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An *in vitro* and *in vivo* evaluation of the immuno- and neurotoxicological effects of Fusaric Acid on altered protein kinase signalling cascades

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DECLARATION I

An *in vitro* and *in vivo* evaluation of the Immuno- and Neurotoxicological effects of Fusaric Acid on altered protein kinase signalling cascades

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This thesis is submitted to the School of Laboratory Medicine and Medical Science, College of Health Science, University of KwaZulu-Natal, Howard College, to satisfy the requirements for the degree of PhD in Medical Biochemistry.

This is the thesis, in which the chapters are written as a set of discrete research publications that have either been accepted or submitted to internationally recognized ISI-rated peer-reviewed journals. This includes an introduction and summary.

This is to certify that the contents of this thesis are the original research work of **Ms Shanel Dhani**, carried out under our supervision at the Department of Medical Biochemistry, University of KwaZulu-Natal, Howard College Campus.

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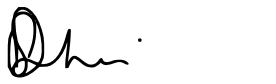

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PUBLICATIONS

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
δ	Delta
γ	Gamma
µg	Microgram
µl	Microliter
µM	Micromolar
ºC	Degree celsius
%	Percentage
3HB	3-ß-Hydroxybutyrate
ADP	Adenosine triphosphate
AHRI	Africa health research institute
Akt	Protein kinase B
ALS	Amyotrophic lateral sclerosis
AMPK	AMP-activated protein kinase
AP-1	Activator protein 1
APC	Antigen presenting cell
ASK1	Apoptosis signal-regulating kinase 1
ATM	Ataxia telangiectasia mutated

ATP	Adenosine triphosphate
BBB	Blood brain barrier
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
BDNF	Brain derived neurotrophic factor
BHT	Butylated hydroxytoluene
BRU	Biomedical Resource Unit
BSA	Bovine serum albumin
BW	Body weight
Ca^{2+}	Calcium
CaMK	Calmodulin-dependent protein kinase
CAMKK2	Calcium/calmodulin-dependent protein kinase kinase 2
CAT	Catalase
CBP	CREB binding protein
cDNA	Complementary DNA
cm^3	Centimetre cube
CNS	Central nervous system
CO_2	Carbon dioxide
CRE	cyclic AMP response element

CREB	cyclic AMP response element binding
CSF	Cerebrospinal fluid
Da	Dalton
dH_2O	Distilled water
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
ECL	Enhanced Chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ETC	Electron transport chain
FA	Fusaric Acid
FCS	Foetal calf serum
FoxO	Forkhead box O
g	Gram
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter
GPCR	G protein coupled receptors
GSK	Glycogen synthase kinase
h	Hour

HCl	Hydrogen Chloride
HIF-1α	Hypoxia inducible factor-1α
HIV	Human immunodeficiency virus
HM	Hydrophobic motif
HRP	Horseradish peroxidase
hrs	Hours
HSP70	Heat shock protein 70
HSV-1	Herpes simplex virus-1
HSV-2	Herpes simplex virus-2
HVA	Homovanillic acid
IC_{50}	half maximum inhibition
i.e.	that is
IGFIR	Insulin-like growth factor I receptor
IgG	Immunoglobulin G
IL-1ß	Interleukin-1ß
IL-3	Interleukin-3
IL-6	Interleukin-6
IL-10	Interleukin-10
ILK	Integrin-linked kinase
JNK	c-Jun N-terminal kinase

KCl	Potassium chloride
kDa	Kilo Dalton
kg	Kilogram
KID	kinase inducible domain
LD_{50}	Lethal dose 50%
LKB1	Liver kinase B1
LPS	Lipopolysaccharides
M	Molar
mA	Milliampere
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MDA	Malonaldehyde
MEK	MAP/ERK kinase
MEKK1	MAPK/ERK kinase kinase 1
mg	Milligram
MHPG	3-Methoxy-4-hyroxy-phenylglycol
min	Minute
MK	MAPK-activated protein kinase
MKK3	Mitogen-activated protein kinase kinase 3

MKK4	Mitogen-activated protein kinase kinase 4
MKK6	Mitogen-activated protein kinase kinase 6
MKK7	Mitogen-activated protein kinase kinase 7
ml	Millilitre
MLK	Mixed-lineage kinase
mM	Millimolar
MNK	MAPK-interacting kinase
mPTP	Mitochondrial permeability transition pore
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger RNA
MSK	Mitogen-and-stress-activated kinase
mTOR	Mammalian target of rapamycin complex
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
n	Number
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine diphosphate
NADPH	Nicotinamide adenine diphosphate phosphate hydrogen
NFDM	Non-fat dry milk
NF-κB	Nuclear factor-kappa B

ng	Nanogram
NGF	Nerve growth factor
NK	Natural killer
nm	Nanometer
Nrf1	Nuclear respiratory factor 1
Nrf2	Nuclear respiratory factor 2
p	Phosphorylated
p70S6K	p70 ribosomal s6 protein kinase
PA	Picolinic acid
PARP-1	Poly (ADP-ribose) polymerase 1
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pg	Picogram
PGC1-α	Peroxisome proliferator-activated receptor gamma coactivator 1-α
PDHE1ß	Pyruvate dehydrogenase E1ß
PDK1	Pyruvate dehydrogenase kinase 1
PH	Pleckstrin homology
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase

$PI3P_3$	Phosphatidylinositol-3-phosphate
PIP3	Phosphatidylinositol 3,4,5- triphosphate
PKA	Protein kinase A
PKM2	Pyruvate kinase isozymes M2
PMA	Phorbol 12-myristate 13-acetate
PPARα	Peroxisome proliferator-activated receptor α
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
RBD	Relative band density
RBI	Relative band intensity
RLU	Relative Light Units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute
RSK	Ribosomal s6 kinase
RT	Room temperature
RTK	Receptor tyrosine kinase
S	Serine
SAPK	Stress activated protein kinase
SD	Standard deviation

SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
STAT3	Signal transducer and activator of transcription 3
T	Threonine
T_3	Triiodothyronine
T_4	Thyroxine
TAK1	Transforming growth factor β-activated kinase 1
TB	Tuberculosis
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Tricarboxylic acid
TCR	T cell receptor
TFAM	Mitochondrial transcription factor A
TGFß-1	Transforming growth factor ß-1
Th	T helper
Thp-1	Acute monocytic leukemic cell
TNF-α	Tumour necrosis factor-α
TNFR1	Tumour necrosis factor receptor 1
TPI	Triose-phosphate isomerase

TRADD	TNF-α receptor associated death domain
Treg	T regulatory
TrkB	Tropomyosin receptor kinase B
TSC2	Tuberous sclerosis complex 2
TSH	Thyroid stimulating hormone
TTBS	Tris buffer saline with tween 20
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
WST-1	Water soluble tetrazolium-1

ABSTRACT

Mycotoxins are naturally occurring toxins produced by moulds which contaminate numerous crops and foodstuffs including cereals, nuts and fruits. When consumed, mycotoxins pose a serious threat to both human and animal well-being, causing acute poisoning or chronic effects such as immune deficiency and cancer. Fusaric acid (FA), a ubiquitous mycotoxin produced by *Fusarium* species, is a frequent contaminate of staple grain-based foods, and is a specific inhibitor of dopamine-β-hydroxylase in human and animal hosts, thereby affecting the autonomic nervous system by causing significant alterations in catecholamine metabolism. The latter effect is believed to occur through the competitive binding of FA with tryptophan to albumin in circulation. Although several studies have reported the oral activity of FA in circulation and the nervous system, the immuno- and neurotoxic effects of FA are unknown. Therefore, in this study we aimed to investigate the immunotoxic and neurotoxic potential of FA, using *in vitro* human and *in vivo* murine models.

On a daily basis, protein kinases are required to integrate developmental cues and environmental stimuli to decide cell fate (cell death or survival). Thus, alterations to mitogen-activated protein kinases (MAPKs) in the immunotoxicity of FA was assessed *in vitro* on healthy human peripheral blood mononuclear cells (PBMCs) and on the human acute monocytic leukemic (Thp-1) cell line at an acute exposure (1 day) (Chapter 2); with Thp-1 cells (IC_{50}-107.7 μg/ml) showing a greater susceptibility to FA exposure than PBMCs (IC_{50}-240.8 μg/ml). Notably, elevated stress-induced stimuli (increased oxidative stress and ATP depletion) activated the pro-apoptotic signalling of phosphorylated-extracellular signal-regulated kinase (p-ERK), resulting in initiation of intrinsic apoptosis evidenced by the decreased phosphorylation of B-cell lymphoma 2 (p-Bcl-2; an anti-apoptotic protein) and subsequent activation of caspase-9 and caspase-3/7 activities in Thp-1 cells. In contrast, a caspase-independent (reduced caspase -8, -9 and -3/7 activities) form of cell death (paraptosis) was induced in PBMCs that was possibly mediated by ERK and c-Jun N-terminal kinase (JNK) in response to metabolic stress (decreased cellular ATP availability).

The significant ATP depletion in immune cells then led to the assessment of the metabolic effects of FA in the brain; since the brain is exposed to the peripheral effects of FA and is a highly metabolic organ. Therefore, the next chapter (Chapter 3) investigated the neurometabolic effects of FA via the protein kinase B (Akt) and AMP-activated protein kinase (AMPK) signalling pathways (which are central regulators of cellular energy and metabolism) in C57BL/6 mice at acute (1 day) and prolonged exposure (10 days). Acute exposure to FA, augmented Akt signalling following the increased expressions of upstream regulators phosphatidylinositol 3-kinase (PI3K), mammalian target of rapamycin (mTOR) and p70 ribosomal S6 protein kinase (p70S6K). Activated Akt inhibited glycogen

synthase kinase 3 (GSK3) activity with the simultaneous activation of AMPK, p53 phosphorylation and reduced glucose transporter (GLUT)-1 and -4 expression, potentially suppressing neuronal glucose entry. However, following prolonged exposure, FA dampened PI3K/Akt and AMPK signalling, but increased the expression of GLUT transporters (1 and 4) in mice brain. Despite the differential regulation of glucose receptors by the PI3K/Akt and AMPK pathways (at acute and prolonged exposures), neuronal ATP failed to rise despite the increased pyruvate dehydrogenase E1ß (PDHE1β) activity [a regulatory subunit of glycolysis and the tricarboxylic acid cycle (TCA) cycle] at both 1 and 10 days; suggesting that FA mediates ATP depletion independent of metabolic signalling.

Given the evident neurometabolic disturbances mediated by FA and its importance as a risk factor for neurometabolic-related diseases, the next chapter (Chapter 4) investigated the neurotoxic potential of FA in C57/BL6 mice following acute (1 day) and prolonged exposures (10 days) and its influence on cyclic AMP (cAMP) response element binding (CREB) signalling, an essential transcription factor, commonly activated by MAPKs, that is responsible for the brain's neuroprotective responses through the regulation of neurotrophic and metabolic signals in the brain. After an acute administration of FA, CREB signalling was enhanced with a simultaneous increase in brain derived neurotrophic factor (BDNF) expression; whilst FA suppressed CREB/BDNF signalling following a prolonged exposure. In contrast, protein expressions of MAPKs (ERK, JNK and p38) negatively correlated with CREB activity; inferring that FA induced MAPK-independent activation of CREB responses. Consistent with caspase activation in Thp-1 cells, FA increased caspase activities (8, -9 and -3/7) at 1- and 10 days post-exposure, although to a lesser extent at a prolonged treatment. However, despite enhanced caspase activity, microanalysis of brain tissue showed no prominent histological markers of damage to extracellular tissue or neuronal cells. Although FA showed no significant neurotoxicity, alterations in glial cell density patterns at both acute and prolonged exposures were observed. Besides the neuroprotective roles of CREB/BDNF signalling in the brain, CREB and BDNF are also involved in memory development and psychiatric disorders. Therefore, although FA may have not been neurotoxic, dysregulation of CREB/BDNF signalling impacts normal brain functions which potentially plays a role in the development of neurological disorders with longer periods of exposure.

Collectively, although FA demonstrated significant toxicity towards Thp-1 cells, FA did not display cytotoxicity to healthy immune and neuronal cells, suggesting that compromised cellular systems may be more vulnerable to the effects of FA. In addition, while FA did alter MAPK, PI3K/Akt, AMPK and CREB pathways, which are important regulators of cell survival/death and energy homeostasis, these pathways are also involved in several other fundamental cellular processes, including gene expression, cell differentiation, inflammation, and synaptic plasticity; thus, modifications to their activities could have severe outcomes in health and disease.

CHAPTER ONE

1.1. Introduction

With a rapidly increasing global population and warming climates that affect the growth of food crops; food security and its impact on public health and economic stability is an inevitable concern (Abdallah et al. 2018). Regions such as Africa, America, China and India, have emerged as hot spots for climate change, providing conditions which are ideal for fungal contamination (Reilly 2007). This has severely impacted agricultural sustainability and the quality of agricultural products (Reilly 2007). Fungal moulds produce secondary metabolites, known as mycotoxins, that contaminate a large proportion of the world's staple diets including groundnuts, maize, spices, fruits and other essential commodities (Misihairabgwi et al. 2019). For many low-income countries, including Africa, these dietary staples are frequently contaminated by high mycotoxin concentrations (Abdallah et al. 2018; Misihairabgwi et al. 2019). Burdened by poverty and a demanding well-stocked food reserve, has increased the demand for low cost foodstuff, the use of substandard ingredients (Streit et al. 2012). In developing countries, communities are largely unaware of the risks associated with mycotoxin exposure and often follow poor harvesting methods, improper storage systems, and inadequate transportation and marketing, that contribute to fungal growth and the proliferation of mycotoxins (Shephard 2008). Although exposure to mycotoxins occurs mostly through ingestion of contaminated food stuff, human exposure can occur via the dermal and inhalation routes during the pre- and post-harvest storage periods; and therefore, represents a major threat to human and animal health (Zain 2011). Whilst the extent to which mycotoxins affect human health and disease is unclear, mycotoxins which impact human health include aflatoxins, ochratoxins (produced by *Aspergillus* species), trichothecenes, deoxynivalenol, and fumonisins (produced by and *Fusarium* species); and these mycotoxins possess carcinogenic, mutagenic, oestrogenic, gastrointestinal, immunotoxic and neurotoxic properties (Freire and da Rocha 2017; Fung and Clark 2004; Zain 2011).

Most importantly, several *Fusarium* species are rapidly spreading globally due to their acclimatization to environmental changes in various regions arounds the world, increasing their occurrence (Bertero et al. 2018). Among the *Fusarium* metabolites, the mycotoxigenic properties of trichothecenes, deoxynivalenol, zearalenone and T2-toxin are well-characterized based on their impact on public health (Freire and da Rocha 2017); whilst it is not clear how other *Fusarium* mycotoxins, such as enniatins, moniliformin and FA impact the biological system. Fusaric acid, in particular, has a high prevalence in feed and food products worldwide, sometimes at relatively high concentrations, which increases the risks of FA exposure via the food chain (Smith and Sousadias 1993; Streit et al. 2013; Swamy et al. 2002).

Primarily described as a plant pathogen, FA exerts its pathogenicity by altering membrane- and mitochondrial activity, together with the inhibition of ATP synthesis and metalloenzymes which result in apoptotic and necrotic cell death events (López-Díaz et al. 2018; Singh et al. 2017). In animals, acute doses of FA have been shown to cause lethargy, vomiting and appetite suppression, whilst significantly inhibiting dopamine-ß-hydroxylase activity, altering monoamine tissue levels (Diringer et al. 1982; Leung et al. 2007; Osumi et al. 1973); with the latter effect attributing to FA's pharmacological activity in hypertension and behavioural disorders in the human population (Goodwin and Sack 1975; Terasawa and Kameyama 1971; Terasawa et al. 1976). More recently, *in vitro* studies have supported FA's cytotoxic activity on various human cancer cell lines and viral infections, crediting FA with antiproliferative and antiviral properties (Elaasser and El Kassas 2013).

Chemically diverse from its *Fusarium* mycotoxin counterparts, FA is a structural derivative of picolinic acid (PA) and contains a butyl functional group that confers its lipophilic nature and enhances its ability to cross biological membranes (Kim et al. 2014). Additionally, FA's physiochemical properties (being lipophilic and of low molecular weight) contributes to its biological significance which allows for easy passage across the brains' protective, blood brain barrier (BBB), in order to exert its neurological effects. FA also indirectly alters neurological activity due to its effects in peripheral circulation, where immune cells are first exposed to its toxicity. In circulation, FA competitively binds to albumin preventing the binding of tryptophan, which is then shuttled to the brain and alters the production of neurotransmitters via the tryptophan pathway (Chaouloff et al. 1986; Osumi et al. 1973). However, despite FA's ability to directly interact with the immune and nervous systems the exact immuno- and neuro-toxicities of FA remain unknown.

The immune system is a diverse network that is divided into two distinct components, the innate and adaptive immune systems, comprised of various types of immune cells (lymphoid and myeloid) with defence (against foreign particles), surveillance and homeostatic functions (Abbas et al. 2015; Council 1992). Whilst, the brain is a complex and highly compartmentalized organ that is responsible for controlling essential processes such as maintaining heart rate and respiration (brainstem), vision and sight (cerebrum), and voluntary movements (cerebellum) (Aloisi 1999; Kolb and Whishaw 2001). The reciprocal communication between the immune system and the brain using both humoral and neuronal routes of communication for fine-tuning immune (antibody and cytokine production) and nervous (appetite, behaviour and thermoregulation) responses is well-established and has recently gained significant attention for the role of metabolic and immune dysfunction in psychiatric diseases such as Alzheimer's, dementia and major depressive disorder (Louveau et al. 2015; Russo and McGavern 2015; Wilson et al. 2010). This intricate relationship between the immune system and the brain is thought to be influenced by a number of signalling pathways. These pathways include major serine threonine

kinase proteins MAPK, Akt, AMPK and CREB that regulate inflammatory responses, neurotransmitter metabolism, neuroendocrine activity, neural plasticity and alterations of brain circuitry (Walsh et al. 2015).

The activation (inflammatory response), proliferation, differentiation and survival of immune cells are important in directing immunomodulatory responses associated with immune susceptibility and tissue homeostasis (Fang et al. 2009). Several studies have shown that the primary regulation of these critical immune-behavioural responses, are mediated by the MAPK signalling pathway (Fang et al. 2009). The MAPKs are an evolutionary conserved family of serine/threonine protein kinases that are present in various cell compartments, including the nucleus, mitochondrion and cell membrane (Jeanneteau and Deinhardt 2011; Kim and Choi 2010). Grouped into three major components: ERK, JNK and p38, each MAPK signalling axis is activated by a sequential three-step phosphorylation process, notably composed of a MAPK kinase kinase (MAP3K), a MAPK kinase (MAP2K), and a MAPK (Jeanneteau *et al.*, 2011; Kim *et al.*, 2010). MAPKs modulate the activities of several proteins (by phosphorylation of their tyrosine, serine, or threonine amino acids) in response to various stimuli, such as environmental stresses (toxins, ionizing radiation, osmotic shock, oxidative stress), inflammatory cytokines and growth factors, which alters gene expression, mitosis, metabolism, cell differentiation, cellular stress responses and cell death or survival (Schroeter et al. 2002). In addition to their critical roles in key cellular activities, including cell fate and inflammation, the MAPK signalling pathways have been implicated in the pathogenesis of many inflammatory and neurological human diseases such as allergies, hepatitis, rheumatoid arthritis, gliosis, Alzheimer's and Parkinson's disease (Schroeter et al. 2002).

Although the mechanism of FA-induced toxicity has not yet been elucidated, its cytotoxicity has been associated with impaired mitochondrial function, decreased cellular energy availability and increased oxidative stress. As the brain is exposed to FA activity, these characteristic changes could have a significant impact on neuronal vulnerability to stress and may also play a role in the pathogenesis of several neurological diseases (Al Shahrani et al. 2017; Butterfield and Halliwell 2019). While MAPKs are central regulators of many physiological functions, MAPK proteins collaborate with other signalling pathways at several substrate target points to control cellular processes. The PI3K/Akt signalling cascade is a major receptor-mediated metabolic pathway closely related to MAPKs and promote cell proliferation, survival, nutrient uptake and metabolism (Zhou et al. 2015). Apart from the classical activation of receptor tyrosine kinases by growth factors and cytokines, other physiological stresses, such as oxidative stress, glucose deprivation, endoplasmic reticulum (ER) stress, and DNA damage activate PI3K and its downstream mediators, Akt (a serine/threonine protein kinase) and mTOR (an essential kinase required for the full activation of Akt), both key components of the PI3K/Akt signalling

cascade (Manning and Toker 2017; Vincent et al. 2011; Zhao et al. 2017). Following activation, Akt phosphorylates many target proteins, most notably caspase-9, GSK3, and CREB, which explains its involvement in cell proliferation, apoptosis and metabolism (Case et al. 2011; Saxton and Sabatini 2017).

Another primary effector in response to metabolic stress is the serine/threonine AMPK. Referred to as the master regulator of metabolism, AMPK is activated downstream of liver kinase B1 (LKB1) or calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2), by low intracellular energy levels and promotes metabolic reprogramming to increase glucose transport and ATP generation (Kim et al. 2016; Mihaylova and Shaw 2011). In addition, AMPK's activity is negatively regulated by Akt via direct phosphorylation or through the downstream effectors, p70S6K and GSK3. While AMPK and Akt are antagonistic pathways, they share downstream mediators [such as mTOR and forkhead box O (FoxO)] which is important in cell proliferation, survival and metabolism (Richter and Ruderman 2009).

Most of the current molecular knowledge which integrates nutrition, metabolic signalling and neuronal survival are orchestrated through the transcription factor, CREB. Activated by numerous growth factors, neurotransmitters and stress signals, CREB is a focal substrate of various signal transduction pathways, including MAPK and Akt, and has been extensively studied for its transcriptional control of cell proliferation, survival, differentiation, adaptive responses, glucose homeostasis, circadian rhythms, and synaptic plasticity (Lonze and Ginty 2002; Wang et al. 2018; Wen et al. 2010). Notably, in the brain, CREB mediates many of its adaptive (e.g. mitochondrial biogenesis during nutrient starvation) and cytoprotective responses through neurotrophic support (Nakazawa et al. 2002). Brain derived neurotrophic factor (BDNF) is the most widely investigated neurotrophin associated with CREB signalling, whose gene expression is directly regulated by CREB (Luo et al. 2017; Nakazawa et al. 2002). In addition, extracellular BDNF activates the receptor tropomyosin receptor kinase B (TrkB), engaging in downstream CREB signalling by promoting the expression of GLUT-3 (to stimulate neuronal energy metabolism), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (to increase mitochondrial biogenesis) and Bcl-2 (to inhibit apoptotic cell death), either directly or indirectly through CREB (Luo et al. 2017; McGregor and English 2018). Accordingly, BDNF has been suggested to be critical in numerous neuronal processes, including cell metabolism, survival and structure (Wang et al. 2018).

Given that these kinases (MAPK, Akt, AMPK and CREB) collaborate in transmitting signals from numerous stimuli to control precise intracellular processes in different subcellular compartments, this study aimed to evaluate the toxicity and metabolic effects of FA in lymphoid and myeloid immune cells

and the brain using *in vitro* immune cell culture and *in vivo* murine models by investigating FA-induced alterations to major protein kinase signalling cascades.

1.2. Literature review

1.2.1. Mycotoxins and human health

The world is currently inhabited by more than 7.6 billion people, with one in nine experiencing chronic undernourishment or starvation (Belz 2018). With the world population expected to reach 8.2 billion people by the year 2030, food security represents a significant challenge and a global struggle (De Laurentiis et al. 2016; Misihairabgwi et al. 2019). As the world confronts more pressing concerns relating to climate change, energy expenditure, severe poverty, food demand and the global economy, food safety is often neglected and overlooked (Reilly 2007). In continents such as Africa, majority of the population are challenged by extreme climates, decreasing amounts of arable land and face hunger daily (Abdallah et al. 2018; Misihairabgwi et al. 2019). As a result, maize-based commodities, which are easily grown in warmer temperatures, form staple diets and provide sustenance and management of hunger in many households (Reilly 2007). However, maize and small grain (wheat, barley and rye) crops cultivated in temperate regions (high temperatures and humidity) are prone to fungal colonization that produce toxic secondary metabolites, known as mycotoxins (Beukes et al. 2017). In addition to environmental factors, poor harvesting methods, improper storage and inadequate transportation of produce increases the risk of mycotoxin propagation (Shephard 2008). While exposure to mycotoxins most commonly occurs via the ingestion of contaminated foods, exposure can also occur through the dermal and inhalation routes (Abdallah et al. 2018; Bertero et al. 2018; Corrier 1991).

Mycotoxins are low molecular weight natural products that are capable of inducing a toxic response at low concentrations in animals and humans (Beukes et al. 2017). Globally, approximately 25% of grain production is contaminated by mycotoxins which are either destroyed or diverted to animal feed leading to agricultural and economic losses (Alshannaq and Yu 2017). Since mycotoxins are highly stable compounds, their removal is extremely difficult (even with heat and chemical treatment), which allows them to survive food processing methods and potentially form deposits in animal-derived products such as milk, meat, and eggs (Hossam 2013; Zain 2011). Therefore, human exposure occurs at various stages in the food chain.

Notably, the most significant agricultural mycotoxigenic fungi include *Aspergillus*, *Fusarium*, and *Penicillium*, which are known to produce some of the most hazardous toxins such as aflatoxin (*Aspergillus*), ochratoxin A (*Aspergillus*), fumonisin, trichothecenes (*Fusarium*) and patulin

(*Penicillium*) which contaminate a large portion of the world's major food sources, including maize, cereals, nuts and fruits (Bertero et al. 2018; Zain 2011). Moreover, the effects of these mycotoxins on human and animal health are influenced by exposure (dosage and period), nutritional status, exposure to infectious diseases or other mycotoxins (synergistic effects); and have been associated with hepatotoxic, mutagenic, carcinogenic, oestrogenic and teratogenic properties, as well as, gastrointestinal, urogenital, vascular, kidney, immunosuppressive and nervous disorders (Abdallah et al. 2018; Freire and da Rocha 2017; Fung and Clark 2004).

Despite the frequent co-existence of these fungal genera, the synthesis of mycotoxins is strongly influenced by geographic climate patterns (oxygen level, humidity and temperature) (Liew and Mohd-Redzwan 2018; Medina et al. 2015). In tropical or subtropical climates, *Aspergillus* species are the most prevalent agricultural contaminate (Paterson and Lima 2017; Perrone et al. 2014). While temperate regions of America, Europe and Asia, had the greatest frequency of *Fusarium* toxins (deoxynivanelol, zearalenone, T-2 toxin and fumonisins) (Adegoke and Letuma 2013; Hossam 2013). In developing countries, such as those in Africa, that experience warmer conditions, the incidence of fumonisins, aflatoxins and ochratoxin A were reported to be the most significant (Abdallah et al. 2018; Beukes et al. 2017).

With the increasing frequency *Fusarium* species worldwide, *Fusarium* mycotoxins have adapted to moderate climates and represent a growing global health concern (Bertero et al. 2018). While fumonisins, deoxynivanelol, zearalenone and T-2 toxin are recognized as the most important fusariotoxins, recent studies have found the high occurrence of other fusarium toxins, such as enniatins, beauvericin, moniliformin, fusaproliferin and FA, in feed and foodstuffs (Gruber-Dorninger et al. 2016). While many countries have established regulatory limits for known mycotoxins (aflatoxins, fumonisins, ochratoxin A, deoxynivalenol and zearalenone), data related to the toxicity of emerging mycotoxins are limited, preventing proper regulatory and risk assessments (Panasiuk et al. 2019).

1.2.2. Fusaric acid: A *Fusarium* metabolite

Fusaric acid, a naturally occurring *Fusarium* secondary metabolite, was first isolated from *Fusarium heterosporium* as a potent phytotoxin (Devaraja et al. 2013; López-Díaz et al. 2018). Since then, several studies have identified the production of FA by a substantial number of fungal species belonging to the *Fusarium* and *Giberella* genera (Bacon et al. 2006; Misihairabgwi et al. 2019; Streit et al. 2013). Subsequently, various investigations have reported the frequent contamination of FA on grain-based foods and feeds worldwide (Bacon et al. 2006; Streit et al. 2013). While there are a limited number of surveys on the amount of FA produced commercially, FA has been shown to occur naturally in animal

feed and cereal grains in cold (14-26 mg/kg) and warm temperate regions (0.643 mg/kg) at concentrations exceeding those of deoxynivalenol and zearalenone (Streit *et al.* 2013; Swamy *et al.*, 2002). Despite this, FA demonstrates low toxicity to animals when compared to other *Fusarium* mycotoxins; however, FA may have synergistic interactions with co-occurring mycotoxins (Smith and Sousadias 1993). Although moderately toxic to animals, FA has been reported to be an effective hypotensive agent as a result of neurochemical alterations within human and animal hosts (Terasawa and Kameyama 1971; Goodwin and Sack 1975). Notably, the latter effect is similar to the toxicology of trichothecenes.

