated neurons in comparison to vehicle controls under OGD conditions (**Figure 2.4D and E**).

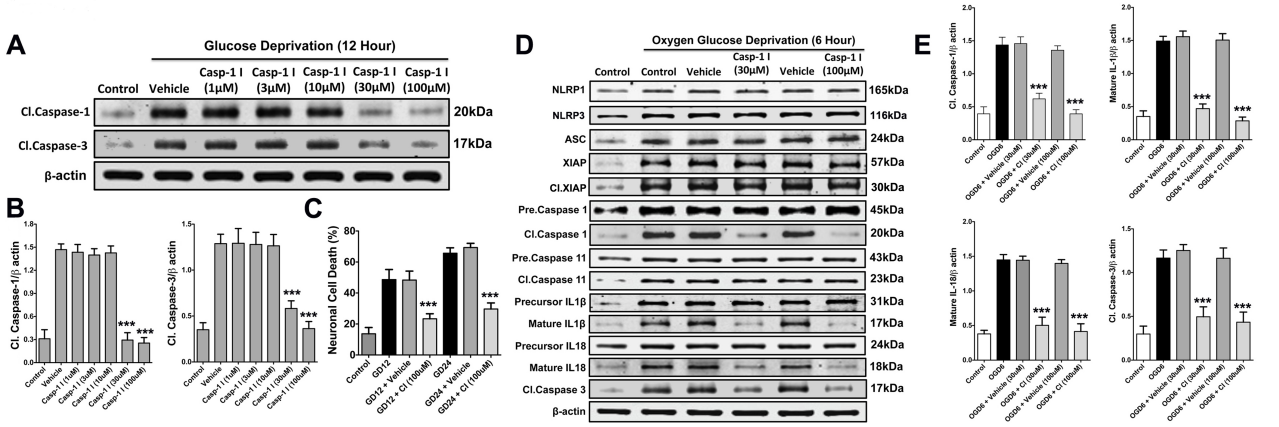


Figure 2.4: **Inhibition of caspase-1 reduces inflammasome activation and cell death in primary cortical neurons subjected to ischemia-like conditions.** (A and B). Representative immunoblots and quantification illustrating the effect of increasing concentrations (μM) of Ac-YVAD.CMK on levels of cleaved caspase-1 and caspase-3 proteins in primary cortical neurons subjected to GD. (C). The effect of Ac-YVAD.CMK treatment on cell death (%) in primary cortical neurons subjected to GD. (D and E). Representative immunoblots and quantification illustrating the effect of 30μM and 100μM Ac-YVAD.CMK treatment on inflammasome proteins, IL-1β, IL-18, and cleaved caspase-3 in primary cortical neurons subjected to OGD. β-actin was used as a loading control. Data are represented as mean ± S.E.M. n=5-6 cultures. ***P<0.001 compared with control.

We next evaluated the potential therapeutic efficacy of a caspase-1 inhibitor in a mouse model of focal ischemic stroke. A dose-response experiment was performed to identify the efficacy of a caspase-1 inhibitor on brain infarct size. It was found that whereas intravenous administration of the two lower doses of the caspase-1 inhibitor (1 and 6 mg/kg) at 3hr after reperfusion had no effect on brain infarct size in comparison to I/R vehicle controls (data not shown), both 10 and 20 mg/kg reduced brain infarct size ($p<0.0001$) and improved functional outcome in comparison to I/R vehicle controls (**Figure 2.5A-C**). Cerebral blood flow measurements obtained immediately before and after middle cerebral artery occlusion (MCAO), and at 60, 120 and 180 min after reperfusion, showed a ~90-95% reduction in blood flow in the cerebral cortex supplied by the middle cerebral artery during ischemia, and flow was not significantly different between groups at up to 180 min of reperfusion (data not shown). In addition, we investigated the effect of a caspase-1 inhibitor (10 mg/kg) on the protein expression levels of the NLRP1 and NLRP3 inflammasome components and both precursor IL-1β and IL-18 in ipsilateral brain tissues 24hr after I/R. Caspase-1 inhibition

downstream had no effect on the expression levels of upstream inflammasome proteins such as NLRP1, NLRP3, ASC, XIAP, cleaved XIAP, precursor caspase-1, caspase-11, and both precursor IL-1β and IL-18 in comparison to vehicle controls **(Figure 2.5D)**. However, the caspase-1 inhibitor at 10mg/kg significantly reduced the levels of cleaved caspase-1 and both mature IL-1β and IL-18 at 24hr following I/R (**Figure 2.5D and E**). Furthermore, levels of cleaved caspase-3 were lower in caspase-1 inhibitor (10 mg/kg) – treated groups in comparison to vehicle controls (**Figure 2.5D and E**).

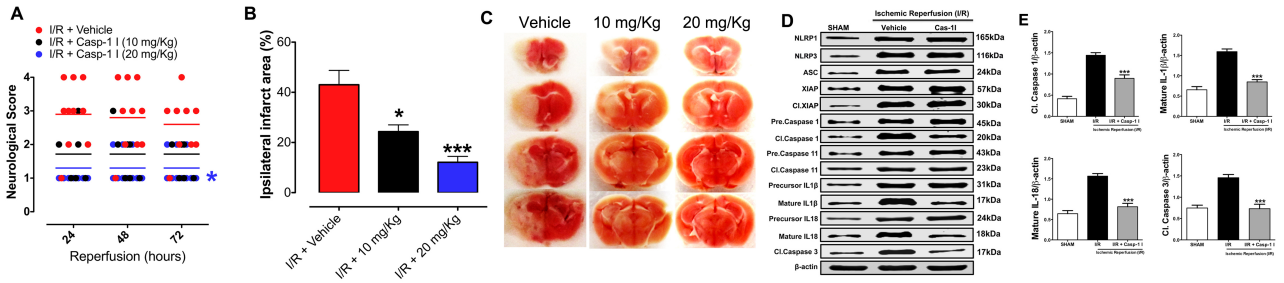


Figure 2.5: **A caspase-1 inhibitor improves neurological outcome, reduces infarct size and suppresses inflammasome activity in a mouse model of focal ischemic stroke.** (A). The effect of Ac-YVAD.CMK (10mg/kg and 20mg/kg) treatment on neurological scores of C57BL6/J mice following MCAO (1hr) and reperfusion at indicated times. *P<0.05. (B). The effect of Ac-YVAD.CMK (10 mg/kg and 20 mg/kg) treatment on ipsilateral infarct area (%) of C57BL6/J mice. n=9-11 animals in each group. *P<0.05, ***P<0.001. (C). Representative images of brains from each treatment group. (D). Representative immunoblots illustrating the effect of Ac-YVAD.CMK (10mg/kg) treatment on the levels of activated inflammasome proteins such as cleaved caspase-1, maturation of IL-1β and IL-18, and cleaved caspase-3 following MCAO (1hr) and reperfusion (24hr) in ipsilateral brain tissues of C57BL6/J mice. (E). Quantification illustrating Ac-YVAD.CMK significantly reducing the levels of activated inflammasome proteins such as cleaved caspase-1, maturation of IL-1β and IL-18, and cleaved caspase-3 in ipsilateral brain tissues following MCAO (1hr) and reperfusion (24hr) in C57BL6/J mice Data are represented as mean ± S.E.M. n=5-6 animals. ***P<0.001 compared with I/R.

IVIg treatment protects primary cortical neurons and brain tissue by decreasing inflammasome activity under *in vitro* and *in vivo* ischemic conditions

We recently identified IVIg as a potent stroke therapy (Arumugam *et al*., 2007; Widiapradja *et al*., 2012). Specifically, we reported that administration of IVIg to mice subjected to experimental stroke significantly reduced brain infarct size and nearly eliminated mortality. Moreover, not only was there a reduced volume of infarct, but within the ischemic region neurons were spared and only occasional cell loss was observed. Recently, it was demonstrated that IVIg could decrease the activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinases (MAPKs) signalling pathways in neurons under ischemic conditions through an unknown mechanism (Widiapradja *et al*., 2012). We therefore investigated the effect of IVIg (5 mg/mL) on levels of the NLRP1 and NLRP3 inflammasome proteins and both IL-1β and

IL-18 in primary cortical neurons under GD conditions over 6, 12 or 24 hrs. Indeed, we found that IVIg treatment significantly decreases levels of NLRP1, NLRP3, ASC, XIAP, caspase-1, caspase-11, IL-1β and IL-18 in comparison to vehicle-treated neurons during GD **(Figure 2.6A and B; Supplementary Figure 2.5)**. Furthermore, levels of cleaved caspase-3 were significantly lower in IVIg (5 mg/mL)–treated, compared to vehicle-treated neurons during GD **(Figure 2.6A and B)**. In addition, immunocytochemical analysis indicated that levels of inflammasome proteins and both IL-1β and IL-18 were lower in IVIg-treated neurons compared to vehicle-treated neurons after 12 hr of GD (**Figure 2.6C**). In addition, we investigated the effect of IVIg (5 mg/mL) on levels of NLRP1 and NLRP3 inflammasome proteins and both IL-1β and IL-18 in primary cortical neurons subjected to transient OGD and reperfusion conditions. We found that IVIg treatment inhibited OGD-induced elevations of NLRP1, NLRP3, ASC, XIAP, cleaved XIAP, caspase-1, precursor caspase-11, IL-18 and cleaved caspase-3 levels **(Figure 2.6D and E; Supplementary Figure 2.6)**. Furthermore, IVIg treatment significantly attenuated the simulated I/R-induced increase in levels of NLRP1, NLRP3, ASC, XIAP, cleaved XIAP, caspase-1, caspase-11, IL-1β, IL-18 and cleaved caspase-3 (**Figure 2.6D and E; Supplementary Figure 2.6**).

We also tested the effect of IVIg treatment on inflammasome activity *in vivo* following experimental stroke. Intravenous administration of 1g/kg IVIg at 3hr following reperfusion was previously reported to reduce brain infarct size and improve neurological outcome in rodent stroke models (Arumugam *et al*., 2007; Widiapradja *et al*., 2012). Here, we investigated the effect of IVIg (1g/kg) on levels of NLRP1 and NLRP3 inflammasome proteins, and both IL-1β and IL-18, in ipsilateral brain tissue at 6hr and 24hr of I/R. IVIg treatment significantly decreased levels of NLRP1 and NLRP3 inflammasome proteins, and both IL-1β and IL-18, in comparison to vehicle-treated mice **(Figure 2.6F and G; Supplementary Figure 2.7)**. Furthermore, levels of cleaved caspase-3 were significantly lower in IVIg (1g/kg)-treated groups in comparison to vehicle controls (**Figure 2.6F and G**).

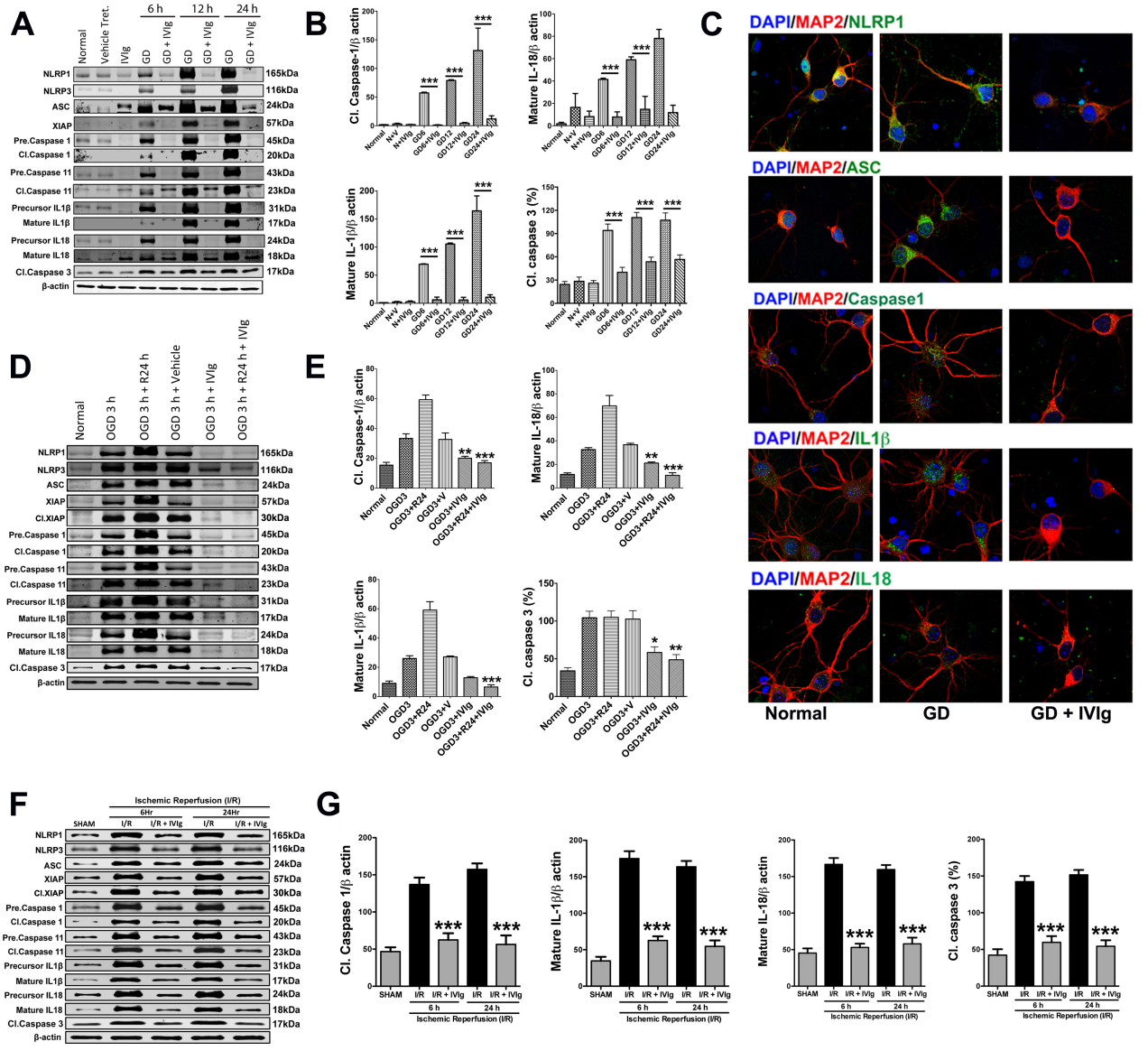


Figure 2.6: **IVIg treatment inhibits the inflammasome in cultured cortical neurons subjected to simulated ischemia, and in a mouse model of focal ischemic stroke.** (A and B). Representative immunoblots and quantification illustrating increases in the levels of inflammasome proteins, and both IL-1β and IL-18 in primary cortical neurons at indicated times during GD. Administration of IVIg (5mg/ml) significantly reduces the levels of inflammasome proteins and both IL-1β and IL-18. Data are represented as mean ± S.E.M. n=6 cultures. ***P<0.001 in comparison with GD. (C). Immunocytochemical analysis of NLRP1, ASC, caspase-1, IL-1β, and IL-18 show localization within the cytoplasm of primary cortical neurons. The levels of inflammasome proteins and both IL-1β and IL-18 are elevated in neurons subjected to GD. Treatment with IVIg (5mg/ml) significantly reduced the levels of inflammasome proteins and both IL-1β and IL-18 in neurons subjected to GD. Magnification x1000. Scale bar, 10mm. Images were taken under identical conditions and exposures. (D and E). Representative immunoblots and quantification illustrating increases in the levels of inflammasome proteins and both IL-1β and IL-18 in primary cortical neurons subjected to simulated I/R. Administration of IVIg (5mg/ml) significantly reduces the levels of inflammasome proteins and both IL-1β and IL-18. Data are represented as mean ± S.E.M. n=6 cultures. *P<0.05; **P<0.01; ***P<0.001 in comparison with cultures not treated with IVIg. (F and G). Representative immunoblots and quantification illustrating increase in the levels of inflammasome proteins and both IL-1β and IL-18 in ipsilateral brain tissues of C57BL6/J mice following MCAO (1hr) and reperfusion (6 and 24hr). β-actin was used as a loading control. Administration of IVIg (1g/kg) significantly reduces the levels of inflammasome proteins and both IL-1β and IL-18. Data are represented as mean ± S.E.M. n=5-6 animals in each group. ***P<0.001 in comparison with I/R (6 and 24hr).

2.4 Discussion:

Inflammation is a major contributor to the pathogenesis of ischemic stroke. The deleterious effects of the inflammatory response following cerebral ischemia are mediated by neurons, glial cells, endothelial cells and infiltrating leukocytes in the brain, which secrete numerous cytokines and chemokines at the site of injury. Numerous studies have shown that pro-inflammatory cytokines such as IL-1β and IL-18 play a significant role in cerebral ischemic damage (Abulafia *et al*., 2009; Caso *et al*., 2007; Deroide *et al*., 2013; Fogal *et al*., 2007; Mallat *et al*., 2001; Savage *et al*., 2012; Wang *et al*., 1997; Yuen *et al*., 2007; Zhang *et al*., 2014). A macromolecular complex, termed the inflammasome, in particular the NLRP1 and NLRP3 inflammasomes, regulate the maturation of these pro-inflammatory cytokines - IL-1β and IL-18. The present study provides strong evidence that the NLRP1 and NLRP3 inflammasomes play a major role in neuronal cell death and cerebral tissue damage in causing neurological and functional deficits following ischemic stroke. The second part of the study investigates the effect of IVIg treatment on ischemic stroke-induced NLRP1 and NLRP3 inflammasome activity. Previous experimental studies have demonstrated that high concentrations of IVIg are able to exert protective effects in neurons and cerebral tissue under *in vitro* and *in vivo* ischemic conditions (Arumugam *et al*., 2007; Chen *et al*., 2014; Tunik *et al*., 2013; Walberer *et al*., 2010; Widiapradja *et al*., 2012). The present study demonstrates for the first time that IVIg is able to decrease ischemic stroke-induced inflammasome activity by attenuating NLRP1 and NLRP3 inflammasome protein expression, with a corresponding down-regulation of pro-inflammatory cytokines IL-1β and IL-18 in neurons and cerebral tissue under *in vitro* and *in vivo* ischemic conditions.

It is proposed that an increase in expression of NLRP1 and NLRP3 inflammasome proteins and both precursor IL-1β and IL-18 in neurons and cerebral tissue under ischemic conditions may involve the activation of plasma membrane pattern recognition receptors (PRRs), such as toll-like receptors (TLRs; TLR-2 and-4), the receptor for advanced glycation end products (RAGE), and the IL-1 receptor 1 (IL-1R1) present on neighboring neurons, glial cells and infiltrating immune cells in the ischemic penumbra, which can detect endogenous danger signals termed damage associated molecular patterns (DAMPs) that are released from necrotic tissue within the ischemic core (Alfonso-Loeches *et al*., 2014; Burm *et al*., 2015; Caso *et al*., 2007; Caso *et al*., 2008; Codolo *et al*., 2013; Eigenbrod *et al*., 2008; Frank *et al*., 2015; Lee *et al*., 2013; Lippai *et al*., 2013; Nagyoszi *et al*., 2015; Nystrom *et al*., 2013; Pradillo *et al*., 2012; Tang *et al*., 2007; Tang *et al*., 2013; Weber *et al*., 2015; Zhao *et al*., 2014; Zheng *et al*., 2013). The activation of PRRs subsequently activates the NF-κB and MAPK(s) signalling pathways that may result in an increased expression of NLRP1 and NLRP3 inflammasome proteins and both precursor IL-1β and IL-18 through a distinct regulatory

process called 'priming' or Signal 1 (Bauernfeind *et al*., 2011a; Bauernfeind *et al*., 2009; Budai *et al*., 2013; Burm *et al*., 2015; Frederick Lo *et al*., 2008; Ghonime *et al*., 2014; Gross *et al*., 2011; Hara *et al*., 2013; He *et al*., 2012; Juliana *et al*., 2010; Kang *et al*., 2000; Legos *et al*., 2001; Liao *et al*., 2012; Liu *et al*., 2004; Liu *et al*., 2013; Mariathasan & Monack, 2007; Okada *et al*., 2014; Qiao *et al*., 2012; Savage *et al*., 2012; Schroder *et al*., 2012; Tamatani *et al*., 2000; Taxman *et al*., 2011; Weber *et al*., 2015; Zhao *et al*., 2013; Zheng *et al*., 2011). In addition, another possible explanation for an increased expression of NLRP1 and NLRP3 inflammasome proteins in ischemic tissue may be associated with the infiltration of peripheral immune cells (i.e. neutrophils, macrophages and T cells) to the site of injury, as immune cells also contain inflammasome proteins that can contribute to the overall expression profile of NLRP1 and NLRP3 inflammasome proteins in the ischemic brain. Following priming, a second regulatory signal may involve the activation and homo-oligomerization of the NLRP1 and NLRP3 receptors in response to DAMPs, or irregularities within the cellular microenvironment from cellular stress, resulting in the formation of the NLRP1 and NLRP3 inflammasome, respectively, which then activates precursor caspase-1 into cleaved caspase-1 through proximity-induced auto-activation (Agostini *et al*., 2004; Boatright *et al*., 2003; Faustin *et al*., 2007; Li *et al*., 2009; Liu *et al*., 2014; Martinon *et al*., 2002; Maslanik *et al*., 2013; Salvesen & Dixit, 1999; Savage *et al*., 2012). Following auto-activation, cleaved caspase-1 facilitates the cleavage of both precursor IL-1β and IL-18 into biologically active pro-inflammatory cytokines – mature IL-1β and mature IL-18, which are then released into the extracellular environment (Bauernfeind *et al*., 2011b). Despite numerous experimental studies showing that priming is required for the expression of NLRP1 and NLRP3 inflammasome proteins and both precursor IL-1β and IL-18 proteins in peripheral immune cells (Bauernfeind *et al*., 2011a,b; Bauernfeind *et al*., 2009; Budai *et al*., 2013; Burm *et al*., 2015; Frederick Lo *et al*., 2008; Ghonime *et al*., 2014; Gross *et al*., 2011; Hara *et al*., 2013; He *et al*., 2012; Juliana *et al*., 2010; Kang *et al*., 2000; Legos *et al*., 2001; Liao *et al*., 2012; Liu *et al*., 2004; Liu *et al*., 2013; Mariathasan & Monack, 2007; Okada *et al*., 2014; Qiao *et al*., 2012; Savage *et al*., 2012; Schroder *et al*., 2012; Tamatani *et al*., 2000; Taxman *et al*., 2011; Weber *et al*., 2015; Zhao *et al*., 2013; Zheng *et al*., 2011), it is not conclusively known whether inflammasome priming occurs similarly in neurons and cerebral tissue during ischemic conditions.