Trichothecenes, such as deoxynivalenol and T-2 toxin, are among the most agriculturally important mycotoxins and have been acknowledged for their impact on human health (Sudakin 2003). Trichothecenes are ubiquitously produced by certain species of *Fusarium, Myrothecium*, and *Stachybotrys* fungi that colonise wheat, rye, barley, oats, and other cereals (Shi et al. 2017). Like FA, trichothecenes alter the brain neurochemistry, although by a different mechanism to that of FA. Adverse effects such as loss of appetite, vomiting, lesions of the intestinal tract, lethargy and ataxia have been reported in experimental animals that were fed a trichothecene contaminated diet (Smith and MacDonald 1991; Smith and Seddon 1998). In addition to its emetogenic properties, trichothecenes have immunomodulatory properties and inhibit protein synthesis at varying degrees; which is likely the cause of many of the human pathologies associated with trichothecene toxicosis (Smith and Seddon 1998; Sudakin 2003). Although FA and trichothecenes are structurally diverse, they share similar neurochemical and behavioural effects (Smith and MacDonald 1991). However, it has not yet been established if FA displays similar cytotoxic and immunomodulatory effects as trichothecenes in humans.

Fusaric acid is a structural derivative of PA also known as 5-butylpicolinic acid (**Figure 1.1**), with PA being produced through the metabolism of L-tryptophan via a sequent side branch of the kynurenine pathway (Grant et al. 2009). Picolinic acid has been detected in various biological mediums including, blood serum, cerebrospinal fluid (CSF), human milk, pancreatic juice and intestinal homogenates (Prodinger et al. 2016). In addition, several investigations have reported the neuroprotective, immunological, and anti-proliferative effects of PA (Prodinger et al. 2016). Like FA, PA is an amphiphilic chelating agent, extensively studied for its efficient chelating properties of nickel, zinc, cadmium, palladium and copper (Prodinger et al. 2016). Due to its potent metal chelating properties, PA-metal complexes are now being exploited as a means of introducing bioactive metals into biological systems (Prodinger et al. 2016). This evidence demonstrates that FA may possess similar pharmacological properties to PA and have widespread biological effects.

1.2.2.1. Structural characteristics of Fusaric acid

Fusaric acid comprises of a carboxylic acid moiety linked to a pyridine ring with a butyl group which enhances FA's ability to permeate cytosolic membranes more efficiently than PA (**Figure 1.1**) (Kim et al. 2014). In addition, FA's biological chelating-activity is believed to be mediated by the nitrogen present in the pyridine ring and the deprotonated, negatively charged oxygen on the carboxylic acid group (**Figure 1.1**) (Gutterman 2005; Kwiatkowski et al. 2000).

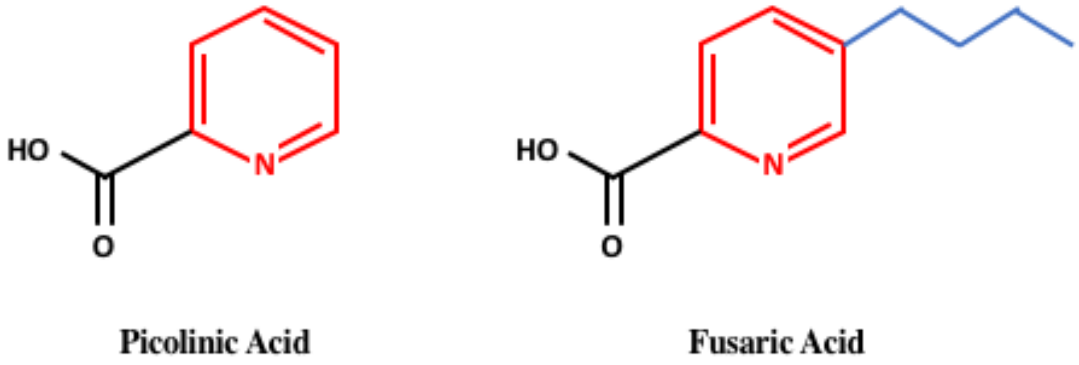

Figure 1.1: Chemical structures of picolinic acid and fusaric acid containing a pyridine ring (red), a carboxylic acid (black) and butyl moiety (blue). (Prepared by author)

Although FA has been described as a neurologically active toxin, no studies have shown its distribution in the brain. However, according to Lipinski's "rule of five" (molecular weight, lipophilicity, polar surface area, hydrogen bonding, and charge), a molecule with a molecular mass less than 500 Da, no more than 5 hydrogen bond donors (-NH-, -OH), 10 or fewer hydrogen bond acceptors (-N-, -O-), and an octanol–water partition coefficient, log P not greater than 5, has a greater likelihood of permeating the BBB (Lipinski et al. 1997). Notably, the low molecular mass (**Table 1**) and high lipid solubility of FA, likely promotes its passage across the lipophilic phospholipid BBB and into the brain (Bacon et al. 2006; Bohni et al. 2016).

Table 1: Molecular properties of fusaric acid. (Prepared by author)

Molecule name	Molecular formula	Molecular mass (Da)
Fusaric acid	$C_{10}H_{13}NO_2$	180.1025

Da: Dalton

1.2.2.2. Pathogenicity of Fusaric acid

1.2.2.2.1. Plants

Among the various plant-pathogenic toxins produced by the genus *Fusarium*, FA is considered the most potent phytotoxin causing significant physiological and biochemical dysfunction leading to wilting, necrosis and plant cell death of many economically important fruit and grain crops such as tomatoes, bananas, cucumbers, corn, rice, wheat, sorghum and barley (Singh et al. 2017; Singh and Upadhyay 2014). Fungi commonly infect their hosts through the roots and clog the xylem vessels with their spores, mycelia or metabolites; thereby, restricting the movement of water in the vascular bundles leading to plant wilt (López-Díaz et al. 2018; Singh et al. 2017). The fungal metabolite FA, then induces mitochondrial dysfunction, inhibits respiration and lowers intracellular ATP levels which stimulates the production of reactive oxygen species (ROS) (Pavlovkin et al. 2004; Singh et al. 2017; Singh and Upadhyay 2014). The toxin further inhibits anti-oxidative enzymes, such as superoxide dismutase (SOD) and catalase (CAT), as well as metal containing enzymes, contributing to respiratory dysfunction, lipid peroxidation, increased electrolyte leakage, and fluctuations in the electrochemical gradients across the plasma membrane (Li et al. 2013; Singh and Upadhyay 2014). As a result, this leads to membrane hyperpolarisation and changes to the membrane permeability which consequently leads to a reduction in root growth (Wang et al. 2015). Additionally, FA is transported to the leaves from the xylem vessels, reducing chlorophyll synthesis along the veins of leaves that result in decreased photosynthesis, disruption of cellular permeability and dysfunction in transpiration (Pavlovkin et al. 2004; Singh et al. 2017). These biological events rapidly lead to leaf epinasty, wilting, necrosis, browning and plant death (Kim and Choi 2010; Pavlovkin et al. 2004; Singh et al. 2017). While most studies have reported the toxic effects of FA at concentrations greater than 10^{-5} M (apoptosis at 50-100 μM; necrosis at >200 μM), FA produced by non-pathogenic *Fusaria* at non-toxic concentrations (below 10^{-6} M) were found to activate signal transduction components essential for plant defence responses that contribute to the biological control against pathogens (Singh et al. 2017; Singh and Upadhyay 2014).

1.2.2.2.2. Animals

Despite its high pathogenicity towards plants, FA has been reported to be only mildly toxic in animals fed a FA-contaminated diet (plant and grain-based feedstuff), with several important pharmacological activities, including cardiovascular and neurological effects (Bacon et al. 1996). These effects are influenced by the inhibitory effect of FA on dopamine-ß-hydroxylase in the synthesis of catecholamine (dopamine, norepinephrine and epinephrine) metabolism (Terasawa and Kameyama 1971). Early investigations in the 1960's, found that FA was an effective, specific inhibitor of dopamine-ß-hydroxylase (a key enzyme responsible for converting dopamine into norepinephrine which is important for memory and stress responses), and had significant hypotensive effects in acute animal studies (rabbits and dogs), which was possibly attributed to decreased peripheral vascular resistance (through the action of adrenergic receptors) and peripheral arteriolar dilation (Hidaka et al. 1969; Terasawa and Kameyama 1971). Additionally, with FA being a physiological metabolite of tryptophan (an amino acid bound to albumin in circulation), Chaouloff *et al.* (1986) discovered its ability to compete with tryptophan for albumin binding sites, thereby, displacing tryptophan and elevating its circulating levels, which is subsequently shuttled across the blood brain barrier to promote serotonin synthesis.

Due to FA's discovery as a potent dopamine-ß-hydroxylase inhibitor, investigations by (Haycock et al. 1978; 1977) determined the effect FA-induced catecholamine alterations on memory retention in rats and mice, reporting reduced norepinephrine and epinephrine concentrations in both animal models. Despite decreased catecholamine levels correlating with deficits in retention performance in rats, FA administration did not affect the memory in mice. Consistent with its inhibitory effect on dopamine-ß-hydroxylase, several other studies have observed significant increases in serotonin, 5-hydroxyindoleacetic acid (a major metabolite of serotonin), dopamine and tyrosine levels with FA in animals; and was also found to have partial inhibitory effects on tyrosine-hydroxylase (Chaouloff et al. 1986; Diringer et al. 1982; Porter et al. 1995). Additionally, the modulatory effect of central catecholamines on animal aggression and motor activity were reportedly decreased at acute doses of FA (mice: 10-75 mg/kg; rat:11-99 mg/kg) (Diringer et al. 1982; Ross and ÖGren 1976; Svensson and Waldeck 1969).

While acute doses of FA have shown therapeutic effects in rats and mice, in swine FA caused vomiting and lethargy at acute doses (200 mg/kg) with simultaneous elevations in tryptophan, serotonin, and 5-hydroxyindoleacetic acid concentrations (Smith and

Sousadias 1993). In dogs, acute administration of FA (20 mg/kg daily via the intramuscular or oral route) displayed no toxic effects on physical and mental activity, body weight, water and food consumption, urinalysis, haematology analysis and auditory function over a 6-month period, with the exception of emesis in some dogs orally dosed (Terasawa and Kameyama 1971). Histological analysis of various organs further showed no significant differences in cellular morphology with FA-treatment. In large doses (125 mg/kg), however, FA caused nausea, loss of appetite, retardation of physical and mental activity, decreases in body weight, gastric and/or duodenal ulcers and slight impairments of some liver functions; which appeared together with its hypotensive activity (Terasawa and Kameyama 1971). In contrast, in poultry FA administration had no effects on feed intake, body weight and frontal cortex mass in animals fed doses between 300-400 mg.kg (Ogunbo et al. 2005); but reduced the cell-mediated cutaneous activation of T lymphocytes between 14-27 µg/g concentrations in a natural feeding blend (Swamy et al. 2002). Whilst FA has many pharmacological activities, at high doses FA has been reported to be lethal in several animals (rats: LD_{50} >210 mg/kg; mice: LD_{50} >75 mg/kg; rabbit: LD_{50} >150 mg/kg) (Matsuzaki et al. 1976).

Notably, a study by Porter et al. (1996), reported the lactational transmission of FA from the feed of nursing dams to their offspring and observed reduced pineal serotonin and tyrosine levels with restricted weight gain in the neonates. A more recent study by Yin et al. (2015) described the teratogenic properties of FA in zebra fish, in which FA induced undulated notochord phenotypes at concentrations between 110-600 µM, that caused abnormal curvature of the spine. Moreover, embryos at early stages post-fertilisation showed a greater sensitivity to low concentrations of FA. Whilst, concentrations above 600 µM resulted in necrosis or death of the embryo. These effects were suggested to be caused by the chelation of copper from the active site of lysyl oxidase, an enzyme important for normal respiratory function, wound healing, and ovulation (Yin et al. 2015). On the other hand, other studies have shown that oral administration of FA (1 mg/mL) slows the progression of squamous cell carcinoma xenografts in Balb/C mice and decreases cardiac fibrosis *in vivo* by altering transforming growth factor ß-1 (TGFß-1)/SMAD, MAPK and P13K/Akt signalling cascades (Li et al. 2017).

In addition, studies have suggested that FA may have synergistic interactions with other naturally occurring mycotoxins. In a study on broilers fed a naturally contaminated diet containing *Fusarium* mycotoxin (FA, deoxynivalenol and zearalenone), erythrocyte

numbers and serum uric acid concentrations were elevated while decreasing serum lipase activity (Swamy et al. 2002). While another study demonstrated no synergistic, additive or antagonistic effects of FA on the sub-chronic toxicity of fumonisin in rats (Voss et al. 1999).

1.2.2.2.3. Clinical

Guided by *in vivo* animal studies, initial clinical investigations have focused on FA as an active inhibitor of dopamine-ß-hydroxylase in the management of hypertension and psychopathological disorders (mania and depression). Consistent with preclinical animal trials, a preliminary study on elderly hypertensive patients using a FA calcium salt showed a hypotensive response in both hypertensive and normotensive patients, despite the simultaneous administration or absence of other antihypertensive agents (Terasawa and Kameyama 1971). Notably, the hypotensive response of FA in normotensive patients was minor compared to its depressive effects in hypertensive patients during the 6-month trial (Terasawa and Kameyama 1971). This outcome was confirmed in a subsequent trial on elderly hypertensive patients over a period of two years, which incited FA as a hypotensive drug in the treatment of hypertension (Terasawa et al. 1976). A study by Matta and Wooten (1973) described the rapid reduction of plasma dopamine-ß-hydroxylase activity after 30 minutes in patients with Parkinson's disease or Huntington's chorea, with almost complete inhibition at 2.5 hours (hrs) post-administration (a single oral dose of 200 mg FA), proving that FA was rapidly absorbed from the gastrointestinal tract. In addition, a reduction in the arterial pressure in the patients was observed, even at 10 hours post-withdrawal of FA treatment (Matta and Wooten 1973).

Several studies have investigated the behavioural effects of FA on psychopathological disorders. While FA showed slight improvements in patients with mild hypo-manic symptoms, patients with severe manic symptoms or with evidence of pre-existing psychotic features became consistently worse (Sack and Goodwin 1974). In conjunction with the exacerbation of manic symptomatology, homovanillic acid (HVA, metabolite of dopamine), and 3-methoxy-4-hydroxy-phenylglycol (MHPG, metabolite of norepinephrine), were increased and decreased in the CSF, respectively, suggesting that brain dopamine levels had risen with norepinephrine levels falling as a result of FA treatment (Sack and Goodwin 1974). On the basis of these findings, Sack and Goodwin (1974) concluded that the improvement of mania was related more to a

reduction in dopamine than to alterations to norepinephrine levels. These effects were further substantiated by Goodwin and Sack (1975) who observed changes in central amine metabolism in the cerebrospinal fluid; specifically, reduced MHPG and increased HVA levels in psychiatric patients following administration of FA. Additionally, 5-hydroxy-indoleacetic acid levels were slightly elevated in the cerebrospinal fluid, indicating increased serotonin turnover as a result of increased brain tryptophan concentrations (Goodwin and Sack 1975). In contrast, treatment with FA in patients with parkinsonism, showed neither improvement or worsening of the condition; although, involuntary adventitious movements in the patients were significantly reduced (Mena and Cotzias 1971). However, the sustained effects of FA following withdrawal was inconsistent amongst subjects. Despite FA's promising pharmacological activity, it was eventually abandoned due to its significant hepatotoxicity (Murphy 2000).

In addition to the cardiovascular and neurological effects of FA, a study by Yoshimura et al. (1977) investigated the effect of FA calcium salts on the release of thyroid hormones [triiodothyronine (T_3) and thyroxine (T_4)], responsible for regulating body temperature, heart rate and metabolism, in patients with hypertension and primary hypothyroidism. While the study did not measure the physiological parameters (body temperature, heart rate and metabolism), FA significantly reduced elevated basal serum thyroid stimulating hormone (TSH) (which acts on the thyroid gland to produce T_3 and T_4) levels in patients suffering from hypothyroidism, with no changes to T_3 and T_4 serum concentrations in healthy and hypertensive subjects; suggesting that FA has the ability to improve endocrine function in hypothyroidic individuals.

1.2.2.2.4. In vitro

In vitro studies have demonstrated that FA effectively inhibits 50% of dopamine-ß-hydroxylase activity at a concentration of 23 ng/ml and approximated the biological half-life of FA to be 12 hrs. While several studies were centred around FA's cardiovascular and psychiatric activity, Devaraja and colleagues (2013) investigated FA's effects on blood coagulation and showed that at low concentrations (10-50 ng) FA promoted clotting, whilst functioning as an anti-coagulant at concentrations above 100 ng. In addition to its circulatory effects, FA ($5x10^{-4}$ mol/L) reduced the synthesis of norepinephrine in circulating human lymphocytes with implications on lymphocyte activity (Musso et al. 1996).

Despite failed efforts into the development of FA as a hypotensive or anti-depressant drug; in recent years, a handful of studies have investigated FA's anti-cancer and anti-viral potential. Notably, at low concentrations FA exhibited significant cytotoxicity against several malignant cell lines, including human breast carcinoma (MCF-7, IC_{50}: 15 μg/ml), human colon carcinoma (HCT- 117, IC_{50}: 14 μg/ml), human epidermoid larynx carcinoma (HEp-2, IC_{50}: 4.4 μg/ml) and human hepatocellular carcinoma (HepG2, IC_{50}: 24 μg/ml) (Elaasser and El Kassas 2013). Similarly, Mamur et al. (2018) reported cytotoxic and genotoxic effects of FA in human cervical carcinoma (HeLa) cells, evidenced by the decreased viability (IC_{50}: 200 μg/ml) and mitotic index, albeit at much higher concentrations. Mamur and colleagues (2018) also evaluated the toxicity of FA in healthy human lymphocytes; and observed cytotoxicity at concentrations ranging from 25-400 μg/ml in healthy human lymphocytes, with no accompanying genotoxic effects. Likewise, another study demonstrated the enhanced anti-proliferative effectiveness of FA (0.1, 0.3 and 0.5 mM) in UM-SCC-1 cells [a human head and neck squamous cell carcinoma (HNSCC) cell line] when co-administered with chemotherapeutic drugs, paclitaxel and carboplatin (Jaglowski and Stack Jr 2006).

While the exact mechanism of FA's cytotoxicity in mammalian cells remains unclear, a study by Devnarain et al. (2017) revealed the induction of intrinsic and extrinsic apoptosis in human oesophageal epithelial carcinoma (SNO) cells by FA at an IC_{50} of 78.81 μg/ml. The genotoxic properties of FA were further demonstrated by Devnarain et al. (2017) who showed an increase in DNA damage markers (comet tail length and PARP-1 protein expression). Similar to plants, FA increased oxidative stress (ROS) and induced membrane damage whilst reducing anti-oxidant activity and ATP availability in SNO cells (Devnarain et al. 2017). Subsequently, Abdul and colleagues (2016) confirmed the mitochondrial toxicity of FA in the HepG2 cell line (IC_{50}: 104 μg/ml) with significant reductions to cellular ATP levels. Despite the evident increased metabolic stress, FA decreased mitochondrial biogenetic protein expressions of PGC-1α, p-CREB, nuclear respiratory factor 1 (Nrf1) and heat shock protein 70 (HSP70). This led to an increased production of ROS, which up-regulated the anti-oxidant response protein nuclear respiratory factor 2 (Nrf2). However, exposure to FA induced both apoptosis (increased activity of caspases-3/7) and necrosis in HepG2 cells (Abdul *et al.* 2016). Coupled with the mitochondrial dysfunction induced by FA, Abdul et al. (2019) assessed the cytoprotective potential of anti-oxidant proteins and its association

with inflammatory responses in HepG2 cells. Although oxidative stress can stimulate an inflammatory response, FA suppressed the priming of the pro-inflammatory cytokine, interleukin-1ß (IL-1β), suggesting the immunosuppressive potential of FA (Abdul et al. 2019). Concurrently, the activation of anti-oxidant transcription factors maintained mitochondrial homeostasis by the autophagic removal of damaged mitochondrion (Abdul et al. 2019).

In plants, FA is thought to be active by increasing DNA damage and preventing DNA synthesis and repair mechanisms. Recently, Ghazi et al. (2017) demonstrated the effects of FA on DNA integrity and the post-translational regulation of the stress activated tumour suppressor protein, p53, in HepG2 cells; and found that FA activated p53 in response to DNA damage, despite the inverse correlation of p53 post-translational regulator proteins, which lead to the induction of apoptosis in the cancerous cell line. Further investigations by Ghazi et al. (2019) showed decreased levels of DNA methyltransferases, which act to suppress the gene expressions of several proliferative, apoptotic and cell cycle proteins, including p53. These epigenetic modifications were shown to be mediated by the up-regulation of microRNA-29b (a post-transcriptional regulator of DNA methyltransferases) (Ghazi et al. 2019).

In addition to FA's cytotoxic potential, a preliminary report FA (12 µg/ml) displayed potent anti-viral activity against herpes simplex virus type 1 (HSV-1) when compared to the anti-viral drug, acyclovir (1.8 µg/ml) (Elaasser and El Kassas 2013). However, at a lower concentration of 10 µg/ml, FA showed no inhibitory effect against herpes simplex virus-1 (HSV-1) and herpes simplex virus-2 (HSV-2). Notably, in combination with acyclovir, FA improved acyclovir's anti-viral activity against HSV-1 and HSV-2 (Elaasser and El Kassas 2013).

1.2.3. Relationship between the brain and immune system

1.2.3.1. Immune system: structure and function

The immune system is one of the most vital components of the body and is the main defence system against foreign materials and biologic agents such as bacteria, viruses, toxins, foreign cells and tissues. The immune system can be divided into two distinct, yet integrated systems. These include the innate and adaptive immune systems (**Figure 1.2**) (Abbas et al. 2015). The

innate immune system is activated in response to pathogen entry, or upon recognition of injured or cancerous cells; and its defence mechanisms are characterized by a generic response with no long-term memory (Abbas et al. 2015). The adaptive immune system is highly specific and requires antigen presentation to distinguish between self and non-self (Abbas et al. 2015). The efficacy of the adaptive immune system is due to its immunological memory (Abbas et al. 2015). In order to perform its functions properly, these responses are mediated by specific cell types that either enhance or suppress extrinsic and intrinsic signals.

The immune system contains a diverse population of cells that remain in a latent or inactive state. However, upon infection, inflammation or other perturbations, these cells become activated and rapidly respond to the site of infection. Immune cell types of the innate and adaptive immune systems are derived from the thymus (T) or bone marrow (B) (Abbas et al. 2015; Council 1992). The thymus is responsible for the maturation of T lymphocytes, the cells developed from here are referred to as the lymphoid lineage (Abbas et al. 2015; Council 1992). While, the bone marrow gives rise to the myeloid lineage and includes erythrocytes, platelets, granulocytes, B lymphocytes, monocytes, and macrophages (**Figure 1.2**) (Abbas et al. 2015; Council 1992). A central component of immune functions includes the circulatory and lymphatic systems where lymphocytes, facilitated by other white blood cells, namely, neutrophils, monocytes, macrophages, eosinophils, and basophils, circulate in and out of tissues to maintain tissue and whole-body homeostasis (Abbas et al. 2015).

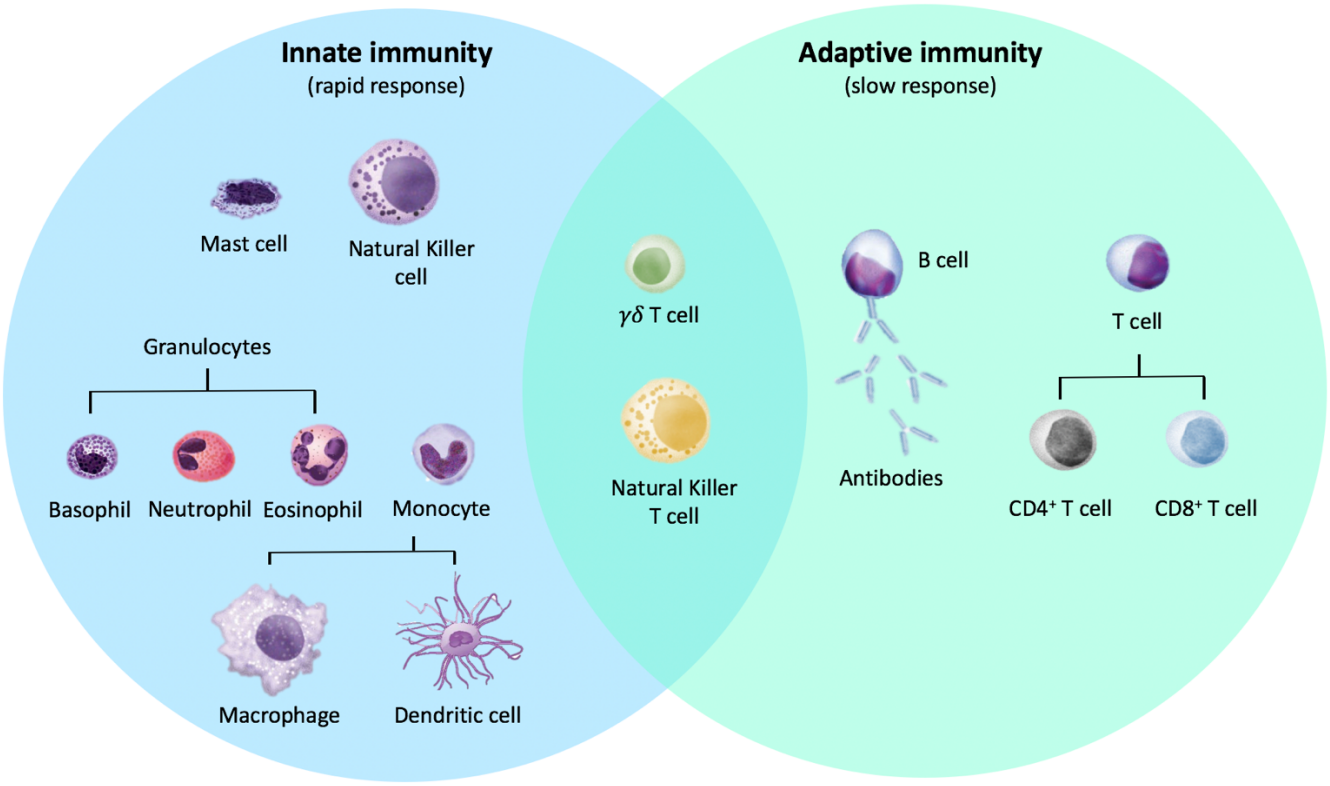

Figure 1.2: Cellular interactions involved in eliciting an immune response. (Prepared by author) (Adapted from Dranoff 2004)

1.2.3.2. Brain: structure and function

The human brain is a highly complex and compartmentalized organ that is responsible for maintaining essential physiological functions as well as processing and interpreting stimuli from the external environment. Its basic functions include controlling respiration, heart rate, memory, learning, vision hearing and movement amongst others (Ackerman 1992). The basic structure of the brain is composed of three primary areas, the cerebrum, cerebellum and the brain stem with each part being unique in its structure and functions (Kolb and Whishaw 2001) (**Figure 1.3**).

The cerebrum is the largest part of the brain and is made up of the left and right hemispheres which are responsible for higher functions such as vision, hearing, speech, reasoning and emotions. The cerebellum's main functions are related to posture, balance and the coordination of muscle movements (Kolb and Whishaw 2001). While the brainstem serves as a connection between the spinal cord and the brain, thereby controlling autonomic activities such as heart rate, breathing, body temperature, wake and sleep, and digestion (Kolb and Whishaw 2001).

Within the brain there also exists a number of specialized structures such as the thalamus and hypothalamus which are found below the cerebral cortex which act as a relay station for sensory inputs and a point of convergence between the endocrine and nervous systems, respectively (Funk et al. 2012). In addition, the hypothalamus plays an essential role in controlling the release of hormones, appetite, body temperature and physiological cycles (Aloisi 1999). The corpus callosum connects the left and right cerebral hemispheres. While the pons and the medulla, the two major components of the brainstem, controls vital functions and voluntary movements (Ackerman 1992) (**Figure 1.3**).

The different areas of the brain are made up of two cell types, neurons and glial cells. The glial cells are further divided into i) astrocytes – which have various roles as the caretaker cells, regulators of the BBB, maintaining homeostasis, nerve cell repair and scar formation ii) oligodendrocytes – secrete myelin that insulates nerve cells to allow for impulse transduction iii) ependymal cells – which secrete CSF and iv) microglia – which are resident immune cells of the brain, and are responsible for removing debris from the brain (Abbott et al. 2010).

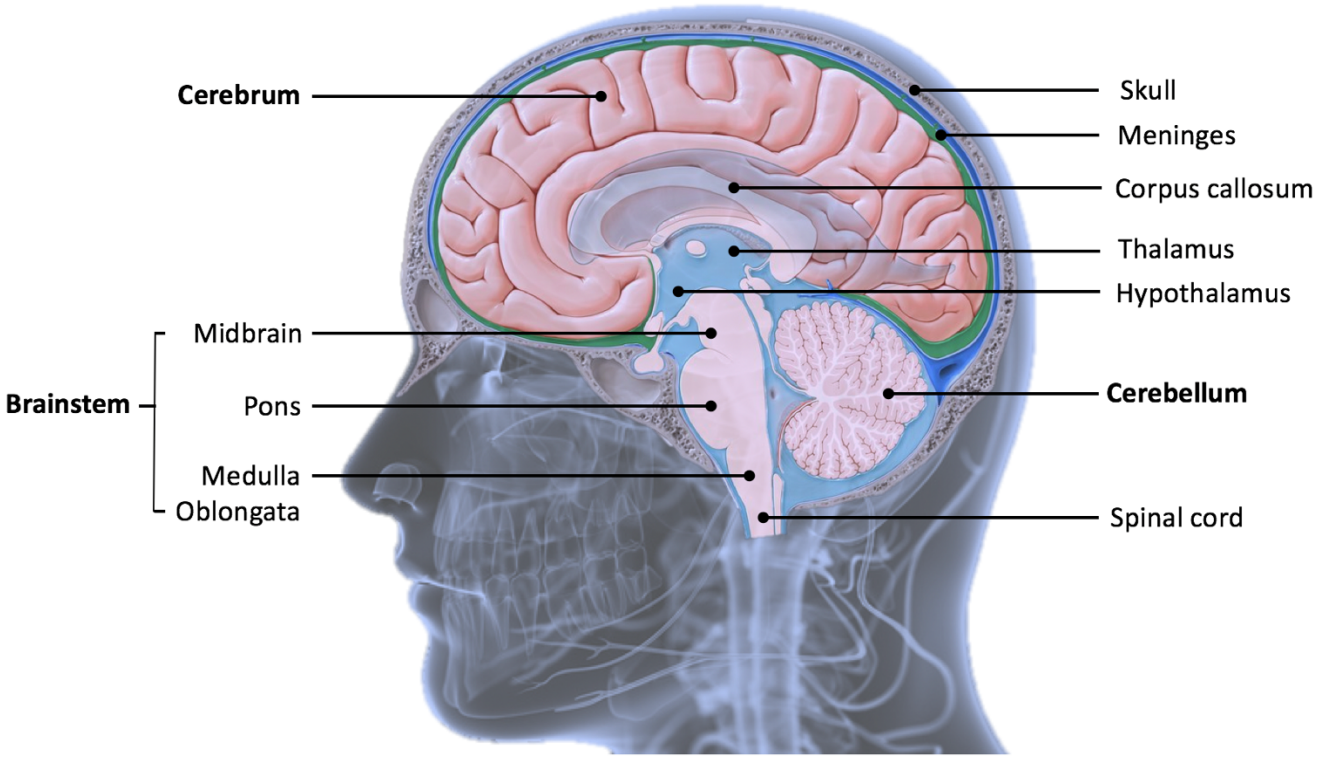

Figure 1.3: Schematic depicting the major structures of the brain. (Prepared by author) (Adapted from Ackerman 1992)

For decades the brain was considered to be an "immune privileged organ" protected from aggressive immunological responses (Aloisi 1999; Ousman and Kubes 2012). This was attributed to the BBB that protected the brain from various pathogens that could enter through the circulatory system, thus implying that there was no need for the immune system to be present in the brain (Aloisi 1999; Ousman and Kubes 2012). Additionally, in response to foreign bacteria, viruses, tumours, and transplant tissue, the immune system becomes active targeting pathogens, compromised cells and foreign tissue for destruction; it was believed that the BBB protects the brain from this hostile immunological response (Aloisi 1999; Ousman and Kubes 2012).

Therefore, the central nervous system was seen as existing separately from the peripheral immune system, left to wield its own less aggressive immune defences (Aloisi 1999; Ousman and Kubes 2012). An example of this was demonstrated in a study in which foreign tissue was grafted into most parts of the body and resulted in an immunologic attack; whilst tissue grafted into the central nervous system sparked a far less hostile response (Carson et al. 2006); which had suggested that the brain possessed its own complex immune response to foreign bodies.

Efficient immune surveillance and tissue repair within the central nervous system (CNS) are achieved through a complex and highly regulated network of interactions between resident brain cells and the immune system (Ousman and Kubes 2012; Russo and McGavern 2015) (**Figure 1.4)**. Due to its unique structure and environment, the CNS exerts strict control on the development of inflammatory and immune reactions that can be extremely injurious to the brain (Ousman and Kubes 2012; Russo and McGavern 2015). Major features that contribute to the immune privilege of the CNS include: the presence of structural barriers such as the tight endothelial junctions of the brain vasculature restricting the passage of circulating immune cells and molecules; the belief that the brain lacks a lymphatic drainage system and the absence of potent antigen presenting cells (APC) such as dendritic cells that capture potential antigens in the tissues and transport them to regional lymph nodes to initiate immune responses (Ousman and Kubes 2012; Russo and McGavern 2015).

In order to achieve immunity, the brain is routinely patrolled by blood-borne lymphocytes and that antigens within the brain can be carried, through the lymphatics, into the periphery (Russo and McGavern 2015; Tamura et al. 2019) (**Figure 1.4)**. To protect this vital system, the healthy brain and spinal cord are under continual immune surveillance to detect and eliminate potential mediators of infection and damage (Russo and McGavern 2015; Tamura et al. 2019). Both resident microglia and immune cells from the general circulation function as primary guardians

of the CNS, and their sentinel duties contribute to the maintenance of homeostasis and thus optimal functioning of the brain and spinal cord (Russo and McGavern 2015; Tamura et al. 2019) (**Figure 1.4)**.

Previously, the brain was believed to lack a lymphatic system. Lymphatic vessels return intracellular fluid to the bloodstream while lymph nodes – stationed periodically along the vessel network – serve as storehouses for immune cells (Tamura et al. 2019; Wilson et al. 2010) (**Figure 1.4)**. In most parts of the body, antigens are presented to white blood cells in our lymph nodes causing an immune response. But it was assumed that this doesn't occur in the brain given its lack of lymphatic structures (Tamura et al. 2019; Wilson et al. 2010). However, a recent study by Louveau and colleagues (Louveau et al. 2015) caused a shift in this paradigm when they discovered the presence of a meningeal lymphatic network in the central nervous system of mice that was responsible for shuttling fluid and immune cells from the CSF to a group of lymph nodes in the neck. Another study by Walsh *et al*., (2015) provided further evidence of a unique CNS immune system that showed peripheral T cell–derived interleukin-4 (IL-4) protects and induces recovery of injured neurons by activation of neuronal IL-4 receptors, which potentiated neurotrophin signalling via the Akt and MAPK pathways.

Experimental and clinical evidence has now described the close association between the immune and nervous systems, and their influence on each other's function via morphological and molecular-mediated communication (Kioussis and Pachnis 2009). Accordingly, the nervous system influences immune function by the direct innervation of lymphoid organs that allow the immune system to recruit local neuronal elements for fine tuning of immune responses (Dantzer 2017; Jeon and Kim 2016). In addition, the secretion of neurotransmitters (e.g., epinephrine, norepinephrine and serotonin) and circulating hormones produced by the neuroendocrine system (e.g., cortisol, growth hormone and prolactin) regulate immune activity and cytokine balance (Jeon and Kim 2016). Reciprocally, peripheral immune cells secrete numerous cytokines that regulate synaptic plasticity, neuroendocrine function and neurotransmitter metabolism in the brain, which impacts appetite, sleep and body temperature (Dantzer 2017). Notably, several studies have linked alterations between the brain and peripheral immune system communication with the development of neuropsychiatric disorders and immune-mediated pathogenic infections (Baganz and Blakely 2012; Kempuraj et al. 2017; Misiak et al. 2019).

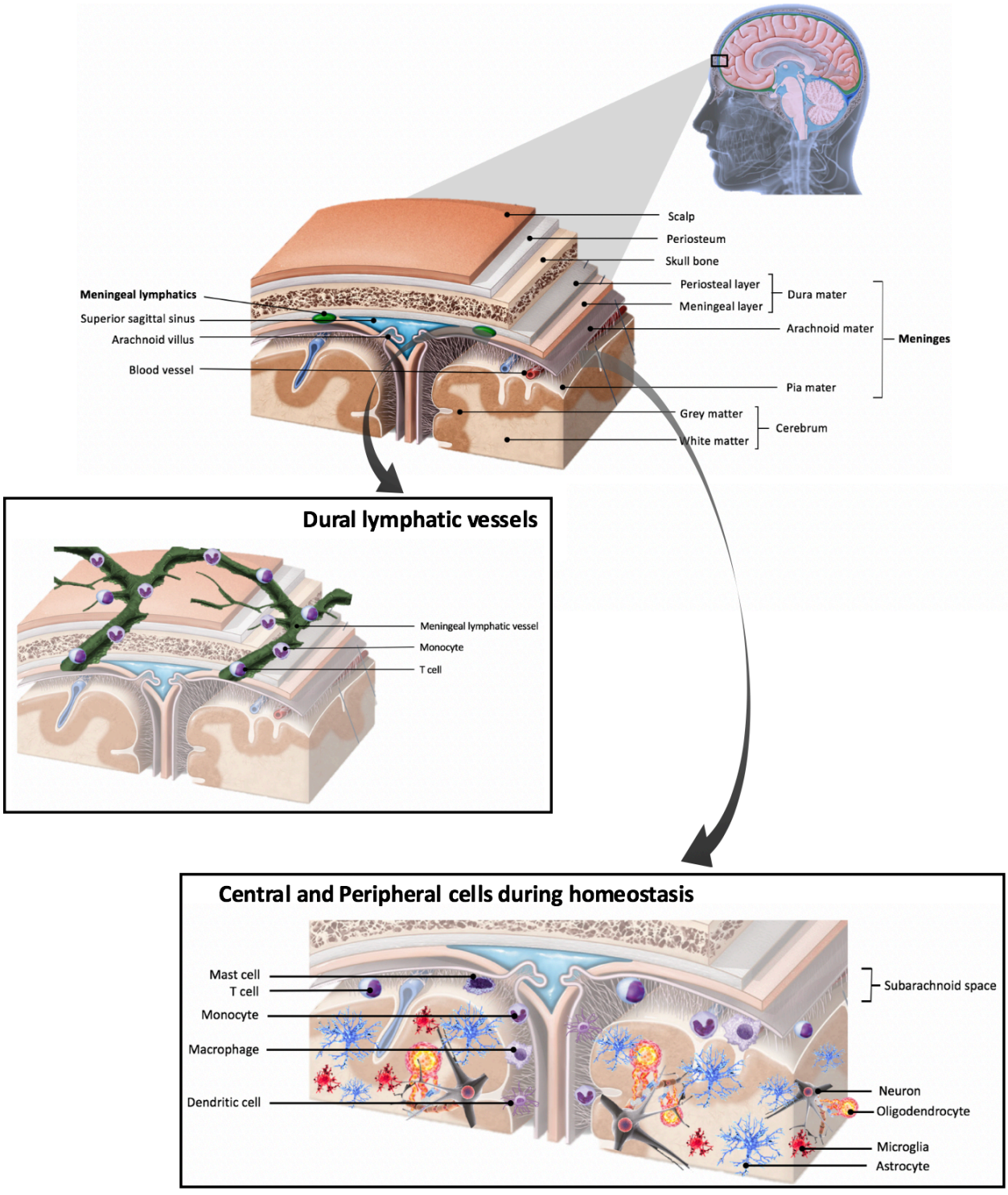

Figure 1.4: Anatomical and cellular interactions between the central and immune systems. (Prepared by author) (Adapted from Smith et al. 2014)

1.2.4. Kinase signalling

Although the bi-directional interaction between the immune and nervous system is complex, significant evidence have shown the profound influence of aberrant protein kinase signalling on peripheral immune responses and neurological behaviour with the onset and progression of immuno- and neuropathological conditions (Jeanneteau and Deinhardt 2011; Schroeter et al. 2002; Zhao et al. 2017).

1.2.4.1. Activation of Mitogen-Activated Protein Kinase (MAPK)

Mitogen-activated protein kinases are serine-threonine kinases ubiquitously expressed in all tissues including the central nervous and immune systems. MAPK signal transduction integrates a wide range of extracellular signals (e.g. osmotic stress, radiation, neurotrophins, cytokines and neurotransmitters) to initiate several precise intracellular programs including cell proliferation, differentiation, cell survival/death, inflammation, metabolism and synaptic plasticity (Jeanneteau and Deinhardt 2011; Kim and Choi 2010). The diversity and specificity of MAPKs' biological functions are orchestrated through three sequential phosphorylation events consisting of a MAP3K, a MAP2K, and a MAPK (Jeanneteau and Deinhardt 2011; Kim and Choi 2010). Three distinct groups of MAPKs are extensively studied, these include the ERKs (isoforms: ERK1-ERK8), the JNKs (isoforms: JNK1-JNK3) and the p38 (isoforms: α, -β, -γ, and -δ) MAPKs (Jeanneteau and Deinhardt 2011). Commonly, activation of ERK integrates survival programs induced by growth factors, cytokines, osmotic stress and microtubule disorganization; whilst, JNK and p38 [also known as stress-activated protein kinases (SAPKs)] respond to oxidative stress, hypoxia and inflammatory cytokines amongst others (Jeanneteau and Deinhardt 2011; Kim and Choi 2010). For full activation of MAPKs, phosphorylation of threonine-X-tyrosine motifs by MAP2Ks are required [ERK: MAP/ERK kinase 1/2 (MEK1/2); JNK: mitogen-activated protein kinase kinase 4 (MKK4), mitogen-activated protein kinase kinase 7 (MKK7); p38: mitogen-activated protein kinase kinase 3 (MKK3), mitogen-activated protein kinase kinase 6 (MKK6)] (Jeanneteau and Deinhardt 2011; Kim and Choi 2010). In turn, MAP2Ks are phosphorylated and activated by MAP3Ks, which are the serine/threonine kinases, [ERK: A-Raf, B-Raf, C-Raf, G protein coupled receptors (GPCRs); JNK: MAPK/ERK kinase kinase 1 (MEKK1), apoptosis signal-regulating kinase 1 (ASK1), mixed-lineage kinase (MLK), transforming growth factor ß-activated kinase 1 (TAK1); p38: ASK1, TAK1] (Jeanneteau and Deinhardt 2011; Kim and Choi 2010; Schroeter et al. 2002). Once activated, MAPKs modulate various transcription factor-associated activities found in the nucleus, cytoplasm, mitochondria, Golgi apparatus, and the ER, including those of CREB, p53, c-Jun, ribosomal s6 kinase ½ (RSK1/2), signal transducer and activator of

transcription 3 (STAT3), c-Myc, activator protein 1 (AP-1), mitogen-and-stress-activated kinase 1/2 (MSK1/2), nuclear factor-kappa B (NF-κB), Tau, etc. (**Figure 1.5**) (Funk et al. 2012; Jeanneteau and Deinhardt 2011; Schroeter et al. 2002).

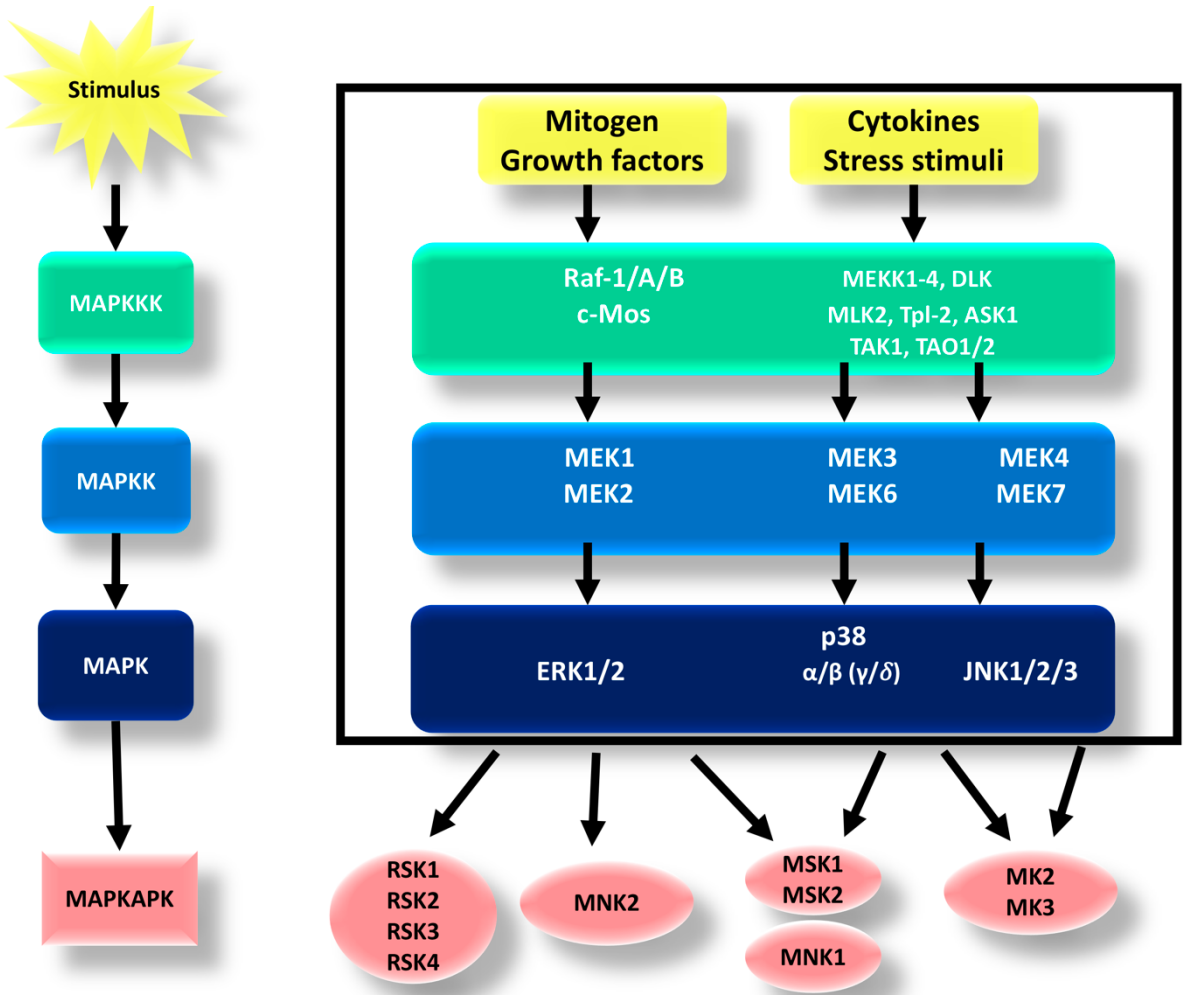

Figure 1.5: Mechanism of MAPK activation and downstream targets responsible for MAPK signalling functions. (Prepared by author)

1.2.4.1.1. The role of MAPK signalling in cell survival and apoptosis

Over the past decades, several studies have investigated ERK survival mechanisms and demonstrated the inhibition of apoptosis by post-translational phosphorylation of apoptotic regulatory molecules such as Bad, Bim, Mcl-1, caspase-9 and Bcl-2 (Mebratu and Tesfaigzi 2009; Wada and Penninger 2004). In myeloid progenitor cells, ERK directly phosphorylated transcription factors such as Elk1, CREB and Fos and

promoted the transcription of certain genes, such as *interleukin-3* (*IL-3*), that stimulated the growth and survival of early myeloid progenitor cells (Chang et al. 2003). Furthermore, neuronal apoptosis was reported in neonate mice following the suppression of ERK activity by the MAP/ERK kinase 1/2 (MEK1/2) inhibitor, SL327 (Thei et al. 2018). In addition, impaired memory and social behavior deficits were also observed by the preventative phosphorylation of ERK in the developing mice (Thei et al. 2018).

Likewise, JNK has been extensively studied in mice genetic models for its contribution to cell fate and has been reported to be a central activator of transcription factors (c-Jun, ATF2, Elk-1, p53, and c-Myc) and non-transcription factors such as Bcl-2 and Bcl-xL involved in cell death and survival (Guma and Firestein 2012). In adult mice, the genetic deletion of JNK resulted in increased resistance to CD3-induced apoptosis in $CD4^+$ splenic and thymocyte cells (Rincón et al. 1998). Similarly, JNK deficient mice injected with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (a neurotoxin) prevented neurodegeneration of brain tissue (Jiao et al. 2015; Youdim and Arraf 2004). Moreover, multiple studies with antioxidant and anti-inflammatory compounds have also shown the neuroprotective roles of JNK inhibition against MPTP and 6-hydroxydopamine (6-OHDA) (a neurotoxin that selectively destroy dopaminergic and noradrenergic neurons) Parkinson's induced models (Duty and Jenner 2011; Pérez-Hernández et al. 2016).

p38 signalling pathways are involved in a variety of cellular responses, and the outcomes of these responses are varied and complicated. Similar to JNK pathways, the involvement of p38 in apoptosis is also diverse (Wada and Penninger 2004). While several studies have implicated p38 in promoting cell death by the phosphorylation of Bcl-2 family members, other studies have shown its role in enhancing survival, cell growth, and differentiation (Mathew Loesch 2008; Munshi and Ramesh 2013). This was demonstrated in $CD4^+$ T cells in which initiation of apoptosis by T cell receptor (TCR) stimulation was prevented upon p38 inhibition with SB203580 (Merritt et al. 2000). In contrast, other studies have reported the execution of apoptosis by antigen receptors despite the suppression of p38 activity with SB203580 (Deschesnes et al. 2001; Wada and Penninger 2004). Moreover, *in vitro* and *in vivo* studies with neurotoxins such as rotenone (an inhibitor of the mitochondrial complex I) and montelukas (a leukotriene inhibitor) induced the degeneration of glial cells through the activation of p38, p53 and Bax (Bohush et al. 2018; Mansour et al. 2018; Sharma and

Ebadi 2014). These data therefore indicate that the p38-MAPK pathway is required for apoptosis and survival depending on cell types and conditions.

Additionally, the collective contribution of each MAPK family member to cell death was investigated in PC-12 pheochromocytoma cells treated with nerve growth factor (NGF) (Le-Niculescu et al. 1999). Withdrawal of NGF led to the sustained activation of JNK and p38 and inhibition of ERK which resulted in cell death (Le-Niculescu et al. 1999). These results suggest further establish ERK as a survival factor, and JNK and p38 as key components for the execution of cell death.

1.2.4.2. Activation of Protein kinase B (Akt) and Adenosine 5' monophosphate-activated protein kinase (AMPK)

An extensive body of literature have demonstrated numerous intracellular signalling cascades linked with MAPK pathways (Zhou et al. 2015). The MAPK and PI3K/ Akt signalling pathways converge at several points making them the major serine/threonine kinases implicated in cellular dynamics (survival, metabolism, differentiation, plasticity, etc.) (Zhou et al. 2015). Like MAPKs, the PI3K/Akt pathway is controlled by a multistep process. PI3K, a lipid kinase, acts downstream of receptor tyrosine kinases (RTKs) and GPCRs (activated by numerous growth factors and cytokines), and generates the phospholipid phosphatidylinositol-3-Phosphate ($PI3P_3$) (Grozinsky-Glasberg et al. 2008; Vincent et al. 2011; Yang et al. 2012; Zhao et al. 2017). In turn, the serine-threonine kinase, Akt (isoforms: Akt1-3), binds to $PI3P_3$ allowing phosphoinositide-dependent kinase-1 (PDK1) access to phosphorylate Akt's threonine-308 (T308) residue leading to the partial activation of Akt (Manning and Toker 2017). For full activation of Akt, a second phosphorylation at serine-473 (S473) by hydrophobic motif (HM) kinase (also called PDK2) [PDK2: integrin- linked kinase (ILK), ataxia telangiectasia mutated (ATM) and mammalian target of rapamycin complex 2 (mTORC2)] is required (Grozinsky-Glasberg et al. 2008; Huang et al. 2015; Vincent et al. 2011; Yang et al. 2012). Once Akt is activated at both sites, it mediates its functions via the phosphorylation of transcription factors, namely, GSK3, FoxOs and mTORC1 (**Figure 1.6**) (Case et al. 2011; Saxton and Sabatini 2017; Zhao et al. 2017).