We demonstrated that when primary cortical neurons were subjected to ischemia-like conditions (GD, OGD or simulated I/R), NLRP1 and NLRP3 inflammasome proteins were increased, which was accompanied by elevated levels of cleaved caspase-1 and 11, and maturation of both precursor IL-1β and IL-18 proteins, an indication of inflammasome activation. Furthermore, immunofluorescence data showed expression and localization of these proteins in the cytoplasm of

neurons following ischemic conditions. The aforementioned findings suggest that the NLRP1 and NLRP3 inflammasome complex are formed in the cytoplasm of neurons following cerebral ischemic damage. Currently, the precise molecular and cellular stimuli(s) for NLRP1 and NLRP3 receptor activation during cerebral ischemia are unknown. Despite the extensive list of stimuli(s) described to be capable of activating the NLRP1 and NLRP3 receptor, there is no evidence of direct ligand binding (Petrilli *et al*., 2007a). Hence, it is now proposed that the NLRP1 and NLRP3 receptor is a sensor for abnormal changes in the intracellular environment in times of cellular stress (Davis *et al*., 2011; Kersse *et al*., 2011; Schroder & Tschopp, 2010). Although a fully defined mechanism leading to NLRP1 and NLRP3 receptor activation has not been elucidated during cerebral ischemia, numerous contributing cellular events are considered plausible, including energy depletion, acidosis, cathepsin release, increased reactive oxygen species (ROS) production, oxidized mitochondrial DNA, increased intracellular calcium (Ca^{2+}) concentration, cell swelling, and protein kinase R (PKR) activation in neurons and cerebral tissue under ischemic conditions (Compan *et al*., 2012; Lee *et al*., 2012; Liao & Mogridge, 2012; Lu *et al*., 2012; Nakahira *et al*., 2011; Rajamaki *et al*., 2013; Rossol *et al*., 2012; Shimada *et al*., 2012; Zhou *et al*., 2010; Zhou *et al*., 2011). However, recent evidence now suggests that adenosine triphosphate (ATP) released from both stressed and/or necrotic neurons in culture, and the ischemic core in the brain under *in vitro* and *in vivo* ischemic conditions, respectively, may be a significant factor in mediating cellular and tissue damage by binding onto P2X4 receptors on the plasma membrane of neighboring neurons and glial cells to open these ligand-gated ion channels in order to facilitate an increased efflux of potassium (K^+) ions from the cytoplasm into the extracellular environment (Carta *et al*., 2015; Cauwels *et al*., 2014; Chen *et al*., 2013; De Rivero Vaccari *et al*., 2012; Ferrari *et al*., 2006; Hung *et al*., 2013; Iyer *et al*., 2009; Mariathasan *et al*., 2006; Schwab *et al*., 2005; Wilhelm *et al*., 2010). In addition, necrotic cells in the ischemic core will passively release potassium (K^+) ions into the extracellular environment. Therefore, these mechanisms will collectively increase potassium (K^+) ions in the extracellular environment and activate Pannexin 1 channels on the plasma membrane (Silverman *et al*., 2009). Opening of Pannexin 1 channels will lead to further release of ATP and activation of P2X4 and P2X7 receptors, creating a positive feedback loop by leading to additional potassium (K^+) ion efflux (Adamson & Leitinger, 2014; Ayna *et al*., 2012; Babelova *et al*., 2009; De Rivero Vaccari *et al*., 2012; Ferrari *et al*., 2006; Franchi *et al*., 2007; Hung *et al*., 2013; Kahlenberg & Dubyak, 2004; Kahlenberg *et al*., 2005; Le Feuvre *et al*., 2003; Locovei *et al*., 2007; Pelegrin & Surprenant, 2006; Raouf *et al*., 2007; Riteau *et al*., 2012; Shestopalov & Slepak, 2014; Stoffels *et al*., 2015). The latter activation of P2X7 receptors is due to P2X4 receptors being more sensitive (approximately 100 times) to ATP than P2X7 receptors in the brain and spinal cord (North & Surprenant, 2000; Raouf *et al*., 2007). Hence, it is subsequently proposed that a decreased

intracellular potassium (K^+) ion concentration and/or an increased extracellular potassium (K^+) ion concentration in neurons and glial cells may create an environment that is favourable for activating the NLRP1 and NLRP3 receptors, either directly or indirectly through an unknown mechanism(s) during ischemic conditions (Franchi *et al*., 2014; Katsnelson *et al*., 2015; Lindestam Arlehamn *et al*., 2010; Munoz-Planillo *et al*., 2013; Petrilli *et al*., 2007b; Silverman *et al*., 2009). In addition, recent studies have suggested that stimulation of astrocytes with ATP results in activation of the NLRP2 inflammasome, and that ATP-induced activation of the NLRP2 inflammasome were inhibited by a pannexin 1 inhibitor and a P2X7 receptor antagonist (Minkiewicz *et al*., 2013; Silverman *et al*., 2009). The ATP-dependent oligomerization of NLRPs and formation of the inflammasome complex will then promote cleavage of precursor caspase-1 into cleaved caspase-1, which in turn cleaves IL-1β and IL-18 into their mature forms (Duncan *et al*., 2007; Faustin *et al*., 2007; Koonin & Aravind, 2000; Levinsohn *et al*., 2012; Martinon *et al*., 2002).

The current data show an increase in levels of the NLRP1 and NLRP3 inflammasome proteins and both precursor IL-1β and IL-18, in addition to effectors of inflammasome activation in primary cortical neurons and cerebral tissue subjected to ischemia. However, whether activation of the NLRP1 and NLRP3 inflammasome in neurons and cerebral tissue under *in vitro* and *in vivo* ischemic conditions is a result of ATP release from necrotic neurons and cerebral tissue remains to be fully determined. The increase in levels of both mature IL-1β and IL-18 under *in vitro* ischemia-like conditions supports findings in which both extracellular IL-1β and IL-18 are implicated in causing autocrine, paracrine and endocrine effects by binding to their respective receptors on the plasma membrane of neighboring neurons and glial cells, and/or peripheral immune cells, and activating NF-κB and MAPK(s) signaling pathways in the target cell (Calkins *et al*., 2002; Dinarello, 1998; Dinarello, 2002; Dinarello, 2009; Dinarello & Van der Meer, 2013; Dinarello *et al*., 2012; Dinarello *et al*. 2013; Garlanda *et al*., 2013; Gracie *et al*., 2003; Lee *et al*., 2004; Novick *et al*., 2013; Rider *et al*., 2011; Sedimbi *et al*., 2013; Srinivasan *et al*., 2004). Consequently, this may lead to increased priming, which would be expected to further increase production of NLRP1 and NLRP3 inflammasome proteins and both precursor IL-1β and IL-18 in surrounding neurons and glial cells, in addition to possibly inducing secondary transcription of multiple inflammation-associated genes, including: pro-inflammatory cytokines (e.g. TNFα, IL-1β, IL-6 and IL-18); chemokines (e.g. CXC-chemokine ligand 8, CXCL8 aka IL-8, CX_3C-chemokine ligand 1, CX_3CL1 aka fractalkine); and adhesion molecules (e.g. E-selectin and ICAM-1), all contributing to ischemic reperfusion injury resulting in neuronal and glial cell death (Allan *et al*., 2005; Allan & Rothwell, 2001; Arumugam *et al*., 2004; Denes *et al*., 2008; Ehrensperger *et al*., 2005; Huang *et al*., 2000; Vila *et al*., 2000; Yilmaz & Granger, 2008; Zhang *et al*., 1998). Furthermore, both mature IL-1β

and IL-18 may contribute to secondary injuries by inducing leukocyte recruitment, which can in turn lead to an increased production and release of ROS and additional pro-inflammatory cytokines at the site of injury, thus exacerbating neuronal cell death and tissue damage during cerebral ischemia (Calkins *et al*., 2002; De Rivero Vaccari *et al*., 2008; Denes *et al*., 2012; Kong *et al*., 2014; McColl *et al*., 2007; Netea *et al*., 2000; Sonnino *et al*., 2014).

Our findings indicate increased levels of not only cleaved caspase-1 but also cleaved caspase-11, in neurons under ischemia-like conditions. Recent evidence suggests that cleaved caspase-1 may require the presence of cleaved caspase-11 for the maturation of precursor IL-1β and IL-18 proteins (Kayagaki *et al*., 2011). In addition, cleaved caspase-11 was shown to activate caspase-3 and cause apoptotic cell death in neurons and glial cells under ischemic conditions (Kang *et al*., 2000; Kang *et al*., 2002; Kang *et al*., 2003). It was previously reported that administration of a caspase-1 inhibitor (Ac-YVAD.CMK) induced long-lasting neuroprotection through a decrease in pro-inflammatory cytokine production and attenuation of apoptosis in a permanent MCAO stroke model (Rabuffetti *et al*., 2000; Zhang *et al*., 2003). Our study found that Ac-YVAD.CMK and IVIg inhibited activation of caspase-3. This link between the inflammasome and apoptotic cascades supports the idea that increased expression levels of cleaved caspase-1 may mediate a number of pleiotropic effects (Erener *et al*., 2012; Frederick Lo *et al*., 2008; Guegan *et al*., 2002; Liu *et al*., 2004; Walsh *et al*., 2011; Zhang *et al*., 2003). A major effect of cleaved caspase-1 is that it is able to directly cleave and activate both executioner caspase-3 and 7, and Bid (BH3 Interacting Domain Death Agonist) into its truncated form, inducing intrinsic and extrinsic apoptotic cell death, respectively (Erener *et al*., 2012; Frederick Lo *et al*., 2008; Guegan *et al*., 2002; Liu *et al*., 2004; Walsh *et al*., 2011; Zhang *et al*., 2003). Hence, our data further supports the role of NLRP1 and NLRP3 inflammasomes in mediating apoptotic cell death in neurons under ischemic conditions. Besides apoptosis, pyroptosis is another form of cell death directly linked to inflammasome activation. Numerous studies suggest that pyroptosis is exclusively regulated by cleaved caspase-1, which initiates the formation of pores in the plasma membrane of cells through an unknown mechanism(s), thereby allowing dissipation of cellular ionic gradients (such as Na^+ and K^+) and subsequently inducing osmotic movement of water through aquaporins into the cell causing lysis, which releases its pro-inflammatory contents into the extracellular environment exacerbating the inflammatory response (Bergsbaken *et al*., 2009; Fink & Cookson, 2006; Fink *et al*., 2008). However, it remains to be determined whether neurons undergo pyroptosis during cerebral ischemia.

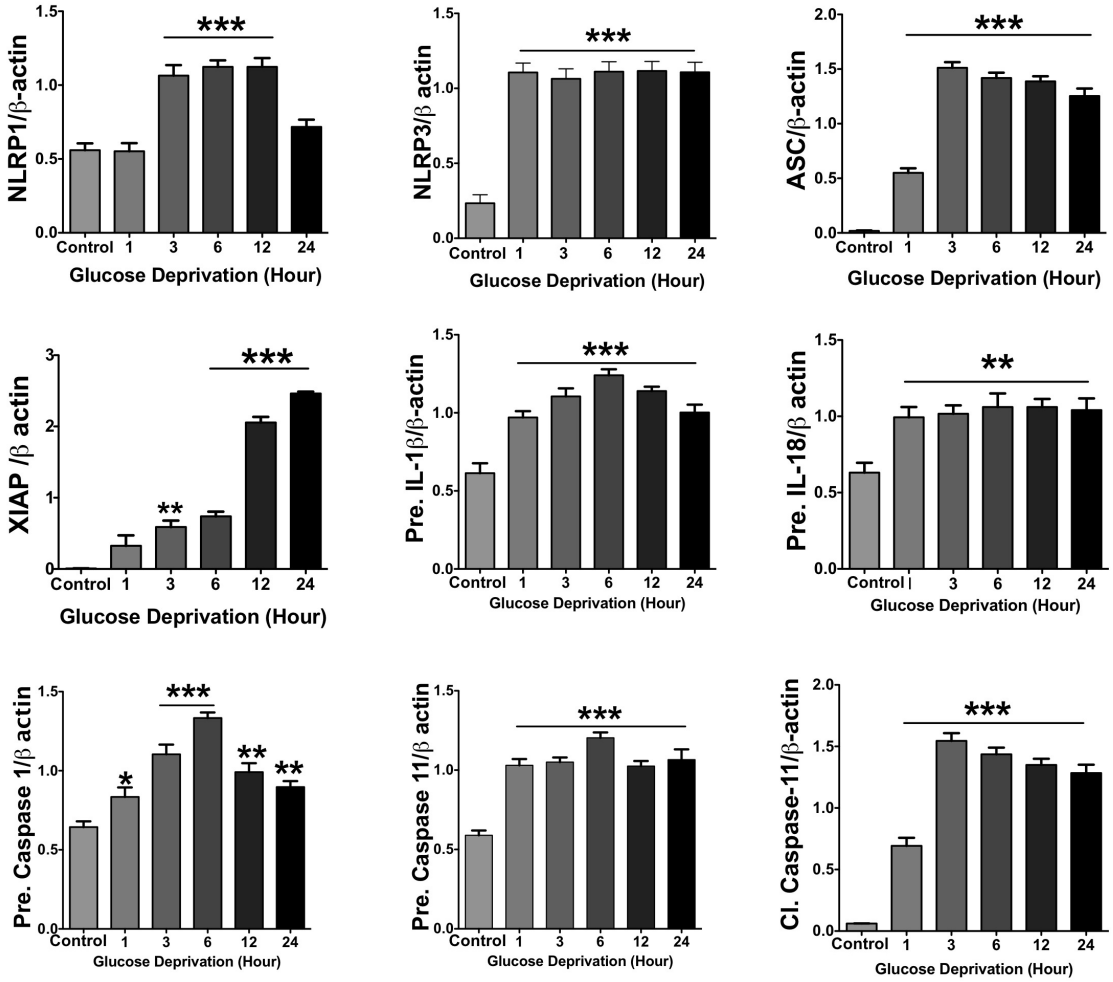
In a previous study, we demonstrated that treatment of cultured neurons with IVIg reduced ischemic neuronal cell death, in part, by inhibiting the complement cascade (Arumugam *et al*.,

2007). The present data demonstrates neuroprotective effects of IVIg on ischemia-induced NLRP1 and NLRP3 inflammasome activity in primary cortical neurons. We found evidence that the neuroprotective effects of IVIg are associated with a significant reduction in the levels of NLRP1 and NLRP3 inflammasome proteins and both precursor IL-1β and IL-18 during simulated ischemia under *in vitro* conditions and in a mouse model of focal ischemic stroke. IVIg was previously shown to reduce activation of caspase-3 and to protect neurons from undergoing apoptotic cell death under ischemic conditions (Arumugam *et al*., 2007; Widiapradja *et al*., 2012). Although the molecular and cellular neuroprotective mechanism(s) of IVIg in ischemic stroke-induced NLRP1 and NLRP3 inflammasome activity in neurons and cerebral tissue remains to be established, the present data fits a model in which IVIg inhibits inflammasome priming by decreasing the activity of both intracellular NF-κB and MAPK(s) signaling pathways in neurons, a possibility consistent with a recent report that IVIg protects neurons from cell death under ischemic conditions by inhibiting the phosphorylation levels of NF-κB, p38, and JNK (Widiapradja *et al*., 2012). This effect would be expected to attenuate the production of NLRP1 and NLRP3 inflammasome proteins and both precursor IL-1β and IL-18, thereby decreasing the production of both cleaved caspase-1 and caspase-11 and hence mature IL-1β and IL-18. Indeed, we found that both a caspase-1 inhibitor and IVIg blocked maturation of precursors IL-1β and IL-18. Hence, we speculate that cleaved caspase-1-dependent apoptosis and pyroptosis would be reduced by IVIg treatment. Consistent with the latter possibility, IVIg can increase the expression of the anti-apoptotic protein, Bcl-2, in cultured cortical neurons and cerebral tissue under *in vitro* and *in vivo* ischemic conditions (Supplementary Figure 2.8; Widiapradja *et al*., 2012). Studies have demonstrated that Bcl-2 can directly bind and inhibit the NLRP1 and NLRP3 receptor in macrophages by specifically preventing ATP from binding onto the nucleotide-binding domain (NBD) of both receptors (Bruey *et al*., 2007; Faustin *et al*., 2009; Shimada *et al*., 2012). Therefore, inhibiting the oligomerization of the NLRP1 and NLRP3 receptors is expected to attenuate inflammasome formation and reduce both caspase-1 activation and maturation of both IL-1β and IL-18 (Bruey *et al*., 2007; Faustin *et al*., 2009; Shimada *et al*., 2012). In addition, it was shown that Bcl-xL, another anti-apoptotic protein was able to directly bind and inhibit the NLRP1 receptor in macrophages through a similar mechanism but whether Bcl-xL is able to inhibit the NLRP3 receptor remains to be established (Bruey *et al*., 2007; Faustin *et al*., 2009). Accordingly, it appears that Bcl-2 is a tight regulator of NLRP1 and NLRP3 receptor activation; however, whether Bcl-xL regulates NLRP3 receptor activation, and how and whether IVIg increases Bcl-2 and Bcl-xL levels, respectively, in neurons and cerebral tissue under *in vitro* and *in vivo* ischemic conditions remains to be fully determined.

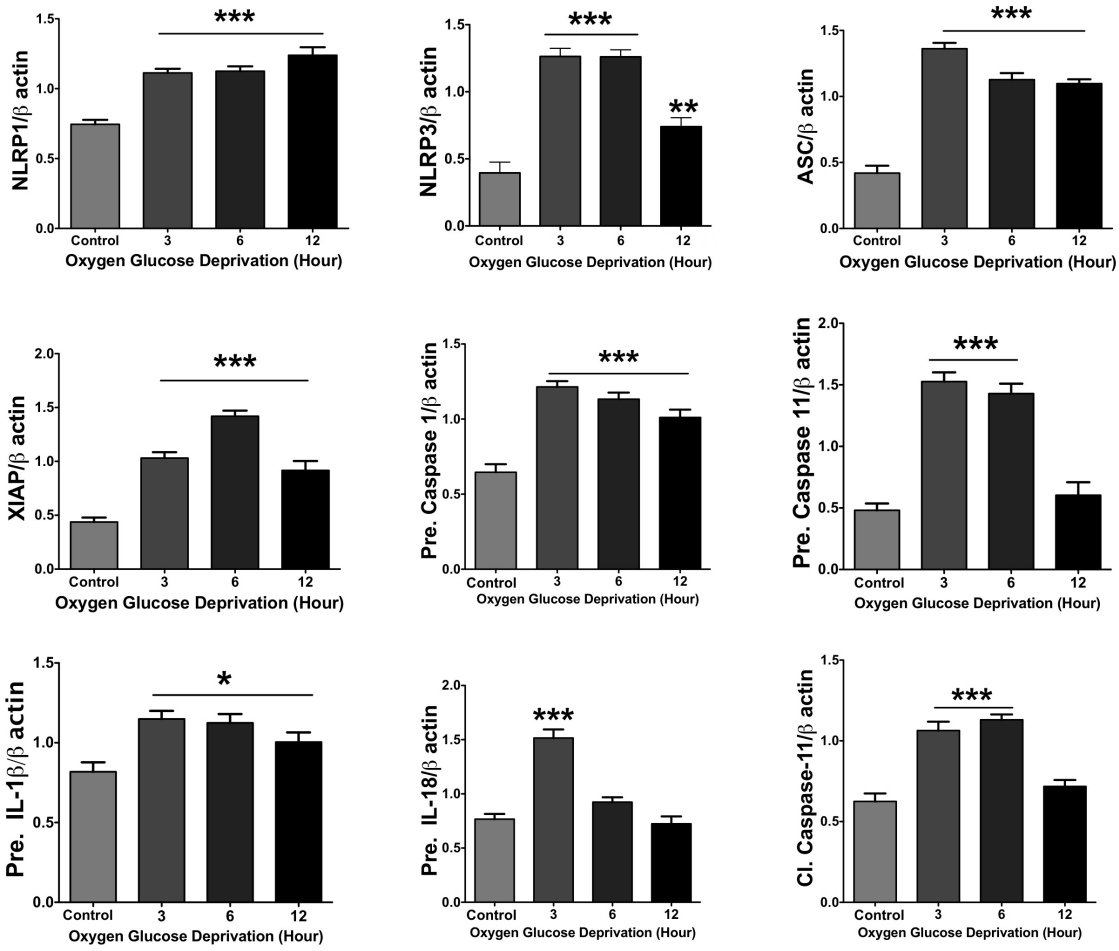
2.5 Conclusion:

Previous reports have suggested IVIg to be a promising therapeutic modality for targeting a number of injury mechanisms in multiple cell types under ischemic conditions (Arumugam *et al*., 2007; Arumugam *et al*., 2008; Widiapradja *et al*., 2012; Widiapradja *et al*., 2014). The present study demonstrated that ischemia-like conditions increased the levels of NLRP1 and NLRP3 inflammasome proteins and both IL-1β and IL-18 in primary cortical neurons. Similarly, levels of NLRP1 and NLRP3 inflammasome proteins and both IL-1β and IL-18 were elevated in ischemic brain tissues of mice subjected to ischemic stroke. In addition, identified a novel mechanism by which Ac-YVAD.cmk and IVIg treatment protected primary cortical neurons and brain tissue by a mechanism(s) involving Caspase-1 inhibition and suppression of NLRP1 and NLRP3 inflammasome activity, respectively, under *in vitro* and *in vivo* ischemic conditions. These findings suggest that therapeutic interventions targeting inflammasome expression and activity during cerebral ischemia may offer substantial promise. Hence, continued investigation into the mechanism(s) underlying NLRP1 and NLRP3 inflammasome activity in neurons and glial cells in settings of brain tissue injury and neurodegeneration is warranted in potential future treatments of ischemic stroke.

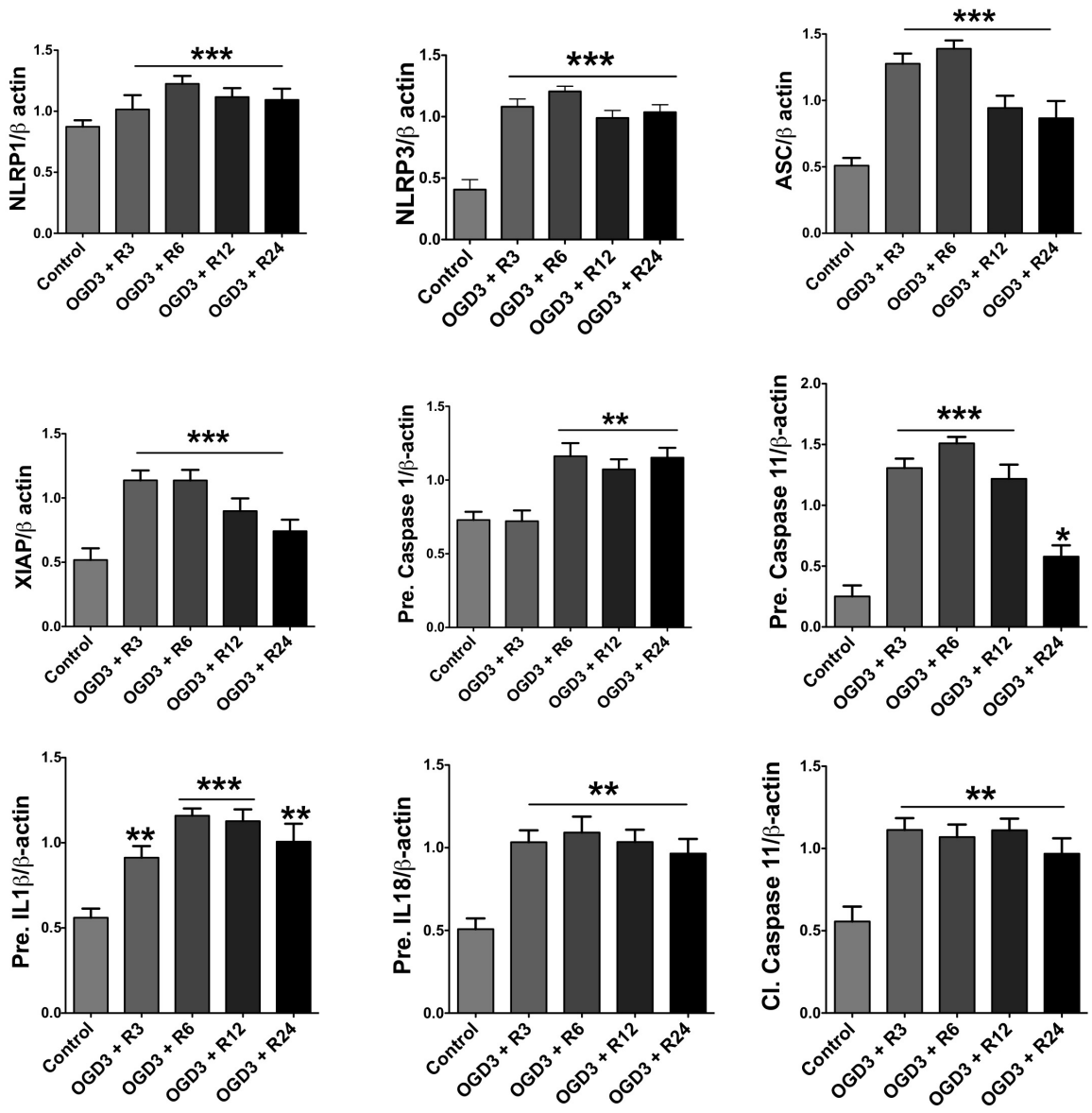
2.6 Supplementary Figures:



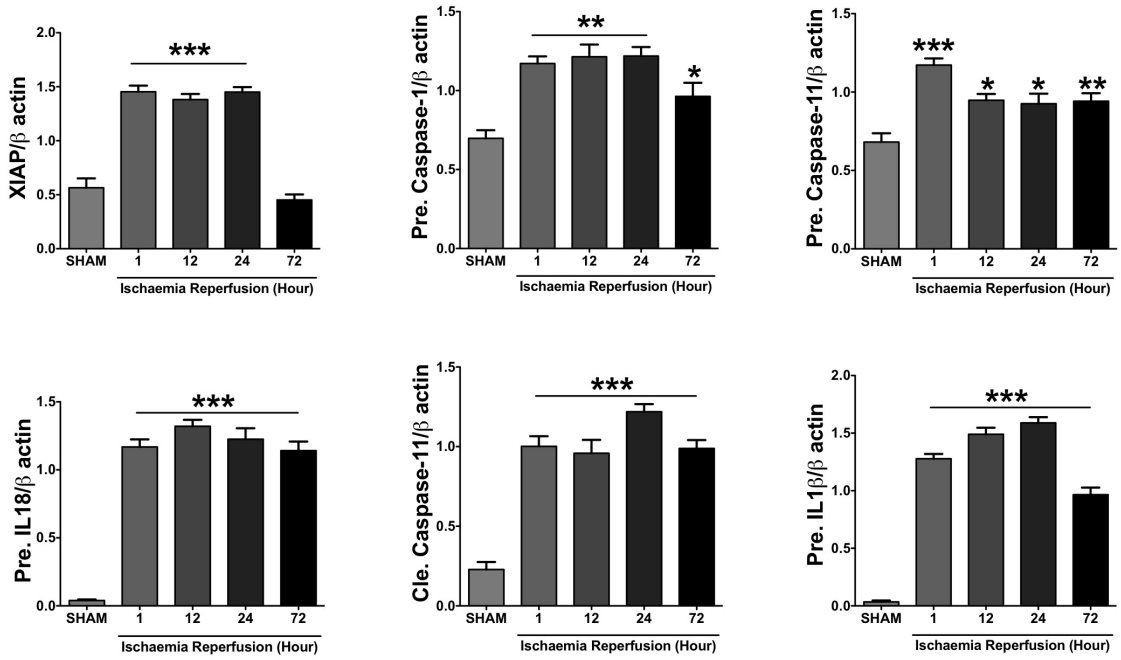
Supplementary Figure 2.1: Glucose deprivation (GD) increases levels of inflammasome proteins, IL-1β and IL-18 in primary cortical neurons of C57BL/6J mice. Quantification of inflammasome proteins, and IL-1β and IL-18, in cortical lysates of neurons at the indicated times periods of GD. β-actin was used as a loading control. Data are represented as mean ± SEM. n= 5 cultures. **p < 0.01; ***p < 0.001 compared with Control.



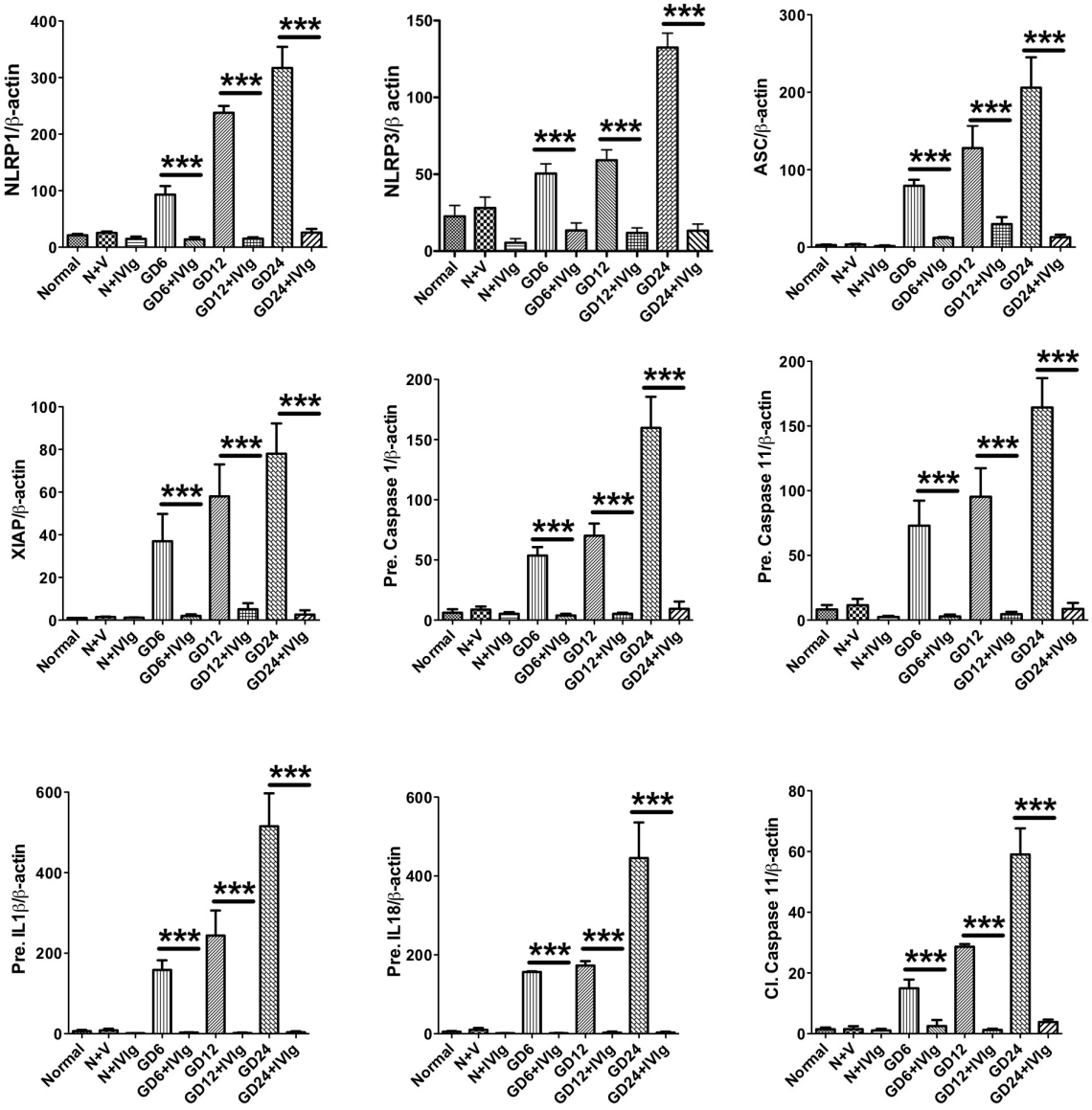
Supplementary Figure 2.2: Combined oxygen and glucose deprivation (OGD) increases the levels of inflammasome proteins, IL-1β and IL-18 in primary cortical neurons of C57BL/6J mice. Quantification of inflammasome proteins and IL-1β and IL-18 in cortical lysates of neurons at indicated times under OGD. β-actin was used as a loading control. Data are represented as mean ± SEM. n= 5 cultures. *p < 0.05; ***p < 0.001 compared with Control.



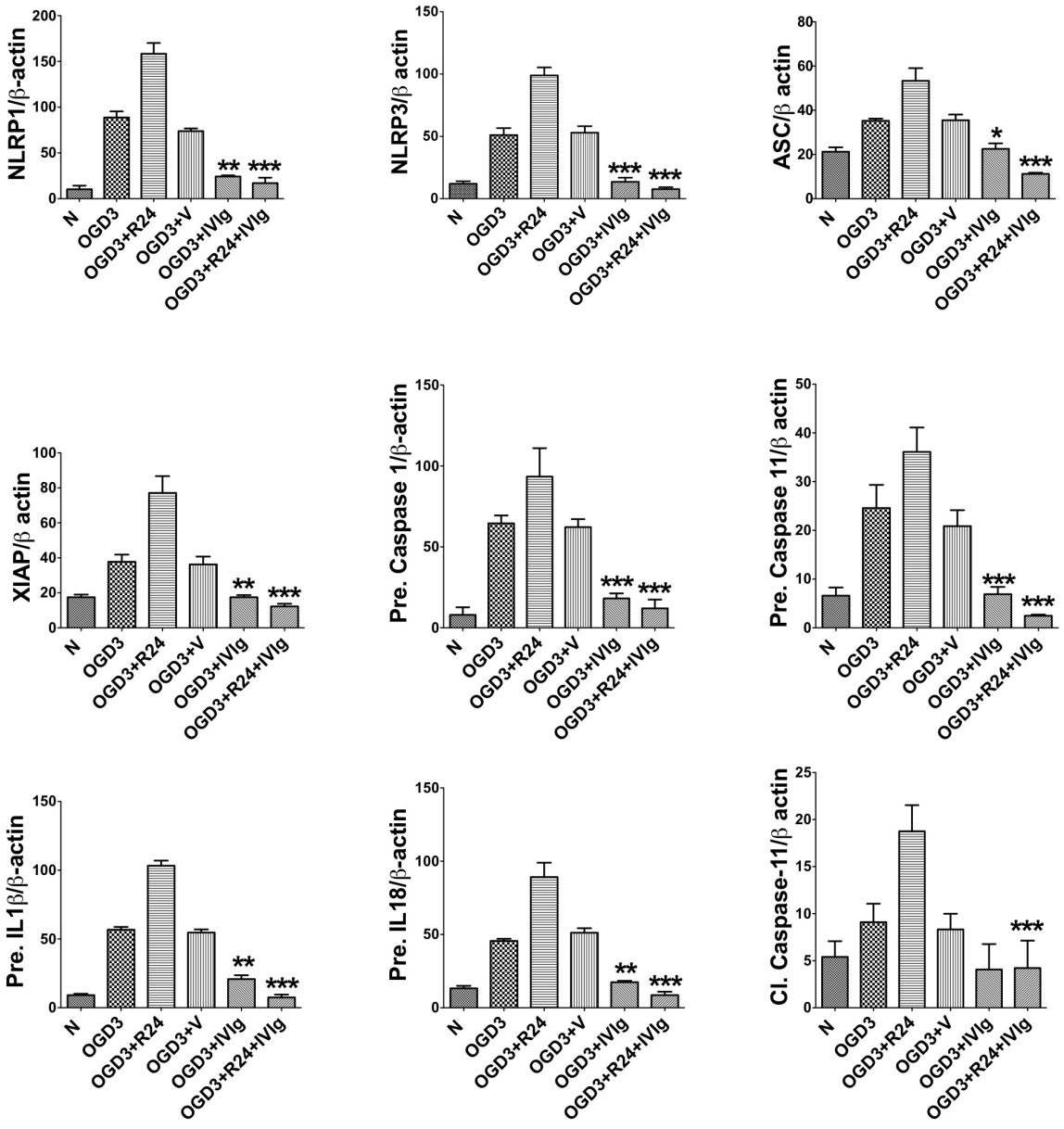
Supplementary Figure 2.3: Simulated ischemia/reperfusion increases levels of inflammasome proteins and IL-1β and IL-18 in primary cortical neurons of C57BL/6J mice. Quantification of inflammasome proteins and both IL-1β and IL-18 in cortical lysates of neurons under OGD_{3hr} with neurobasal reperfusion at indicated times. β-actin was used as a loading control. Data are represented as mean ± SEM. n= 5-6 cultures. **p < 0.01; ***p < 0.001 compared with Control.



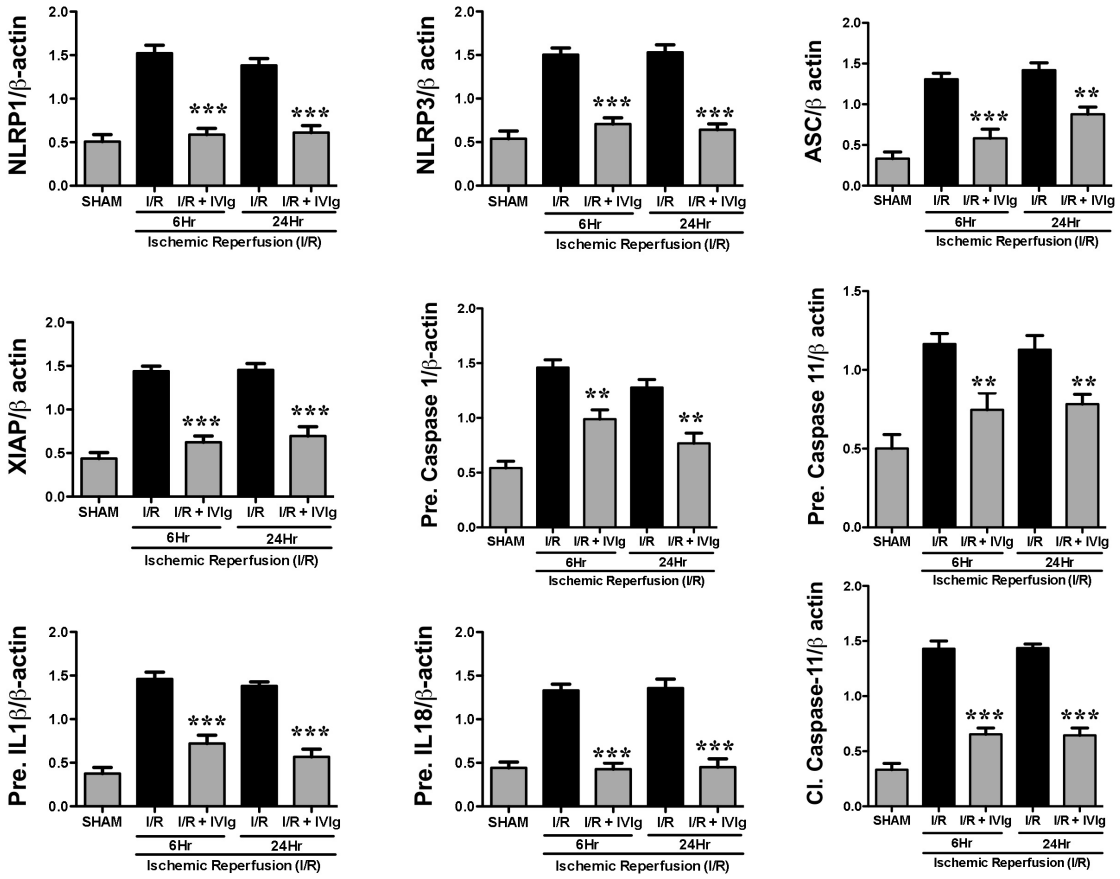
Supplementary Figure 2.4: Levels of inflammasome proteins and IL-1β and IL-18 are elevated in response to middle cerebral artery occlusion/reperfusion in ipsilateral brain tissues of C57BL/6J mice. Quantification of inflammasome proteins and both IL-1β and IL-18 in ipsilateral brain lysates at indicated times. β-actin was used as a loading control. Data are represented as mean ± SEM. n= 5-6. *p < 0.05; **p < 0.01; ***p < 0.001 compared with SHAM (control).



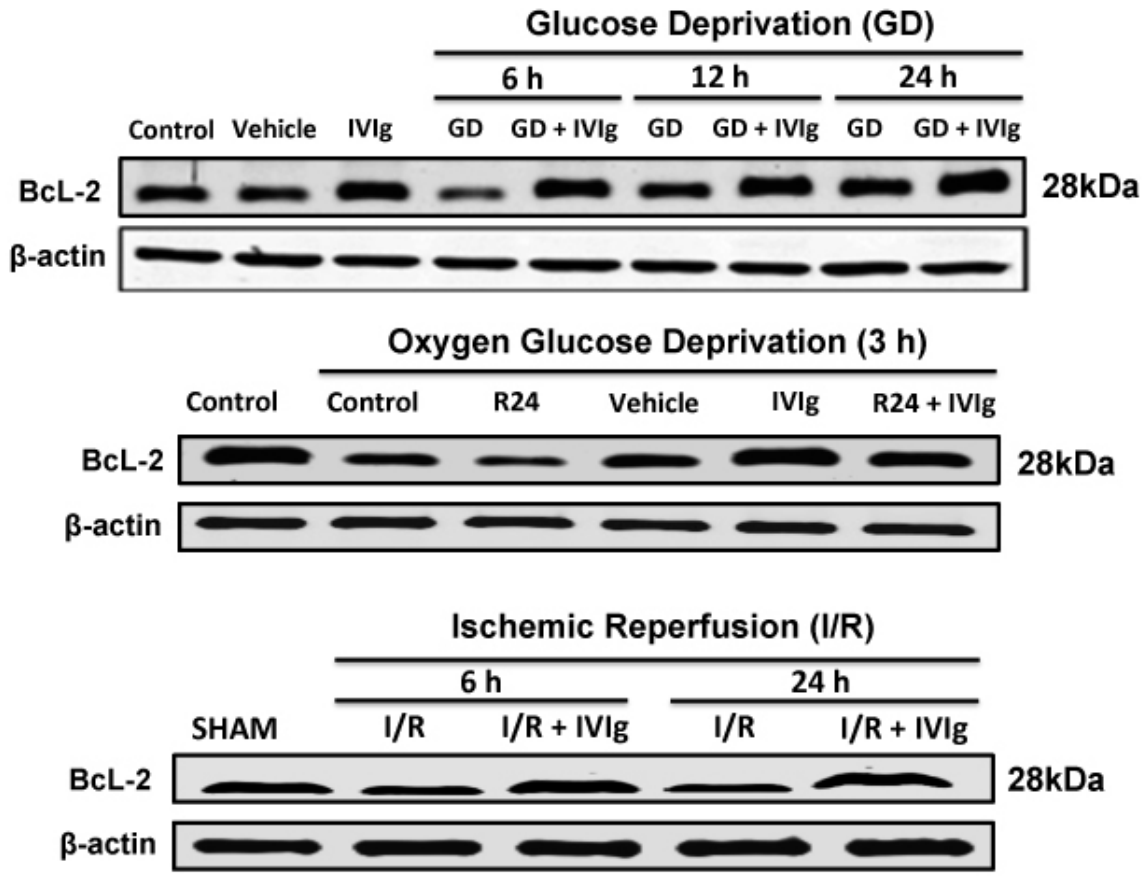
Supplementary Figure 2.5: IVIg treatment suppresses the inflammasome in cultured cortical neurons subjected to glucose deprivation (GD). Quantification illustrating increase in the expression levels of inflammasome proteins and IL-1β and IL-18 in primary cortical neurons at indicated times after subjection to GD. β-actin was used as a loading control. Administration of IVIg (5 mg/mL) significantly reduces the expression levels of inflammasome proteins and both IL-1β and IL-18. Data are represented as mean ± SEM. n= 5-6 cultures. ***$p < 0.001$ in comparison to GD.



Supplementary Figure 2.6: IVIg treatment suppresses the inflammasome in cultured cortical neurons subjected to oxygen glucose deprivation (OGD) or simulated ischemia/reperfusion (IR). Quantification illustrating increase in the expression levels of inflammasome proteins and IL-1β and IL-18 in neurons subjected to OGD (3 hours) or simulated I (3 hours)/R (24 hours). β-actin was used as a loading control. Administration of IVIg (5mg/mL) significantly reduces the expression levels of inflammasome proteins and both IL-1β and IL-18. Data are represented as mean ± SEM. n= 5-6 cultures. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ in comparison to OGD_{3hr} and OGD_{3hr} + neurobasal reperfusion (24hr).



Supplementary Figure 2.7: IVIg treatment suppresses the inflammasome in a mouse model of focal ischemic stroke. Quantification illustrating increases in the expression levels of inflammasome proteins and both IL-1β and IL-18 in ipsilateral brain tissues of mice following middle cerebral artery occlusion (1 hour) and reperfusion (6 or 24 hours). β-actin was used as a loading control. Administration of IVIg (2g/Kg) significantly reduces the expression levels of inflammasome proteins and IL-1β and IL-18. Data are represented as mean ± SEM. n=3-5. ***$p < 0.001$; **$p < 0.01$ in comparison to I/R_{6hr} and I/R_{24hr}.



Supplementary Figure 2.8: IVIg treatment increases the levels of BcL-2 in cultured cerebral cortical neurons subjected to ischemia-like conditions. (A). Representative immunoblot illustrating increase in the expression levels of BcL-2 in primary cortical neurons subjected to GD for the indicated time periods. Administration of IVIg (5 mg/mL) significantly increases the expression levels of BcL-2 in comparison to GD. n=3-6 (B). Representative immunoblot illustrating the expression levels of BcL-2 in primary cortical neurons subjected to OGD for 3 hours, or simulated I (3 hours)/R (24 hours. (C). Representative immunoblot illustrating the expression levels of BcL-2 in ipsilateral brain tissues of mice following middle cerebral artery occlusion (1 hour) and reperfusion (6 or 24 hours). Administration of IVIg (2 g/Kg) significantly increases the expression levels of BcL-2 in comparison to vehicle-treated control mice. n=5-6. β-actin was used as a loading control.

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CHAPTER 3:

Evidence that NF-κB and MAPK(s) Signalling Promotes NLRP Inflammasome Expression and Activation in Neurons Following Ischemic Stroke

3.1 Introduction:

Stroke is the second leading cause of death worldwide and a major cause of permanent disability. The molecular and cellular mechanisms responsible for neuronal cell death following stroke are complex and remains to be fully understood, especially during post-stroke inflammation involving multi-protein complexes termed inflammasomes. In a previous study, we established that ischemia-like conditions increased the levels of NOD (nucleotide-binding oligomerization domain)-like receptor (NLR) Pyrin domain containing 1 and 3 (NLRP1 and NLRP3) inflammasomes in primary cortical neurons (Fann *et al*., 2013). The NLRP inflammasomes are cytosolic macromolecular complexes composed of the NLRP1/3 receptor, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), precursor caspase-1 and/or both precursor caspase-11 (homologous to precursor caspase-4 or 5 in humans) and XIAP (X-linked inhibitor of apoptosis) (Agostini *et al*., 2004; De Rivero Vaccari *et al*., 2008; De Rivero Vaccari *et al*., 2009; De Rivero Vaccari *et al*., 2012; Schroder & Tschopp, 2010; Silverman *et al*., 2009). The activation and homo-oligomerization of NLRP receptors will lead to the formation of NLRP inflammasomes, which converts precursor caspase-1 into cleaved caspase-1 (Agostini *et al*., 2004; Martinon *et al*., 2002). Cleaved caspase-1 cleaves precursors interleukin (IL)-1β and IL-18 into biologically active mature pro-inflammatory cytokines that are then released into the extracellular environment (Bauernfeind *et al*., 2011a). Furthermore, cleaved caspase-1 may induce apoptosis and a particular type of cell death known as pyroptosis (Bergsbaken *et al*., 2009; Erener *et al*., 2012; Fink & Cookson, 2006; Fink *et al*., 2008; Lamkanfi, 2011; Sagulenko *et al*., 2013; Walsh *et al*., 2011; Zhang *et al*., 2003). The NLRP1 and NLRP3 inflammasomes in neurons and glial cells may play an important role in detecting cellular damage and mediating inflammatory responses to sterile tissue injury following ischemic stroke (Abulafia *et al*., 2009; Deroide *et al*., 2013; Ito *et al*., 2015; Savage *et al*., 2012; Fann *et al*., 2013; Zhang *et al*., 2014). It was established that the levels of NLRP1 and NLRP3 inflammasome proteins and both IL-1β and IL-18 were elevated in neurons and ipsilateral brain tissues in both cerebral ischemic mice and stroke patients (Fann *et al*., 2013). Furthermore, we have recently shown that caspase-1 inhibitor treatment protected cultured cortical neurons and brain cells under simulated *in vitro* and *in vivo* experimental stroke models (Fann *et al*., 2013).