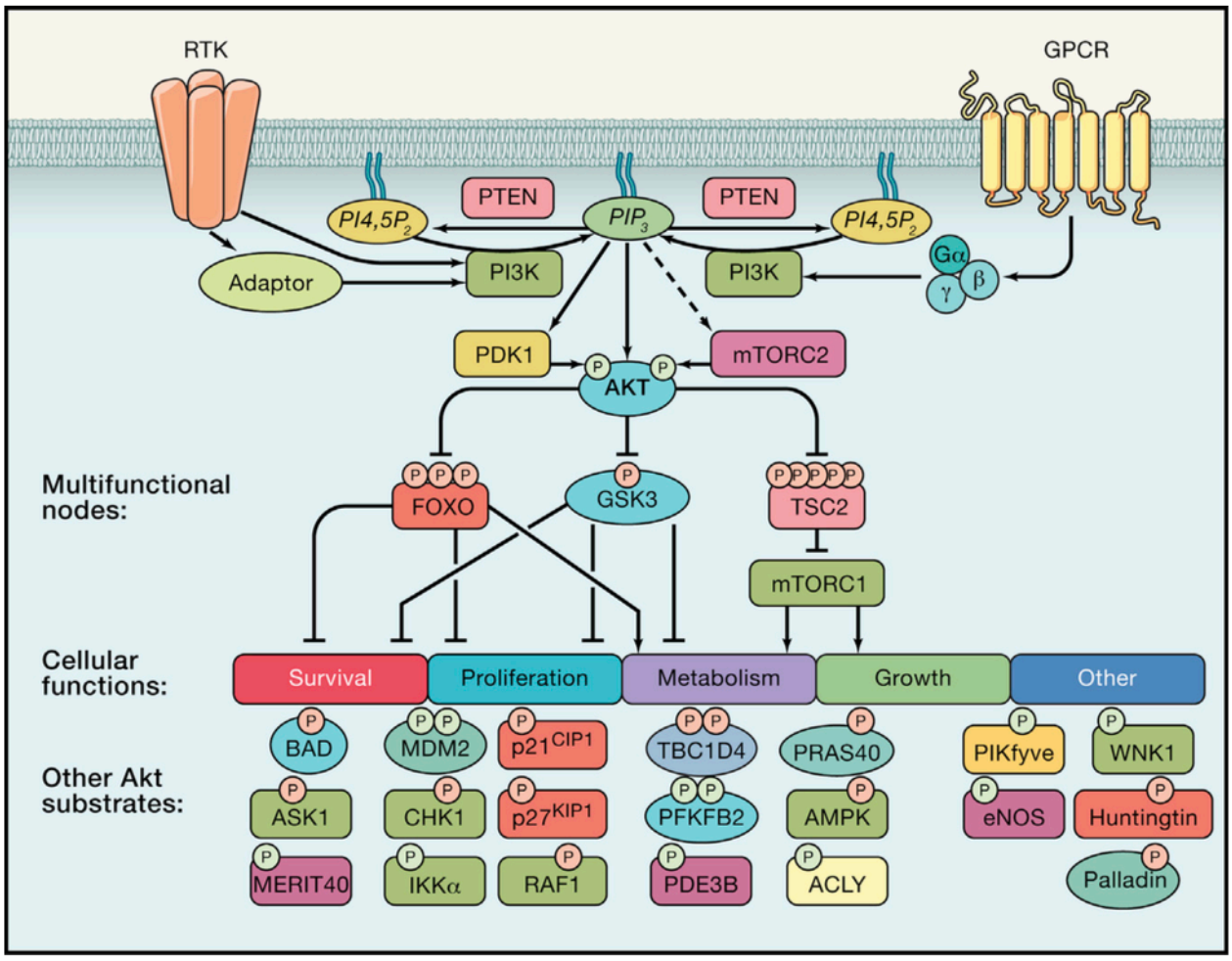

Figure 1.6: Mechanism of Akt activation and downstream targets responsible for Akt signalling functions. (Manning and Toker 2017)

The maintenance of energy homeostasis (i.e. ATP production: ATP expenditure) is a fundamental requirement of all cells. Adenosine 5′ monophosphate-activated protein kinase, acts as a cellular energy sensor and is activated in response to numerous conditions that reduce cellular ATP levels, such as nutrient starvation (especially glucose deprivation), hypoxia and exposure to toxins that inhibit the mitochondrial respiratory chain complex (Kim et al. 2016; Mihaylova and Shaw 2011; Richter and Ruderman 2009). Adenosine 5′ monophosphate-activated protein kinase is a heterotrimeric serine-threonine kinase, composed of a catalytic α-subunit (isoforms: α1and α2) and regulatory β (isoforms: β1and β2) and γ subunits (isoforms: γ 1, γ 2 and γ3), responds to increased AMP and ADP levels (Kim et al. 2016; Richter and Ruderman 2009). According to literature, AMP or ADP binds to the γ-subunit triggering a conformational change that promotes the phosphorylation of threonine-172 (T172) on the α-subunit by upstream kinases (Kim et al. 2016; Manning and Toker 2017; Richter and Ruderman

2009). The two major upstream kinases responsible for AMPK activation are the tumour suppressor LKB1 and CaMKK2. Liver kinase B1 activates AMPK during energy stress, whereas CaMKK2 activity is induced by increased intracellular Ca^{2+} levels, regardless of the energy status of the cells (**Figure 1.7**) (Kim et al. 2016; Richter and Ruderman 2009). Once activated, AMPK maintains energy equilibrium by suppressing non-essential anabolic pathways that consume ATP, such as lipid and protein synthesis, and cell growth and proliferation; whilst, promoting catabolic programs that increase ATP levels, including fatty acid oxidation and glucose transport, that is mediated by the phosphorylation of transcription factors and co-activators (**Figure 1.8**) (Kim et al. 2016; Zhao et al. 2017). In addition to AMPK's critical role in metabolic reprogramming, increasing evidence has demonstrated a much wider range of AMPK actions which regulate diverse events such as mitochondrial biogenesis, angiogenesis, cell polarity and hypothalamic control of food intake and whole-body energy expenditure (Hardie 2013; Huang et al. 2015; Kim et al. 2016; Richter and Ruderman 2009). The latter was described in a study by Hurtado-Carneiro *et al*., (2012) by which glucagon-like peptide 1 (GLP-1) induced fluctuations in hypothalamic glucose levels and inhibited AMPK and p70S6K (a downstream target of mTOR) activities, in response to feeding behaviour in lean and obese male rats. Despite opposing AMPK (catabolic) and Akt (anabolic) functions, accumulating evidence has established a cross-talk between the AMPK and Akt pathways by mutual phosphorylation (either directly or indirectly) of downstream targets suggesting their synergistic influence on cellular processes (**Figure 1.8**) (Han et al. 2018; Saha et al. 2018; Zadra et al. 2015; Zhao et al. 2017).

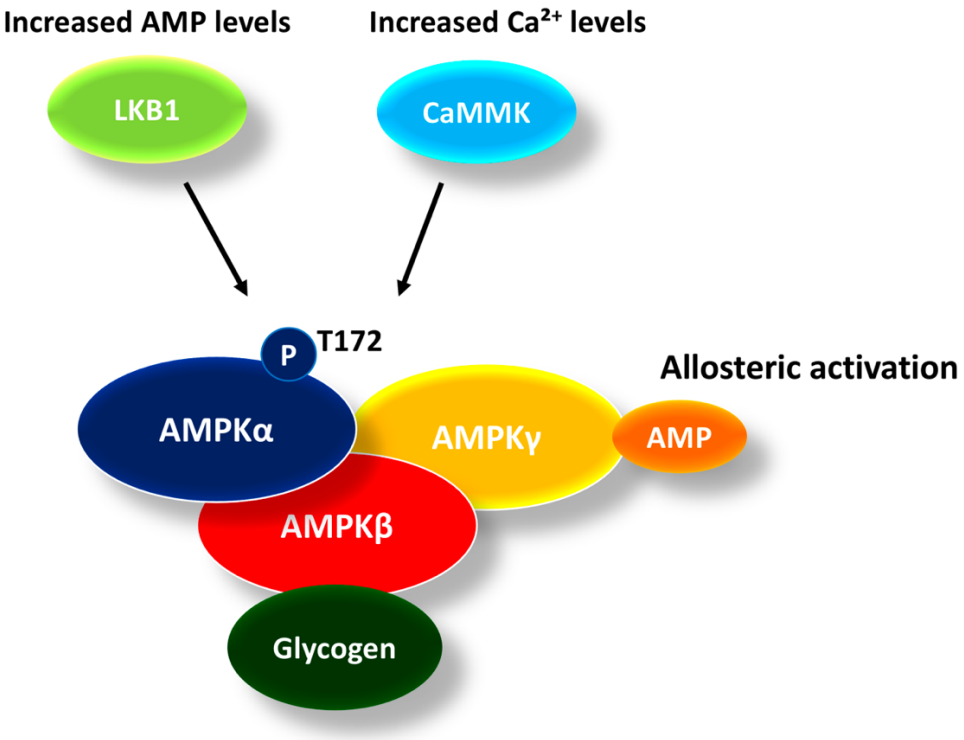

Figure 1.7: Mechanism of AMPK activation. (Prepared by author)

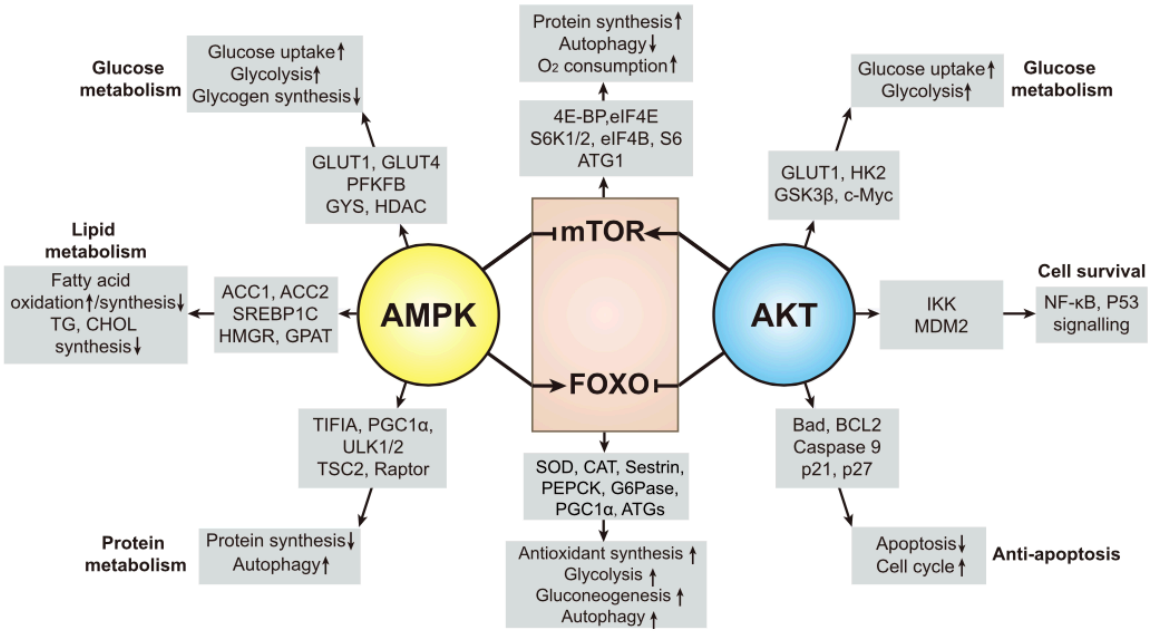

Figure 1.8: Cross-talk between AMPK and Akt signalling pathways on cellular responses. (Zhao et al. 2017)

1.2.4.2.1. The role of Akt and AMPK in metabolic signalling

Glucose deprivation is one of the main patterns of metabolic stress due to its crucial function in glycolysis (breakdown of glucose for cellular energy production) (Cui et al. 2017). Thus, a deficiency in glucose directly reduces ATP production which impacts cellular function and survival (Caro-Maldonado et al. 2010; Cui et al. 2017). The Akt and AMPK signalling pathways are central regulators of cellular metabolism (Zhao et al. 2017).

The PI3K/Akt pathway plays an important role in regulating aerobic glycolysis by increasing glucose uptake via the up-regulation of cell surface glucose transporters and the activation of several glycolytic enzymes such as hexokinase (Coloff and Rathmell 2006; Whiteman et al. 2002). Additionally, Akt promotes the activation of ATP citrate lyase, which enhances the conversion of citrate to acetyl-CoA for entry into the TCA cycle (Koundouros and Poulogiannis 2018). In addition to the direct role in glycolytic metabolism, Akt indirectly regulates glycolysis through downstream control of GSK3 and mTORC activity (Jason and Cui 2016; Khorami et al. 2015). Glycogen synthase 3 is a direct target of Akt and is inactivated upon phosphorylation, which consequently reduces the activation of glycogen synthase (Khorami et al. 2015; Manning and Toker 2017). Likewise, activation of mTORC1 by Akt promotes glycolysis by upregulating the expression of hypoxia-inducible factor 1 (HIF1) (Jason and Cui 2016; Manning and Toker 2017). Moreover, mTORC1 positively regulates mitochondrial function and oxidative metabolism by selectively promoting the translation of nucleus-encoded mitochondria-related mRNAs (Hoxhaj and Manning 2019; Jason and Cui 2016). While data from several studies in mammalian cells have shown the hyperactivation of Akt and its downstream targets with the accumulation of ATP; other studies have found an increased susceptibility to metabolic stress and cell death with prolonged Akt activity (under glucose deprivation) (Robey and Hay 2009). Thus, implying the dual roles of Akt in metabolic survival and stress resistance.

Under glucose deprivation, AMPK is activated in cells to mediate metabolic reprogramming in order to survive. Notably, AMPK phosphorylates the regulatory-associated protein of mTOR (Raptor) and tuberous sclerosis complex 2 (TSC2), which indirectly leads to inhibition of mTORC1; thereby decreasing protein synthesis and increasing autophagy (Zhao et al. 2017). The decreased anabolism reduces ROS production, while enhanced autophagy and glycolysis increases the resilience of cells to ROS (Zhao et al. 2017). In addition, multiple studies have shown an enhanced mitochondrial biogenesis and oxidative phosphorylation mediated by AMPK through activation of the p38/ PGC1α pathway (Herzig and Shaw 2018). This indicates AMPK is not only involved in glycolytic regulation, but also participates in ATP generation from oxidative phosphorylation (Herzig and Shaw 2018).

1.2.4.3. Activation of cyclic AMP response element-binding protein (CREB) and brain derived neurotrophic factor (BDNF)

Cyclic AMP response element-binding protein, an important transcription factor recognized for its roles in cell growth and survival, differentiation, learning and memory, and synaptic plasticity is activated in response to a vast array of physiological stimuli (stressors, growth factors, cytokines, neurotransmitters and neurotrophins) (Lonze and Ginty 2002; Wang et al. 2018). Phosphorylated at its serine-133 (S133) residue [located in the kinase inducible domain (KID)] by several receptor-activated protein kinases, such as protein kinase A (PKA), calmodulin-dependent protein kinase (CaMK) and MAPKs, CREB binding protein (CBP) facilitates the binding of the transcriptional coactivator, to the KID domain initiating the transcription of numerous target genes containing a cAMP-responsive element (CRE) (**Figure 1.9A**) (Wang et al. 2018). The diversity of CREB responses are facilitated by specific initiation or inhibitory transcriptional gene expressions involved in metabolism (*PEPCK, cytochrome c*), transcription factors (*STAT3, c-fos, NF-κB*), cell survival proteins (*bcl-2, cyclin D1*, and *cyclin A*) and growth factors (*BDNF, IGF-1, NGF*) (**Figure 1.9B**) (Wang et al. 2018; Wen et al. 2010; West et al. 2002).

Over the past decade, CREB has been extensively studied for its role in cognitive and emotional disorders, with BDNF being the most important target (Luo et al. 2017). Brain derived neurotrophic factor is a central neurotrophin that promotes the development of neuronal cell populations and confers neuroprotection under different conditions (McGregor and English

2018). Furthermore, the mutual relationship between CREB and BDNF are well documented whereby BDNF promotes the phosphorylation of CREB, which, in turn, promotes the transcription of the BDNF gene. Numerous studies have validated BDNF-induced CREB activation in neurons through the activation of CaMK, Trk B receptors and the Ras/ERK/RSK pathway (Luo et al. 2017). In several other investigations, CREB phosphorylation was independent of BDNF stimuli and was activated by monoamine neurotransmitter (dopamine and serotonin) receptor signalling, promoting the expression of BDNF (Huang et al. 2015; Lonze and Ginty 2002; Luo et al. 2017; Nakazawa et al. 2002; Wang et al. 2018).

1.2.4.3.1. The role of CREB and BDNF in neurometabolism and neuroprotection

While the relationship between CREB and cell survival is well-established, recent studies have demonstrated CREB's role as a metabolic sensor and regulator of glucose homeostasis in brain, liver and fat tissue; and has also been shown to be a key component in the control of appetite and food intake in the hypothalamus (Altarejos and Montminy 2011; Yadav et al. 2011). In its active form, CREB regulates metabolism in several ways; namely, through the up-regulation of glucose receptor expressions and glycolytic enzymes [Pyruvate kinase isozymes M2 (PKM2), triose-phosphate isomerase (TPI), alpha-enolase], and by enhancing mitochondrial respiration and biogenesis via the expression of several genes (i.e. *BDNF, PGC-1α, Nrf1, PPARα, and TFAM*) (Steven et al. 2017).

Apart from CREB's role in metabolic homeostasis, the proliferative and anti-apoptotic characteristics of CREB extend to its protective ability. During lymphocyte development, analysis of *Bcl-2* transcription revealed the direct binding of CREB to its promoter region, thereby up-regulating Bcl-2 gene expression; and whose transcription was blocked upon CREB mutation (Walton and Dragunow 2000). Similarly, in PC-12 pheochromocytoma cells, *Bcl-2* transcription was dependent on IGF1R-induced CREB activation; suggesting that various stimuli that activate receptor mediated CREB activity, such as neurotrophins, may also promote survival (Zheng and Quirion 2006). In particular, endogenous BDNF has been shown to stimulate CREB phosphorylation via the activation via of kinase-regulated pathways, directly regulate *BDNF* transcription; suggesting that a positive-feedback loop might promote resistance to brain injury (Pizzorusso et al. 2000). The cytoprotective roles of BDNF induced by metabolic stress was further evidenced by the effects of exercise and intermittent fasting on neuronal bioenergetics and stress resistance; and was shown to stimulate

neuronal energy metabolism by increasing the expression of GLUT 3, sodium-dependent amino acid transport and protein synthesis, ketone utilization and expression of peroxisome PGC-1α to increase mitochondrial biogenesis (Duan et al. 2001; Rothman et al. 2012; Wrann et al. 2013).

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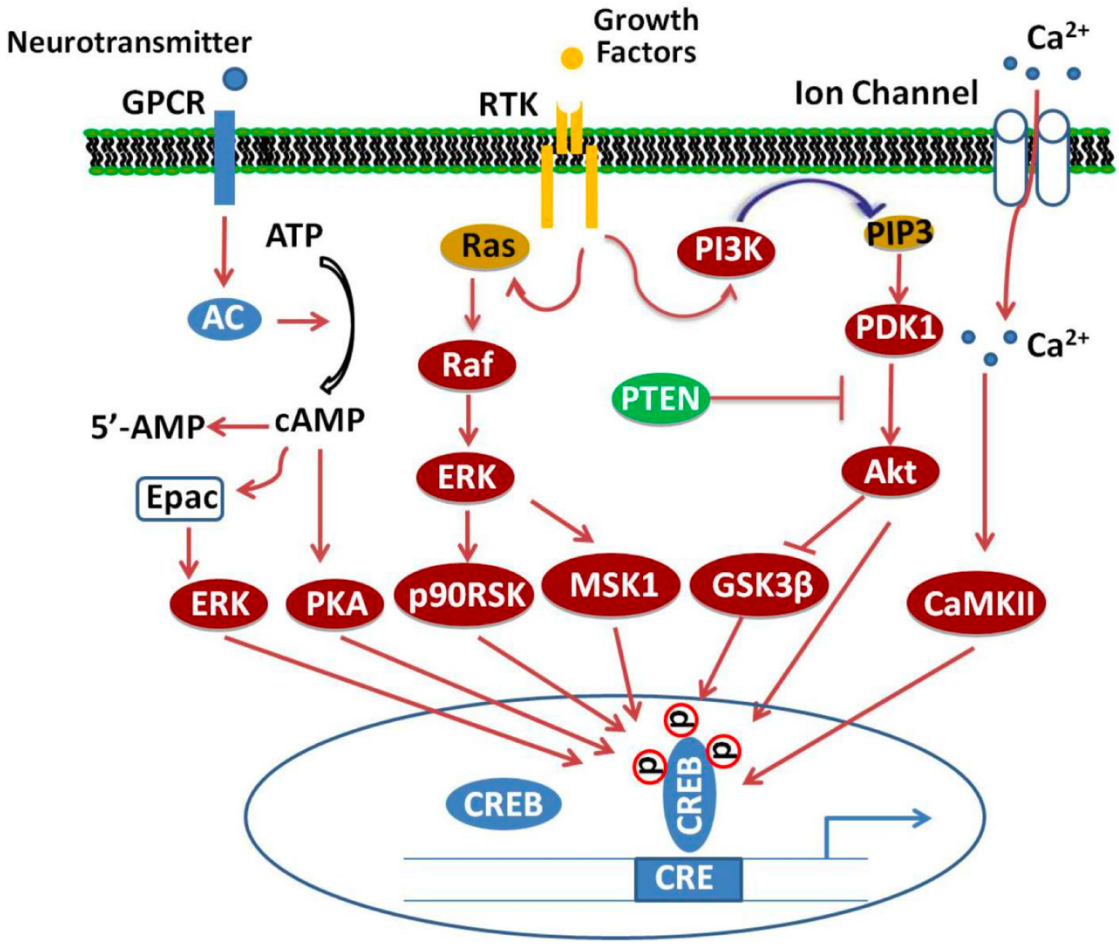

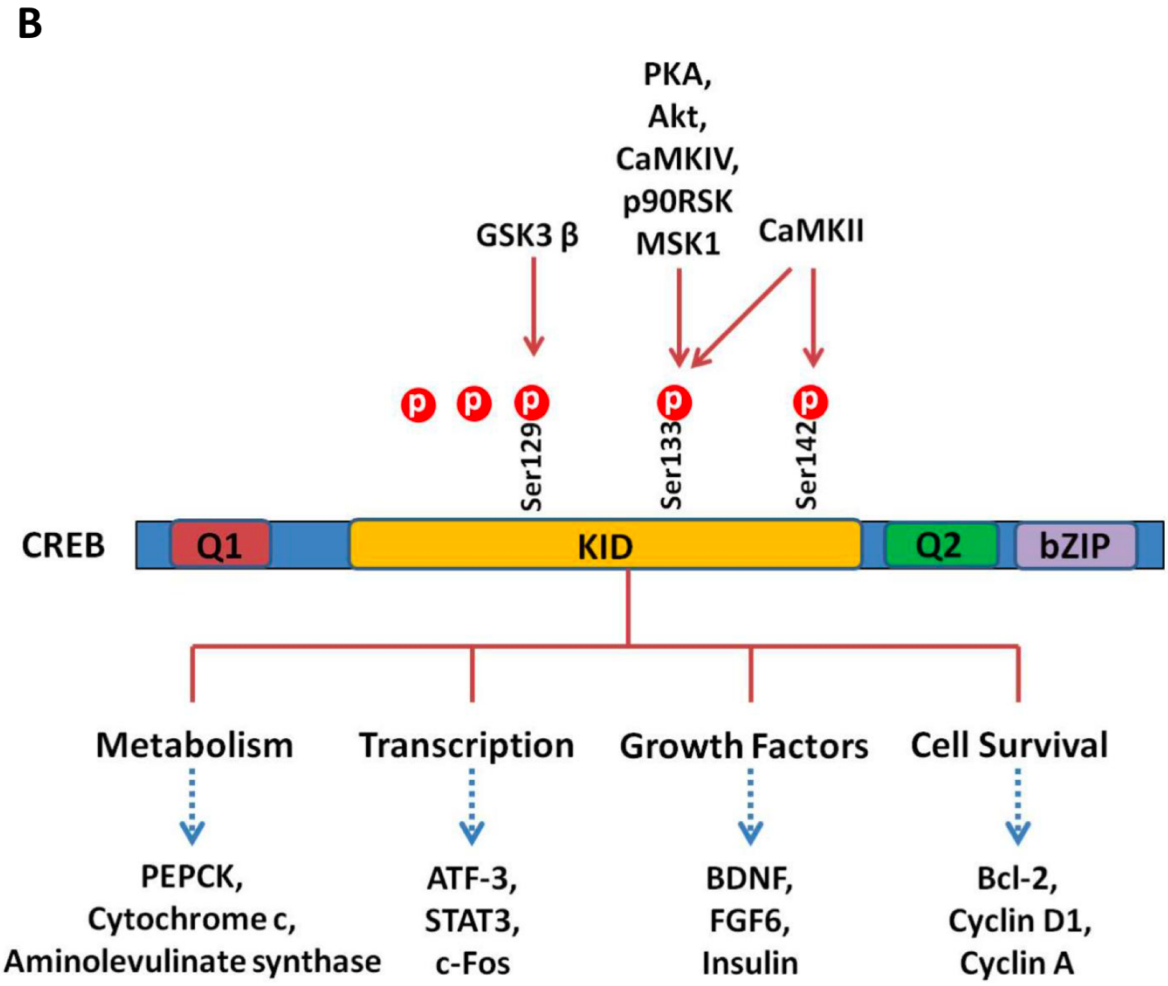

Figure 1.9: Activation of CREB signalling (A) and its downstream target genes (B). (Wang et al. 2018)

1.2.5. Aim and objectives

Despite the toxicity of FA being well-established in plants, its cellular effects on the mammalian immune and nervous system is unknown. Therefore, the main aim of this study was to evaluate the immuno- and neurotoxic effects of FA and its alterations to major kinase signalling pathways associated with survival and metabolic functions in *in vitro* cell culture [normal human peripheral blood mononuclear cells (PBMCs) and monocytic leukaemia cells (Thp-1)] and *in vivo* murine (C57BL/6 mice) models.

The immuno- and neuro-toxic effects of FA were determined by assessing immune and neuronal cell viability. Metabolic alterations and influences to kinase signalling pathways were also measured. The immunotoxicity of FA was determined (*in vitro*) at an acute (1 day) exposure. Whilst the assessment of FA neurotoxicity was investigated (*in vivo*) at acute (1 day) and prolonged (10 days) exposures. The specific objectives of this study were to determine:

- The immunotoxicity of FA on normal PBMCs and Thp-1 cell viability and expression of cell death markers
- The effect of FA exposure on the oxidative status and intracellular energy levels in PBMCs and Thp-1 cells
- The regulation of MAPK survival signalling pathways in PBMCs and Thp-1 cells treated with FA
- The metabolic effect of acute and prolonged FA-exposure on intracellular energy levels and alterations to the Akt and AMPK signalling pathways in murine brain
- The neurotoxic potential of FA following acute and prolonged treatment on microscopic tissue structure in murine brain
- Differential regulation of CREB/BDNF signalling by MAPK pathways following acute and prolonged exposure to FA in murine brain

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CHAPTER TWO

Manuscript title: Fusaric Acid immunotoxicity and MAPK activation in normal peripheral blood mononuclear cells and Thp-1 cells

The nervous and immune systems share a complex bi-directional communication network, with the nervous system influencing immune function via the secretion of neurotransmitters (e.g., epinephrine, norepinephrine and serotonin) and circulating hormones produced by the neuroendocrine system (e.g., cortisol, growth hormone and prolactin) and through the direct regulation of immune organs which allows the immune system to recruit local neuronal elements for the fine tuning of immune responses. Conversely, the immune system modulates brain activity (body temperature, sleep and feeding behaviour) by secretion of several chemical messengers, among which cytokines are the most well recognized. Although the brain was classically considered "immunologically privileged", it is now acknowledged that peripheral immune cells may be active in the brain and shifts in the peripheral immune system may be an important factor which could initiate or stabilize neuropathological conditions. The complexity of this communication network and responses are regulated by key intracellular components. Of these various systems, the MAPK signalling pathway regulates several fundamental cellular functions including growth, cell migration, differentiation, apoptosis, immune responses and synaptic plasticity indicating its high relevance. FA, a neurologically active mycotoxin, induces significant cytotoxicity in plants and *in vitro* models; whilst it is moderately toxic to animals upon dietary exposure. Although mycotoxins have varying effects and target organs, they all interact with the immune system (the primary defence system). While the neurobiochemical effects of FA has been recognized, it's impact on the immune system remains largely unknown. Therefore, this chapter determined the immunotoxicity of FA and its effect on MAPK activity in lymphoid and myeloid cells.

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Fusaric Acid immunotoxicity and MAPK activation in normal peripheral blood mononuclear cells and Thp-1 cells

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Fusaric acid (FA), a food-borne mycotoxin, is a potent divalent metal chelator. The human immune system is complex and susceptible to environmental insults, however, the immunotoxity of FA remains unknown. We investigated the immunotoxicity of FA on human peripheral blood mononuclear cells (PBMCs) and Thp-1 cells. FA was cytotoxic to PBMCs (IC_{50}-240.8μg/ml) and Thp-1 (IC_{50}-107.7μg/ml) cells at 24h. FA induced early apoptosis but significantly decreased caspase activity in PBMCs, a characteristic of paraptosis. In Thp-1 cells, FA induced apoptosis and increased caspase -9 and -3/7 activities. In PBMCs, FA maintained mitochondrial membrane potential and decreased protein expression of Bax whilst increasing expression of p-Bcl-2; FA induced oxidative stress and depleted ATP levels in both cell types. In Thp-1 cells, FA increased mitochondrial membrane depolarization and decreased p-Bcl-2 expression. In PBMCs, FA significantly up-regulated the MAPK protein expression of p-ERK and p-JNK but down-regulated p-p38 expression. In Thp-1 cells, FA up-regulated MAPK protein expression of p-ERK whilst p-JNK and p-p38 expression were down-regulated. In conclusion FA induced programmed cell death and altered MAPK signaling in healthy PBMCs and Thp-1 cells strongly suggesting a possible mechanism of FA induced immunotoxicity *in vitro*.

Keywords-10: Fusaric Acid; immunotoxicity; ROS; MAPK; intrinsic apoptosis; paraptosis

Introduction

Endophytic fungi produce mycotoxins that are toxic to animals and humans [1]. Fusaric acid (FA, 5-butylpicolinic acid), is a picolinic acid derivative produced by several strains of *Fusarium* species [2,3]. These fungal strains are ubiquitous in soil and are known to parasitize maize and many other cereal grains [4,5].

FA contains a pyridine ring with a butyl side chain that allows it to easily permeate cell membranes [6]. The toxicity of FA is also due to its ability to chelate divalent ions such as magnesium, calcium, zinc and iron [2,7]. The nitrogen in the pyridine ring and the deprotonated, negatively charged oxygen on the carboxylic acid group are responsible for FA's divalent metal chelating ability [8,9].

The human immune system functions in host defense against environmental exposure to bacteria, viruses, parasites, fungi and other perturbations, and in acquiring immunity against invading pathogens [10,11]. In response to foreign particle or pathogen, several signaling pathways are activated in immune cells [12]. Foremost of these pathways, is the activation of mitogen-activated protein kinases (MAPKs) [12]. MAPK activity directs diverse immune responses ranging from stress, cell death/survival and immune defense [12-14].