Despite a fully defined mechanism(s) leading to NLRP1 and NLRP3 receptor activation has not been elucidated during cerebral ischemia, numerous contributing cellular events are considered plausible, including energy depletion, acidosis, cathepsin release, decreased intracellular potassium (K^+) concentration, increased reactive oxygen species (ROS) production, cytosolic oxidized mitochondrial DNA, increased intracellular calcium (Ca^{2+}) concentration, cell swelling, and protein kinase R (PKR) activation (Compan *et al*., 2012; Lee *et al*., 2012; Liao & Mogridge, 2012; Lindestam Arlehamn *et al*., 2010; Lu *et al*., 2012; Munoz-Planillo *et al*., 2013; Nakahira *et al*., 2011; Petrilli *et al*., 2007; Rajamaki *et al*., 2013; Rossol *et al*., 2012; Shimada *et al*., 2012; Zhou *et al*., 2010; Zhou *et al*., 2011). In addition, emerging evidences suggest that plasma membrane receptors such as toll-like receptors (TLRs) and receptor for advanced glycation products (RAGE) may play a role in the expression of NLRP inflammasome proteins and both IL-1β and IL-18 via activating nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPKs) signaling pathways (Alfonso-Loeches *et al*., 2014; Burm *et al*., 2015; Caso *et al*., 2007; Caso *et al*., 2008; Codolo *et al*., 2013; Eigenbrod *et al*., 2008; Frank *et al*., 2015; Lee *et al*., 2013; Lippai *et al*., 2013; Lok *et al*., 2015; Nagyoszi *et al*., 2015; Nystrom *et al*., 2013; Tang *et al*., 2007; Tang *et al*., 2013; Weber *et al*., 2015; Zhao *et al*., 2014; Zheng *et al*., 2013). Both NF-κB and MAPK(s) signaling pathways are known to modulate the expression of NLRP inflammasome proteins and both IL-1β and IL-18 under inflammatory conditions in immune cells (Bauernfeind *et al*., 2011b; Bauernfeind *et al*., 2009; Budai *et al*., 2013; Burm *et al*., 2015; Frederick Lo *et al*., 2008; Ghonime *et al*., 2014; Hara *et al*., 2013; He *et al*., 2012; Juliana *et al*., 2010; Kang *et al*., 2000; Legos *et al*., 2001; Liao *et al*., 2012; Liu *et al*., 2004; Liu *et al*., 2013; Mariathasan & Monack, 2007; Okada *et al*., 2014; Qiao *et al*., 2012; Savage *et al*., 2012; Schroder *et al*., 2012; Tamatani *et al*., 2000; Weber *et al*., 2015; Zhao *et al*., 2013). Recently, we have demonstrated that administration of intravenous immunoglobulin (IVIg); a highly purified blood preparation containing immunoglobulin G (IgG) was able to decrease the expression of NLRP1 and NLRP3 inflammasome proteins, and both IL-1β and IL-18, and thus inflammasome activity by conceivably attenuating the activation of the NF-κB (i.e. p-p65) and MAPK(s) (i.e. p-P38 and p-JNK) pathway via an unknown mechanism(s) in neurons and brain tissue under ischemic conditions (Fann *et al*., 2013; Widiapradja *et al*., 2012). Despite numerous experimental evidences in peripheral immune cells, the connection between both the NF-κB and MAPK(s) signaling pathways with inflammasome protein expression and activation in neurons under simulated ischemic conditions remains unclear. Here we provide evidence that the NF-κB and MAPK(s) signaling pathways play an essential role in the regulation of NLRP1 and NLRP3 inflammasome expression and activation in neurons following ischemic conditions. Furthermore, we provide supporting evidence that suppression of NF-κB and MAPK(s) signaling

pathways may be responsible for reducing NLRP inflammasome expression and activation in neurons following IVIg treatment under ischemic conditions.

3.2 Material and Methods:

Pharmaceuticals

NF-κB inhibitor (Bay-11-7082), P38-MAPK inhibitor (SB 203580), JNK inhibitors (SP 600125 and JNK inhibitor V) and ERK-inhibitor (U-0126), were purchased from Cayman Chemical, Ann Arbor, USA. Intravenous immunoglobulin (IVIg; KIOVIG) was purchased from Baxter, UK.

Primary Cortical Neuronal Cultures

Dissociated neuron-enriched cell cultures of cerebral cortex were established from Day 16 C57BL6/J mouse embryos, as described (Okun *et al*., 2007). Experiments were performed in 7 to 9 day-old cultures. Approximately 95% of the cells in such cultures were neurons, and the remaining cells were astrocytes. For oxygen and glucose deprivation (OGD), neurons were incubated in glucose-free Locke's buffer in an oxygen-free chamber for 6 hours. For simulated ischemic and reperfusion (I/R) experiments, neurons were incubated in glucose-free Locke's medium in an oxygen-free chamber for 3 hours followed by the medium being replaced with Neurobasal medium for 24 hours. To observe the effect of IVIg (KIOVIG, Baxter, UK), a NF-κB inhibitor (Bay-11-7082, Cayman Chemical, Ann Arbor, USA), a P38-MAPK inhibitor (SB 203580, Cayman Chemical, Ann Arbor, USA), a JNK inhibitor (SP 600125, Cayman Chemical, Ann Arbor, USA), and an ERK inhibitor (U-0126, Cayman Chemical, Ann Arbor, USA), each drug were added to cultures during and after OGD or simulated I/R. Control conditions included exposure to neurobasal medium alone or vehicle.

Cell Viability

Neuronal cell viability was determined by trypan blue exclusion assay. The assay is based on the principle that live cells possess intact cell membranes, which will exclude the dye trypan blue, while the membrane of injured or dead cells is permeable to trypan blue. Hence, injured or dead cells are stained blue whereas live cells will show no staining. Following incubation with trypan blue, the plates were emptied and the cells fixed with 4% paraformaldehyde for 20 min at room temperature. The cells were then washed with PBS three times and stored in PBS for latter observation under a light microscope to quantify the percentage of cells that were trypan-blue positive in each culture.

Cell Lysis and Protein Quantitation

In order to extract protein, primary cortical neurons were homogenized in cell lysis buffer (Radio-Immunoprecipitation Assay (RIPA)) containing protease and phosphatase inhibitor in 1:100 ratio, respectively, (Thermo Scientific, Rockford, IL, USA) using a cell disruptor (Biospec Products, Inc.**,** Bartlesville, OK, USA). Samples were centrifuged at 15,000 rpm at 4°C for 15 minutes and the supernatant collected. Total protein concentration of each sample was measured in a microplate using the Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Bovine serum albumin (BSA) standards (20-2,000µg/mL) were prepared as per the manufacturer's instructions to generate a standard curve with known concentrations. Absorbance was measured at 562nm using the Tecan 26 Sunrise Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland) and data was analysed using Graphpad Prism 5 software (Graphpad Software, San Diego, CA, USA) by comparing samples to the standard curve to determine the concentration and volume of protein required to be loaded for separation by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Western Blot Analysis

Protein samples were subjected to sodium dodecyl sulfate–polyacrylamide (10%) gel electrophoresis using a Tris-glycine running buffer. Gels were then electro-blotted using a transfer apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in transfer buffer containing 0.025 mol/L Tris base, 0.15 mol/L glycine, and 10% (v/v) methanol for 2 hr at 80V onto a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membrane was then incubated in blocking buffer (5% non-fat milk in 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.2 % Tween-20) for 1hr at 23°C. The membrane was then incubated overnight at 4°C with primary antibodies including those that selectively bind phosphorylated P-65 (Cell Signaling), total P-65 (Cell Signaling), phosphorylated JNK (Cell Signaling), Total JNK (Cell Signaling), phosphorylated P38 (Cell Signaling), Total P38 (Cell Signaling), phosphorylated ERK (Cell Signaling), Total ERK (Cell Signaling), phosphorylated c-JUN (Cell Signaling), Total c-JUN (Cell Signaling), NLRP1 (Novus Biologicals), NLRP3 (Novus Biologicals), ASC (Abcam, Cambridge, UK), Caspase-1 (Abcam), Caspase-11 (Abcam), XIAP (Novus Biologicals), IL-1β (Abcam), IL-18 (Abcam), Bcl-2 (Cell Signaling), BcL-XL (Cell Signaling), cleaved Caspase-3 (Cell Signaling), Caspase-3 (Cell Signaling) and β-actin (Sigma-Aldrich). After washing three times (10 min per wash) with Tris-buffered saline-T (20 mM Tris-HCL, pH 7.5, 137 mM NaCl, 0.2 % Tween-20), the membrane was incubated with secondary antibodies against the primary antibody for 1 hour at room temperature. The membrane was washed with Tris-Buffered saline-T and scanned using the Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Quantification of protein levels was

achieved by densitometry analysis using Image J v1.46 software (National Institute of Health, Bethesda, MD, USA).

Statistical Analysis

All experimental data obtained are expressed as mean ± standard error of the mean (SEM). Statistical analysis of all data were performed using one-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc analysis to determine between-group differences. Statistical difference was taken as $p<0.05$. Statistical analyses were performed using GraphPad Prism 5.02 software.

3.3 Results:

Inhibition of NF-κB and MAPK(s) signaling pathways protects primary cortical neurons following simulated ischemic conditions.

In order to establish the role of the NF-κB and MAPK(s) signaling pathways in neuronal inflammasome expression and activation, we tested the effect of NF-κB and MAPK(s) inhibition against simulated ischemic conditions such as oxygen and glucose deprivation (OGD) and OGD plus reperfusion. We first evaluated the efficacy of NF-κB and MAPK(s) inhibitors in primary cortical neurons under OGD conditions. Increasing concentrations (1-100μM) of NF-κB (Bay-11-7082), JNK (SP 600125), P38 (SB 203580) and ERK (U-0126) inhibitors were administered and neurons were then analyzed for phosphorylated-P65-NF-κB, phosphorylated-JNK, phosphorylated-P38 and phosphorylated-ERK, respectively. In addition, cleaved caspase-3, an indicator of apoptosis was analyzed to observe the effect of these inhibitors against OGD-induced cell death. NF-κB inhibitor concentrations above 30 μM were significantly effective in reducing levels of phosphorylated-P65-NF-κB, in addition to, cleaved caspase-3 following 6 hours of OGD compared with the vehicle control group (**Figure 3.1A and B**). Similarly, JNK inhibitor (SP 600125) concentrations above 30 μM, P38 inhibitor concentrations above 10 μM and ERK inhibitor concentrations above 30 μM significantly reduced the levels of phosphorylated-JNK (**Figure 3.1C and D**), phosphorylated-P38 (**Figure 3.1E and F**) and phosphorylated-ERK, (**Figure 3.1G and H**) respectively, in addition to, cleaved caspase-3 following 6 hours of OGD compared to the vehicle control group.

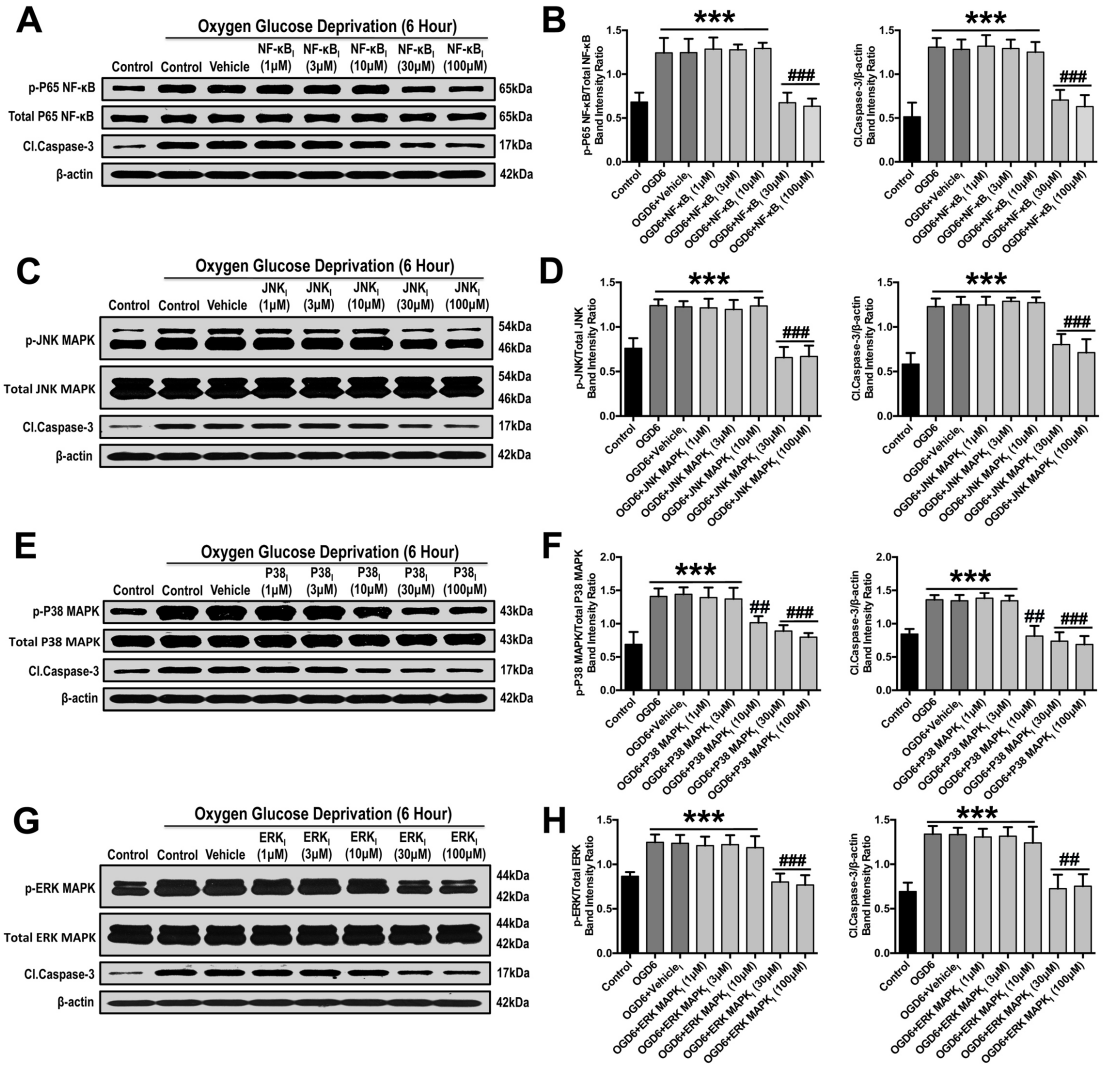

Figure 3.1: Inhibition of the NF-κB and MAPK(s) signalling pathway and cell death in primary cortical neurons following simulated ischemic-like conditions. (A and B). Representative immunoblots and quantification illustrating the effect of increasing concentrations (μM) of a NF-κB inhibitor (Bay-11-7082) on levels of p-P65 NF-κB and cleaved caspase-3 proteins in primary cortical neurons subjected to oxygen and glucose deprivation (OGD_{6hr}). (C and D). Representative immunoblots and quantification illustrating the effect of increasing concentrations (μM) of a JNK MAPK inhibitor (SP600125) on levels of p-JNK MAPK and cleaved caspase-3 proteins in primary cortical neurons subjected to oxygen and glucose deprivation (OGD_{6hr}). (E and F). Representative immunoblots and quantification illustrating the effect of increasing concentrations (μM) of a P38 MAPK inhibitor (SB203580) on levels of p-P38 MAPK and cleaved caspase-3 proteins in primary cortical neurons subjected to oxygen and glucose deprivation (OGD_{6hr}). (G and H). Representative immunoblots and quantification illustrating the effect of increasing concentrations (μM) of an ERK MAPK inhibitor (U-0126) on levels of p-ERK MAPK and cleaved caspase-3 proteins in primary cortical neurons subjected to oxygen and glucose deprivation (OGD_{6hr}). β-actin was used as a loading control. Data are represented as mean ± S.E.M. n = 4 cultures. $^{***}P < 0.001$ compared with control; $^{\#\#}P < 0.01$ compared with vehicle; $^{\#\#\#}P < 0.001$ compared with vehicle.

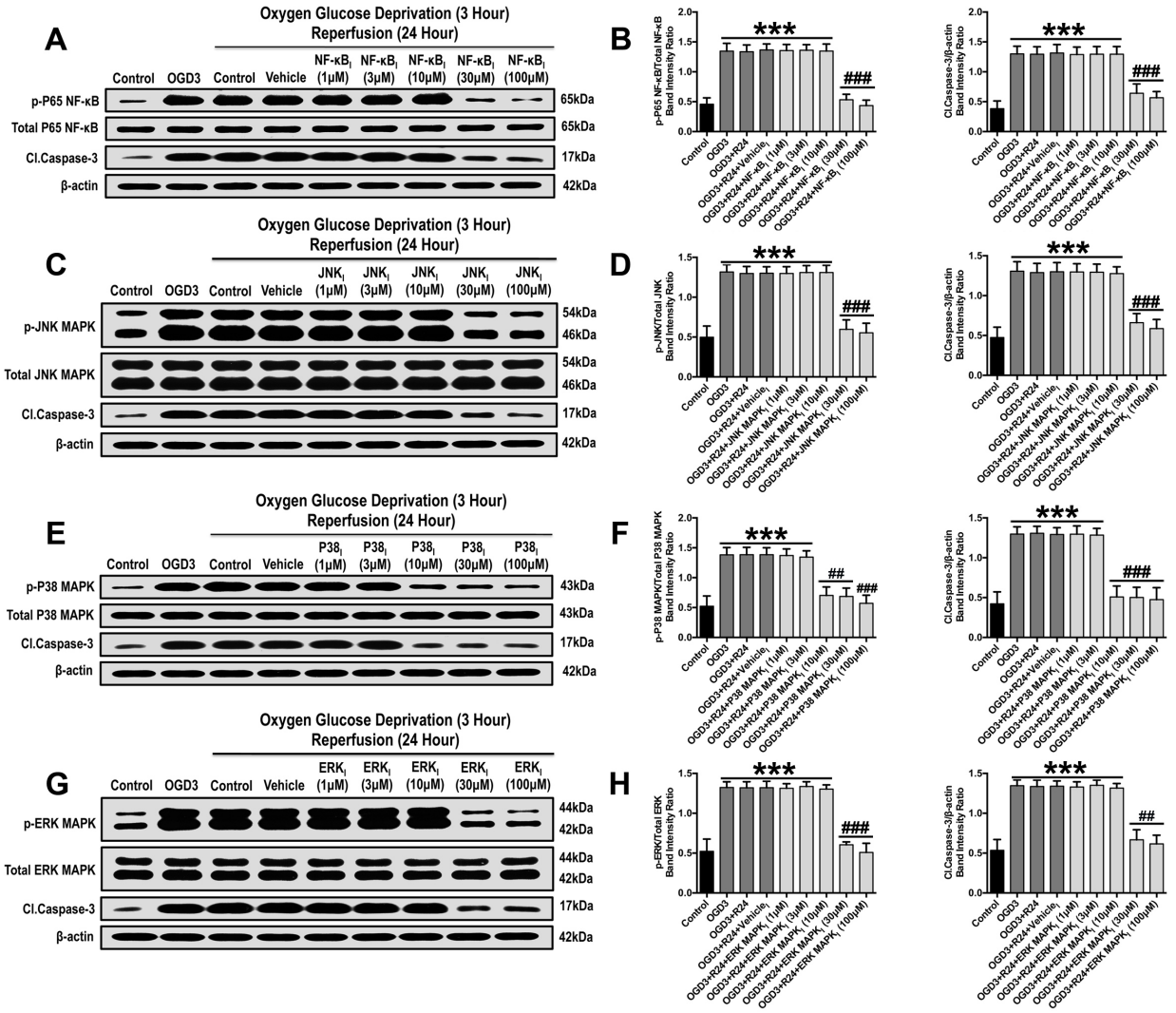

Figure 3.2: Inhibition of the NF-κB and MAPK(s) signalling pathway and cell death in primary cortical neurons following simulated ischemic/reperfusion (I/R) conditions. (A and B). Representative immunoblots and quantification illustrating the effect of increasing concentrations (μM) of a NF-κB inhibitor (Bay-11-7082) on levels of p-P65 NF-κB and cleaved caspase-3 proteins in primary cortical neurons subjected to oxygen and glucose deprivation (OGD_{3hr}) followed by neurobasal reperfusion (24 hour). (C and D). Representative immunoblots and quantification illustrating the effect of increasing concentrations (μM) of a JNK MAPK inhibitor (SP600125) on levels of p-JNK MAPK and cleaved caspase-3 proteins in primary cortical neurons subjected to oxygen and glucose deprivation (OGD_{3hr}) followed by neurobasal reperfusion (24 hour). (E and F). Representative immunoblots and quantification illustrating the effect of increasing concentrations (μM) of a P38 MAPK inhibitor (SB203580) on levels of p-P38 MAPK and cleaved caspase-3 proteins in primary cortical neurons subjected to oxygen and glucose deprivation (OGD_{3hr}) followed by neurobasal reperfusion (24 hour). (G and H). Representative immunoblots and quantification illustrating the effect of increasing concentrations (μM) of a ERK MAPK inhibitor (U-0126) on levels of p-ERK MAPK and cleaved caspase-3 proteins in primary cortical neurons subjected to oxygen and glucose deprivation (OGD_{3hr}) followed by neurobasal reperfusion (24 hour). β-actin was used as a loading control. Data are represented as mean ± S.E.M. n = 4 cultures. $^{***}P < 0.001$ compared with control; $^{\#\#}P < 0.01$ compared with vehicle; $^{\#\#\#}P < 0.001$ compared with vehicle.

In order to further confirm the efficacy of NF-κB and MAPK(s) inhibitors and to test the protective effect against ischemic cell death; we next tested NF-κB and MAPK(s) inhibitors in primary cortical neurons under OGD plus reperfusion conditions. The results were similar to OGD

conditions obtained in Figure 1. NF-κB inhibitor concentrations above 30 μM were significantly effective in reducing levels of phosphorylated-P65-NF-κB, in addition to cleaved caspase-3 following 3 hours of OGD and 24 hours of reperfusion compared to the vehicle control group (**Figure 3.2A and B**). Similarly, JNK inhibitor concentrations above 30 μM, P38 inhibitor concentrations above 10 μM and ERK inhibitor concentrations above 30 μM significantly reduced the levels of phosphorylated-JNK (**Figure 3.2C and D**), phosphorylated-P38 (**Figure 3.2E and F**) and phosphorylated-ERK (**Figure 3.2G and H**), respectively, in addition to cleaved caspase-3 following 3 hours of OGD and 24 hours of reperfusion compared to the vehicle control group.