Optimal cellular mitochondrial function increases ATP synthesis and reactive oxygen species (ROS) that mediate cell signaling pathways [8]. The amount of intracellular ROS will significantly influence the MAPK pathway [6]. The MAPK family comprises of three universal serine/threonine protein kinases; these include the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 kinase [15,16]; each group of MAPK is activated

via a series of phosphorylation events [16]. The first event involves the phosphorylation and activation of a MAPK kinase kinase (MAPKKK), which in turn, phosphorylates and activates a MAPK kinase (MAPKK). MAPKKs activate MAPKs through dual phosphorylation on both threonine and tyrosine residues located within the tri-peptide motif of the MAPK [14,15,17,18]. Once activated, MAPKs phosphorylate several transcription factors, thereby regulating gene expression and cellular functions [13,14].

Apoptosis is executed by immune cells to maintain homeostasis of the immune system[19-21]. Apoptosis occurs via two main pathways, the intrinsic and extrinsic apoptotic pathways [19,22,23]. Both the intrinsic and extrinsic pathways are activated by caspases; the initiator caspases (-8 and -9) are involved in the intrinsic pathway, whilst the executioner caspases (-3/7) are integral to the extrinsic pathway [19,24]. Paraptosis is distinct from necrotic and apoptotic cell death and its features are defined by the lack of apoptotic morphology and independent of caspase activation [19,22,23,25-27].

The phytotoxicity of FA is well documented and includes altered mitochondrial membrane potential and inhibition of ATP synthesis [28,29]. In animals, FA inhibits the activity of dopamine-β-hydroxylase, synthesis of nucleic acids (zinc finger proteins involved in DNA repair) and impairs protein synthesis [30]. In young swine, FA showed moderate toxicity, induced vomiting and increased concentration levels of tryptophan and serotonin in the brain [31]. Elevated levels of serotonin results from its impaired regulation and consequently amplifies behaviors distinctive of the firing of serotonergic neurons such as loss of appetite and lethargy [32]. In zebrafish, FA had teratogenic effects by inhibition of lysyl oxidase (a copper-dependent enzyme) [33]. FA also decreased norepinephrine levels in the brain, heart, spleen and adrenal gland of rats [34].

To date, no study has investigated the effect of FA on the mammalian immune system. In this study, we assessed the immunotoxicity of FA associated with MAPK activity in healthy human peripheral blood mononuclear cells (PBMCs) and the acute monocytic leukemic (Thp-1) cell line. It was hypothesized that FA altered MAPK signaling was immunotoxic in both cell types. This study shows that FA, a common food borne mycotoxin, is toxic to the human immune system. This data may help develop a better understanding of the immune risks associated with FA consumption. This is of importance in South Africa, the epicenter of infectious diseases, where the majority population relies on maize as a food staple.

Results

- Cell viability of PBMCs and Thp-1 cells

The WST-1 assay showed that FA induced a dose dependent decrease in PBMC and Thp-1 cell viability over 24 h (Supplementary Tables: S3-PBMC, S4-Thp-1). Thp-1 cells were more susceptible than PBMCs to FA toxicity. An IC_{50} of 240.8 μg/ml (Fig. 1A) and 107.7 μg/ml (Fig. 1B) determined for PBMCs and Thp-1 cells respectively; and was used in all subsequent assays.

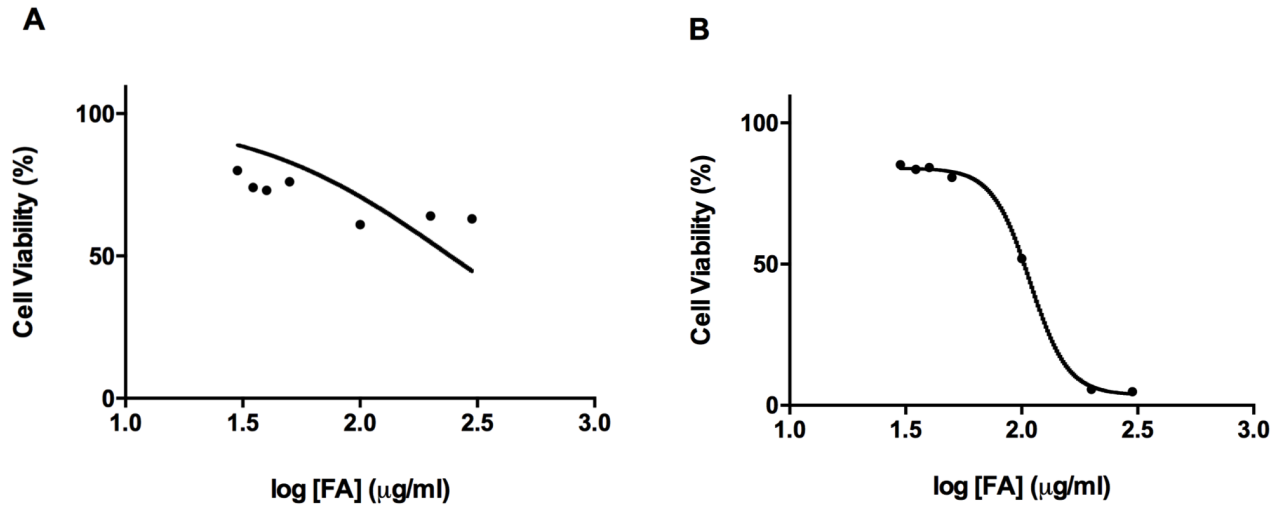

Figure 1: Cytotoxicity of FA on PBMCs and Thp-1 cells. FA induced a dose dependent decrease in PBMC **(A)** and Thp-1 **(B)** cell viability.

- FA activates caspase-independent cell death in PBMCs and intrinsic apoptosis in Thp-1 cells

To confirm the toxicity of FA, we assessed the externalization of phosphatidylserine (PS) on the plasma membranes of PBMCs and Thp-1 cells. FA significantly increased the externalization of PS in PBMCs and Thp-1 cells by 1.42 (18.43 + 0.006% vs. 26.16 + 0.003%; p=0.0003) and 2.27 (8.03 + 0.004% vs.18.19 + 0.002%; p<0.0001) fold, respectively (Fig. 2). Propidium iodide (PI) staining showed a decreased percentage of necrotic cells by FA as compared to the controls in both PBMCs and Thp-1 cells (Supplementary Fig. S1). This was confirmed by quantifying the release of lactate dehydrogenase (LDH). FA did not induce membrane leakage in both PBMCs and Thp-1 cells (Supplementary Fig. S2), therefore, necrotic cell death was excluded.

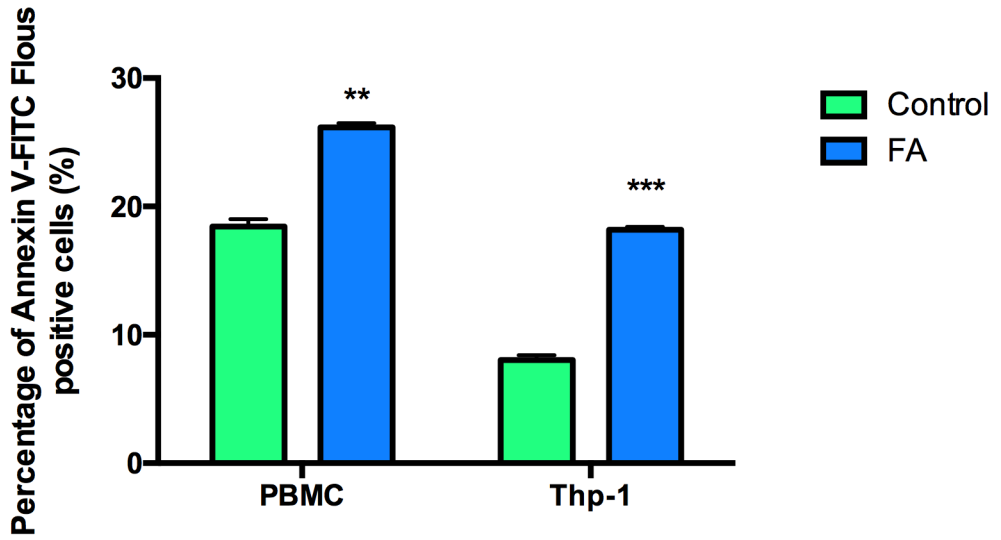

Figure 2: Translocation of PS in PBMCs and Thp-1 cells. FA induced PS externalization in both PBMCs and Thp-1 cells. Data are expressed as mean ± SD. ** $p<0.005$ relative to respective control; *** $p<0.0001$ relative to respective control.

Next, we measured caspase -8, -9 and -3/7 activities to determine the type of programmed cell death induced by FA in PBMCs and Thp-1 cells. Interestingly, despite the increase in PS externalization, FA significantly decreased caspase -8 activity by 0.81 fold ($1.09 \pm 0.001 \times 10^4$ RLU vs. $0.88 \pm 0.042 \times 10^4$ RLU; $p=0.0022$), caspase -9 by 0.73 fold ($10.89 \pm 0.609 \times 10^4$ RLU vs. $7.92 \pm 0.241 \times 10^4$ RLU; $p=0.0070$) and caspase -3/7 activities by 0.10 fold ($1.19 \pm 0.258 \times 10^4$ RLU vs. $0.12 \pm 0.026 \times 10^4$ RLU; $p=0.0035$) in PBMCs, relative to the control (Table 1). This result suggests that FA induced caspase-independent cell death in PBMCs. In Thp-1 cells, however, FA significantly decreased caspase -8 activity by 0.74 fold ($4.52 \pm 0.306 \times 10^4$ RLU vs. $3.3265 \pm 0.021 \times 10^4$ RLU; $p=0.0211$), but significantly increased both

caspase -9 activity (1.43 fold; 62.67 ± 3.701x10^4 RLU vs. 89.37 ± 0.590x10^4 RLU; p=0.0065) and caspase -3/7 activity (5.33 fold; 0.82 ± 0.482x10^4 RLU vs. 4.38 ± 0.604x10^4 RLU; p=0.0041) when compared to the control (Table 2); an indicator of activation of intrinsic apoptosis.

Table 1: Effect of FA on caspase (-8, -9, -3/7) activity in healthy PBMCs

	Mean ± SD (RLU x10^4)		**Fold change**	***p* value**
	PBMC			
	Control	FA		
Caspase -8	1.0918 ± 0.0007	0.8831 ± 0.0419	0.81	0.0022**
Caspase -9	10.8855 ± 0.6094	7.9185 ± 0.2409	0.73	0.0070*
Caspase -3/7	1.1858 ± 0.2581	0.1218 ± 0.0261	0.10	0.0035**

SD: standard deviation**; RLU:** relative light units**;** *p<0.05; **p<0.005.

Table 2: Effect of FA on caspase (-8, -9, -3/7) activity in Thp-1 cells

	Mean ± SD (RLU x10^4)		**Fold change**	***p* value**
	Thp-1			
	Control	FA		
Caspase -8	4.5235 ± 0.3055	3.3265 ± 0.0206	0.74	0.0211*

Caspase -9	62,6683 ± 3.7013	89.3652 ± 0.5900	1.43	0.0065*
Caspase -3/7	0.8210 ± 0.4816	4.3758 ± 0.6041	5.33	0.0041**

SD: standard deviation**; RLU:** relative light units; **p*<0.05; ***p*<0.005.

- FA induces oxidative stress in PBMCs and Thp-1 cells

Increased intracellular ROS is known to promote oxidative stress, that is not only injurious to cells but may also regulate cell signaling pathways [8]. Lipid peroxidation, a marker for oxidative stress, was measured by quantifying malonaldehyde (MDA) using the Thiobarbituric acid (TBARS) assay. FA significantly elevated MDA levels in PBMCs (7.59 fold; 0.02 ± 0.010 μM vs. 0.16 ± 0.016 μM; p=0.0006) and Thp-1 cells (1.59 fold; 0.18 ± 0.020 μM vs. 0.28 ± 0.010 μM; p=0.0039) (Fig. 3A). FA induced oxidative stress in both normal human PBMCs and Thp-1 cells.

Mitochondria are important in maintaining cellular redox homeostasis and activation of the intrinsic apoptotic pathway. The evaluation of FA induced mitochondrial membrane integrity by flow cytometry in PBMCs showed that despite increased MDA levels, there was no effect on the mitochondrial membrane potential; however, in Thp-1 cells, mitochondrial membrane depolarization was significantly increased (1.95 fold; 33.58 ± 1.425% vs. 65.48 ± 0.329%; p=0.0007) when compared to the controls (Fig. 3B).

Further, FA significantly depleted ATP levels in both PBMCs (0.16 fold; 38.97 ± 1.183x10^4 RLU vs. 6.11 ± 0.266x10^4 RLU; p=0.0002) and in Thp-1 cells (0.52 fold; 225.21 ± 8.014x10^4 RLU vs. 117.26 ± 10.017x10^4 RLU; p=0.0007) relative to controls (Fig. 3C).

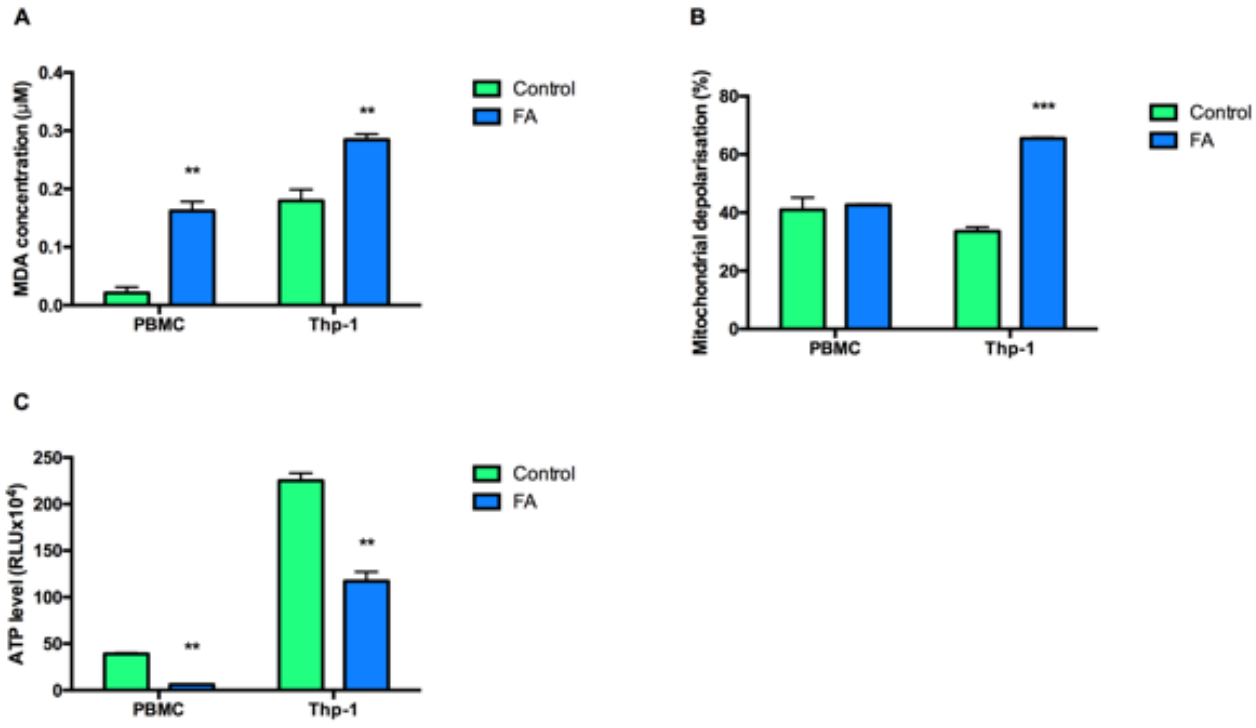

Figure 3: Effect of FA on the oxidative status and mitochondrial function in PBMCs and Thp-1 cells. FA increased MDA levels in both PBMCs and Thp-1 cells **(A),** had no effect on the mitochondrial membrane potential in PBMCs but increased depolarization of the mitochondrial membrane in Thp-1 cells **(B).** FA depleted ATP levels in PBMCs and Thp-1 cells **(C).** Data are expressed as mean ± SD. **p<0.005 relative to respective control; ***p<0.0001 relative to respective control.

- Effect of FA on Bax and p-Bcl-2 protein expression in PBMCs and Thp-1 cells

To validate caspase-dependent and –independent cell death, protein expressions of pro-apoptotic Bax and anti-apoptotic p-Bcl-2 was determined. FA significantly decreased Bax protein expression (0.71 fold; 100 ± 10.33 % vs. 70.90 ± 7.34 %; p=0.0201) and increased p-Bcl-2 protein expression (1.18 fold; 100 ± 6.40 % vs. 118.30 ± 2.83 %; p=0.0455) in PBMCs compared to the control (Figure 4A and 4B), whilst it only significantly decreased the protein expression of p-Bcl-2 (0.78 fold; 99.39 ± 0.89 % vs. 77.40 ± 2.82 %; p=0.0007) in Thp-1 cells (Fig. 4C and 4D).

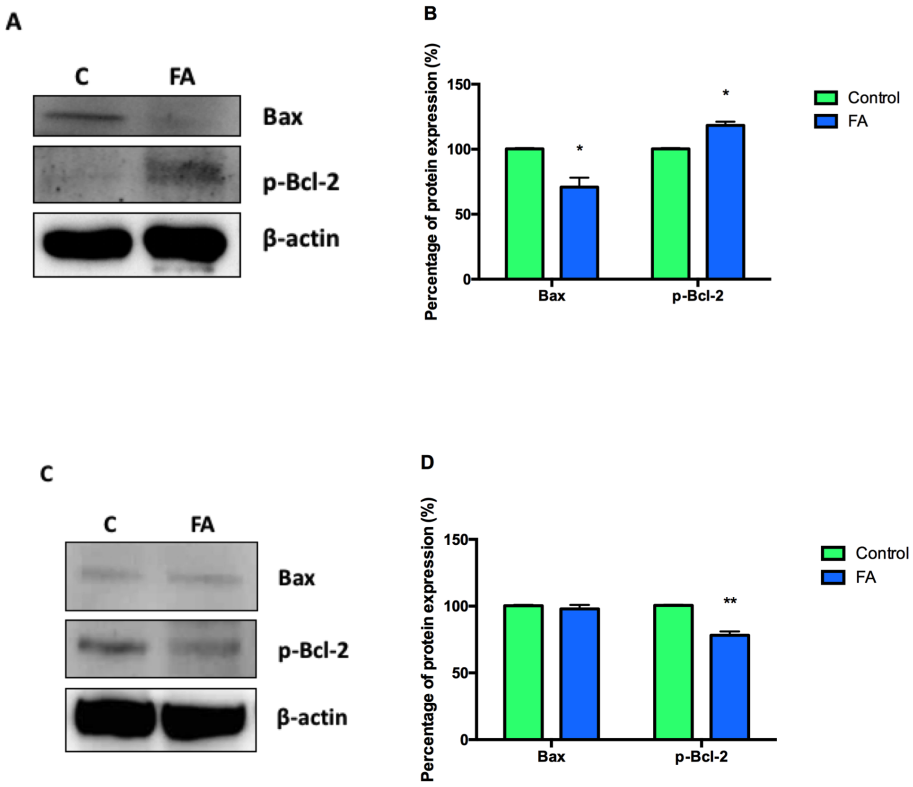

Figure 4: Effect of FA on apoptotic regulator proteins Bax and p-Bcl-2 in PBMCs and Thp-1 cells. Protein expressions of Bax and p-Bcl-2 in healthy PBMCs **(A)** and Thp-1 cells **(C).** Percentage of Bax and p-Bcl-2 protein expression in healthy PBMCs **(B)** and Thp-1 cells

(D). Percentage of protein expressions were represented as mean ± SD. *p<0.05 relative to respective control; **p<0.005 relative to respective control.

- Effect of FA on MAPKs expression in PBMCs and Thp-1 cells

Finally, to evaluate the effect of FA on MAPK signaling, phosphorylation of MAPK protein expressions was determined using western blotting. In PBMCs, FA significantly increased expression of p-ERK (42 kDa fragment and 44 kDa fragment; 1.94 fold; 100 ± 5.73 % vs. 194.19 ± 26.83 %; p=0.0271 and 1.36 fold; 99.80 ± 0.68 % vs. 136.20 ± 5.29 %; p=0.0006, respectively) and p-JNK (46 kDa fragment and 54 kDa fragment; 1.46 fold; 97.10 ± 10.06 % vs. 141.39 ± 0.92 %; p=0.0035 and 1.13 fold; 96.56 ± 11.92 % vs. 108.77 ± 2.45 %; p=0.0454, respectively) (Fig. 5A and 5B). In Thp-1 cells, FA also significantly increased p-ERK (42 kDa and 44 kDa fragments; 1.35 fold; 100 ± 3.54 % vs. 135.13 ± 15.06 %; p<0.0001 and 1.05 fold; 100 ± 5.15 % vs. 104.68 ± 6.93 %; p=0.0006, respectively) (Fig. 5C and 5D), whilst significantly decreasing p-JNK (46 kDa and 54 kDa fragments; 1.02 fold; 105.85 ± 20.25 % vs. 108.37 ± 0.75 %; p=0.0461 and 0.62 fold; 103.48 ± 12.05 % vs. 63.98 ± 7.03 %; p=0.0055, respectively) (Figure 5C and 5D). Furthermore, FA significantly decreased the expression of p-p38 in PBMCs and Thp-1 cells (0.75 fold; 100 ± 9.17 % vs. 74.73 ± 11.08 %; p=0.0401 and 0.70 fold; 100 ± 17.40 % vs. 69.86 ± 9.89 %; p=0.0033, respectively) (Fig. 5A, 5B, 5C and 5D).

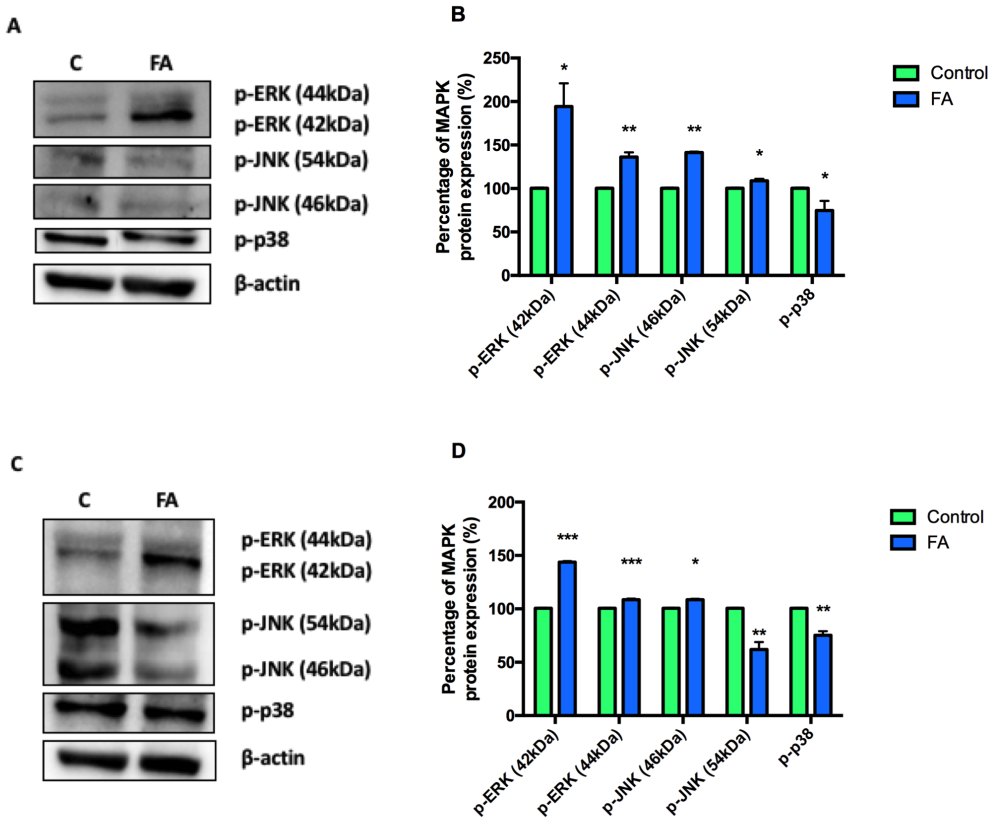

Figure 5: Effect of FA on MAPK protein expression. MAPK protein expression in healthy PBMCs **(A)** and Thp-1 cells **(C).** Percentage of MAPK protein expression in healthy PBMCs **(B)** and Thp-1 cells **(D)**. Percentage of protein expressions were represented as mean ± SD. *p<0.05 relative to respective control; **p<0.005 relative to respective control; ***p<0.0001 relative to respective control.

Discussion

The promising role of divalent ion chelators in proliferative and virulent diseases has led to growing interest [35-37]. FA, a picolinic acid analogue and potent divalent metal chelator, has shown potential as an anti-cancer, anti-microbial and anti-viral agent [35-37]. Recently, the role

of divalent ion chelators in proliferative and virulent diseases has been extensively studied. However, the toxicity of FA, a food-borne mycotoxin, on the immune system containing a diverse population of cells, has to date not been studied.

In this study, we showed the immunotoxic potential of FA to both healthy PBMCs (diverse population of immune cells) and the distinct immune Thp-1 cells. Interestingly, FA inhibited Thp-1 cell proliferation at an IC_{50} value less than half that of PBMCs (Fig. 1A and 1B). This result is in agreement with other studies that showed cytotoxic effects of FA on WI-38 cells (fibroblastic cells), LoVo cells (colorectal adenocarcinoma cells) and MDA-468 cells (human breast adenocarcinoma cells) in which FA had preferentially inhibited the proliferation of cancerous cells (LoVo and MDA-468) when compared to the normal cells (WI-38) [35]. Our data suggests that FA may exert selective toxicity to distinct immune cell types as evidenced by the Thp-1 response, albeit a leukemic cell line. Additionally, in comparison to the anti-neoplastic drug, ellipticine failed to inhibit PBMC and Thp-1 cell viability; which further exemplifies the potency of FA.

Contrary to the study by Fernandez-Pol (1998), FA significantly increased the externalization of PS in both PBMCs and Thp-1 cells by 26.16% and 18.19%, respectively (Fig. 2). Given that the externalization of PS occurs during both apoptosis and paraptosis, activities of caspases -8, -9 and -3/7 were assayed to determine type of cell death induced by FA in both cell lines; apoptosis requires caspase activation whilst paraptosis is independent of caspase activation. FA substantially decreased caspase activities in PBMCs (Table 1), strongly suggesting that paraptosis was the preferred mode of cell death. In Thp-1 cells, however, FA significantly increased caspase -9 and -3/7 activities were (Table 2), indicating the induction of intrinsic apoptosis in Thp-1 cells.

Immune cells respond to stimuli by activating MAPK signaling to amplify other signals to elicit an appropriate physiological response for programmed cell death [14,38]. Previous studies showed that prolonged activation of MAPK signaling induced cell death via ROS-activation of MAPK signaling pathways [16]. ROS are continuously generated by cellular processes, with the mitochondrion being the major source [6]. Excessive ROS generated during oxidative phosphorylation can cause oxidative damage to proteins, DNA and phospholipids [16]; oxidative degradation of lipids results in the formation of lipid peroxides such as MDA [39]. FA significantly increased MDA levels in both PBMCs and Thp-1 cells (Fig. 3A), indicative of a prolonged oxidative stress environment. Furthermore, FA disrupted mitochondrial membrane potential by increasing mitochondrial depolarization in Thp-1 cells (Fig. 3B). This could be due to the weak acidic nature of the carboxylic acid group of FA [40]. Weak acids act as proton carriers across lipid membranes, thereby disrupting the proton gradient along the electron transport chain (ETC) [41,42]. Interestingly, FA did not disrupt the mitochondrial membrane potential in normal PBMCs (Fig. 3B) despite the significant depletion of ATP levels in both normal PBMCs and Thp-1 cells (Fig. 3C). In Thp-1 cells, this is substantiated by the decreased cell viability (decreased redox potential) and increased mitochondrial membrane depolarization. Also, the activation of ATP dependent caspases -9 and -3/7 may further deplete ATP levels [43]. In PBMCs, however, the decreased ATP levels may be due to increased activation and prolonged activation of ATP dependent protein kinases.

Intracellular ROS not only alters cellular integrity but is also important to MAPK signaling cascades [6]; FA induced increased ROS production and up-regulated protein expression of ERK in Thp-1 cells (Fig. 5C and 5D). Although ERK signaling pathways are well known for their role in promoting cell survival, recent studies have demonstrated their ability to

potentiate apoptosis [44]. Prolonged activation of ERK may be due to the inhibition of tyrosine phosphatases, a group of enzymes responsible for the removal of phosphate groups on phosphorylated tyrosine residues, hence inactivating the protein [3]. However, tyrosine phosphatases are sensitive to increased ROS and become oxidized, thereby inhibiting their activity and prolonging ERK activation [3,45].