Intravenous immunoglobulin (IVIg) attenuates NF-κB and MAPK(s) signaling and c-Jun in primary cortical neurons following ischemic conditions

Using animal models of ischemic stroke, we recently identified IVIg as a potent stroke therapy (Arumugam *et al*., 2007). Specifically, we reported that administration of IVIg to mice subjected to experimental stroke significantly reduced brain infarct size and eliminated mortality. In addition, we established that IVIg treatment protects neurons in simulated ischemic conditions by a mechanism involving suppression of NLRP1 and NLRP3 inflammasome activity (Fann *et al*., 2013). It was also demonstrated that IVIg could decrease the activity of NF-κB and MAPK(s) signaling pathways in neurons under ischemic conditions through an unknown mechanism(s) (Lok *et al*., 2015; Widiapradja *et al*., 2012). In order to investigate whether IVIg-mediated suppression of inflammasome expression and activity is due to inhibition of NF-κB and MAPK(s) signaling, we next analyzed the expression levels of NF-κB and MAPK(s) and compared them with the effect of NF-κB and MAPK(s) inhibitors following both OGD and OGD plus reperfusion conditions. Indeed, we confirmed again that IVIg treatment (5mg/mL) significantly decreased levels of phosphorylated-P65-NF-κB, phosphorylated-P38, phosphorylated-JNK, and phosphorylated-ERK following both 6 hours OGD and 3 hours OGD plus 24 hours of reperfusion compared to the vehicle control group (**Figure 3.3 A-D**). In addition, we have also found that IVIg treatment (5mg/mL) significantly reduced the levels of phosphorylated-c-Jun compared to the vehicle control group (**Figure 3.3 A-D**). In order to determine whether NF-κB and MAPK(s) inhibitors are specific to their corresponding signaling pathway, we next analyzed the level of all four proteins (P65, P38, JNK and ERK) following treatment with NF-κB and MAPK(s) inhibitors in both OGD and OGD plus reperfusion conditions. Our data show that pharmacological inhibitors of the NF-κB and MAPK(s) signalling pathways utilized were specific to their corresponding pathway by selectively reducing the phosphorylation protein expression levels associated with that particular pathway (**Figure 3.3**

A-D). In addition, combined (ALL) administration of NF-κB and MAPKs inhibitors significantly reduced the activation levels of NF-κB (p-P65) and MAPKs such as p-P38, p-JNK, p-ERK and p-c-Jun compared to the vehicle control group. However, there were no additive effects by combining all inhibitors (**Figure 3.3 A-D**).

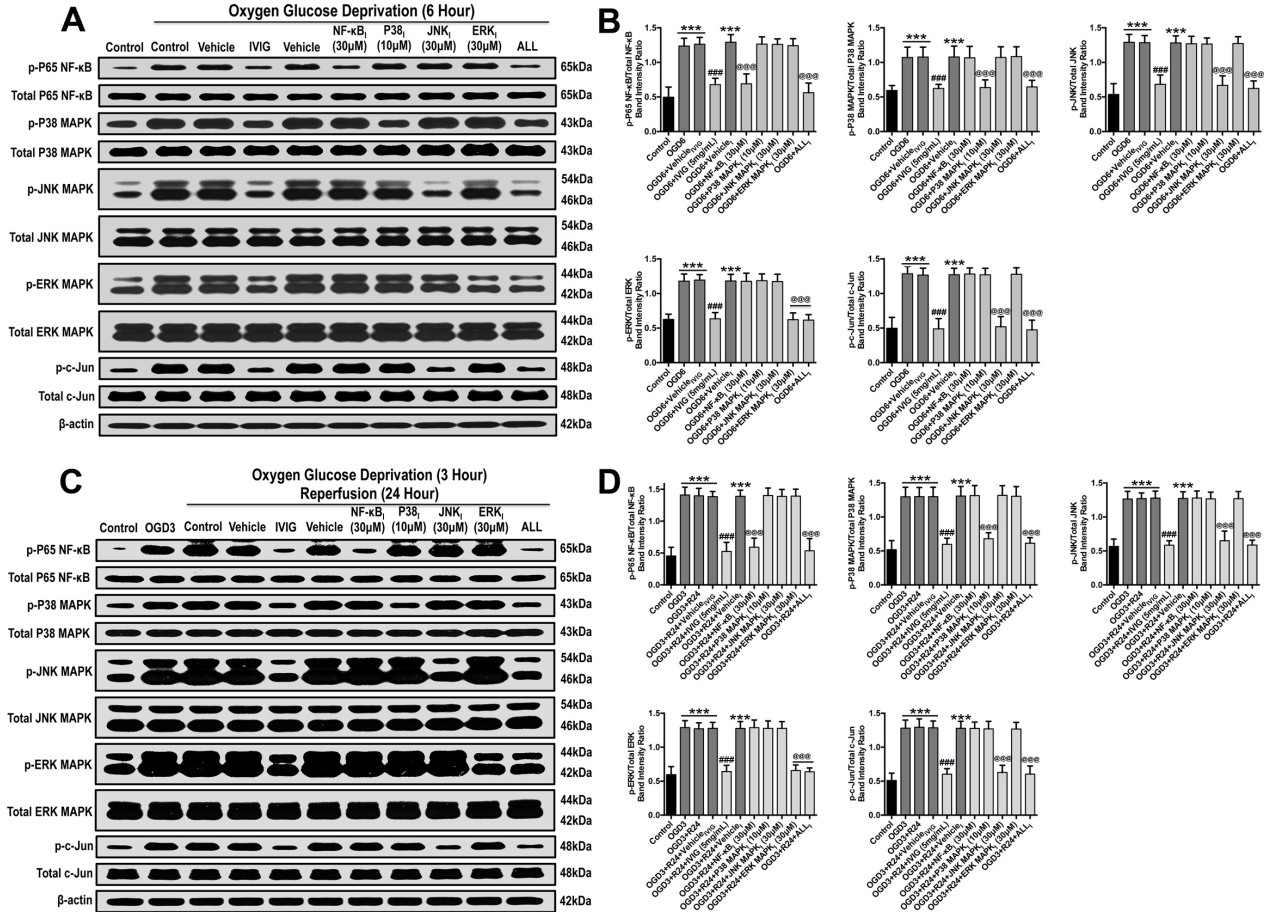

Figure 3.3: Intravenous immunoglobulin (IVIG) and both NF-κB and MAPK(s) inhibitors attenuate NF-κB and MAPK(s) signalling pathway activation in primary cortical neurons following simulated ischemic conditions. (A and B). Representative immunoblots and quantification illustrating increases in the activation levels of NF-κB (p-P65) and MAPKs such as p-P38, p-JNK, p-ERK and p-c-Jun in primary cortical neurons subjected to oxygen and glucose deprivation (OGD_{6hr}). The administration of intravenous immunoglobulin (IVIG; 5mg/mL) and both NF-κB (30μM) and MAPKs inhibitors (P38 inhibitor, 10μM; JNK inhibitor, 30μM; ERK inhibitor, 30μM) significantly reduced the activation levels of NF-κB (p-P65) and MAPKs such as p-P38, p-JNK, p-ERK and p-c-Jun. Combined (ALL) administration of NF-κB and MAPKs inhibitors significantly reduced the activation levels of NF-κB (p-P65) and MAPKs such as p-P38, p-JNK, p-ERK and p-c-Jun. (C and D). Representative immunoblots and quantification illustrating increases in the activation levels of NF-κB (p-P65) and MAPKs such as p-P38, p-JNK, p-ERK and p-c-Jun in primary cortical neurons subjected to oxygen and glucose deprivation (OGD_{3hr}) followed by neurobasal reperfusion (24 hour). The administration of intravenous immunoglobulin (IVIG; 5mg/mL) and both NF-κB (30μM) and MAPKs inhibitors (P38 inhibitor, 10μM; JNK inhibitor, 30μM; ERK inhibitor, 30μM) significantly reduced the activation levels of NF-κB (p-P65) and MAPKs such as p-P38, p-JNK, p-ERK and p-c-Jun. Combined (ALL) administration of NF-κB and MAPKs inhibitors significantly reduced the activation levels of NF-κB (p-P65) and MAPKs such as p-P38, p-JNK, p-ERK and p-c-Jun. β-actin was used as a loading control. Data are represented as mean ± S.E.M. n=5 cultures. $^{***}P < 0.001$ compared with control; $^{###}P < 0.001$ compared with $OGD6+Vehicle_{IVIG}$ or $OGD3+R24+Vehicle_{IVIG}$; $^{@@@}P < 0.001$ compared with $OGD6+Vehicle_{Inhibitor}$ or $OGD3+R24+Vehicle_{Inhibitor}$.

Inhibition of the NF-κB and MAPK(s) signaling pathway attenuates the expression levels of inflammasome proteins and both IL-1β and IL-18 in primary cortical neurons following simulated ischemic conditions

We have previously shown that IVIg treatment significantly reduced the expression levels of NLRP1, NLRP3, ASC, XIAP, precursor-caspase-1, precursor caspase-11, and both precursor-IL-1β and IL-18 in comparison with vehicle-treated neurons following *in vitro* and *in vivo* ischemic conditions (Fann *et al*., 2013). Here, we have reconfirmed that IVIg treatment significantly reduces the expression levels of the above-mentioned inflammasome proteins and both precursor IL-1β and IL-18 following OGD for 6 hours (**Figure 3.4A and B**) or OGD for 3 hours plus 24 hour reperfusion conditions compared to the vehicle control group (**Figure 3.4C and D**). In order to establish the molecular mechanism(s) responsible for inflammasome protein expression in neurons following ischemic conditions, we analyzed the expression levels of inflammasome proteins such as NLRP1, NLRP3, ASC, XIAP, precursor-caspase-1 and 11, and both precursor-IL-1β and IL-18 following treatment with NF-κB and MAPKs inhibitors. We selected concentrations of inhibitors either 10 μM (P38) or 30 μM (NF-κB, JNK and ERK) based on results from Figure 1 and 2. Our data shows that all inhibitors significantly reduced the expression levels of NLRP1, NLRP3, ASC, XIAP, precursor-caspase-1, precursor caspase-11 and both precursor-IL-1β and IL-18 in both OGD and OGD plus reperfusion conditions compared to vehicle control groups (**Figure 3.4 A-D**). The effect of NF-κB and MAPKs inhibitors were similar to the effect of IVIg as no significant difference was observed between the IVIg treatment group with either the NF-κB and MAPKs inhibitors treatment groups under both OGD and OGD plus reperfusion conditions. Furthermore, combined (ALL) administration of NF-κB and MAPKs inhibitors significantly reduced the expression levels of NLRP1, NLRP3, ASC, XIAP, precursor-caspase-1, precursor-caspase-11, and both precursor-IL-1β and IL-18 compared to the vehicle control group. However, there was no additive effect by combining all inhibitors (**Figure 3.4 A-D)**.

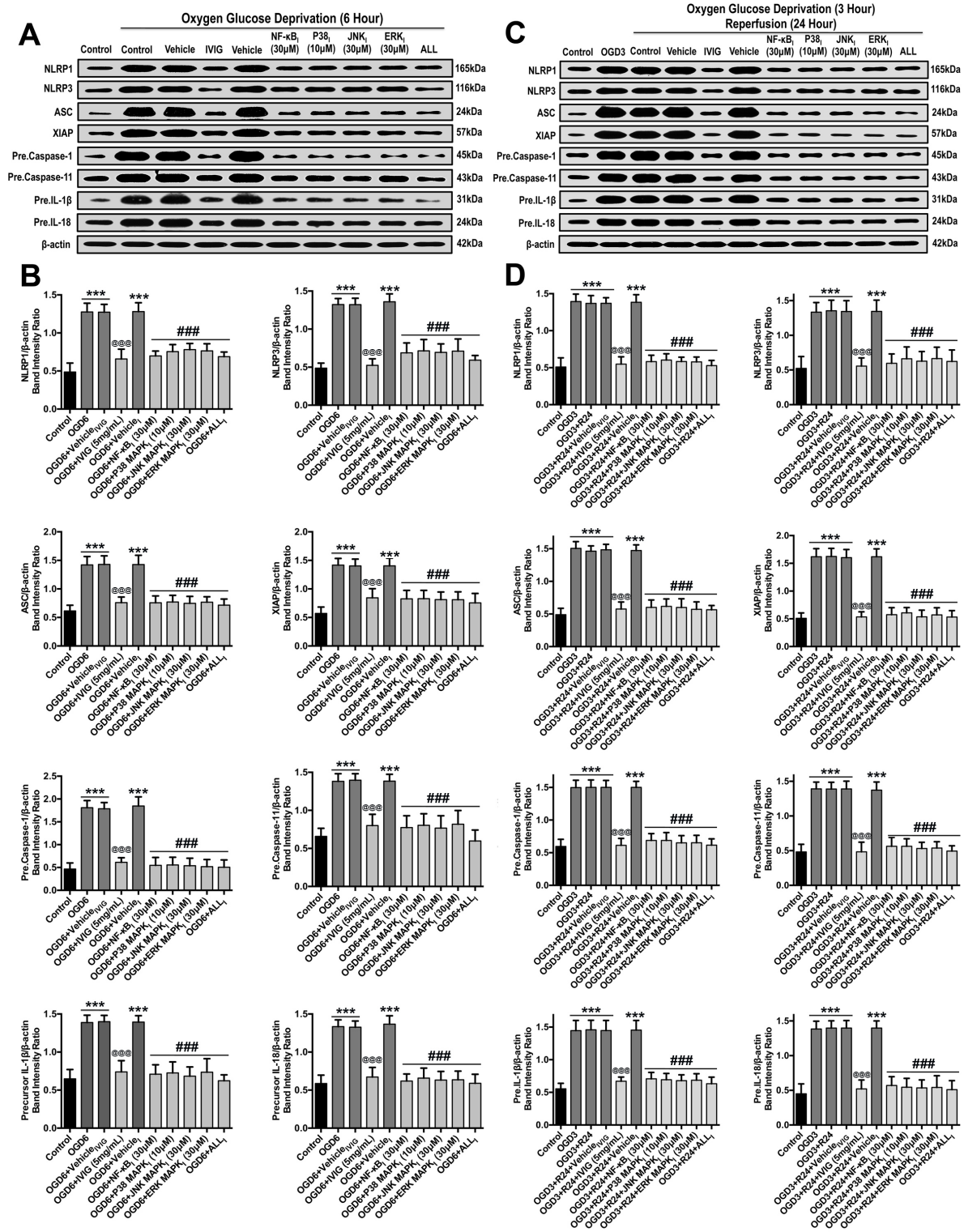

Figure 3.4: Intravenous immunoglobulin (IVIG) and both NF-κB and MAPK(s) inhibitors attenuate the expression of inflammasome proteins and both IL-1β and IL-18 in primary cortical neurons following simulated ischemic conditions. (A and B). Representative immunoblots and quantification illustrating an increase in the expression levels of inflammasome proteins and both IL-1β and IL-18 in primary cortical neurons subjected to oxygen and glucose deprivation (OGD_{6hr}). The administration of intravenous immunoglobulin (IVIG; 5mg/mL) and both NF-κB (30μM) and MAPKs inhibitors (P38 inhibitor, 10μM; JNK inhibitor, 30μM; ERK inhibitor, 30μM) significantly reduced the expression levels of inflammasome proteins and both IL-1β and IL-18. Combined (ALL) administration of NF-κB and MAPKs inhibitors significantly reduced the expression levels of inflammasome proteins and both IL-1β and IL-18. (C and D). Representative immunoblots and quantification illustrating an increase in the expressions levels

of inflammasome proteins and both IL-1β and IL-18 in primary cortical neurons subjected to oxygen and glucose deprivation (OGD_{3hr}) followed by neurobasal reperfusion (24 hour). The administration of intravenous immunoglobulin (IVIG; 5mg/mL) and both NF-κB (30μM) and MAPKs inhibitors (P38 inhibitor, 10μM; JNK inhibitor, 30μM; ERK inhibitor, 30μM) significantly reduced the expression levels of inflammasome proteins and both IL-1β and IL-18. Combined (ALL) administration of NF-κB and MAPKs inhibitors significantly reduced the expression levels of inflammasome proteins and both IL-1β and IL-18. β-actin was used as a loading control. Data are represented as mean ± S.E.M. n=5 cultures. $^{***}P < 0.001$ compared with control; $^{@@@}P < 0.001$ compared with $OGD6+Vehicle_{IVIG}$ or $OGD3+R24+Vehicle_{IVIG}$; $^{###}P < 0.001$ compared with $OGD6+Vehicle_{Inhibitor}$ or $OGD3+R24+Vehicle_{Inhibitor}$.

Inhibition of the NF-κB and MAPK(s) signaling pathway attenuates inflammasome activation in primary cortical neurons following simulated ischemic conditions

Our group has previously established that IVIg treatment significantly decreases NLRP1 and NLRP3 inflammasome activation and maturation of both IL-1β and IL-18 in neurons under ischemic conditions in comparison to the vehicle control group (Fann *et al*., 2013). In order to support our data that IVIg treatment may mediate this effect by reducing NF-κB and MAPKs activation; we have again tested the effect of IVIg treatment against ischemia-induced inflammasome activation along with NF-κB and MAPKs inhibitors.

IVIg treatment significantly reduced the expression levels of cleaved XIAP, cleaved-caspase-1, cleaved-caspase-11, mature-IL-1β and mature-IL-18 following OGD for 6 hours (**Figure 3.5A and B**) or 3 hours OGD and 24 hours reperfusion conditions (**Figure 3.5C and D**) compared to the vehicle control group. NF-κB and MAPKs inhibitor treatment equally reduced the expression levels of cleaved XIAP, cleaved-caspase-1, cleaved-caspase-11, mature-IL-1β and mature-IL-18 following 6 hours OGD (**Figure 3.5A and B**) or 3 hours OGD and 24 hours reperfusion conditions (**Figure 3.5C and D**) compared to the vehicle control group. The effect of NF-κB and MAPKs inhibitors were comparable to the effect of IVIg treatment as there was no significant difference observed between the IVIg treatment group with either the NF-κB and MAPKs inhibitor treatment groups following 6 hours OGD conditions (**Figure 3.5A and B**) or 3 hours OGD and 24 hours reperfusion conditions (**Figure 3.5C and D**). Combined (ALL) administration of NF-κB and MAPKs inhibitors significantly reduced the activation levels of cleaved XIAP, cleaved-caspase-1, cleaved-caspase-11, mature-IL-1β and mature-IL-18, whereas no additive effect was achieved by combining all inhibitors (**Figure 3.5A-D)**.

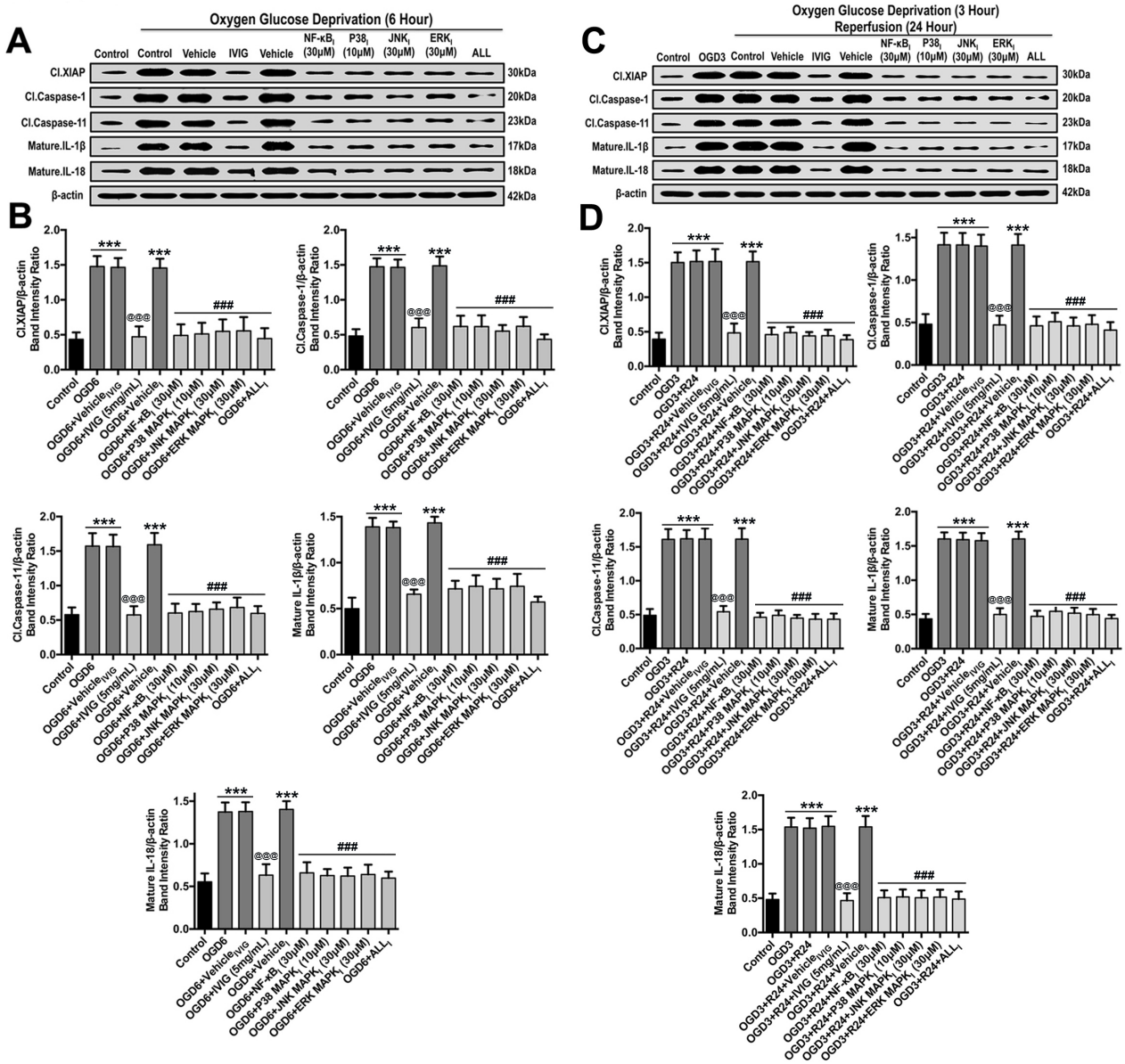

Figure 3.5: Intravenous immunoglobulin (IVIG) and both NF-κB and MAPK(s) inhibitors attenuate inflammasome activation in primary cortical neurons following simulated ischemic-like conditions. (A and B). Representative immunoblots and quantification illustrating an increased expression level of activated inflammasome proteins such as cleaved XIAP, cleaved caspase-1 and -11 and maturation of IL-1β and IL-18 in primary cortical neurons subjected to oxygen and glucose deprivation (OGD_{6hr}). The administration of intravenous immunoglobulin (IVIG; 5mg/mL) and both NF-κB (30μM) and MAPKs inhibitors (P38 inhibitor, 10μM; JNK inhibitor, 30μM; ERK inhibitor, 30μM) significantly reduced the expression levels of cleaved XIAP, cleaved caspase-1 and -11 and maturation of IL-1β and IL-18. Combined (ALL) administration of NF-κB and MAPKs inhibitors significantly reduced the expression levels of cleaved XIAP, cleaved caspase-1 and -11 and maturation of IL-1β and IL-18. (C and D). Representative immunoblots and quantification illustrating an increased expression level of activated inflammasome proteins such as cleaved XIAP, cleaved caspase-1 and -11 and maturation of IL-1β and IL-18 in primary cortical neurons subjected to oxygen and glucose deprivation (OGD_{3hr}) followed by neurobasal reperfusion (24 hour). The administration of intravenous immunoglobulin (IVIG; 5mg/mL) and both NF-κB (30μM) and MAPKs inhibitors (P38 inhibitor, 10μM; JNK inhibitor, 30μM; ERK inhibitor, 30μM) significantly reduced the expression levels of cleaved XIAP, cleaved caspase-1 and -11 and maturation of IL-1β and IL-18. Combined (ALL) administration of NF-κB and MAPKs inhibitors significantly reduced the expression levels of cleaved XIAP, cleaved caspase-1 and -11 and maturation of IL-1β and IL-18. β-actin was used as a loading control. Data are represented as mean ± S.E.M. n=5 cultures. $^{***}P < 0.001$ compared with control; $^{@@@}P < 0.001$ compared

with OGD6+Vehicle$_{IVIG}$ or OGD3+R24+Vehicle$_{IVIG}$; $^{###}P < 0.001$ compared with OGD6+Vehicle$_{Inhibitor}$ or OGD3+R24+Vehicle$_{Inhibitor}$.