JNK and p38 MAPK signaling pathways are generally directed towards initiating cell death upon activation by stress signals. Recently, however, these signaling pathways have been associated in both cell death and survival [38]. In Thp-1 cells, FA significantly decreased p-JNK activation and p-p38 protein expressions (Fig. 5C and 5D). A study by Pedram et al. (1998) documented the cross-talk between the ERK and JNK MAPKs where the activation of JNK by ERK MAPK was followed by the activation of ERK by vascular endothelial growth factor (VEGF) whilst JNK stimulated ERKs proliferative signaling. Therefore, it can be inferred that a decrease in JNK activity hinders the cross-talk between JNK and ERK MAPKs, preventing survival signaling by ERK [46].

Additionally, JNK and ERK MAPKs regulate the expression of Bcl-2 family proteins that are central in regulating the mitochondrial apoptotic death pathway [3,47]. Bcl-2 inhibits apoptosis by forming a complex with pro-apoptotic proteins such as Bax [47,48]. Phosphorylation of Bcl-2 compromises it's protein stability and affects dimerization with Bax [49]. Thus, dissociation from the complex at the mitochondrial membrane leads to the formation of mitochondrial permeability transition pore (mPTP) and subsequent caspase activation [50]. In support of the increased caspase -9 and -3/7 activities in Thp-1 cells, FA decreased p-Bcl-2 expression resulting in apoptotic cell death (Fig. 4C and 4D). JNK signaling regulates the expression of Bcl-2 and is up-regulated in response to JNK activation. FA activated ERK death signaling,

decreased p-Bcl-2 expression and induced apoptosis in Thp-1 cells, but had no significant effect on Bax expression (Fig. 4C and 4D). This may be due to the deletion of the p53 gene in the Thp-1 cell line as p53 acts as a transcription factor for Bax expression and recruitment to the mitochondrial membrane [24,51-55]. Additionally, increased ERK activity regulates mitochondrial membrane potential [56] and corresponds with the increased caspase -9 and -3/7 activities, and the subsequent activation of cell death in Thp-1 cells by FA. In PBMCs, FA increased p-Bcl-2 expression (Fig. 4A and 4B) and decreased Bax expression (Fig. 4A and 4B), with a corresponding decrease in caspase -8, -9 and -3/7 activities. Increased p-Bcl-2 expression helps maintain the mitochondrial membrane integrity and subsequent mitochondrial membrane potential by preventing the release of cytochrome c, activation of caspase -9 and the initiation of intrinsic apoptosis, further validating the induction of paraptosis in normal PBMCs by FA.

Although the molecular activation of paraptosis remains unknown, studies have suggested the involvement of MAPK signaling in the induction of cell death. In PBMCs, FA significantly increased the expression of ERK and JNK whilst decreasing p38 expression (Fig. 5A and 5B). Sperandio et al., (2000) reported that ERK and JNK activity mediated paraptosis stimulation by insulin-like growth factor 1 receptor, and that inhibition of these MAPKs prevented the induction of paraptosis in 293T cells [57]. Another study by Yumnam et al., (2014) showed the involvement of ERK MAPK in hesperidin-induced paraptosis of human hepatocellular carcinoma (HepG2) cells [58]. Sugimori and colleagues (2015) recently showed that activated JNK induced paraptosis induction in HL-60 and NB4 human promyelocytic leukemic cell lines and in bone marrow blasts treated with benfotiamine [59]. Contrary to the studies by Sperandio et al., (2000) and Yumnam et al., (2014), benfotiamine inhibited the activity of ERK in bone marrow blasts and had no effect on ERK activity in HL-60 and NB4

cell lines [57,58]. This suggests that the involvement of MAPK in the induction of paraptosis may be dependent on the cell line and type of activation. Additionally, caspase -9 was reported to be a direct target of ERK MAPK, and that phosphorylation at threonine 125 on caspase -9 inhibits its pro-apoptotic activity [60]. These findings support the activation of MAPK signaling pathways in the induction of paraptosis in PBMCs treated by FA.

Conclusion

FA is immunotoxic to both healthy PBMCs and Thp-1 cells, albeit at a higher concentration in PBMCs. The cancerous Thp-1 cells are highly susceptible to FA toxicity. Collectively, the results show that the host response to FA exposure augmented MAPK signaling and induction of apoptosis in Thp-1 cells (via the mitochondrial apoptotic pathway) and paraptosis in PBMCs. This study shows that FA, a common food borne mycotoxin, is toxic to the human immune system. This data may help develop a better understanding of the immune risks associated with FA consumption. This has great importance in socio-economically challenged countries where the majority population relies on corn as a food staple.

Material and methods

- Materials

Cell culture reagents for PBMC maintenance and FA (*Gibberella fujikuroi*) were purchased from Sigma Aldrich (Johannesburg, SA). The Thp-1 cells and media were purchased from ATCC (University Boulevard Manassas, USA) and Scientific group (Johannesburg, SA), respectively. Luminometry reagents were obtained from Promega (Madison, USA).

- Cell culture

PBMCs were isolated from whole blood using Histopaque 1077 (Sigma Aldrich) and gradient centrifugation from young healthy males following institutional ethical approval (BE057/15) and written informed consent. Collection and use of blood was in compliance with relevant institutional guidelines and procedures. Isolated PBMCs were maintained at 37 ^{0}C with 5% CO_2 in RPMI 1640 medium (supplemented with 10% FCS, 1% L-glutamine and 1% pencillin-streptomycin). Thp-1 cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 1% L-glutamine, 1% pencillin-streptomycin, 1 mM sodium pyruvate and 0.05 mM β-mecaptoethanol. Thp-1 cells were maintained at 3 x10^5 cells/ml in 75 cm^3 ventilated flasks at (37 ^{0}C, 5% CO_2) and were split at a cell count of 8 x10^5 cells/ml. Viability of cells was assessed using trypan blue exclusion.

- Cell viability

The cytotoxicity of FA on PBMCs and Thp-1 cells was analyzed using the WST-1 reagent. Briefly, PBMC and Thp-1 cells (20,000 cells/well) were seeded into a 96-well microtitre plate. The cells were incubated with varying FA concentrations (30-300 µg/ml) in triplicate (200 µl/well) for 24 h (37 ^{0}C, 5% CO_2). Ellipticine, anti-neoplastic agent, was used as a positive control to FA toxicity (Data shown in Supplementary Tables: S1 PBMC; S2 Thp-1). A positive control of cells with RPMI only and a negative control with RPMI/WST-1 reagent solution was also seeded. Following incubation, the plate was centrifuged at 24 ^{0}C, 400 *xg* for 10 min. The supernatant was then aspirated and 110 µl/well of a RPMI/WST-1 reagent solution (1:10) was added and incubated for 3 h (37 ^{0}C, 5% CO_2). The optical density of the

colorimetric reaction was measured at a wavelength of 450 nm and reference wavelength of 620 nm using a spectrophotometer (Bio-Tek uQuant, Winooski, VT, USA). The percentage cell viability was calculated by standardizing untreated (control) cells to 100% and then comparing FA treated cells to the control cells (Detailed calculation shown as supplementary information). The concentration of half maximum inhibition (IC_{50}) was determined using GraphPad Prism v5.0 software. All assays were performed in triplicate, twice independently.

- Cell death parameters

- *PS Externalization*

Flow cytometry was performed to determine the externalization of PS. Following treatment, 100 µl of an Annexin V-FITC Fluos solution (1:1:50; annexin V-FITC: PI: staining buffer) was added to each sample (200,000 cells in 100 µl PBS) and incubated in the dark at room temperature (RT, 15 min). Thereafter, the samples (20,000 events) were analyzed for apoptosis on the AccuriTM C6 flow cytometer. The cells were gated to exclude cellular debris using the Fl-1 channel (525 nm) (BD Biosciences, Johannesburg, SA). The results were expressed as a percentage.

- *LDH activity*

The LDH cytotoxicity detection kit (Roche, Mannheim, Germany) was used to confirm damaged/necrotic cells. In brief, cell homogenates (100 µl) were seeded into a 96-well opaque polystyrene microtitre plate in triplicate. Subsequently, 100 µl of a substrate mixture containing a catalyst (diaphorase/NAD^+) and dye solution (INT/sodium lactate) was added to

each homogenate and incubated in the dark for 25 min (RT). The optical density was measured (500 nm) using a spectrophotometer (Bio-Tek uQuant, Winooski, VT, USA). The results were reported as mean optical density.

- *Caspase activity*

Caspase activities of -8, -9 and -3/7 were determined using luminometry. Cells (20,000 cells/well) were seeded into a 96-well opaque polystyrene microtitre plate in triplicate. 20 µl/well of the reagent (Caspase-Glo® 3/7, Caspase-Glo® 8 and Caspase-Glo® 9 Assays) was added to each sample and incubated in the dark for 30 min (RT). Thereafter, the luminescence was measured on a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA). All data was expressed as relative light units (RLU).

- Evaluation of oxidative stress

- *Lipid peroxidation*

The TBARS was used to determine FA generation of ROS [61]. Briefly, cell homogenates (400 µl) were added to a 7% phosphoric acid (400 µl) and a thiobarbituric acid (1%w/v)/ butylated hydroxytoluene (0.1 mM) (TBA/BHT) solution. A positive control containing MDA (1 µl) and a negative control containing 3 mM hydrogen chloride were prepared. All samples were heated in a water bath (100 ^{0}C, 15 min) and allowed to cool (RT). Thereafter, lipids were extracted with butanol (1.5 ml) and were measured on a spectrophotometer (Bio-Tek uQuant, Winooski, VT, USA) at 532 nm with reference wavelength of 600 nm. The mean optical

density for each sample was calculated and divided by the absorption coefficient (156 mM^{-1}). The results were expressed in μM.

- *Mitochondrial membrane potential*

Mitochondrial membrane potential was measured using the JC-1 Mitoscreen kit (BD Biosciences, Johannesburg, SA) and flow cytometry. Briefly, 100 μl of a JC-1 working solution was added to each sample (200,000 cells in 100 μl PBS) and incubated in the dark for 30 min (RT). Following incubation, 100 μl flow cytometry sheath fluid was added to each sample and were analyzed on the AccuriTM C6 flow cytometer. A total of 20 000 events were gated using AccuriTM C6 flow cytometer Fl-1 channel (525 nm) (BD Biosciences, Johannesburg, SA). The results were expressed as a percentage.

- *ATP levels*

Intracellular ATP levels were measured using the ATP CellTitre Glo reagent (Promega, Madison, USA). Following treatment, 20,000 cells/well were seeded into a 96-well opaque polystyrene microtitre plate in triplicate. The reagent (20 μl/well) was added to each sample and incubated in the dark for 30 min (RT). Thereafter, the luminescence was measured on a ModulusTM microplate luminometer (Turner Biosystems, Sunnyvale, USA). The data was expressed as relative light units (RLU).

- Western blotting

Western blots were performed to analyze the protein expressions of p-ERK, p-JNK, p-p38, Bax and p-Bcl-2. Briefly, total protein was isolated using Cytobuster^{TM} reagent (Novagen, San Diego, CA, USA). Cells were re-suspended in 200 µl Cytobuster and incubated on ice for 30 min. Following incubation, the cells were centrifuged for 10 min (180 xg, 4 ^{0}C). Protein samples were quantified by the bicinchoninic acid (BCA) assay and standardized to 0.2 mg/ml (PBMCs) and 1.0 mg/ml (Thp-1). The samples were then boiled in Laemmli Sample buffer (dH_20, 0.5 M Tris-HCl (pH 6.8), glycerol, 10% SDS, β-mercaptoethanol, 1% bromophenol blue) for 5 min (100 ^{0}C). Thereafter, the samples (25 µl- Thp-1; 40 µl- PBMC) were electrophoresed in sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gels (4% stacking gel, 7.5% resolving gel) for 1.5 h at 150 V (Bio-Rad compact power supply). The separated proteins were electro-transferred onto nitrocellulose membranes for 30 min (400 mA) using the Trans-Blot Turbo Transfer system (Bio-Rad). The membranes were then blocked for 1 h (RT) with 5% bovine serum albumin (BSA) in Tris Buffer Saline with tween 20 (TTBS- NaCl, KCl, Tris, dH_2O, 0.5% tween 20, pH 7.5) or 5% Non-Fat Dry Milk (NFDM) in TTBS for phospho- and non-phospho- antibodies, respectively. Thereafter, the membranes were incubated with primary antibody [mouse anti-p-ERK (9106), mouse anti-p-JNK (9255), rabbit anti-Bax (5023), rabbit anti-p-Bcl-2 (2827), Cell Signalling, 1: 1000; mouse anti-p-p38 (M8177), β-actin (A3854), Sigma Aldrich, 1: 5000] for 1 h (RT) and then overnight at 4 ^{0}C. The membranes were washed 5 times with TTBS (10 min intervals) and incubated (RT) with horseradish peroxidase (HRP)- conjugated secondary antibody [goat anti-rabbit (ab6112), anti-mouse (ab97046), Abcam, 1: 5000] for 1 h. Once more, the membranes were washed 5 times with TTBS (10 min intervals). Protein band images were detected using Clarity Western ECL Substrate (Bio-Rad) and captured using Alliance 2.7 Image documentation system (UViTech, Cambridge, UK). The expression of protein bands was analyzed using UViBand Advanced Image Analysis software v12.14 (UViTech,

Cambridge, UK). All proteins were normalized to β-actin before comparison (i.e. control vs. FA treatment. The data was expressed as relative band intensity (RBI).

- Statistical analysis

Statistical analysis was performed using GraphPad Prism v5.0 software (GraphPad Software Inc., La Jolla, USA). GraphPad Prism Software was used for the unpaired t-test with Welch's correction to assess the differences between samples. Level of significance (p) was established at a $p<0.05$.

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S.D. performed all experiments and analyzed data. S.D., S.N. and A.A.C. wrote main manuscript text. A.A.C. designed the study. D.B.N. assisted in cell culture and analyzed data. All authors reviewed the manuscript.

Disclosure of conflict of interest

The authors declare that there are no competing interests.

CHAPTER THREE

Manuscript title: Fusaric acid alters Akt and AMPK signalling in C57BL/6 mice brain tissue

The previous chapter (Chapter 2) demonstrated FA acute toxicity against lymphoid (PBMCs) and myeloid cells (Thp-1 cells), showing moderate toxicity to healthy PBMCs, with significant energy deficits in both PBMCs and Thp-1 cells. At the organism level, immune hypometabolic states not only significantly impact immune function but also immune interactions with several effector organs in order to maintain systemic homeostasis. In particular, compromised immune activity may lead to impaired neuronal regulation of body temperature, sleep and appetite. FA-induced immunotoxicity and its potential impact on the nervous system, could result in concurrent hypometabolic effects in neuronal cells which are critically important due to the brains' high energy demand. Glucose is the primary energy source for the brain and facilitates cellular ATP production that is regulated by the AMPK and PI3K/Akt signalling cascades. AMPK is a major metabolic sensor that is activated in response to ATP depletion or by alterations in intracellular calcium levels, acting as a nuclear regulator of energy metabolism in order to shut down ATP-consuming pathways; Whilst, the PI3K/Akt pathway and its downstream mediators (GSK3, mTORC1) have been shown to be central players in regulating neuronal survival, cellular energy and metabolism, independent of cellular metabolic states. Given the emerging importance of metabolic dysregulation underlying several brain diseases, including amyotrophic lateral sclerosis (ALS), Alzheimer's, Parkinson's and Huntington's disease; and that FA targets the brain and induces significant immuno-metabolic toxicity, the following chapter aimed to determine the neurometabolic effects of FA and its influences on metabolic AMPK and Akt signalling pathways in murine brain tissue.

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FUSARIC ACID ALTERS AKT AND AMPK SIGNALLING IN C57BL/6 MICE BRAIN TISSUE

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ABSTRACT

The brain is a highly metabolic organ and requires regulatory mechanisms to meet its high energy demand, with the PI3K/Akt and AMPK signalling pathways being central regulators of cellular energy and metabolism, also making them major targets for the development of neurometabolic disorders. Fusaric acid (FA), a toxin of fungal origin, was found to be a potent hypotensive agent *in vivo* and in clinical trials by altering brain neurochemistry thus demonstrating its neurological effects. Notably, FA is a putative mitochondrial toxin, however, the metabolic effects of FA in the brain remains unknown. Therefore, this study investigates the neurometabolic effects of FA via alterations to Akt and AMPK signalling pathways in C57BL/6 mice at acute (1 day) and prolonged exposure (10 days). Following 1 day exposure, FA augmented Akt signalling by increasing Akt S473 phosphorylation and the upstream regulators PI3K, mTOR and p70S6K. Activated Akt showed inhibition of GSK3 activity with the simultaneous activation of AMPK, p53 phosphorylation and reduced GLUT-1 and -4 receptor expressions, potentially suppressing neuronal glucose entry. However, after 10 days exposure, FA dampened PI3K/Akt and AMPK signalling, but increased the expression of GLUT receptors (1 and 4) in mice brain. Further, FA significantly depleted ATP levels, at 10 days exposure, despite increased PDHE1β activity (at both 1 and 10 days), strongly suggesting that FA mediates ATP depletion independent of metabolic signalling. In conclusion, FA mediates neurometabolic disturbances, at 1 and 10 day exposures, which may negatively influence normal brain aging and predisposes to neurodegenerative disorders.[1]

[1] Abbreviations:
Fusaric acid (FA); Phosphoinositide 3-kinase (PI3K); Protein kinase B (Akt); mammalian target of rapamycin (mTOR); Ribosomal protein S6 kinase (p70S6K); Glycogen synthase kinase 3 (GSK3); pyruvate dehydrogenase E1β (PDHE1β); pyruvate dehydrogenase kinase 1 (PDK1); AMP-activated protein kinase (AMPK); glucose transporter (GLUT); tricarboxylic acid (TCA)

Keywords: Fusaric acid, Akt, AMPK, ATP, GLUT4, brain

1. INTRODUCTION

Fusaric acid (FA, 5-butylpicolinic acid), a picolinic acid derivative, is a mycotoxin produced by the *Fusarium* species and was first recognised in the 1960's, for its potency against dopamine-beta-hydroxylase activity (Goodwin and Sack, 1975; Hidaka *et al.*, 1969; Nagasaka *et al.*, 1985; Sack and Goodwin, 1974; Toshiharu *et al.*, 1970). In animals, FA toxicity is associated with the induction of emesis, loss of appetite and lethargy (Smith, 1992). Studies have also reported FA's role in plant pathogenesis and metabolic toxicity in both plants and animals, resulting in alterations in mitochondrial activity and inhibition of ATP synthesis, cell proliferation and DNA synthesis (Bharathiraja *et al.*, 2010; D'Alton and Etherton, 1984; Pavlovkin *et al.*, 2004). Despite its toxic potential, FA's ability to alter brain neurochemistry by increasing tryptophan and serotonin levels have proven beneficial against hypertension and neurological diseases (Goodwin and Sack, 1975; Matta and Wooten, 1973; Terasawa and Kameyama, 1971; Terasawa *et al.*, 1976). Although FA has been shown to be neurologically active, it's metabolic effects on the brain remains unknown.

While the brain only constitutes about 2% of whole body weight, it requires a consistent and substantial supply of energy (Bélanger *et al.*, 2011; Magistretti and Allaman, 2015; Raichle and Gusnard, 2002). Although glucose is an obligatory energy source, the brain has the ability to efficiently utilize additional neuronal substrates, as well as, foster blood-derived

energy substrates to meet its energy requirements (Bélanger, *et al*., 2011; Magistretti and Allaman, 2015). At the same time, the brain is highly sensitive to glucose and oxygen deficiency hence an intricate control of neurometabolism is imperative (Ronnett *et al.*, 2009; Wang *et al.*, 2017). Over the past decade, several studies highlighted the role of AMP-activated protein kinase (AMPK) in the regulation of neuronal energy homeostasis (Claret *et al*., 2007; Fu *et al*., 2012). AMPK is a cellular energy sensor that responds to increases in the AMP:ATP ratio. Due to this unique capability, AMPK is considered a master regulator of metabolism. Activation of AMPK via phosphorylation on the threonine-172 (T172) residue (Rosso *et al.*, 2016) enhances energy generating processes [glucose uptake via glucose transporter (GLUT) recruitment and fatty acid oxidation] and dampens energy consuming processes (protein synthesis). With regard to neuronal activity, AMPK regulates mitochondrial metabolism and biogenesis (Fu *et al.*, 2012). A study done on Neuro2α cells reported AMPK activation by resveratrol and increased transcripts of mitochondrial biogenesis markers - peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1-α) and mitochondrial transcription factor A (TFAM), and Mitofusin 2, a mitochondrial protein marker (Amato and Man, 2011).

In addition to being induced by low ATP levels, AMPK activity can also be controlled by protein kinase B (Akt) through the alteration of glycogen synthase kinase 3 (GSK3) and p70 ribosomal S6 protein kinase (p70S6K) activity (Bhattacharya, 2018; Longnus *et al.*, 2005). Under basal conditions, Akt is expressed at low levels in the brain, but amplifies substantially upon neuronal stress (Dávila and Torres-Aleman, 2008; Yang *et al.*, 2018). Activation of Akt is dependent on the phosphorylation of threonine-308 (T308) and serine-473 (S473) residues at its catalytic domain by phosphoinositide 3-kinase (PI3K) and mammalian target of rapamycin complex 2 (mTORC2), respectively (Hassan *et al*., 2013). Several studies have

shown that disturbances in these regulatory pathways may lead to the development of diabetes, obesity, Alzheimer's disease and Parkinson's disease (Fu *et al.*, 2012; Rosso *et al.*, 2016). Although, the effect of FA on mitochondrial function and metabolism have been described, its metabolic effect and influence on these crucial pathways have not been investigated in the brain. Therefore, in this study we aimed to determine the effects of FA on neurometabolism through its role in the alteration of the Akt and AMPK metabolic signalling pathways in C57BL/6 mice brain.

2. MATERIALS AND METHODS

2.1. Ethics statement

This study was performed in accordance to the standard approved guidelines of the institutional Animal Research Ethics Committee of the University of KwaZulu-Natal (AREC/079/016) and in accordance with the ARRIVE N3R guidelines (McGrath *et al.*, 2010).

2.2. Animal and experimental design

C57BL/6 black male mice were obtained from the Biomedical Resource Unit (BRU) at the University of KwaZulu-Natal and housed at the Africa Health Research Institute (AHRI) animal facility (University of KwaZulu-Natal, Durban) under standard conditions [temperature (23 ± 1 ℃) and humidity (40-60 %), a 12-hour light/dark cycle] with *ad libitum* access to a commercially available animal feed and normal drinking water. Mice with mean

body weights (BW) of 18-22 g (6-8 weeks old) were randomly divided into four groups (n=3 per group) for short- (1 day) and prolonged (10 days) treatments: two control groups (1 and 10 days) and two FA-dosed groups (1 and 10 days).

Fusaric Acid (*Gibberella fujikuroi*) was purchased from Sigma Aldrich (Johannesburg, SA). FA was administered orally via gavage (0.25 ml) once daily at a dose of 50 mg/kg (Reddy *et al.*, 1996). Control groups received phosphate buffer saline (0.1 M PBS; 0.25 ml) (by oral gavage). At the end of the experimental periods (1 and 10 days), mice were euthanised by gas inhalation anaesthesia (Isofor; Safeline Pharmaceuticals). Brain tissue was surgically removed, rinsed well in PBS, and stored (-80 ℃) in Cytobuster ™ (Novagen, San Diego, CA, USA) and QIAzol lysis reagent (Qiagen, Germantown, MD) for protein and mRNA analysis respectively.

2.3. Intracellular ATP levels

Intracellular ATP levels were determined using the CellTiter-Glo kit (Promega, Madison, USA). Briefly, fresh brain tissue was dissociated (1-2 mm pieces) in Dulbecco's Modified Eagle Medium (DMEM) containing collagenase type II (15 mg/ml) and incubated at 37 ℃ (40 min). The digest was centrifuged (500 *xg*, 10 min, 4 ℃), and the supernatant was aspirated. The pellet composed of live cells was re-suspended and centrifuged (1000 *xg*, 20 min, 4 ℃) in DMEM containing 20% bovine serum albumin (BSA). The lipid layer was carefully aspirated and the pellet was re-suspended in serum-free DMEM (with 0.1% BSA)

for 30 min (37 ℃). Once more, the digest was re-suspended and centrifuged (500 *xg*, 10 min, 4 ℃). The pellet was washed once with PBS and the density of viable cells (viable cells/ml) was assessed using the trypan blue exclusion method described by Strober (2015). Subsequently, the cells (20,000 cells/well) were seeded into a 96-well opaque polystyrene microtitre plate (triplicate) and incubated with the reagent (20 µl/ml) for 30 min (dark, RT). The luminescent signal was measured on a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA) and was expressed as relative light units (RLU).

2.4. Brain tissue preparation and protein phosphorylation array

Brain tissue stored in Cytobuster ™ reagent supplemented with protease and phosphatase inhibitors was homogenized mechanically and centrifuged at 10,000 *xg* (4 ℃, 10 min). Cytoplasmic protein fractions were aspirated and quantified by the bicinchoninic acid (BCA) assay. Phosphorylation of Akt/mTOR signalling proteins (p-Akt, p-mTOR, p-p70S6K, p-GSK3α, p-GSK3β and p-p53) were detected using a Human/Mouse MAPK protein phosphorylation array (RayBiotech Inc., Norcross, USA) according to the manufacturer's protocol. Briefly, membranes were incubated in blocking buffer (30 min, RT) before being incubated overnight (4 ℃) with protein lysates (standardised to 1 mg/ml in blocking buffer). Thereafter, the membranes were washed and incubated (overnight, 4 ℃) with a detection antibody cocktail. The membranes were subsequently washed and incubated at 4 ℃ (overnight) with an HRP-Anti-Rabbit IgG concentrate. The membranes were washed once more and incubated with a chemiluminescent detection buffer (Clarity™ ECL Western

detection reagent, Bio-Rad, 2 min, RT) before being captured on ChemiDoc XRS$^+$ system (Bio-Rad, Johannesburg, SA). Signal spot intensities of phosphorylated proteins were quantified with Image Lab™ software 6.0.1 (Bio-Rad, Johannesburg, SA) and normalized against positive control spots for comparison (i.e. control vs. FA). This ratio (phosphorylated protein/positive control) was expressed as relative protein expression to the positive control.

2.5. Immunoblotting

Cytoplasmic protein was standardised to 1 mg/ml (in Cytobuster ^{TM} reagent) and boiled in Laemmli buffer (dH_2O, 0.5 M Tris-HCl (pH 6.8), glycerol, 10 % SDS, β-mercaptoethanol, 1 % bromophenol blue) for 5 min (100 ℃). Proteins were resolved by SDS-PAGE (7,5 %) and transferred to a nitrocellulose membrane. The membranes were blocked with 5 % BSA in Tween20 Tris-Buffer Saline (TTBS) for 1 hour (RT) and incubated (overnight, 4 ℃) with primary antibodies (Table 1). The membranes were washed (5x10 min) with Tris Buffer Saline with Tween 20 (TTBS- NaCl, KCl, Tris, dH_2O, 0.5 % tween 20, pH 7.5) before an additional incubation (2 hrs, RT) in secondary antibody: anti-rabbit (1:5,000; 7074S), anti-mouse (1:5,000; 7076S), Cell signalling. Chemiluminescent protein expressions were visualized using Clarity Western ECL Substrate (Bio-Rad, Johannesburg, SA) and captured on ChemiDoc XRS$^+$ system (Bio-Rad, Johannesburg, SA). Protein band densities were quantified with Image Lab™ software 6.0.1 (Bio-Rad, Johannesburg, SA). All protein expressions were normalized against β-actin (1:5,000, Sigma Aldrich) before comparison to the respective control. The intensity ratios of the target proteins (measured relative to β-actin) and phosphorylated protein isoforms (measured relative to the total protein form) were expressed as relative protein expression.