Inhibition of the NF-κB and MAPK(s) signaling pathway does not change anti-apoptotic protein expression but attenuates cell death in primary cortical neurons following simulated ischemic conditions

We have previously shown that IVIg treatment can increase the expression of anti-apoptotic protein BcL-2 in cultured cortical neurons following simulated ischemic conditions and in an animal stroke model (Fann *et al*., 2013; Widiapradja *et al*., 2012). It was also established that anti-apoptotic proteins Bcl-2 and Bcl-xL directly bind and inhibit the oligomerization of the NLRP receptor (Bruey *et al*., 2007; Faustin *et al*., 2009). Thus, BcL-2 and BcL-xL are likely to reduce caspase-1 activation and maturation of both IL-1β and IL-18. In order to investigate whether an IVIg-dependent increase in BcL-2 and BcL-xL expression are mediated by NF-κB and MAPKs signaling, we next analyzed the expression levels of both BcL-2 and BcL-xL following simulated ischemic conditions. Treatment with IVIg significantly increased the expression levels of both BcL-2 and BcL-xL following 6 hours OGD (**Figure 3.6A and B**) or 3 hours OGD and 24 hours reperfusion (**Figure 3.6D and E**) compared to the vehicle control group. However, treatment with either NF-κB and MAPKs inhibitors or Combined (ALL) administration of NF-κB and MAPKs inhibitors failed to reverse OGD (**Figure 3.6A and B**) or OGD plus reperfusion (**Figure 3.6D and E**) induced decline in the expression levels of BcL-2 and BcL-xL. Finally, we investigated whether a reduction in cell death following treatment with NF-κB and MAPKs inhibitors was comparable to the protection observed with IVIg treatment in ischemic conditions. Both the levels of cleaved caspase-3 and cell death (trypan blue exclusion assay) following treatment with NF-κB and MAPKs inhibitors were significantly lower compared to vehicle treated groups following both following 6 hours OGD (**Figure 3.6C**) or 3 hours OGD and 24 hours reperfusion (**Figure 3.6F**). The protection obtained following treatment with NF-κB and MAPKs inhibitors was equivalent to the levels observed following IVIg treatment in OGD or OGD plus reperfusion.

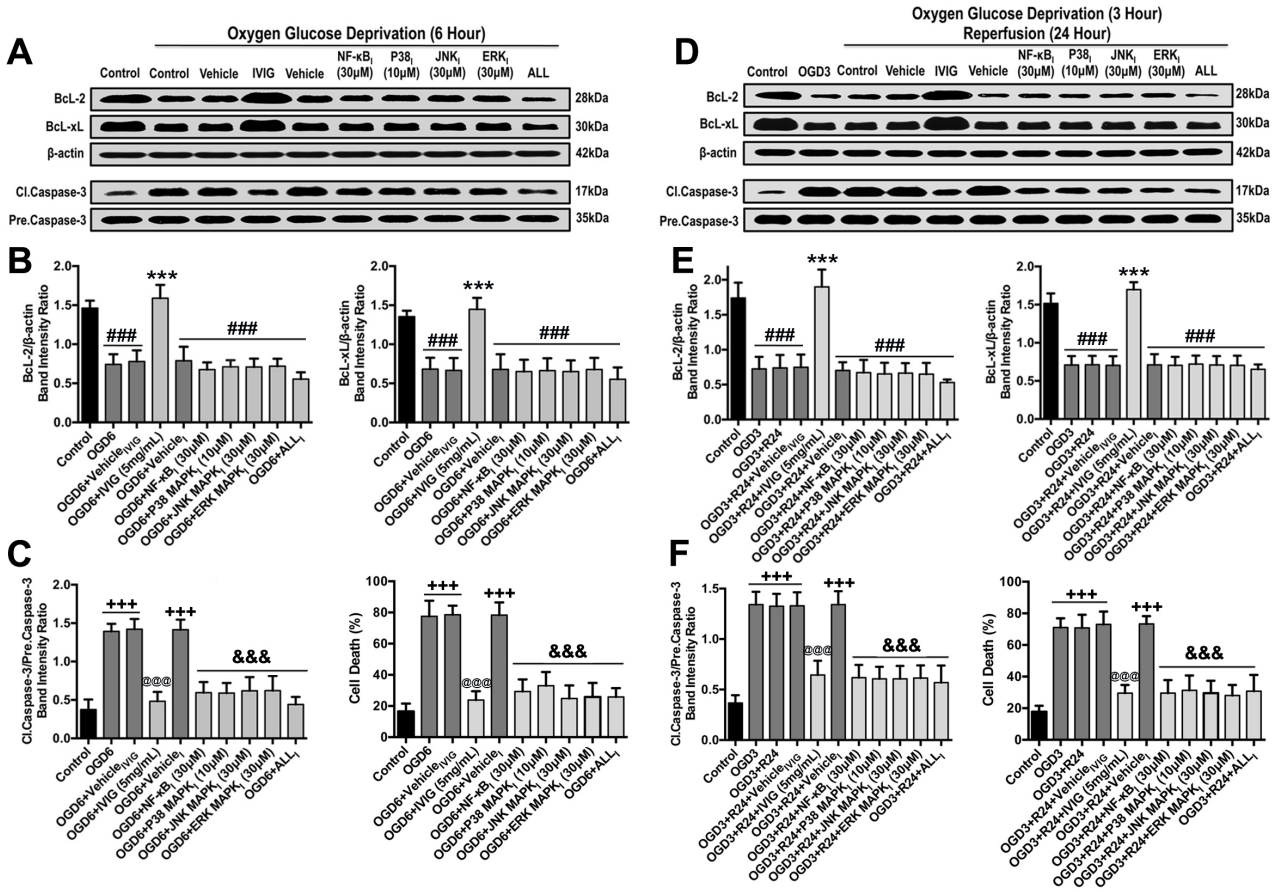

Figure 3.6: Intravenous immunoglobulin (IVIG) and both NF-κB and MAPK(s) inhibitors attenuate cell death in primary cortical neurons following simulated ischemic conditions. (A-C). Representative immunoblots and quantification illustrating increased levels of pro-apoptotic protein cleaved caspase-3 and reduced levels of anti-apoptotic proteins Bcl-2 and Bcl-xL in primary cortical neurons subjected to oxygen and glucose deprivation (OGD_{6hr}). The administration of intravenous immunoglobulin (IVIG; 5mg/mL) and both NF-κB (30μM) and MAPKs inhibitors (P38 inhibitor, 10μM; JNK inhibitor, 30μM; ERK inhibitor, 30μM) significantly reduced the levels of cleaved caspase-3 and cell death; however, intravenous immunoglobulin (IVIG; 5mg/mL) alone increased the levels of Bcl-2 and Bcl-xL. Combined (ALL) administration of NF-κB and MAPKs inhibitors significantly reduced the levels of cleaved caspase-3 and cell death; however, the levels of Bcl-2 and Bcl-xL remained unchanged. (D-F). Representative immunoblots and quantification illustrating increased levels of pro-apoptotic protein cleaved caspase-3 and reduced levels of anti-apoptotic proteins Bcl-2 and Bcl-xL in primary cortical neurons subjected to oxygen and glucose deprivation (OGD_{3hr}) followed by neurobasal reperfusion (24 hour). The administration of intravenous immunoglobulin (IVIG; 5mg/mL) and both NF-κB (30μM) and MAPKs inhibitors (P38 inhibitor, 10μM; JNK inhibitor, 30μM; ERK inhibitor, 30μM) significantly reduced the levels of cleaved caspase-3 and cell death; however, intravenous immunoglobulin (IVIG; 5mg/mL) alone increased the levels of Bcl-2 and Bcl-xL. Combined (ALL) administration of NF-κB and MAPKs inhibitors significantly reduced the levels of cleaved caspase-3 and cell death; however, the levels of Bcl-2 and Bcl-xL remained unchanged. β-actin was used as a loading control. Data are represented as mean ± S.E.M. n=5 cultures. $^{###}P < 0.001$ compared with control; $^{***}P < 0.001$ compared with $OGD6+Vehicle_{IVIG}$ or $OGD3+R24+Vehicle_{IVIG}$; $^{+++}P < 0.001$ compared with control; $^{@@@}P < 0.001$ compared with $OGD6+Vehicle_{IVIG}$ or $OGD3+R24+Vehicle_{IVIG}$; $^{\&\&\&}P < 0.001$ compared with $OGD6+Vehicle_{Inhibitor}$ or $OGD3+R24+Vehicle_{Inhibitor}$.

3.4 Discussion:

The NLRP inflammasomes are multi-protein complexes that activate and convert precursor caspase-1 into cleaved caspase-1, which cleave precursors IL-1β and IL-18 into biologically active

mature pro-inflammatory cytokines that are then released into the extracellular environment (Bauernfeind *et al*., 2011a). Both these pro-inflammatory cytokines have been shown to stimulate immune responses and mediate active roles in the initiation of neuroinflammation that is responsible for inducing neuronal and glial cell death following an ischemic stroke (Abulafia *et al*., 2009; Deroide *et al*., 2013; Fann *et al*., 2013; Ito *et al*., 2015; Savage *et al*., 2012; Zhang *et al*., 2014). Despite activating precursors IL-1β and IL-18 into biologically active mature pro-inflammatory cytokines, a major pleiotropic effect of cleaved caspase-1 is that it is able to induce pyroptosis, a highly inflammatory form of cell death characterised by rapid plasma membrane rupture and release of pro-inflammatory contents into the extracellular environment due to the development of pores in the plasma membrane allowing an osmotic movement of water into the cell causing cell lysis mediated by cleaved capase-1 through an unknown mechanism(s) (Bergsbaken *et al*., 2009; Fink & Cookson, 2006; Fink *et al*., 2008). In addition to inducing pyroptosis, cleaved caspase-1 has been shown to directly cleave and activate both executioner caspase-3 and 7, and Bid (BH3 interacting death domain agonist), into their active forms inducing intrinsic and extrinsic apoptotic cell death, respectively (Erener *et al*., 2012; Frederick Lo *et al*., 2008; Guegan *et al*., 2002; Liu *et al*., 2004; Walsh *et al*., 2011; Zhang *et al*., 2003). We recently established that the levels of NLRP1 and NLRP3 inflammasome proteins and both precursors IL-1β and IL-18 were increased in primary cortical neurons under simulated ischemic conditions, and brain tissues in response to cerebral ischemic and reperfusion (I/R) injury in mice and humans following ischemic stroke (Fann *et al*., 2013). These inflammasome components included the NLRP1 and NLRP3 receptors, ASC, XIAP, and precursors caspase-1 and 11. In addition, it was established that both NLRP1 and NLRP3 inflammasomes were activated due to elevated levels of cleaved XIAP, cleaved caspases-1 and 11, and maturation of both IL-1β and IL-18 found in primary cortical neurons and brain tissues following simulated *in vitro* and *in vivo* ischemic conditions. Furthermore, we were able to demonstrate that caspase-1 inhibitor treatment was able to reduce neuronal cell death and brain infarct size, and improve functional outcome by targeting inflammasome activation under simulated *in vitro* and *in vivo* experimental stroke models (Fann *et al*., 2013).

Despite establishing a role for NLRP1 and NLRP3 inflammasomes in stroke-induced neuronal cell death and brain tissue injury following *in vitro* and *in vivo* ischemic conditions, our previous study did not explore the molecular mechanism(s) responsible for ischemia-induced NLRP1 and NLRP3 inflammasome expression and activation in neurons (Fann *et al*., 2013). A major finding of this present study is that both NF-κB and MAPK(s) signaling pathways played a major role in the expression and activation of NLRP1 and NLRP3 inflammasomes in primary cortical neurons, and that the expression and activation of neuronal NLRP1 and NLRP3

inflammasomes was able to be attenuated by treatment with either NF-κB and MAPKs inhibitors under simulated *in vitro* ischemic conditions.

Several previous studies have provided evidence that activation of both NF-κB and MAPK(s) signaling pathways occur in neurons following ischemic stroke (Cheng *et al*., 2014; Gladbach *et al*., 2014; Liang *et al*., 2014; Liu *et al*., 2009; Lok *et al*., 2015; Tang *et al*., 2007). In the present study, we again provide supporting evidence that activation of both NF-κB and MAPK(s) signaling pathways are detrimental to neuronal survival and pharmacological inhibition of either the NF-κB and MAPK(s) signaling pathways were able to significantly protect neurons under ischemic conditions. It was previously established from numerous experimental studies that both NF-κB and MAPKs signaling pathways are known to modulate the expression of NLRP inflammasome proteins and both precursors IL-1β and IL-18 in immune cells under inflammatory conditions (Bauernfeind *et al*., 2011b; Bauernfeind *et al*., 2009; Budai *et al*., 2013; Burm *et al*., 2015; Frederick Lo *et al*., 2008; Ghonime *et al*., 2014; Hara *et al*., 2013; He *et al*., 2012; Juliana *et al*., 2010; Kang *et al*., 2000; Legos *et al*., 2001; Liao *et al*., 2012; Liu *et al*., 2004; Liu *et al*., 2013; Mariathasan & Monack, 2007; Okada *et al*., 2014; Qiao *et al*., 2012; Savage *et al*., 2012; Schroder *et al*., 2012; Tamatani *et al*., 2000; Weber *et al*., 2015; Zhao *et al*., 2013; Zheng *et al*., 2011). The present study indeed confirms that pharmacological inhibition of either the NF-κB, P38, JNK and ERK signaling pathways was able to significantly reduce the expression levels of NLRP inflammasome proteins and both precursors IL-1β and IL-18 in neurons, and hence provide evidence for the first time that activation of either the NF-κB and MAPKs signaling pathways are responsible for inducing the expression of NLRP inflammasome proteins and both precursors IL-1β and IL-18 in neurons under simulated ischemic conditions. Furthermore, we demonstrated that pharmacological inhibition of both the NF-κB and MAPKs signaling pathways was able to directly attenuate NLRP inflammasome activation and maturation of both IL-1β and IL-18 in neurons under ischemic conditions. In addition, here we provide supporting evidence for the first time that a novel neuroprotective effect of IVIg treatment is associated with a significant reduction in the activation of the NF-κB and MAPKs signaling pathways, which is suggested to be responsible for reducing the expression and activation of NLRP inflammasome proteins and both precursors IL-1β and IL-18 in neuronal cells following ischemic conditions.

Commercial IVIg is a purified polyclonal blood preparation of natural antibodies primarily containing immunoglobulin G (IgG) that is derived from the plasma of several thousand healthy human individuals in order to ensure the preparation is homogenous but functionally heterogeneous (Arumugam *et al*., 2008; Rezaei *et al*., 2011; Saeedian & Randhawa, 2014; Schwab & Nimmerjahn,

2013). IVIg is a therapeutic modality that is approved by the US Food and Drug Administration (FDA) to treat a number of autoimmune and inflammatory conditions such as primary immune deficiency diseases, immune (idiopathic) thrombocytopenia purpura (ITP), Kawasaki's disease, and neurological conditions such as Guillian-Barre syndrome, chronic inflammatory demyelinating polyneuropathy (CIDP) and multifocal motor neuropathy (Arumugam *et al*., 2008; Dash *et al*., 2014; Hahn *et al*., 2013; Kuitwaard *et al*., 2009; Leger *et al*., 2013; Rezaei *et al*., 2011; Sakata *et al*., 2007; Wasserman *et al*., 2012). In addition, off-label uses of IVIg treatment following randomized controlled trials of efficacy include dermatomyositis, Lambert-Eaton syndrome, Myasthenia Gravis and Stiff-Pearson syndrome (Dalakas, 2005; Katz *et al*., 2011; Miyasaka *et al*., 2012; Rezaei *et al*., 2011; Rich *et al*., 1997; Zinman *et al*., 2007). Consequently, IVIg has potential to diminish inappropriate inflammatory and immune activation through a number of mechanisms by inhibiting complement fragments, pro-inflammatory cytokine production and infiltration of leukocytes, which are all useful properties that may offer neuroprotection. Hence, these pleiotropic effects of IVIg in inhibiting multiple components of inflammation in different cell types within the neurovascular unit make it an attractive candidate for use in stroke therapy (Arumugam *et al*., 2007; Fann *et al*., 2013; Lok *et al*., 2015; Lux *et al*., 2010; Walberer *et al*., 2010; Widiapradja *et al*., 2012; Widiapradja *et al*., 2014). This was elegantly confirmed in a previous study from our laboratory for the first time that administration of IVIg was able to significantly attenuate brain infarct size (50-60%) and eliminate mortality, and improve functional outcome in mice subjected to experimental ischemic stroke (Arumugam *et al*., 2007). In a subsequent study, our group investigated the effect of IVIg on downstream signaling pathways involved in neuronal cell death under simulated *in vitro* experimental models of stroke and Alzheimer's disease (Widiapradja *et al*., 2012). It was shown that treatment of cultured primary cortical neurons with IVIg significantly reduced simulated ischemic and amyloid β peptide (Aβ)-induced phosphorylation of cell death-associated NF-κB (i.e. p-p65) and MAPK(s) (i.e. p-P38 and p-JNK) signaling pathways and activation of pro-apoptotic protein caspase-3 under *in vitro* conditions. In addition, IVIg treatment significantly up-regulated the expression of anti-apoptotic protein Bcl-2 in primary cortical neurons under simulated ischemic-like conditions and exposure to Aβ (Widiapradja *et al*., 2012). As previously mentioned, we recently demonstrated the effect of IVIg on the expression and activation levels of NLRP1 and NLRP3 inflammasome proteins and both precursors IL-1β and IL-18 in primary cortical neurons and brain tissues under simulated *in vitro* and *in vivo* ischemic conditions. It was established that administration of IVIg was able to significantly attenuate the expression of NLRP1 and NLRP3 inflammasome proteins, and both precursors IL-1β and IL-18, and thus inflammasome activity in primary cortical neurons and brain tissues under *in vitro* and *in vivo* ischemic conditions (Fann *et al*., 2013). While the molecular and cellular mechanism(s) in how IVIg reduces NLRP1 and NLRP3

inflammasome expression and activation levels remains to be fully determined in primary cortical neurons and brain tissues under *in vitro* and *in vivo* ischemic conditions, the present study provides compelling evidence to suggest for the first time that a novel neuroprotective mechanism(s) of IVIg may be mediating its protective effects through the attenuation of inflammasome priming by decreasing the activation of either the NF-κB and MAPK(s) signaling pathway in primary cortical neurons under *in vitro* ischemic conditions. In another recent study, our group investigated the effect of IVIg on the expression levels of plasma membrane pattern recognition receptors (PRRs) such as TLR-2, TLR-4 and RAGE, and its downstream adaptor proteins such as myeloid differentiation primary response gene 88 (MyD88) and tumor necrosis factor receptor-associated factor 6 (TRAF6) that are associated in activating major downstream signaling pathways such as the NF-κB and MAPK(s) pathways in primary cortical neurons and brain tissues under *in vitro* and *in vivo* ischemic conditions (Lok *et al*., 2015). It was shown that IVIg treatment significantly reduced the expression levels of TLR-2, TLR-4 and RAGE, and its downstream adaptor proteins, MyD88 and TRAF6, in primary cortical neurons and brain tissues subjected to ischemic conditions (Lok *et al*., 2015). Hence, provides supporting evidence that both a decrease in expression of PRRs (i.e. TLR-2, TLR-4 and RAGE) and TLR adaptor and signaling proteins (i.e. MyD88 and TRAF6) may provide an explanation for IVIg's ability to decrease activation of either the NF-κB and MAPKs signaling pathways in primary cortical neurons and brain tissues under *in vitro* and *in vivo* ischemic conditions (Fann *et al*., 2013; Lok *et al*., 2015; Widiapradja *et al*., 2012). Consequently, a decreased activation of the NF-κB and MAPKs signaling pathways from IVIg and both NF-κB and MAPK(s) inhibitors observed in the present study is expected to decrease the expression of NLRP inflammasome components (i.e. NLRP1, NLRP3, ASC, precursor caspase-1, precursor caspase-11 and XIAP) and both precursors IL-1β and IL-18, thereby decrease the number of inflammasome complexes formed and subsequent production of activated proteins such as cleaved XIAP, cleaved caspase-1 and -11, and both mature IL-1β and IL-18, demonstrating that pharmacological inhibition of the NF-κB and MAPK(s) signaling pathway and IVIg may mediate its protective effects through the attenuation of inflammasome priming in primary cortical neurons under *in vitro* ischemic conditions.

Our current data supports findings from previous studies that IVIg can increase the expression of anti-apoptotic proteins, Bcl-2 and Bcl-xL, in primary cortical neurons and cerebral tissues under *in vitro* and *in vivo* ischemic conditions through an unknown mechanism(s), although it is postulated from the present study that IVIg may be increasing the expression of Bcl-2 and Bcl-xL by activating alternate pathway(s) that is responsible for elevating the expression of Bcl-2 and Bcl-xL independent of the NF-κB and MAPKs signaling pathways in neurons under ischemic

conditions (Fann *et al*., 2013; Lok *et al*., 2015; Widiapradja *et al*., 2012). It was shown that Bcl-2 can directly bind and inhibit the NLRP1 and NLRP3 receptors in macrophages by specifically preventing ATP from binding onto the nucleotide-binding domain (NBD) of both receptors (Bruey *et al*., 2007; Faustin *et al*., 2009; Shimada *et al*., 2012). Therefore, inhibiting the oligomerization of the NLRP1 and NLRP3 receptors would be expected to attenuate inflammasome formation and reduce both caspase-1 activation and maturation of both IL-1β and IL-18 (Bruey *et al*., 2007; Faustin *et al*., 2009; Shimada *et al*., 2012). In addition, it was shown that Bcl-xL, another anti-apoptotic protein was able to directly bind and inhibit the NLRP1 receptor in macrophages through a similar mechanism as Bcl-2, but whether Bcl-xL is able to inhibit the NLRP3 receptor remains to be determined (Bruey *et al*., 2007; Faustin *et al*., 2009). Hence, it appears that Bcl-2 is a tight regulator of NLRP1 and NLRP3 receptor activation; however, whether Bcl-xL regulates NLRP3 receptor activation, and the precise mechanism(s) behind IVIg increasing Bcl-2 and Bcl-xL levels in neurons under *in vitro* ischemic conditions remains to be established. In this study, we demonstrate that IVIg can target NLRP inflammasome expression and activation not only by suppressing the activation of the NF-κB and MAPKs signaling pathways, but possibly via the aforementioned mechanism by increasing the expression levels of anti-apoptotic proteins, Bcl-2 and Bcl-xL, in primary cortical neurons under *in vitro* ischemic conditions.

3.5 Conclusion:

In summary, the present findings provide compelling evidence that both the NF-κB and MAPKs signaling pathways play a pivotal role in regulating the expression and activation of NLRP1 and NLRP3 inflammasome proteins and both precursors IL-1β and IL-18 in primary cortical neurons under simulated *in vitro* ischemic conditions. In addition, it was demonstrated that IVIg was able to attenuate the activation of the NF-κB and MAPK(s) signaling pathways, which decreased the expression and activation of NLRP1 and NLRP3 inflammasome proteins and both precursors IL-1β and IL-18 in neurons under ischemic conditions. Furthermore, it was also established that IVIg was able to induce an increased expression of anti-apoptotic proteins, Bcl-2 and Bcl-xL, possibly providing another mechanism in targeting NLRP inflammasome activation in primary cortical neurons under simulated *in vitro* ischemic conditions. Hence, these findings suggest that therapeutic interventions that target inflammasome signaling such as inflammasome priming (i.e. the NF-κB and MAPK(s) signaling pathways), and inflammasome activation; may provide new opportunities in the future treatment of neuronal cell death in ischemic stroke.

3.6 References:

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CHAPTER 4:

Intermittent Fasting Attenuates Inflammasome Activity in Ischemic Stroke

4.1 Introduction:

Ischemic stroke is the second leading cause of mortality and a major cause of morbidity worldwide (Donnan *et al*., 2008). The molecular and cellular mechanisms responsible for ischemic stroke-induced neuronal cell death involve bioenergetic failure, ionic imbalance, excitotoxicity, metabolic and oxidative stress, and inflammatory processes, including activation of resident glial cells and infiltration of leukocytes (Arumugam *et al*., 2005; Brouns & De Deyn, 2009; Broughton *et al*., 2009; Dirnagl, 2012; Sims & Muyderman, 2010). Inflammasomes are involved in a newly discovered multi-protein complex signaling pathway that contributes to inflammation and cell death in various pathological conditions. Nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) pyrin domain containing (NLRP) inflammasomes play a role in the inflammatory response during ischemic stroke (Abulafia *et al*., 2009; Deroide *et al*., 2013; Fann *et al*., 2013a; Fann *et al*., 2013b; Savage *et al*., 2012; Zhang *et al*., 2014). The NLRP inflammasomes are cytosolic macromolecular complexes composed of the NLRP receptor, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), precursor caspase-1, precursor caspase-11 and/or XIAP (X-linked inhibitor of apoptosis) (Agostini *et al*., 2004; Boyden & Dietrich, 2006; De Rivero Vaccari *et al*., 2009; Martinon *et al*., 2002). Activation and homo-oligomerization of NLRP receptors induce formation of the NLRP inflammasome, which converts precursor caspase-1 into cleaved caspase-1 via proximity-induced auto-activation (Agostini *et al*., 2004; Boatright *et al*., 2003; Liu *et al*., 2014; Martinon *et al*., 2002; Salvesen & Dixit, 1999; Schroder & Tschopp, 2010). Cleaved caspase-1 converts precursors interleukin (IL)-1β and IL-18 into biologically active mature pro-inflammatory cytokines that are released into the extracellular environment (Bauernfeind *et al*., 2011a; Schroder & Tschopp, 2010). In addition, cleaved caspase-1 can initiate cell death directly via apoptosis or pyroptosis (Aachoui *et al*., 2013; Adamczak *et al*., 2014; Alfonso-Loeches *et al*., 2014; Sagulenko *et al*., 2013; Tan *et al*., 2014; Tan *et al*., 2015; Yin *et al*., 2015; Zhang *et al*., 2015). Furthermore, we have recently demonstrated that ischemia-like conditions increased the levels of NLRP1 and NLRP3 inflammasome proteins, and both IL-1β and IL-18, in primary cortical neurons and cerebral tissue (Fann *et al*., 2013a). An increase in expression of NLRP1 and NLRP3 inflammasome proteins and both precursor IL-1β and IL-18 proteins in brain cells under ischemic conditions may be induced by the activation of intracellular NF-κB and MAPK(s) signaling pathways via a regulatory process called 'priming' or Signal 1 that is similarly observed in immune cells (Bauernfeind *et al*., 2009; Bauernfeind *et al*., 2011b; Budai *et al*., 2013; Burm *et al*., 2015;

Frederick Lo *et al*., 2008; Ghonime *et al*., 2014; Gross *et al*., 2011; Hara *et al*., 2013; He *et al*., 2012; Juliana *et al*., 2010; Kang *et al*., 2000; Legos *et al*., 2001; Liao *et al*., 2012; Liu *et al*., 2004; Liu *et al*., 2013; Mariathasan & Monack, 2007; Okada *et al*., 2014; Qiao *et al*., 2012; Savage *et al*., 2012; Schroder *et al*., 2012; Tamatani *et al*., 2000; Weber *et al*., 2015; Zhao *et al*., 2013a).

Dietary restriction in the form of daily calorie reduction (CR) or intermittent fasting (IF) are dietary protocols, which have been proven to extend lifespan and decrease the development and severity of age-related diseases, including cardiovascular (e.g. myocardial infarction and stroke) and neurodegenerative (e.g. Alzheimer's disease, Parkinson's disease and Huntington's disease) diseases demonstrated in a number of animal models (Belkacemi *et al*., 2011; Bruce-Keller *et al*., 1999; Duan *et al*., 2003; Halagappa *et al*., 2007; Katare *et al*., 2009; Longo & Mattson, 2014; Manzanero *et al*., 2011; Manzanero *et al*., 2014; Mattson, 2000; Mattson *et al*., 2003; Mattson, 2005; Mattson, 2014; Mattson & Wan, 2005; Patterson *et al*., 2015; Pedersen *et al*., 1999; Wan *et al*., 2010). CR and IF have been shown to reduce circulating markers of oxidative stress and inflammation, and can improve cardiovascular disease risks (Harvie *et al*., 2011; Johnson *et al*., 2007; Mager *et al*., 2006; Mattison *et al*., 2012; Weiss & Fontana, 2011). Several studies suggest dietary restriction may promote neuronal survival and plasticity in ischemic stroke, by inducing the expression of neuroprotective factors and suppressing inflammatory pathways (Arumugam *et al*., 2010; Manzanero *et al*., 2011; Manzanero *et al*., 2014; Yu & Mattson, 1999). Major pro-inflammatory cytokines implicated in ischemic brain injury are tumor necrosis factor-α (TNF-α), IL-1β and IL-6 (Arumugam *et al*., 2010; Lambertsen *et al*., 2012). IF appears to protect the brain against ischemic injury by preconditioning neurons and glial cells with energy restriction, which act as a mild metabolic stressor that effectively upregulates the expression of several key neuroprotective proteins including neurotrophic factors, protein chaperones, and antioxidant enzymes, and downregulation of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β (Arumugam *et al*., 2010). Furthermore, we recently reported that levels of NLRP1/3 inflammasome proteins, IL-1β and IL-18 were elevated in ipsilateral brain tissues of cerebral I/R mice and stroke patients, and that caspase-1 inhibitor treatment protected cultured cortical neurons and cerebral tissue under *in vitro* and *in vivo* models of ischemic stroke (Fann *et al*., 2013a). Since IF is neuroprotective and reduces pro-inflammatory cytokines in stroke, and inflammasomes are involved in the production of pro-inflammatory cytokines such as IL-1β, we tested the hypothesis whether IF impairs stroke-induced inflammasome expression and activation. Here we demonstrate for the first time that a neuroprotective effect of IF in experimental stroke involves suppression of inflammasome activity.

4.2 Material & Methods:

Animals and Diets

Male C57BL/6J mice were obtained from the Animal Resources Centre in Canning Vale, Australia, and group housed upon arrival at The University of Queensland Animal Facility. At ten weeks of age, mice were randomly assigned to either the *ad libitum* (AL) or intermittent fasting (IF) diet conditions. Mice in the IF condition were fed for 8hrs out of every 24-hour period, with food available between 07:00 and 15:00 (lights on at 06:00, lights off at 18:00) for four months. In addition, mice on the IF diet were housed using non-edible bedding (Aspen Chips; Tapvei Ltd., Kuopio, Finland) to prevent calorie intake during the IF period. Following the dietary protocol, half the mice from both the AL and IF diets were randomly selected and subjected to focal cerebral ischemia/reperfusion (I/R) injury, while the remaining half of the mice from both the AL and IF diets underwent a Sham operation. Following Sham operation or I/R injury, IF mice were no longer subjected to the IF diet and had AL access to food and water. The Animal Care and Use Committee of The University of Queensland approved all experimental procedures.

Focal Cerebral Ischemia/Reperfusion (I/R) Stroke Model

Three-month-old C57BL/6J male mice were subjected to transient middle cerebral artery ischemia and reperfusion (I/R) injury, as described previously (Arumugam *et al*., 2004). Briefly, after making a midline incision in the neck, the left external carotid and pterygopalatine arteries were isolated and ligated with a 6-0 silk thread. The internal carotid artery (ICA) was occluded at the peripheral site of the bifurcation with a small clip and the common carotid artery (CCA) was ligated with a 5-0 silk thread. The external carotid artery (ECA) was cut, and a 6-0 nylon monofilament with a tip that was blunted (0.20-0.22mm) with a coagulator was inserted into the ECA. After the clip at the ICA was removed, the nylon thread was advanced to the origin of the middle cerebral artery (MCA) until light resistance was evident. The nylon thread and the CCA ligature were removed after 1hr to initiate reperfusion. In the Sham group, surgery was performed until the arteries were visualized but not disturbed for a period of 1hr under isofluorane-induced anaesthesia In a separate set of experiments, anesthetized animals from all groups (5-6 mice per group) underwent cerebral blood flow (CBF) measurements using a Laser Doppler Perfusion Monitor (Moor Lab, Moor Instruments, Axminster, UK). The University of Queensland Animal Care and Use Committee approved all *in vivo* experimental procedures.

Tissue Lysis and Protein Quantitation

In order to extract protein, the contralateral (non-damaged) and ipsilateral (damaged) brain

tissues were homogenized separately in tissue lysis buffer (Tissue Protein Extraction Reagent (TPER) containing protease and phosphatase inhibitor in 1:100 ratio) (Thermo Scientific, Rockford, IL, USA) using a Tissue-Tearer (Biospec Products, Inc., Bartlesville, OK, USA). Samples were centrifuged at 15,000 rpm at 4°C for 15 minutes and the supernatant collected. Total protein concentration of each sample was measured in a microplate using the Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Bovine serum albumin (BSA) standards (20-2,000µg/mL) were prepared as per the manufacturer's instructions to generate a standard curve with known concentrations. Absorbance was measured at 562nm using the Tecan 26 Sunrise Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland) and data was analyzed using Graphpad Prism 5 software (Graphpad Software, San Diego, CA, USA) by comparing samples to the standard curve to determine the concentration and volume of protein required to be loaded for separation by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Western Blot Analysis

Protein samples from the cerebral cortex were subjected to Tris-HCl polyacrylamide gel (7.5%, 10% and 12.5%) electrophoresis and run at 80V using 1X Tris/glycine/sodium dodecyl sulphate buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) until the proteins and ladder (ProSieve Colour Protein Marker ladder; Lonza Rockfield, Inc., Rockfield, ME, USA) were optimally spread. Gels were then electro-blotted using a transfer apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in 1X transfer buffer containing 0.025 mol/L Tris base, 0.15 mol/L glycine, and 10% (v/v) methanol for 2hrs at 80V onto a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, US). The membrane was then incubated in blocking buffer (5% non-fat milk in 20 mM Tris–HCl, pH 7.5, 137mMNaCl, 0.2% Tween-20) for 1hr at 23°C. The membrane was then incubated overnight at 4°C with primary antibodies including those that selectively bind p-P65 NF-κB (Cell Signaling Technology, Danvers, MA, USA), P65 NF-κB (Cell Signaling), p-P38 (Cell Signaling), P38 (Cell Signaling), p-JNK (Cell Signaling), JNK (Cell Signaling), p-ERK (Cell Signaling), ERK (Cell Signaling), p–c-Jun (Cell Signaling), c-Jun (Cell Signaling), NLRP1 (Novus Biologicals, Littleton, CO, USA), NLRP3 (Novus Biologicals), ASC (Abcam, Cambridge, UK), caspase-1 (Abcam), caspase-11 (Abcam), IL-1β (Abcam), IL-18 (Abcam), caspase-3 (Cell Signaling), Cleaved caspase-3 (Cell Signaling), Bcl-2 (Cell Signaling), Bcl-xL (Cell Signaling) and β-actin (Sigma-Aldrich, St. Louis, MO, USA). After washing three times (10 min per wash) with Tris-buffered saline-T (20 mM Tris–HCL, pH 7.5, 137 mM NaCl, 0.2% Tween-20), the membrane was incubated with secondary antibodies against the primary antibody and β-actin for 1hr at room temperature. The membrane was washed with Tris-buffered saline-T and scanned using the Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln,

NE, USA). Quantification of protein levels was achieved by densitometry analysis using Image J v1.46 software (National Institute of Health, Bethesda, MD, USA). In detail, the densitometry of phosphorylated NF-κB and MAPK(s) signaling proteins was determined and divided by the densitometry of its corresponding "Total protein" indicating activation. The densitometry of the inflammasome proteins, IL-1β and IL-18, and caspase-3, Bcl-2 and Bcl-xL was determined and divided by the densitometry of its corresponding β-actin, which was used as a loading control.

4.3 Results:

In addition to our previously published study (Arumugam *et al*., 2010), recently, we have tested the functional consequences of transient focal stroke in the IF mice that were fed for 8hrs out of every 24-hour period. Similar to 24-hour IF mice (Arumugam *et al*., 2010), these mice also exhibited smaller infarcts three days after ischemia and reperfusion injury, relative to mice on the *ad libitum* diet (Manzanero *et al*., 2014). In this study, we subsequently investigated the effects of IF on the activation of intracellular NF-κB and MAPK(s) signaling pathways, and the expression of NLRP1 and NLRP3 inflammasome proteins and both IL-1β and IL-18 in ipsilateral brain tissue (cerebral cortex) following 24hr of I/R. IF significantly attenuated the ischemia-induced increase in levels of phosphorylated-P65-NF-κB and phosphorylated MAPK(s) such as p-P38, p-JNK, p-ERK and p–c-Jun compared to the AL group in ipsilateral brain tissue following 24hr of I/R (**Figures 4.1A and B**). However, no difference was observed in these proteins under Sham conditions between AL and IF groups. Furthermore, levels of total NF-κB and MAPK(s) were similar in AL and IF groups under both Sham and I/R conditions (**Figure 4.1A**). Next, we analyzed the levels of NLRP1 and NLRP3 inflammasome components such as NLRP1, NLRP3, ASC, precursor caspase-1 and 11, as well as the precursors of IL-1β and IL-18 in ipsilateral brain tissues 24hr after I/R. Similar to both total NF-κB and MAPK(s) levels, no differences in inflammasome proteins and precursors of IL-1β and IL-18 were observed under Sham conditions between AL and IF groups. However, ischemia-induced increases in inflammasome proteins and precursors of IL-1β and IL-18 were significantly reduced in the IF group compared to the AL group in ipsilateral brain tissues following 24hr of I/R (**Figures 4.1C and D**). We further investigated the effect of IF on inflammasome activation by measuring the levels of cleaved caspases-1 and 11, and mature forms of IL-1β and IL-18, in ipsilateral brain tissue at 24hr of I/R. IF significantly decreased the levels of both cleaved caspase-1 and 11, and both mature IL-1β and IL-18 in the ischemic cortex (**Figures 4.2A and B**). In addition, the data indicated that inflammasome activity in the contra-lateral brain hemisphere was lower in comparison to the ipsilateral brain hemisphere in *ad libitum* ischemic mice. However, no change was evident between the contra-lateral and ipsilateral brain hemispheres in IF ischemic mice (**Figures 4.2A and B**). Furthermore, levels of pro-apoptotic cleaved caspase-3

was significantly lower and anti-apoptotic protein Bcl-xL was significantly higher in the IF group in comparison to AL controls in the ischemic cortical brain tissue at 24hr after I/R. However, no significant difference was observed in the levels of precursor caspase-3 and Bcl-2 in the IF group in comparison to AL controls in the ischemic cortical brain tissue at 24hr following I/R (**Figures 4.2C and D**).

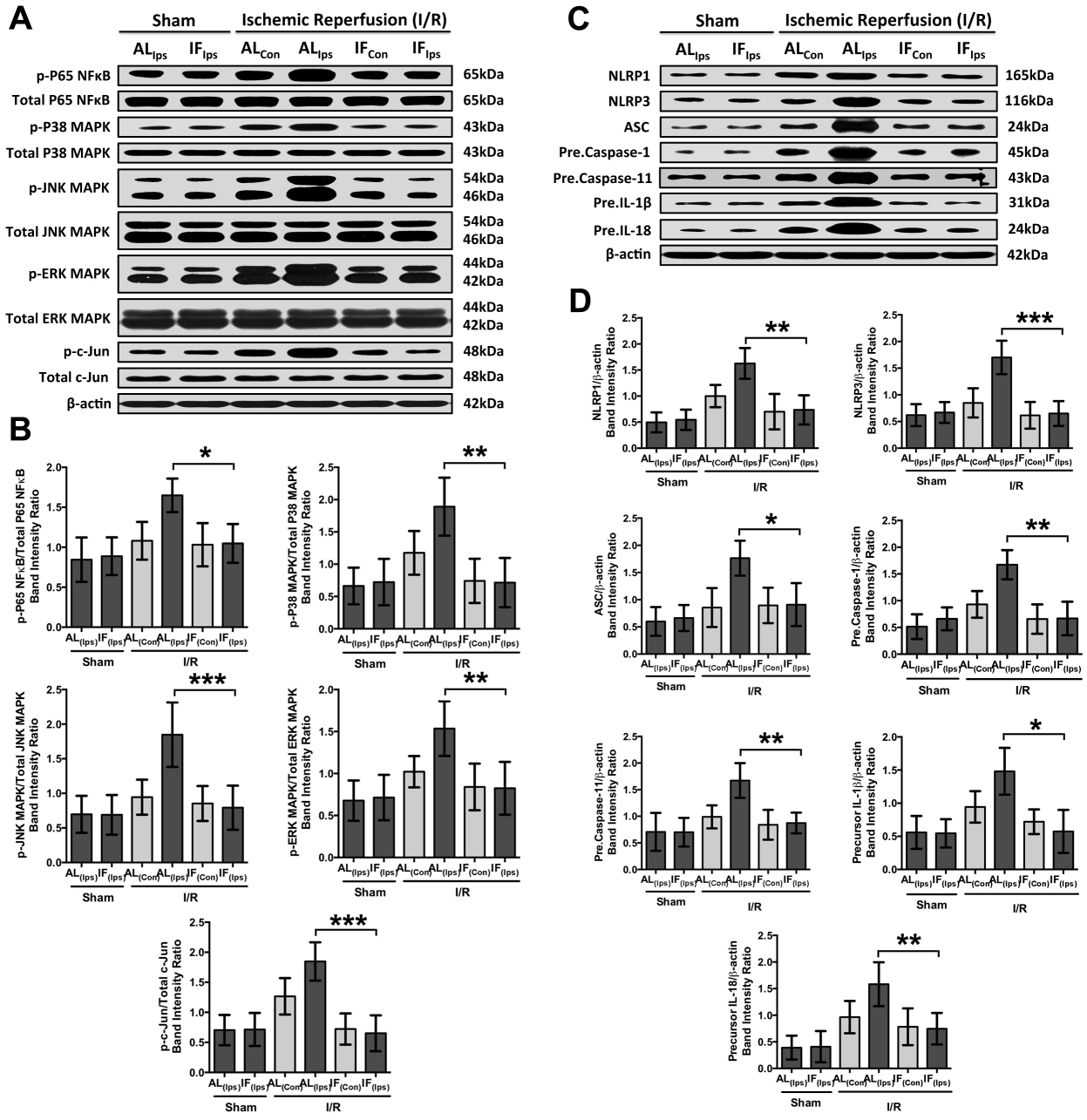

Figure 4.1: Intermittent fasting reduces NF-κB, MAPK(s) and inflammasome expression in a mouse model of focal ischemic stroke. (A & B) Representative immunoblots and quantification illustrating increases in the activation levels of NF-κB (p-P65) and MAPK(s) such as p-P38, p-JNK and p-ERK and p–c-Jun in ipsilateral brain tissues of C57BL6/J mice following middle cerebral artery occlusion (1hr) and reperfusion (24hr). Intermittent fasting (IF) significantly reduced the activation levels of NF-κB (p-P65) and MAPK(s) such as p-P38, p-JNK and p-ERK and p–c-Jun. Data are represented as mean ± S.D. n=5-6 animals in each group. *$p < 0.05$ in comparison to AL $I/R_{(Ips)}$; **$p < 0.01$ in comparison to AL $I/R_{(Ips)}$; ***$p < 0.001$ in

comparison to AL $I/R_{(Ips)}$ group. (C & D) Representative immunoblots and quantification illustrating increases in the levels of inflammasome proteins such as NLRP1, NLRP3, ASC, pre-caspase-1, pre-caspase-11, pre-IL-1β and pre-IL-18 in ipsilateral brain tissues of C57BL6/J mice following middle cerebral artery occlusion (1hr) and reperfusion (24hr). Intermittent fasting (IF) significantly reduced the levels of all inflammasome proteins as well as pre-IL-1β and pre-IL-18. Data are represented as mean ± S.D. n=5–6 animals in each group. *p < 0.05 in comparison to AL $I/R_{(Ips)}$; **p < 0.01 in comparison to AL $I/R_{(Ips)}$; ***p < 0.001 in comparison to AL $I/R_{(Ips)}$ group. β-actin was used as a loading control.

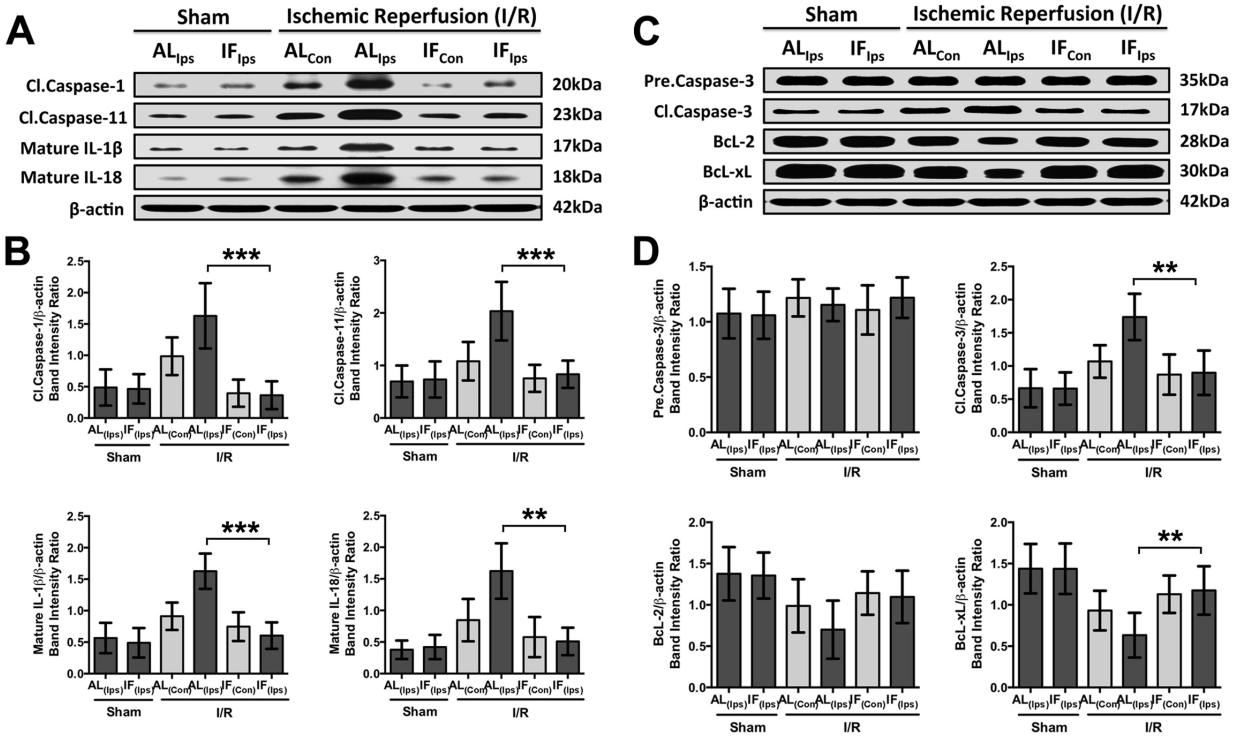

Figure 4.2: Intermittent fasting reduces inflammasome activity and cell death in a mouse model of focal ischemic stroke. (A & B) Representative immunoblots and quantification illustrating increases in the levels of activated inflammasome proteins such as Cl.caspase-1 and Cl-caspase-11 and maturation of IL-1β and IL-18 in ipsilateral brain tissues of C57B6/J mice following middle cerebral artery occlusion (1hr) and reperfusion (24hr). Intermittent fasting (IF) significantly reduced the levels of Cl.caspase-1 and Cl-caspase-11 and maturation of IL-1β and IL-18. Data are represented as mean ± S.D. n=5-6 animals in each group. **p < 0.01 in comparison to AL $I/R_{(Ips)}$; ***p < 0.001 in comparison to AL $I/R_{(Ips)}$ group. (C & D) Representative immunoblots and quantification illustrating increased levels of pro-apoptotic protein Cl.caspase-3 and reduced levels of anti-apoptotic protein Bcl-xL in ipsilateral brain tissues of C57BL6/J mice following middle cerebral artery occlusion (1hr) and reperfusion (24hr). Intermittent fasting (IF) significantly reduced the levels of Cl.caspase-3 and increased the levels of Bcl-xL. Data are represented as mean ± S.D. n = 5-6 animals in each group. **p < 0.01 in comparison to AL $I/R_{(Ips)}$ group. β-actin was used as a loading control.