Table 1: **Primary antibodies for densitometric protein analyses by immunoblots**

Manufacturer	Antibody name	Host species	Catalogue number	Concentration
Cell signalling Technology	PI3 Kinase p110α	Rabbit	4249S	1:1,000
	Akt	Rabbit	9272S	1:1,000
	Phospho-AMPKα (Thr172)	Rabbit	2335S	1:1,000
	AMPKα	Rabbit	2532S	1:1,000
	Phospho-GSK-3α/β (Ser21/9)	Rabbit	9331S	1:1,000
	GSK-3α/β	Rabbit	5676S	1:1,000
Abcam	Glucose Transporter GLUT4	Rabbit	ab654	1:1,000
	PDK1	Rabbit	ab207450	1:1,000
	Phospho-Pyruvate Dehydrogenase E1-ß subunit (Ser293)	Mouse	ab177461	1:1,000
	Phospho-Akt (Ser473)	Rabbit	ab81283	1:1,000

2.6. *GLUT* mRNA expression

Total RNA from whole brain tissue was extracted with QIAzol reagent. Complementary DNA (cDNA) was synthesized from RNA (1,000 ng) using an iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA) in accordance with manufacturer's instructions. Quantitative detection of GLUT mRNA [*GLUT1*: Sense 5'GGGAGAGCAAACGGAACCG3', Anti-sense 5'CTGCCGAATTTTTCGCTGTCG3'; *GLUT4*: Sense 5'CTGAAGGTGGAATACTTGGAGC3', Anti-sense 5'GCCCAGGAACTGTGAGAAGA3'] levels were detected using the iQ™ SYBR® Green Supermix (Bio-Rad) as per manufacturer's protocol and performed on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Differences in gene expressions were normalized against housekeeping gene *GAPDH* [*GAPDH*: Sense 5'ATGTGTCCGTCGTGGATCTGAC3', Anti-sense 5'AGACAACCTGGTCCTCAGTGTAG3'] and analysed according to the methods described by Livak and Schmittgen, (2001). The data was represented as relative fold change.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism v6.0 software (GraphPad Software Inc., La Jolla, USA). GraphPad Prism Software was used for the unpaired t-test with Welch's correction. Results were expressed as mean ± standard deviation (SD). Level of significance (p) was established at a $p < 0.05$.

3. RESULTS

3.1. FA depletes intracellular neuronal ATP levels

Given that FA decreased ATP levels in plants, animals and *in vitro* human models (Abdul *et al.*, 2016; Bouizgarne *et al.*, 2006; Dhani *et al.*, 2017; Telles-Pupulin *et al.*, 1998), we determined its metabolic effect in whole brain tissue following a once daily 50 mg/kg.b.w dose by measuring intracellular ATP levels using luminometry. Consistent with previous reports, there was a minimal decrease in ATP levels in mice brain by FA following a 1 day exposure ($p < 0.3177$); however, ATP levels decreased significantly after 10 days exposure ($p < 0.0116$) (Table 2).

Table 2: The effect of FA exposure (1 and 10 days) on mice neuronal ATP levels

Treatment period	Mean ± SD ($RLUx10^4$)		Fold change	*p* value
	Control	FA		
1 day	6.235 ± 0.964	5.690 ± 0.824	0.91	0.3177
10 days	3.555 ± 0.324	2.356 ± 0.771	0.66	$0.0116^{\#}$

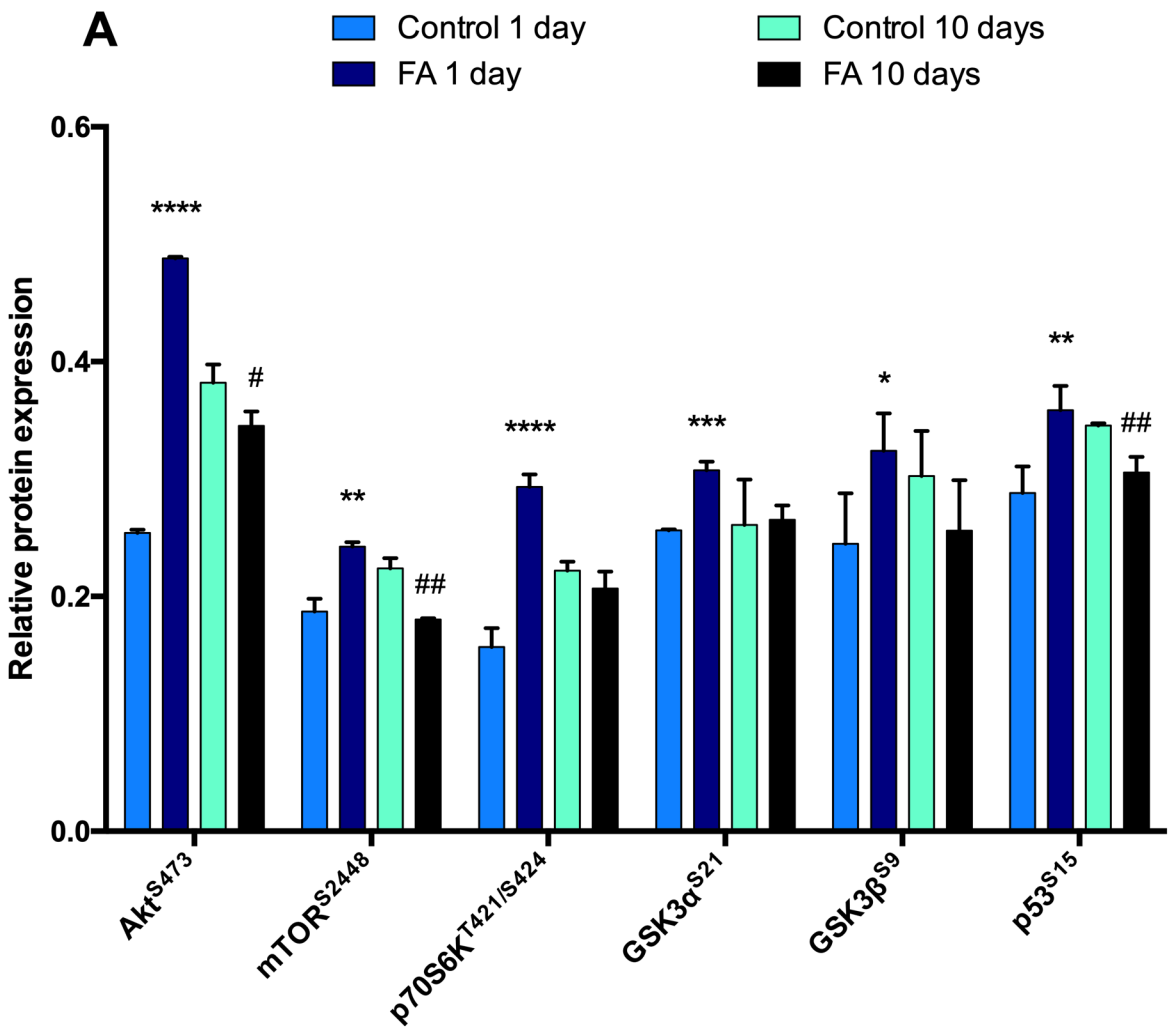
The data are represented as the mean ± SD obtained from experiments performed in triplicate (n=3). RLU: relative light units. # $p < 0.05$ (FA relative to control 10 days).

3.2. Effect of FA on neuronal PI3K/Akt/mTOR signalling

As a result of FA-induced ATP depletion, we investigated its subsequent effect on the neurometabolic energy sensor AMPK, and metabolic regulator Akt. We first evaluated phosphorylation of PI3K/Akt/mTOR signalling cascade using a commercial MAPK protein phosphorylation array. After 1 day of FA exposure, Akt signalling was significantly activated

as evidenced by the increased S473 phosphorylation ($p < 0.0001$), its upstream regulator, mTOR at serine-2448 (S2448) ($p < 0.0010$) and, threonine-421/serine-424 (T421/S424) phosphorylation on p70S6K ($p < 0.0001$) (Figures 1A and 1B). However, mice treated with FA over 10 days showed decreased phosphorylation of both mTOR (S2448) ($p < 0.0018$) and p70S6K (T421/S424) ($p < 0.1257$). These results paralleled the significantly reduced S473 Akt phosphorylation (10 days) ($p < 0.0107$) as observed from the protein arrays (Figures 1A and 1B).

To validate the protein array results, we then analysed the ratio between phosphorylated/total Akt and its upstream activator, PI3K by immunoblotting. Densitometric analysis illustrated a significant increase in PI3K expression at day 1 ($p < 0.0001$) (Figures 2A and 2B) and confirmed the activation of Akt (array), but no significant differences were noted with Akt S473 phosphorylation (1 and 10 days, $p < 0.6070$ and $p < 0.2242$, respectively) (Figures 2A and 2B). The data demonstrates that FA induced activation of Akt signalling following an acute exposure (1 day) whilst dampening Akt signalling at a prolonged exposure (10 days).


A
Control 1 day
FA 1 day
Control 10 days
FA 10 days
Relative protein expression
0.6
0.4
0.2
0.0
****
#
**
##
****
***
*
**
##
AktS473
mTORS2448
p70S6KT421/S424
GSK3αS21
GSK3βS9
p53S15

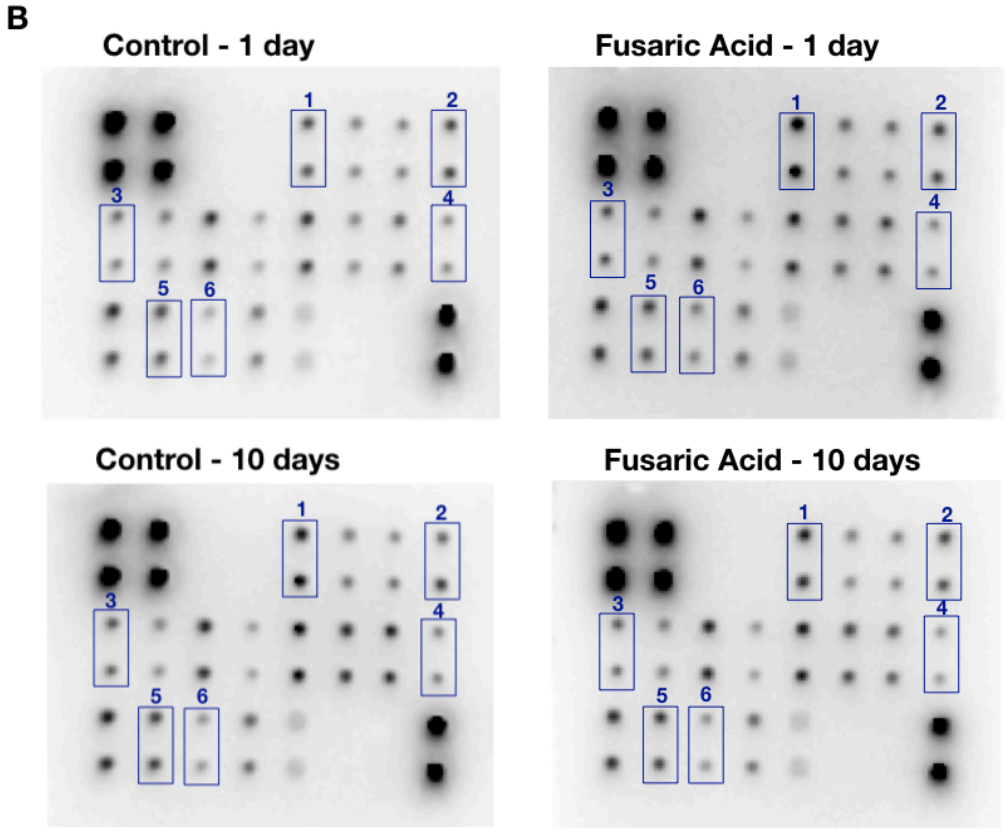


Figure 1: Effect of FA exposure on Akt signalling in mice brain tissue at 1 and 10 days exposure. Densitometric analysis of PI3K/Akt/mTOR signalling pathway proteins **(A)** were analysed using a MAPK protein phosphorylation array **(B)** for p-Akt^{S473} (1), p-GSK3α^{S21} (2), p-GSK3β^{S9} (3), p-mTOR^{S2448} (4), p-p53^{S15} (5) and p-p70S6K$^{T421/S424}$ (6). The data are represented as the mean ± SD obtained from experiments performed in triplicate (n=3). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ **** $p < 0.0001$ (FA relative to control 1 day); # $p < 0.05$, ## $p < 0.005$ (FA relative to control 10 days).

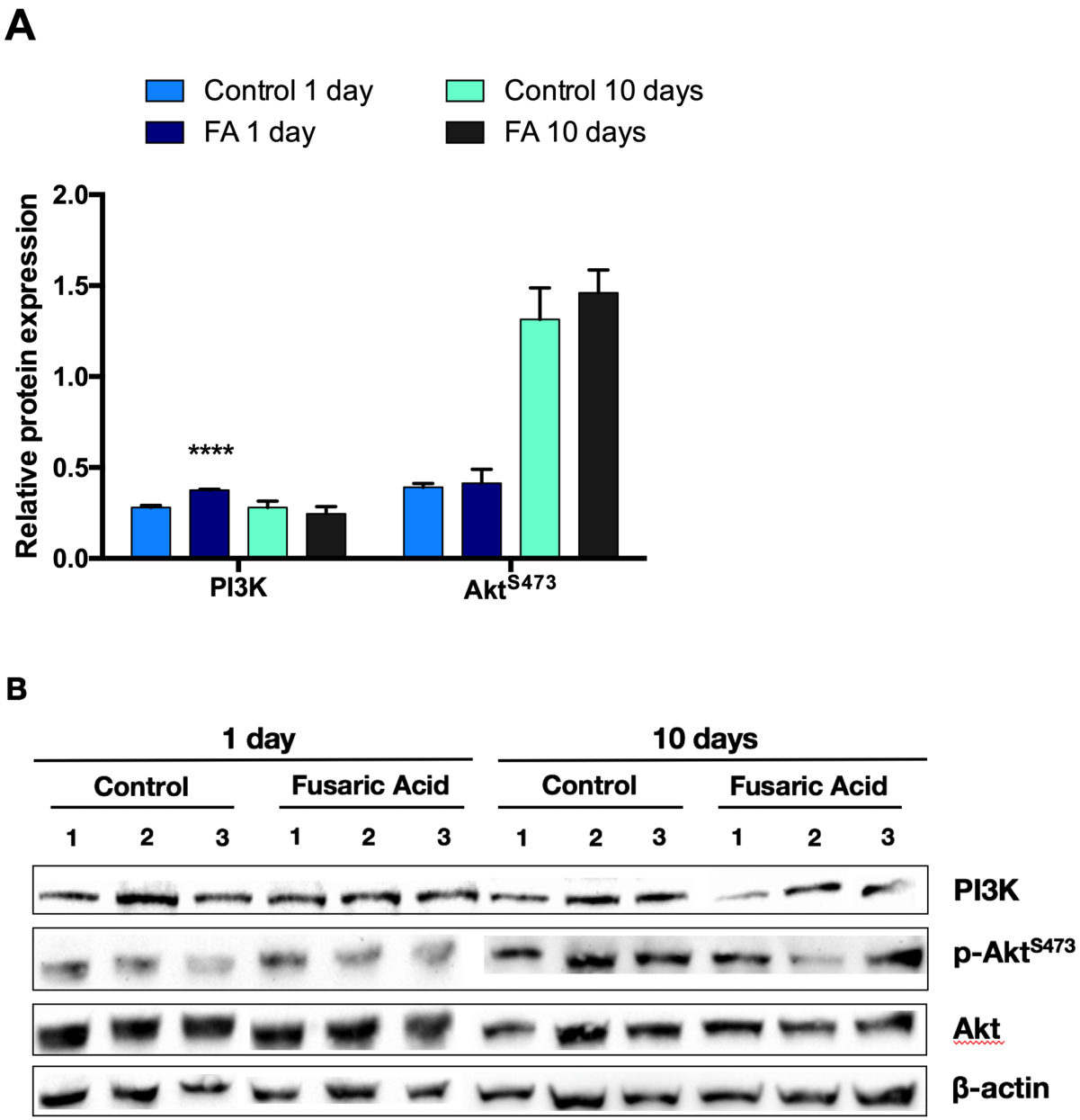


Figure 2: Validations on the effect of FA on the activation of Akt signalling in mice brain tissue at 1 and 10 days exposure. Relative densitometric (**A**) and protein expressions of PI3K, p-Akt^{S473} and Akt were quantified by immunoblotting (**B**). Detection of β-actin was used as an internal control. The data are represented as the mean ± SD obtained from experiments performed in triplicate (n=3). **** $p < 0.0001$ (FA relative to control 1 day).

3.3. FA regulation of GSK3α/β phosphorylation through Akt signalling and attenuates phosphorylation of PDHE1β

To elucidate the downstream mechanism by which Akt phosphorylation regulates neuroenergetics, we investigated the expression of GSK3, a downstream component of Akt signalling, known for its role in regulating mitochondrial energy metabolism (Case *et al.*, 2011; Maixner and Weng, 2013; Manning and Toker, 2017; Martin *et al.*, 2018; Medina and Wandosell, 2011; Salcedo-Tello *et al.*, 2011; Uranga *et al.*, 2013; Vincent *et al.*, 2011). In response to FA-induced Akt phosphorylation and activation, both isoforms of GSK3 (GSK3α and GSK3β) were phosphorylated and inhibited at serine-21 ($p < 0.0006$) and serine-9 ($p < 0.0275$), respectively (1 day) (Figures 1A and 1B), while FA decreased serine phosphorylation on GSK3α (S21) ($p < 0.8450$) and GSK3β (S9) ($p < 0.1602$) at 10 days, thereby increasing its activity (Figures 1A and 1B). Changes in FA-induced GSK3α/β phosphorylation were confirmed by assessing differences in the ratio between phosphorylated GSK3α/β over total GSK3α/β by immunoblotting. Western blotting revealed a decreased S21 expression on GSK3α following acute FA exposure ($p < 0.0497$) whilst its expression remained unchanged at 10 days ($p < 0.7384$) (Figures 3A and 3B). Additionally, FA increased S9 phosphorylation on GSK3β at both 1 ($p < 0.0006$) and 10 days ($p < 0.0001$) (Figures 3A and 3B). These discrepancies could be due to minor differences in total protein expression.

GSK3 has been shown to regulate mitochondrial function by modulating the activity of PGC1-α in human H4 neuroglioma cells (Martin, *et al*., 2018). Thus, as a result of decreased ATP levels, we assessed the expression of pyruvate dehydrogenase E1β (PDHE1β), a regulatory subunit of glycolysis and the tricarboxylic acid (TCA) cycle and, its aerobic

glycolytic regulator and a PGC1-α gene target, pyruvate dehydrogenase kinase 1 (PDK1). PDK1 expression was significantly decreased at 1 day ($p < 0.0006$) but increased at 10 days ($p < 0.0018$) following exposure to FA (Figures 3A and 3B). Next, we measured serine-293 (S293) phosphorylation, an inhibitory site on PDHE1β targeted by PDK1. Interestingly, FA significantly reduced S293 phosphorylation of PDHE1β at both exposure times ($p < 0.0338$, $p < 0.0125$) respectively (Figures 3A and 3B). This suggests that PDHE1β was still functional despite the increased PDK1 protein levels at 10 days.

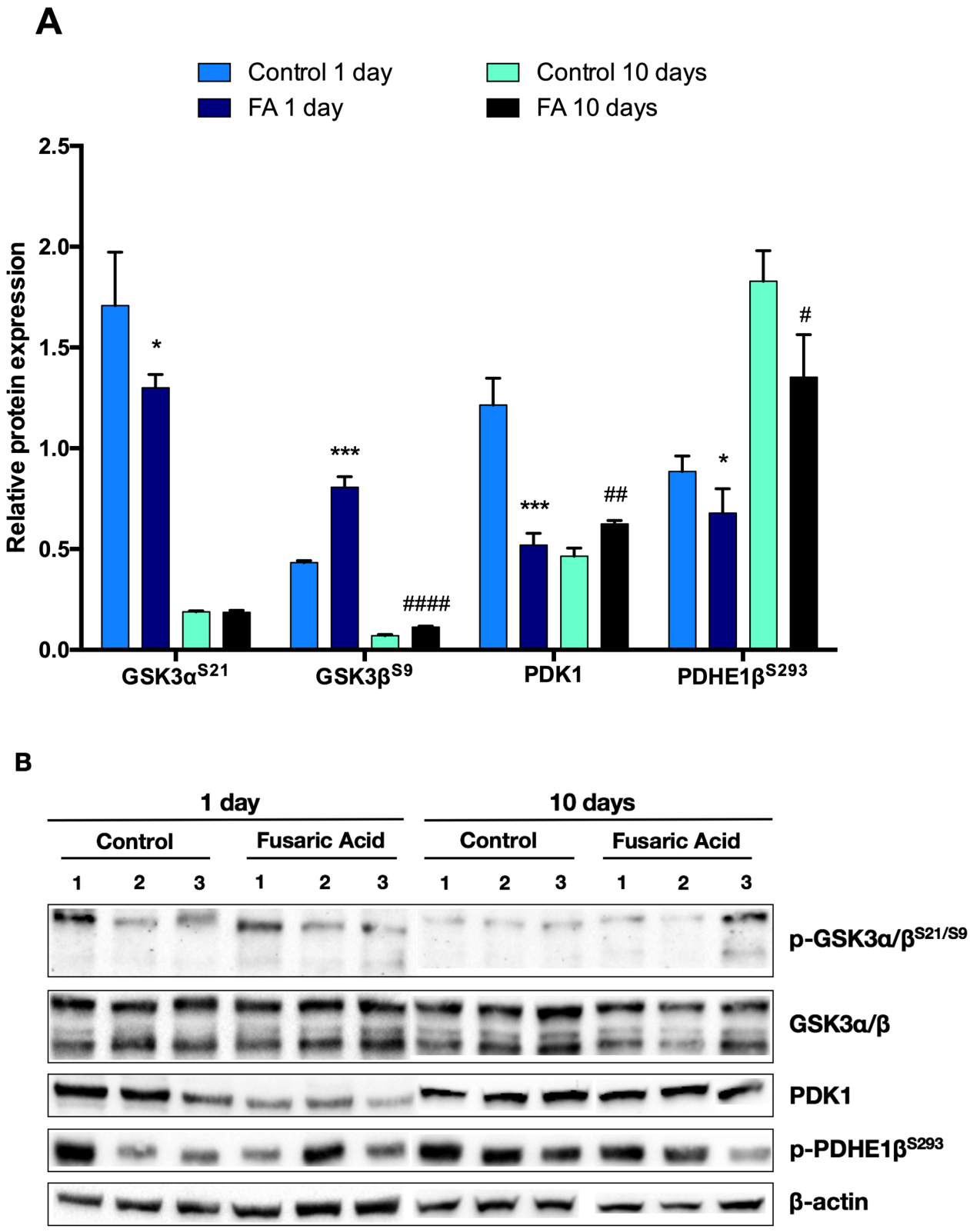


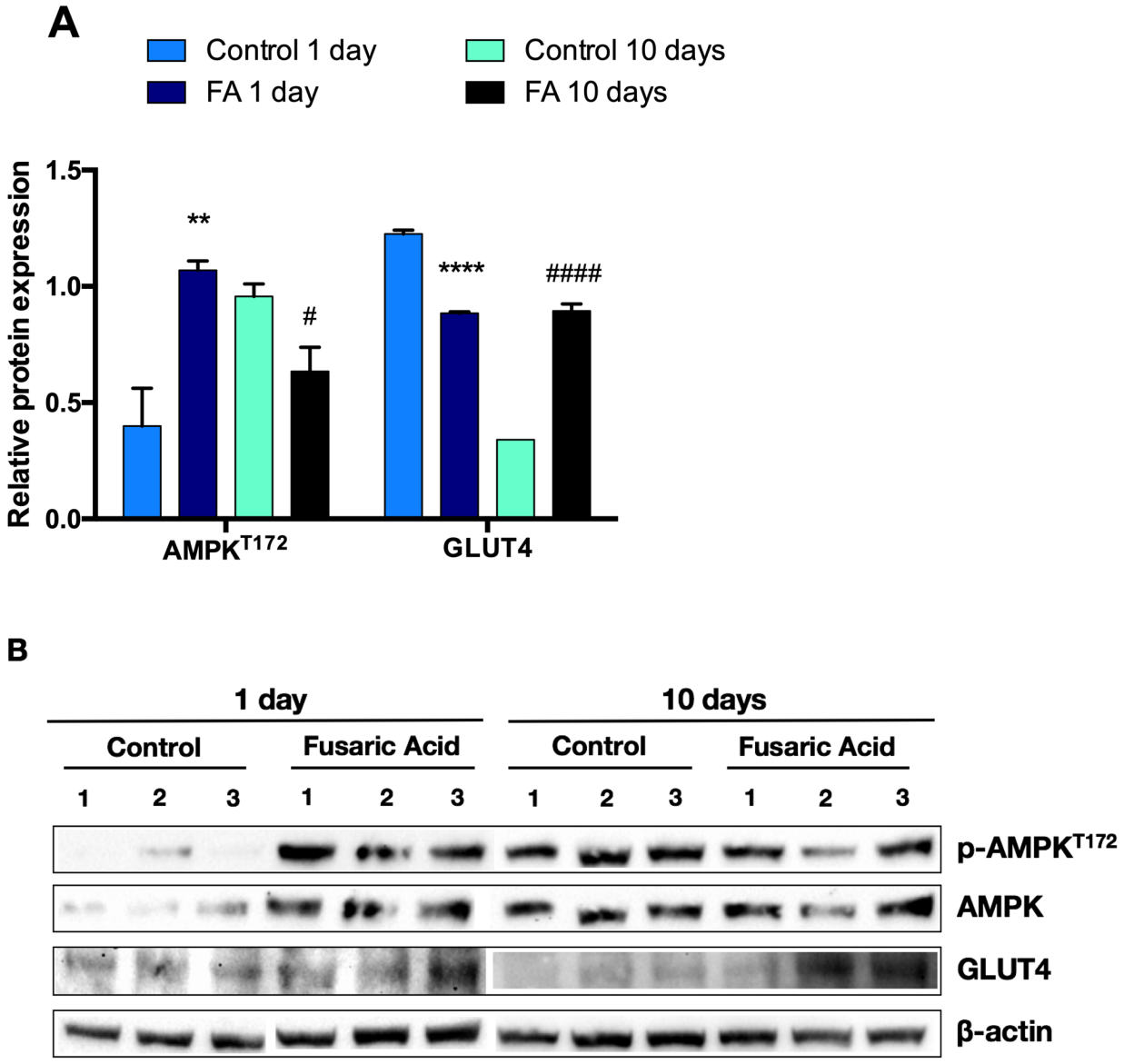
Figure 3: FA regulates Akt-GSK3α/β phosphorylation and prevents PDHE1β inhibition in mice brain tissue at 1 and 10 days exposure. Densitometric protein quantification **(A)**

and expression **(B)** of p-GSK3α/$\beta^{S21/S9}$, PDK1 and p-PDHE1β^{S293} were detected by immunoblotting. Expression of β-actin was used as an internal control. The data are represented as the mean ± SD obtained from experiments performed in triplicate (n=3). * $p < 0.05$, *** $p < 0.0005$ (FA relative to control 1 day); # $p < 0.05$, ## $p < 0.005$, #### $p < 0.0001$ (FA relative to control 10 days).

3.4. FA modulation of AMPK-mediated p53 metabolic signalling and GLUT4 expression

It is well-established that several points of cross-regulation exist between the PI3K/Akt pathway and other major signalling pathways in maintaining cellular energy homeostasis. Therefore, the effect of FA on the energy sensor AMPK in mice brain tissue was investigated. Although, ATP levels were slightly decreased (1 day), AMPK activity was amplified as indicated by the increased threonine-172 (T172) phosphorylation ($p < 0.0026$) (Figures 4A and 4B). Surprisingly, despite a significant decline in ATP levels at 10 days, T172 phosphorylation was decreased ($p < 0.0169$) thus reducing the activity of AMPK (Figures 4A and 4B). Since glucose is the primary source of energy in the brain, and variances in its entry and metabolism could alter energy levels, we assessed the expression of the transcription factor p53 in response to the disparities in ATP levels and AMPK activation. The metabolic regulation of p53 is directed by GSK3 and AMPK; consistent with AMPK activation, p53 serine-15 (S15) phosphorylation was increased (1 day) ($p < 0.0038$) and decreased (10 day) ($p < 0.0084$) accordingly (Figures 1A and 1B), indicating that activation of p53 was targeted by AMPK and not GSK3. To understand the interaction of AMPK and p53, we measured the expression of GLUT1 and GLUT4 receptors, whose expression is negatively regulated by p53. As expected, GLUT4 transcript and protein expressions were

decreased (1 day; $p < 0.0001$) and increased (10 day; $p < 0.0001$) in correlation with p53 activity (Figures 4A, 4B and 4C). Likewise, GLUT1 transcript levels were down- (1 day; $p < 0.0001$) and up-regulated (10 day; $p < 0.0054$) after exposure to FA (Figure 4C).