4.4 Discussion:

A macromolecular complex termed the inflammasome, in particular, the NLRP1 and NLRP3 inflammasomes, regulate the maturation of pro-inflammatory cytokines such as IL-1β and IL-18. Several lines of evidence suggest that activation of inflammasomes may contribute to cell death via apoptosis or pyroptosis following brain injury (Aachoui *et al*., 2013; Adamczak *et al*., 2014; Alfonso-Loeches *et al*., 2014; Lamkanfi & Dixit, 2012; Sagulenko *et al*., 2013; Tan *et al*.,

2014; Tan *et al*., 2015; Yin *et al*., 2015; Zhang *et al*., 2015). Apoptosis and pyroptosis are both programmed cell death mechanisms, with pyroptosis being highly inflammatory and involving cytoplasmic swelling and early plasma membrane rupture (Fink & Cookson, 2006; Fink *et al*., 2008; Lamkanfi & Dixit, 2012). We have recently demonstrated that the NLRP1 and NLRP3 inflammasomes play a major role in neuronal cell death and cerebral tissue damage resulting in neurological functional deficits in a mouse model of focal ischemic stroke (Fann *et al*., 2013a). The present results demonstrate for the first time that dietary restriction in the form of intermittent fasting can attenuate expression levels of NLRP1 and NLRP3 inflammasome proteins and activity, together with a corresponding down-regulation of pro-inflammatory cytokines such as IL-1β and IL-18, and pro-apoptotic protein cleaved caspase-3 in cerebral tissue following ischemic stroke.

An increase in expression of NLRP1 and NLRP3 inflammasome proteins and precursors of IL-1β and IL-18 in the brain following ischemic stroke may be induced by the activation of pattern recognition receptors (PRRs) located on the plasma membrane of neurons, glial and microvascular endothelial cells, which can detect endogenous danger signals termed damage associated molecular patterns (DAMPs) that are released from necrotic tissue within the infarct core (Alfonso-Loeches *et al*., 2014; Burm *et al*., 2015; Caso *et al*., 2007; Caso *et al*., 2008; Codolo *et al*., 2013; Eigenbrod *et al*., 2008; Frank *et al*., 2015; Lee *et al*., 2013; Lippai *et al*., 2013; Lok *et al*., 2015; Nagyoszi *et al*., 2015; Nystrom *et al*., 2013; Pradillo *et al*., 2012; Tang *et al*., 2007; Tang *et al*., 2013; Weber *et al*., 2015; Zhao *et al*., 2014; Zheng *et al*., 2013). It is proposed that DAMPs stimulate PRRs such as toll-like receptors (TLRs; TLR-2 and TLR-4), the receptor for advanced glycation end products (RAGE), and the IL-1 receptor 1 (IL-1R1), which activate intracellular NF-κB and MAPK(s) signaling pathways resulting in an upregulation of NLRP1 and NLRP3 inflammasome proteins and both precursor IL-1β and IL-18 through a distinct regulatory process known as 'priming' or Signal 1 (Bauernfeind *et al*., 2009; Bauernfeind *et al*., 2011b; Budai *et al*., 2013; Burm *et al*., 2015; Fann *et al*., 2013b; Frederick Lo *et al*., 2008; Ghonime *et al*., 2014; Gross *et al*., 2011; Hara *et al*., 2013; He *et al*., 2012; Juliana *et al*., 2010; Kang *et al*., 2000; Legos *et al*., 2001; Liao *et al*., 2012; Liu *et al*., 2004; Liu *et al*., 2013; Mariathasan & Monack, 2007; Okada *et al*., 2014; Qiao *et al*., 2012; Savage *et al*., 2012; Schroder *et al*., 2012; Tamatani *et al*., 2000; Weber *et al*., 2015; Zhao *et al*., 2013a). Several studies have provided evidence that activation of NF-κB and MAPK(s) signaling pathways occur in neurons and glial cells during ischemic stroke (Arumugam *et al*., 2011; Cheng *et al*., 2014; Guan *et al*., 2006; Legos *et al*., 2001; Lok *et al*., 2015; Murata *et al*., 2012; Namura *et al*., 2001; Piao *et al*., 2003; Tang *et al*., 2007; Wang *et al*., 2004; Zhang *et al*., 2005, Zhao et al., 2013b). Our current data shows that I/R-induced activation of both NF-κB and MAPK(s) signaling pathways were significantly down regulated by IF. This was supported by numerous studies that

alternate-day fasting is cardioprotective and neuroprotective against age-induced inflammation by inhibiting NF-κB and MAPK(s) activation and oxidative damage via inhibition of the DNA binding activity of phosphorylated-p65-NF-κB and activator protein 1 (AP-1) (Castello *et al*., 2010; Jung *et al*., 2009; Tajes *et al*., 2010). Consequently, the NF-κB and MAPK(s) signaling pathways may induce the expression of NLRP1 and NLRP3 inflammasome proteins and the precursors of IL-1β and IL-18 in the brain under ischemic conditions (Bauernfeind *et al*., 2009; He *et al*., 2012; Kang *et al*., 2000; Liu *et al*., 2004; Zhao *et al*., 2013a). Our previous findings indicate that cerebral ischemia increased the expression of NLRP1 and NLRP3 inflammasome proteins and precursors of IL-1β and IL-18, and increased inflammasome activation demonstrated by an accumulation of cleaved caspases-1 and 11, and mature IL-1β and IL-18 (Fann *et al*., 2013a). Hence, our present findings suggest that the neuroprotective effects of IF are associated with a significant reduction in the levels of NLRP1 and NLRP3 inflammasome proteins as well as precursors of IL-1β and IL-18 in a mouse model of focal ischemic stroke.

Our data indicate that IF significantly attenuated ischemia-induced activation of caspase-3, which was associated with a decreased production of cleaved caspase-1 and 11 and both mature pro-inflammatory cytokines, IL-1β and IL-18. Cleaved caspase-1 has been shown to induce apoptotic cell death by cleaving and activating both executioner caspases-3 and 7, and Bid (BH3 interacting death domain agonist) into its truncated form, inducing intrinsic and extrinsic apoptotic cell death, respectively (Erener *et al*., 2012; Frederick Lo *et al*., 2008; Guégan *et al*., 2002; Liu *et al*., 2004; Walsh *et al*., 2011; Zhang *et al*., 2003). Furthermore, cleaved caspase-11 can activate caspase-3 and cause apoptosis in neurons and glial cells under ischemic conditions (Kang *et al*., 2000; Kang *et al*., 2002; Kang *et al*., 2003; Kayagaki *et al*., 2011). In addition, it was shown that cleaved caspase-1 might require the presence of cleaved caspase-11 for the maturation of precursors IL-1β and IL-18 (Kang *et al*., 2000, Kang *et al*., 2002; Kayagaki *et al*., 2011; Wang *et al*., 1998).

While the neuroprotective mechanism(s) behind IF reducing inflammasome signaling in the brain following ischemic stroke remained to be fully determined, the present data fits a model whereby IF may be able to inhibit inflammasome priming by decreasing the activity of both intracellular NF-κB and MAPK(s) signaling pathways through the following plausible mechanisms including - a down regulation in the expression of PRRs such as TLR-2, TLR-4 and RAGE on neurons and glial cells in the ischemic penumbra via an unknown mechanism(s) and/or an increase in the expression and activity of silent information regulator-1 (SIRT1) induced by IF, which deacetylates key regulatory proteins associated with the NF-κB and MAPK(s) signaling pathway in the brain rendering them inactive (Aris *et al*., 2010; Singh *et al*., 2015; Sun *et al*., 2001; Tajes *et al*., 2010; Vasconcelos *et al*., 2014). Hence, these potential mechanisms induced by IF would be

expected to reduce the expression of NLRP1 and NLRP3 inflammasome proteins and both precursor IL-1β and IL-18, thereby decrease the number of inflammasome complexes formed and subsequent production of activated proteins such as cleaved caspase-1 and 11, and maturation of precursors IL-1β and IL-18 in cerebral tissue following ischemic stroke.

Dietary restriction in the form of CR and IF were both shown to substantially increase the levels of anti-apoptotic proteins Bcl-2 and Bcl-xL in cardiomyocytes and hepatocytes (Katare *et al*., 2009; Niemann *et al*., 2010; Peart *et al*., 2012; Sokolovic *et al*., 2013). Numerous studies have demonstrated that Bcl-2 can directly bind and inhibit the NLRP1 and NLRP3 receptors in immune cells such as macrophages by specifically preventing ATP from binding onto the nucleotide-binding domain (NBD) of both receptors in order to form the central core of the inflammasome complex, which is an ATP-dependent process (Bruey *et al*., 2007; Fann *et al*., 2013a; Faustin *et al*., 2009; Shimada *et al*., 2012). Therefore, inhibiting the activation and subsequent oligomerization of the NLRP1 and NLRP3 receptors is expected to reduce the number of NLRP1 and NLRP3 inflammasomes formed and thereby attenuate the activation of caspase-1 and 11, and maturation of both precursor IL-1β and IL-18 in the cytosol (Bruey *et al*., 2007; Fann *et al*., 2013b; Faustin *et al*., 2009; Shimada *et al*., 2012). In addition, it was shown that Bcl-xL, another anti-apoptotic protein was able to directly bind and inhibit the NLRP1 receptor in macrophages through a similar mechanism as Bcl-2, but whether Bcl-xL is able to inhibit the NLRP3 receptor remains to be established (Bruey *et al*., 2007; Faustin *et al*., 2009). Accordingly, it appears that Bcl-2 and Bcl-xL are both tight regulators of NLRP1 receptor activation; however, whether Bcl-xL regulates NLRP3 receptor activation, and how IF increases both Bcl-2 and Bcl-xL expression levels in cerebral tissue under *in vivo* ischemic conditions remains to be fully determined (Bruey *et al*., 2007; Faustin *et al*., 2009; Shimada *et al*., 2012).

Additional plausible mechanisms behind IF attenuating inflammasome signaling in the brain following ischemic stroke may include IF decreasing inflammasome assembly. This may be achieved by an increased expression and activity of SIRT1/2 induced by IF or activators of SIRT1/2 (e.g. resveratrol) that continuously deacetylate microtubules, in particular, α-tubulin, which in turn would prevent an accumulation of acetylated α-tubulin in the cytosol during times of cellular stress that was demonstrated to be required for mediating inflammasome assembly by transporting ASC on the mitochondria into close proximity to the NLRP3 receptor on the endoplasmic reticulum upon activation in order to facilitate the formation of the NLRP3 inflammasome complex (Misawa *et al*., 2013; Misawa *et al*., 2015). However, it remains to be determined whether IF has a similar effect in decreasing NLRP1 inflammasome assembly mediated by SIRT1/2. Moreover, it was demonstrated in numerous studies that dietary restriction was able to increase the production of ketone bodies, in

particular, β-hyroxybutyrate, which was elegantly shown in a recent study to inhibit the formation of the NLRP3 inflammasome by preventing both potassium (K^+) efflux and ASC oligomerization in macrophages; critical events that are required for NLRP3 receptor activation and ASC-dependent inflammasome formation, respectively (Lin *et al*., 2015; Maalouf *et al*., 2009; Mahoney *et al*., 2006; Nakamura *et al*., 2014; Shimazu *et al*., 2013; Youm *et al*., 2015). However, it remains to be established whether IF has a similar effect in decreasing NLRP1 inflammasome assembly mediated by β-hyroxybutyrate. Hence, these potential neuroprotective mechanisms induced by IF would be expected to reduce the production of cleaved caspase-1 and 11, and maturation of precursors IL-1β and IL-18 in cerebral tissue following ischemic stroke.

4.5 Conclusion:

The present findings demonstrate for the first time that a neuroprotective effect of IF can suppress inflammasome activation in the cerebral cortex in a mouse model of focal ischemic stroke. IF was shown to inhibit the activation of the NF-κB and MAPK(s) signaling pathways, which likely contributed to a reduction in the expression of NLRP1 and NLRP3 inflammasome proteins and both precursor IL-1β and IL-18, thereby decreasing the activation of caspase-1 and 11, and maturation of both precursor IL-1β and IL-18, thereby attenuating apoptotic cell death in cerebral tissue following ischemic stroke. These findings suggest that therapeutic interventions that target inflammasome signaling such as inflammasome priming, assembly or activity in the brain during ischemia may provide new opportunities in the future treatment of ischemic stroke.

4.6 References:

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CHAPTER 5:

Conclusion and Future Directions

Stroke is the second leading cause of mortality worldwide resulting in approximately 6 million deaths every year and is a major cause of long-term disability. Hence, it is without question that stroke poses a major economic and health burden globally. Recent findings have provided insight into a newly described inflammatory mechanism fundamental to the innate immune system that may contribute to neuronal and glial cell death during cerebral ischemia known as sterile inflammation. There is emerging evidence to suggest that plasma membrane pattern recognition receptors (PRRs) on neurons and glial cells can play an important role in activating nuclear factor-kappa B (NF-κB) and mitogen activated protein kinase (MAPKs) pathways. This occurs in response to endogenous danger signals initiated by substances released from necrotic cells at the site of injury, leading to an increased production of pro-inflammatory cytokines and to neuronal and glial cell death mediated by intracellular multi-protein complexes termed inflammasomes. Thus, understanding the role of inflammasome signalling is indeed critical in order to reveal the novel mechanisms that are responsible for inducing neuronal and glial cell death in ischemic stroke.

Currently, intravenous recombinant tissue plasminogen activator (r-tPA) is the only pharmacological agent approved by the US Food and Drug Administration (FDA) for acute stroke therapy by inducing thrombolysis following a thrombotic occlusion. However, there are several limitations towards the use of r-tPA in stroke patients such as patient age, the presence of co-morbidities and the use of concurrent medications (like anti-platelet agents) that may increase the risk of intracerebral haemorrhage in conjunction with r-tPA treatment. Nevertheless, the most limiting exclusion criterion for stroke patients receiving r-tPA is its narrow therapeutic window of 3-4.5 hours from symptom onset to treatment. Hence, urgent scientific research into finding an alternative approach for treating acute ischemic stroke has enabled another dimension of therapeutic intervention to develop known as neuroprotection.

Considerable research has been conducted in the search for an ideal neuroprotective agent for over a decade. In spite of neuroprotective agents decreasing neuronal cell death and infarct size in cell culture and animal stroke models, respectively, each of these agents failed in clinical trials involving stroke patients due to deleterious side effects and/or low efficacy. Despite a number of possible reasons contributing to the failure such as anatomical and physiological differences in the brains of animals and humans, heterogeneity between animal stroke models and the presence of

comorbidities; a common underlying feature is that previous neuroprotective agents only targeted a particular cell injury mechanism in the ischemic cascade, and in either single or multiple cell types. Hence, development of neuroprotective agents that can target multiple cell injury mechanisms in multiples cell types, in particular, the inflammasome signalling pathway may be advantageous as it is responsible for causing a number of cell injury mechanisms in multiple cell types in the brain during cerebral ischemia, and appropriately, scientific research into this area is warranted in the future treatment of ischemic stroke; although it is presently unknown whether any off-target effects on the normal physiology of other systems will be effected.

Novel potential therapies envisaged to target multiple cell injury mechanisms in multiple cell types in the brain following cerebral ischemia include - intravenous immunoglobulin (IVIg) and intermittent fasting (IF). IVIg is a purified polyclonal immunoglobulin preparation obtained from the plasma of several thousand healthy donors, which have been demonstrated to modulate a number of inflammatory mechanisms. It is a therapeutic modality approved by the FDA that is used to ameliorate various autoimmune and inflammatory conditions. Numerous experimental studies by our laboratory for the first time demonstrated that administration of IVIg was able to significantly attenuate brain infarct size (50-60%) and mortality, and improve functional outcome in mice subjected to experimental stroke. The efficacy of IVIg is attributed to a number of mechanisms including its ability to neutralise active complement fragments (C3b) in ischemic brain tissue, which accordingly reduced endothelial cell adhesion molecule (i.e. ICAM-1) production and infiltration of inflammatory cells (i.e. neutrophils), subsequently reducing inflammation and neuronal apoptosis at the site of injury. In addition, IVIg was demonstrated to decrease NF-κB and MAPK(s) signalling pathway activity in primary cortical neurons under ischemic conditions, which reduced neuronal apoptosis through an unknown mechanism(s). Moreover, IF is a form of dietary restriction and encompasses alternate periods of *ad libitum* feeding and fasting, which have been proven to extend lifespan and decrease the development of age-related diseases such as cardiovascular disease. Previous experimental studies by our laboratory demonstrated that IF was able to significantly attenuate brain infarct size and mortality, and improve functional outcome in young (3 months) and middle-aged (9 months) male mice subjected to experimental stroke. The efficacy of IF to protect neurons against ischemic injury involved the coordinate upregulation of multiple neuroprotective proteins such as neurotrophic factors such as BDNF and bFGF; protein chaperones, including Hsp70 and GRP78; antioxidant enzymes, such as SOD and HO-1; and downregulation of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) at the site of injury. However, the precise mechanism(s) in how IVIg and IF directly protect neurons and cerebral tissue

from inflammasome-mediated sterile inflammation following ischemic stroke remains to be determined and is a major focus of this research thesis.

In the first study of this research thesis, we performed a comprehensive investigation into the dynamic expression patterns of the NLRP1 and NLRP3 inflammasome proteins and both IL-1β and IL-18 in mouse primary cortical neurons subjected to simulated ischemia and in a model of focal ischemic stroke in C57BL/6J mice. In addition, determined whether the NLRP1 and NLRP3 inflammasome could be targeted with a Caspase-1 inhibitor (Ac-YVAD.cmk) and IVIg for therapeutic intervention. The study demonstrated that ischemia-like conditions increased the levels of NLRP1 and NLRP3 inflammasome proteins and both IL-1β and IL-18 in primary cortical neurons. Similarly, levels of NLRP1 and NLRP3 inflammasome proteins and both IL-1β and IL-18 were elevated in ischemic brain tissues of mice subjected to ischemic stroke. Moreover, Ac-YVAD.cmk and IVIg treatment protected primary cortical neurons and brain tissue by a mechanism(s) involving Caspase-1 inhibition and suppression of NLRP1 and NLRP3 inflammasome activity, respectively, under *in vitro* and *in vivo* ischemic conditions.

In the second study of this research thesis, we provide evidence that both the NF-κB and MAPK(s) signaling pathways are involved in regulating the expression and activation of NLRP1 and NLRP3 inflammasome proteins and both precursors IL-1β and IL-18 in mouse primary cortical neurons subjected to simulated ischemic conditions. This study established that activation of either the NF-κB and MAPK(s) signaling pathways are responsible for inducing the expression of NLRP1 and NLRP3 inflammasome proteins and both precursors IL-1β and IL-18 in neurons under ischemic conditions. In addition, the present study demonstrated that pharmacological inhibition of both the NF-κB and MAPKs signaling pathways was able to directly attenuate NLRP inflammasome activation and maturation of both IL-1β and IL-18 in neurons under ischemic conditions. Furthermore, this study provided supporting evidence that IVIg treatment was able to significantly decrease NF-κB and MAPK(s) signalling pathway activation, which decreased the production of NLRP1 and NLRP3 inflammasome proteins and both IL-1β and IL-18, and subsequently attenuate NLRP1 and NLRP3 inflammasome activity; in addition to increasing the expression of anti-apoptotic proteins, Bcl-2 and Bcl-xL, in primary cortical neurons following ischemic conditions.

In the third study of this research thesis, we investigated the impact of IF on NLRP1 and NLRP3 inflammasome activation in a model of focal ischemic stroke in C57BL/6J mice. This study demonstrated that IF was able to significantly decrease apoptotic tissue damage by attenuating the

activation of the NF-κB and MAPK(s) signaling pathways, which possibly reduced the expression and activation of NLRP1 and NLRP3 inflammasome proteins, and both IL-1β and IL-18; in addition to increasing the expression of anti-apoptotic proteins, Bcl-2 and Bcl-xL, in ischemic brain tissues.

Despite establishing a number of novel findings from the aforementioned studies in this research thesis, there are still a number of questions that remain to be addressed, which will require considerable research in the future. Nevertheless, these studies have provided a tremendous platform to further conduct additional studies in order to fully understand the pathophysiology of inflammasome signaling and mechanism(s) behind the protective effects of IVIg and IF in the brain following ischemic stroke. Firstly, future research should be conducted into identifying and understanding other molecular and cellular targets in inflammasome signaling or other signaling pathways modulated by IVIg and IF that can protect neurons; in addition to, other cell types such as astrocytes, microglia and endothelial cells individually or in co-cultures with neurons to form the neurovascular unit from undergoing cell death under *in vitro* and *in vivo* ischemic conditions. Secondly, identifying and understanding the potential stimuli(s) and mechanism(s) behind NLRP1 and NLRP3 receptor activation and inflammasome formation in neurons and glial cells under *in vitro* and *in vivo* ischemic conditions is a great research potential avenue to explore as data pertaining to this issue are needed. Thirdly, determining the degree of cell death or tissue damage inflicted by the NLRP1 and NLRP3 inflammasome individually is warranted by either knocking down or overexpressing the NLRP1 and NLRP3 receptors in neurons and glial cells, or utilizing NLRP1 and NLRP3 knockout mice under *in vitro* and *in vivo* ischemic conditions, respectively. Lastly, the use of female and aged mice would serve as excellent models to achieve a more comprehensive understanding of the pathophysiology of inflammasome signaling and mechanism(s) behind the protective effects of IVIg and IF in the brain following ischemic stroke.

In summary, the findings from this research thesis provided evidence of expression and a functional role of the NLRP1 and NLRP3 inflammasome in neuronal apoptosis and cerebral tissue damage under *in vitro* and *in vivo* ischemic conditions. It was demonstrated for the first time that activation of the NF-κB and MAPK(s) signaling pathways are responsible for inducing the expression and activation of NLRP1 and NLRP3 inflammasome proteins and both precursors IL-1β and IL-18 in primary cortical neurons under ischemic conditions. Furthermore, we established for the first time that a neuroprotective effect of IVIg and IF involved suppressing NLRP1 and NLRP3 inflammasome activity through a mechanism(s) associated with decreasing the NF-κB and MAPK(s) signalling pathways, which attenuated production of NLRP1 and NLRP3 inflammasome

proteins and both IL-1β and IL-18 in primary cortical neurons and/or brain tissues under ischemic conditions. In addition, it was demonstrated for the first time that another neuroprotective effect of IVIg and IF involved increasing the expression of anti-apoptotic proteins, Bcl-2 and Bcl-xL, through an unknown mechanism(s) that remain to be established in primary cortical neurons and/or brain tissues under ischemic conditions. Collectively, our findings identified NLRP1 and NLRP3 inflammasome inhibition as a novel mechanism by which IVIg and IF can protect brain cells against ischemic damage, suggesting a potential clinical benefit of therapeutic interventions that can target ischemic stroke-induced inflammasome priming, assembly and activation in future treatments of ischemic stroke.