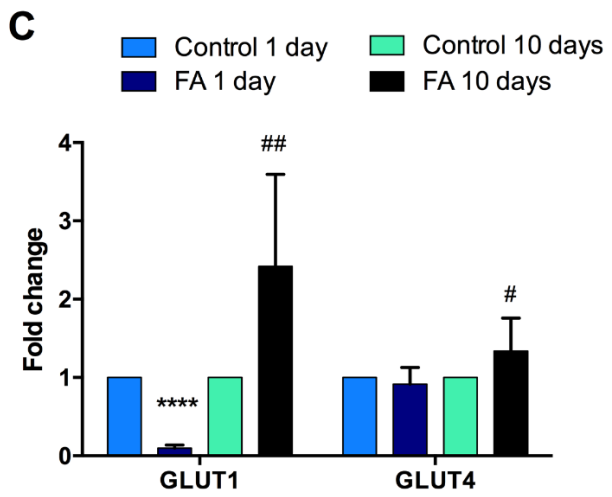


Figure 4: FA regulates p53 metabolic signalling and GLUT4 expression via AMPK signalling in mice brain tissue at 1 and 10 days exposure. Densitometric (**A**) and protein expressions (**B**) of p-AMPK^{T172}, AMPK and GLUT4 were measured by immunoblotting. β-actin was used as an internal control. GLUT1 and GLUT4 transcript levels were quantified using qPCR (**C**). The data are represented as the mean ± SD obtained from experiments performed in triplicate (n=3). ** $p < 0.005$, **** $p < 0.0001$ (FA relative to control 1 day); # $p < 0.05$, ## $p < 0.005$, #### $p < 0.0001$ (FA relative to control 10 days).

4. DISCUSSION

In this study, we investigated the neurometabolic potential of FA and its subsequent alterations in molecular signalling pathways. Several studies have described the metabolically toxic potential of FA, with reports of mitochondrial toxicity in plant tissue (Bouizgarne *et al.*, 2004; D'Alton and Etherton, 1984; Köhler and Bentrup, 1983; Telles-Pupulin *et al.*, 1996a) and catecholamine disturbances in animals (Chowdhury and Smith, 2004; Leung *et al.*, 2007; Raymond *et al.*, 2003; Swamy *et al.*, 2002; Yegani *et al.*, 2006). Consistent with this, ATP levels were reduced in brain tissue upon FA administration (1 day), and decreased more

significantly following a prolonged exposure (10 days). As a result of ATP-depleted levels, glucose-uptake regulatory signalling pathways, Akt and AMPK, were activated (1 day). Since PI3K and mTOR regulate Akt activity by phosphorylation of T308 and S473 residues, respectively, (Abeyrathna and Su, 2015; Islam *et al.*, 2014; Vadlakonda *et al.*, 2013; Vincent, *et al*., 2011; Yung *et al.*, 2011) we assessed their expressions and found an increase in both (PI3K and mTOR) following an acute exposure to FA (1 day) (Figures 1A, 1B, 2A and 2B), confirming the activation of Akt signalling. A study by Polter *et al*., (2009), reported increased brain serotonin levels and activation of PI3K/Akt signalling upon administration of D-fenfluramine in C57BL/6 mice. In support of Akt activation (1 day), various studies showed elevated brain tryptophan and serotonin synthesis by FA (Chaouloff *et al.*, 1986; Diringer *et al.*, 1982; Ogunbo *et al.*, 2005; Porter *et al.*, 1995), which may also contribute to the regulation of Akt signalling. In contrast, 10 day administration of FA suppressed Akt stimulation (Figures 1A, 1B, 2A and 2B); since activation of Akt is ATP-dependent, dampening its activation may further prevent ATP depletion.

Literature indicates an array of metabolic targets regulated by GSK3, making this enzyme among the most extensively studied substrates regulated by Akt (Case, *et al*., 2011; Uranga, *et al*., 2013). In correlation with Akt activation at 1 day, GSK3 activity was negatively regulated by PI3K/Akt/mTOR signalling (Figures 3A and 3B) and likely influenced neurometabolism by modulation of the PGC1-α target PDK1. PDK1 responds to high ATP and low oxygen levels and regulates PDHE1β activity by inhibiting phosphorylation on S293 (Kumar *et al.*, 2012; Rardin *et al.*, 2009). Together with decreased ATP levels and GSK3 inhibition (1 day), PDK1 levels were reduced hence promoting oxidative metabolism by PDHE1β (Figures 3A and 3B). Interestingly, despite the sequestered inhibition of its upstream regulators, GSK3 and PDK1, PDHE1β remained active following prolonged

exposure to FA (10 days) (Figures 3A and 3B), with ATP levels being further depleted (Table 2). Telles-Pupulin and colleagues, showed that FA impairs mitochondrial respiratory activity in maize root mitochondria (1996b) and rat liver mitochondria (1998) at least in part by inhibition of succinate dehydrogenase, α-ketoglutarate dehydrogenase or ATPase/ATP-synthase activity; suggesting that FA affects ATP production more significantly than ATP consumption. Another study by Mailloux *et al*., (2009) revealed that a loss of α-ketoglutarate dehydrogenase and succinate dehydrogenase activity elevated succinate concentrations; while several independent studies have coupled the regulation of hypoxia inducible factor-1α (HIF-1α) (transactivates PDK1 gene expression) with increased succinate levels in the brain (Chinopoulos, 2013; Kim *et al.*, 2006; Lukyanova and Kirova, 2015; Rutter *et al.*, 2010; Semba *et al.*, 2016). Thus, it's possible that the significant reduction of ATP levels seen at prolonged exposure to FA (10 day) led to an accumulation of TCA substrates that induced PDK1 activity, mediated by HIF-1α.

The tumor suppressor protein p53 is commonly known for its role DNA repair (Chang *et al.*, 2012; Merlo *et al.*, 2014; Nagasaka *et al.*, 2006). In the past few decades, its role in metabolism emerged (Berkers *et al.*, 2013; Jiang *et al.*, 2015; Maddocks and Vousden, 2011). In response to metabolic changes such as metabolic stress or glucose deprivation, kinases such as GSK3 and AMPK regulate p53's metabolic activity by phosphorylating and activating it on its S15 residue (Agarwal *et al.*, 2015; Boehme *et al.*, 2008; Jones *et al.*, 2005; Loughery *et al.*, 2014; Qu *et al.*, 2004). Since acute administration of FA inhibited GSK3 activity, it can be insinuated that p53 activity was activated in response to AMPK activation (Figures 4A and 4B). p53 is a negative regulator of glycolysis and transcriptionally represses the expression of GLUT1 and GLUT4 (Gnanapradeepan *et al.*, 2018; Liang *et al.*, 2013; Schwartzenberg-Bar-Yoseph *et al.*, 2004; Zhang *et al.*, 2014). The importance of glucose

transporters is now well-established in facilitating glucose diffusion into the brain; with high expressions of GLUT-1 (in glia) and -3 (in neurons), and to a lesser extent GLUT4; consistent with the high requirement of glucose for normal brain functioning. Correlating with p53 activity, GLUT-1 and -4 expressions were decreased at acute exposure to FA (Figures 4B and 4C). This was further substantiated by the reversal of p53 activation and GLUT-1 and -4 suppression following AMPK attenuation in mice administered with FA for 10 days (Figures 4B and 4C).

Although a key function of AMPK is to recondition energy levels in response to low ATP availability (Dagon *et al.*, 2012; Hurtado-Carneiro *et al.*, 2012; Yang *et al.*, 2012), AMPK prevented glucose receptor expression at an acute exposure to FA. Interestingly, prolonged administration of FA dampened AMPK signalling promoting GLUT4 expression and glucose uptake (Figures 4A, 4B and 4C); likely to promote ATP production as ATP levels were further depleted. Taken together, these observations suggest that FA alters neuronal metabolic homeostasis by decreasing ATP volume and modifying glucose-mediated entry by counter-regulatory cross-talk between the Akt and AMPK signalling pathways (Fig. 5). The implications of these findings are significant with several experimental and clinical studies associating altered glucose transporter gene expressions and decreased ATP levels with premature aging and the progression of neurodegenerative diseases such as Alzheimer's and Parkinson's disease. Multiple studies have also shown impaired mitochondrial function as a common feature in cognitive dysfunction and behavioural disorders. Lastly, it is important to consider further investigations to determine how neuronal tissue may compensate for FA-induced glycolytic reprogramming and energy depletion.

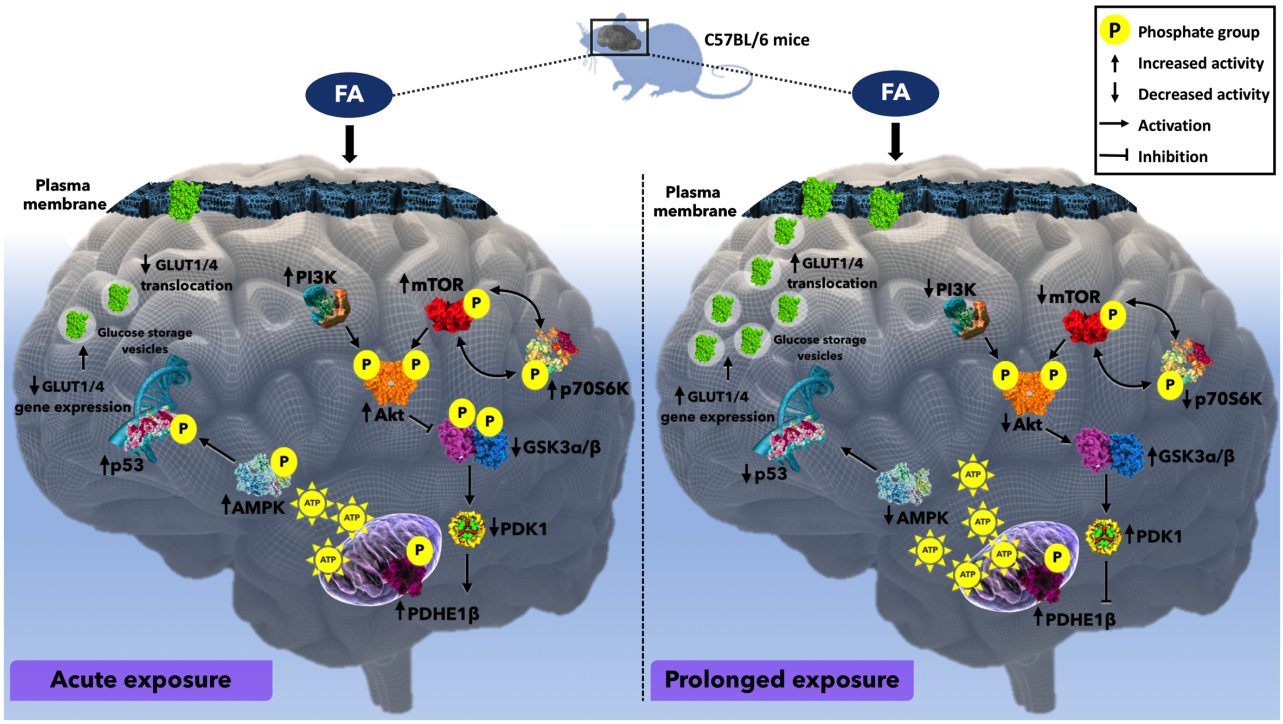


Figure 5: Schematic diagram summarising the alterations to Akt and AMPK signalling pathways by FA in mice brain tissue at acute (1 day) and prolonged (10 day) exposures.

5. CONCLUSION

To conclude, this study demonstrates a mechanism by which FA differentially controls glucose receptors by modulation of Akt and AMPK signalling pathways. This is of particular significance considering the high metabolic activity of the brain and its dependency on glucose as its primary fuel source. Additionally, FA impairs neuronal ATP production, and to a greater extent upon prolonged exposure in mice. Collectively, our findings bring a new dimension of FA's role in metabolism with implications in neuronal-mediated regulation of glucose homeostasis and its influences on several aspects of brain development, aging and behaviour.

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AUTHOR CONTRIBUTIONS

S.D.S and S.B assisted with mice treatment and sacrifice. S.D. performed all molecular experiments and analysed data. T.G assisted with qPCR. S.D., S.N. and A.A.C. composed the manuscript. All authors reviewed the manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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CHAPTER FOUR

Manuscript title: Differential regulation of CREB/BDNF signalling by Fusaric acid in C57BL/6 mice brain tissue

Impaired homeostatic function by immune cells (such as the cytokine mediated regulation of appetite), and cerebral energy deficits are commonly seen in patients who present with psychiatric and neurodegenerative disorders. Although FA was moderately toxic to healthy immune cells (Chapter 2), both the previous chapters (Chapters 2 and 3) revealed substantial immuno- and neuro-metabolic deficits, which have significant implications on brain function and has the potential for the subsequent development of neurological disorders. CREB is an important intracellular protein that regulates the expression of genes involved in neuronal survival, development, inflammation and metabolism such as B Cell Lymphoma-2 (Bcl-2), insulin-like growth factor 1 (IGF-1), leptin and BDNF. Importantly, CREB/BDNF signalling was found to be a critical component of the neuroprotective transcriptional network, and its dysregulation contributes to an array of neuropathological conditions. Furthermore, CREB activity and its target genes are regulated by neurotransmitters, growth factors, neurotrophins and various upstream kinases including MAPKs, calmodulin-dependent protein kinases and protein kinases -A, -B (Akt) and -C. Since FA demonstrates the ability to modify the activity of several protein kinases, including MAPK (Chapter 2) and Akt (Chapter 3), the following chapter aimed to determine the neurotoxicity of FA and its alterations to MAPK/CREB/BDNF signalling in C57BL/6 mice.

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Differential regulation of CREB/BDNF signalling by Fusaric acid in C57BL/6 mice brain tissue

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AUTHOR CONTRIBUTIONS

S.D. performed all molecular experiments and analysed data. S.D., S.N. and A.A.C. conceptualised study and assisted in manuscript preparation. S.D.S and S.B assisted with mice treatment and sacrifices. All authors reviewed the manuscript.

ABSTRACT

Fusaric acid (FA), a *Fusarium* metabolite identified for its potency against dopamine-β-hydroxylase, alters brain neurochemistry and exhibits substantial inhibitory effects on mitochondrial respiration, represents an important risk factor to neurometabolic-related diseases. However, FA's neurotoxicological relevance in human and animal health is limited. cAMP response element binding (CREB) is an essential transcription factor that is responsible for neuroprotective responses through the regulation of neurotrophic and metabolic signals in the brain. Therefore, the present study investigated the neurotoxic potential of FA (50 mg/kg) and its influence on CREB signalling in C57/BL6 mice following short (1 day) and prolonged exposures (10 days). FA increased caspase activities at 1- and to a lesser extent at 10-days post exposure. However, despite enhanced caspase activity, there were no histological markers of cellular damage to extracellular tissue or neuronal cells, evidenced by microanalysis of brain tissue. Further, FA enhanced CREB signalling by increasing CREB serine-133 phosphorylation with a simultaneous increase in brain derived neurotrophic factor (BDNF) expression whilst suppressing CREB/BDNF signalling following 10-day exposure. In contrast, protein expressions of mitogen-activated protein kinases (MAPK) (ERK, JNK and p38) negatively correlated with CREB activity; inferring FA induced MAPK-independent activation of CREB responses. Although FA showed no significant neurotoxicity, alterations in glial cell density patterns at both acute and prolonged exposures were observed. Collectively, FA alters CREB/BDNF signalling with profound influences on neuronal function via glial cell modifications and could impact normal brain functions and may play a role in the development of neurological disorders.

Keywords: Fusaric acid, MAPK, CREB, BDNF, Brain

INTRODUCTION

Fusarium species frequently contaminate grain and cereal products worldwide and produce many secondary chemical metabolites known as mycotoxins that are associated with various acute and chronic diseases in humans, presenting a growing concern to both human and animal health [1-3]. In the 1960's Fusaric acid (FA, 5-butylpicolinic acid), a *Fusarium* mycotoxin widely known for its phytotoxicity, was first identified as a dopamine-β-hydroxylase inhibitor [4-8]. Since then, several *in vivo* behavioural studies have associated FA toxicity with lethargy, loss of appetite and vomiting [8] while other studies (preclinical and clinical) showed elevated levels of tryptophan, serotonin and dopamine as distinct FA-induced neurological effects; a response believed to occur as a result of the competitive binding of tryptophan to albumin in circulation [8-11]. In addition, the lipophilic nature of FA, enables easy penetration of cell membranes and ability to cross the blood brain barrier to exert its neurological effects. However, FA's neurological impact in human and animal health

are poorly documented. Although the precise mechanism of FA toxicity is unclear, various studies have attributed the toxicity of FA to impaired mitochondrial function, ATP synthesis, and cell proliferation [6,4,5].

Several converging reports demonstrate mitochondrial dysfunction and inflammatory changes as key pathological phenomena's in many neurodegenerative disorders including Alzheimers's, Parkinson's and Huntington's diseases [12-14]. Over the past decade, multiple studies have emphasized the significance of the transcription factor CREB in neuropathologies given its functions in neurophysiological processes such as neurodevelopment, synaptic plasticity, and neuroprotection [15-17]. A recent study by Brady *et al*., (2016) showed that neuronal cell loss was associated reduced CREB activity; whilst, increased CREB phosphorylation inhibits neuroinflammation and improves neuronal function in a murine model of Alzheimer's disease. CREB facilitates its neuroprotective properties via neurotrophin-mediated process, with BDNF, an essential neurotrophin associated with neuronal survival, differentiation and synaptic plasticity, being a direct target of CREB [18,19]. Accordingly, accumulating evidence has demonstrated the link between reduced CREB activity and low BDNF levels with increased neuronal injury in neurodegenerative diseases [18,19].

Additionally, CREB is well-established for its role as a metabolic sensor in fat, liver and brain tissue and recently in the hypothalamic control of appetite and food intake [16,15]. A study by Fusco *et al*., [20] reported significant transcriptional reduction of neuronal metabolic genes in CREB deficient dietary-restricted mice. The activity of CREB is largely dependent on the phosphorylation of its serine-133 (S133) residue that is regulated by neurotransmitters and several upstream kinases including MAPKs, calmodulin-dependent protein kinases and receptor-activated protein kinases [protein kinase A (PKA), protein kinase B (Akt) and protein kinase C (PKC)] [21,22,15]. In particular, MAPKs are one of the major regulators of CREB activity and was previously implicated in Alzheimers's, Parkinson's and various cancers [23-25]. Evidently, phosphorylation of CREB is crucial for several neurophysiological responses and alterations to its signal transduction are therefore highly relevant as modulators of neurological pathogenic processes.

We have previously shown altered MAPK activity in FA-associated immunotoxicity, and substantially depleted energy levels whereby immune cells became hypometabolic. This effect has significant implications on brain functioning and subsequent development of neurological disorders. However, the relationship between neurometabolism and neurotoxicity of FA and its influence on its neurological manifestations remain elusive. The current study aimed to determine the neurometabolic toxicity of FA and its alterations to CREB/BDNF signalling in C57BL/6 mice.

EXPERIMETAL DESIGN

Reagents

Fusaric Acid (*Gibberella fujikuroi*) was purchased from Sigma Aldrich (Johannesburg, SA). Luminometry reagents were obtained from Promega (Madison, USA).

Animals

All animal procedures were approved by the institutional Animal Research Ethics Committee of the University of KwaZulu-Natal (AREC/079/016) and were performed in accordance with the Standard Approved Protocols (University of KwaZulu-Natal) guidelines. Experiments were performed using C57BL/6 black male mice obtained from the Biomedical Resource Unit (University of KwaZulu-Natal). Mice were housed at the Africa Health Research Institute (AHRI, University of KwaZulu-Natal) under standard conditions of temperature (23 $\pm$ 1°C) and humidity (40-60%) controlled environment, on a 12-hour (hr) light/dark cycle. All mice received commercially available animal feed (mouse pellet diet) and normal drinking water *ad libitum*.

Experimental procedure and brain tissue harvest

C57BL/6 mice aged 6-8 weeks (mean body weight 18-22 g) were randomly divided into four groups (n=3 per group): with two control groups (1 and 10 days) and two FA-treated groups (1 and 10 days). Experimental groups were administered FA (50 mg/kg.bw [26]; 0.25 ml) by oral gavage (once daily) against a phosphate buffer saline (0.1 M PBS; 0.25 ml) control group for 1- and 10-day treatment periods. Mice were sacrificed at the end of each treatment period by inhalation anaesthesia (Isofor; Safeline pharmaceuticals) and the whole brain tissue samples were harvested and divided into two hemispheres. Each hemisphere was added to Cytobuster ™ (Novagen, San Diego, CA, USA) and QIAzol (Qiagen, SA) reagents, respectively, and stored (-80°C) for molecular analysis. All assays were performed in triplicate, independently.

Caspase activity

Briefly, fresh whole brain tissue was homogenized (~1x2 mm^3 pieces) in Dulbecco's Modified Eagle Medium (DMEM) containing collagenase type II (15 mg/ml) and incubated for 40 min (37℃). Thereafter, the digest was centrifuged (500 x *g*, 10 min, 4℃), and the pellet was washed in DMEM with BSA (20%) and centrifuged (1000 x *g*, 20 min, 4℃) once again. The lipid layer was aspirated and the pellet was re-suspended in serum-free DMEM (with 0.1% BSA) for 30 min (37℃). The digest was then re-suspended by pipetting prior to centrifugation (500 x *g*, 10 min, 4℃) followed by one wash in PBS. Cell viability was assessed using trypan blue staining. Opaque polystyrene microtiter plates (96-well) were prepared with the cell suspension (20,000 cells/well) and 20 µl of each respective reagent (Caspase-Glo® 3/7, Caspase-Glo® 8 and Caspase-Glo® 9 Assays). The plate was incubated in the dark for 30 min (RT) and read on a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA). All data was represented as relative light units (RLU).

Histological assessment of brain tissue

Briefly, whole brain tissue samples were dissected into hemispheres, from which one hemisphere was rinsed with 0.1 M PBS and drop fixed in 10% formaldehyde (RT). Tissue samples were dehydrated in a series of

ethanol solutions and xylene followed by infiltration with paraffin wax. Infiltrated tissues were then embedded and sectioned (3 μm thick sections) using a rotary microtome (microTec® Laborgeräte GmbH, Walldorf, Germany) and routinely stained with Hematoxylin and Eosin (H&E). The sectioned samples were dehydrated in ethanol [95% (once) and 100% (twice) at 5 min intervals each] and cleared in xylene. Sagittal tissue sections were captured with a Leica SCN400 slide scanner (Leica Biosystems, Berkshire, UK) and viewed using SlidePath Gateway Client™ software (SlidePath Ltd, Leica Biosystems, Dublin, Ireland).

Transmission electron microscopy (TEM)

Tissue sections (approximately 1 mm^3 of the cerebral cortex and hypothalamus) were rinsed in 0.1 M PBS and fixed in 2.5% glutaraldehyde (overnight, 4°C). The samples were washed 3 times in 0.1 M phosphate buffer (PO_4, pH 7.2), post-fixed with 0.5% osmium tetroxide on rotation (overnight, RT) and washed (3 times with 0.1 M PO_4, pH 7.2) before being dehydrated through graded acetone solutions [30% acetone, 50% acetone, 75% acetone (twice at 5 min intervals each) and 100% acetone (twice at 10 min intervals)]. Next, the samples were infiltrated with a 1:1 resin and acetone solution (overnight, RT), resuspended in complete resin (overnight, RT), embedded in fresh resin for 5 hrs (on rotation, RT) and polymerized at 60°C (4 hrs). The resin blocks were trimmed with an axe saw and cut into ultrathin sections using an Ultramicrotome equipped with a glass knife. The sections were placed on copper grids (200 mesh) and contrasted with uranyl acetate (2%). The samples were viewed on a JEOL 1010 transmission electron microscope (JEOL, Peabody, MA, USA) at an operating voltage of 100 kV. Images were captured using a Megaview III camera and analysed using iTEM software.

Protein phosphorylation array

Proteomic profiles of phosphorylated CREB was detected using a commercially available Human/Mouse MAPK protein phosphorylation array (RayBiotech, Inc., Norcross, USA) according to the manufacturer's instructions. In brief, membranes were blocked with a blocking buffer for 30 min (RT) and then incubated overnight (4°C) with protein fractions (1 mg/ml; diluted in blocking buffer). Samples were then aspirated and the membranes were washed before incubation with a biotin-conjugated detection antibody cocktail (overnight, 4°C). The membranes were washed once again, followed by additional overnight incubation (4°C) with an HRP-Anti-Rabbit IgG concentrate. Thereafter, membranes were washed and incubated with the chemiluminescent detection buffer (2 min, RT). Images were captured on a ChemiDoc XRS^+ system (Bio-Rad, Johannesburg, SA) and analysed with Image Lab™ software 6.0.1 (Bio-Rad, Johannesburg, SA). Relative protein signal intensities were normalized to positive control spots prior to comparison (i.e. control vs. FA). The data was expressed as relative band density (RBD).

Western blotting

Protein homogenates were quantified by the bicinchoninic acid (BCA) assay and standardised to 1 mg/ml. The samples were resolved on SDS-PAGE gels (7.5%) and transferred to nitrocellulose membranes. The membranes

were incubated (1 hr, RT) with 5% bovine serum albumin (BSA) followed by an overnight incubation (4°C) with primary antibodies (Table 1). The membranes were then washed 5 times with Tris Buffer Saline with tween 20 (TTBS- NaCl, KCl, Tris, dH_2O, 0.5% tween 20, pH 7.5) and incubated with secondary antibodies [anti-rabbit or anti-mouse (1:5,000, Cell signalling)] for 2 hrs (RT). The membranes were washed once more before been captured on a ChemiDoc XRS$^+$ system (Bio-Rad, Johannesburg, SA). Protein band intensities were analysed using Image Lab™ software 6.0.1 (Bio-Rad, Johannesburg, SA). β-actin (1:5,000, Sigma Aldrich) was used for comparison of uniform protein load. The data was expressed as relative band density (RBD).

Table 1: Primary antibodies for immunoblot analysis

Manufacturer	Antibody name	Host species	Catalogue number	Concentration
Cell signalling Technology	Cytochrome c	Rabbit	4280S	1:1,000
	Phospho-p44/42 MAPK (ERK1/2)	Mouse	9106S	1:1,000
	p44/42 MAPK (ERK1/2)	Rabbit	4695S	1:1,000
	Phospho-SAPK/JNK (Thr183/Tyr185)	Mouse	9255S	1:1,000
	SAPK/JNK	Rabbit	9252S	1:1,000
	p38 MAPK	Rabbit	14451S	1:1,000
	Phospho-CREB (Ser133)	Rabbit	9191S	1:1,000
Sigma Aldrich	Phospho-p38	Mouse	M8177	1:1,000
	BDNF	Rabbit	AB1543	1:500

Quantitative PCR

Total RNA was isolated from whole brain tissue with QIAzol reagent and reverse transcribed to complementary DNA (cDNA) using an iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA) according to manufacturer's instructions. Quantitative detection of mRNA [*cytochrome c*: Sense 5'CCCATCTTTGAGCATCTTGGT3', Anti-sense 5'GCCCAGCCTGAGTAGTGAAG3'; *CREB*: Sense 5'GTCCCAGGCTCTCTATCATCTC3', Anti-sense 5'ATAGGCATCAAGACGGCAGAA3'; *BDNF*: sense 5'AAACATCCGAGGACAAGGTG3', Anti-sense 5'AGAAGAGGAGGCTCCAAAGG3'] levels were detected using the iQ™ SYBR® Green Supermix (Bio-Rad, Johannesburg, SA) as per manufacturers protocol and performed on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Johannesburg, SA). Differences in gene expressions were normalized against the housekeeping gene *GAPDH* [Sense 5'ATGTGTCCGTCGTGGATCTGAC3', Anti-sense 5'AGACAACCTGGTCCTCAGTGTAG3'] and analysed according to the methods described by Livak, Schmittgen [27]. The data was represented as relative fold change ($2^{--\Delta\Delta Ct}$).

Data analysis

All data were analysed with GraphPad Prism v6.0 software (GraphPad Software Inc., La Jolla, USA) by the unpaired t-test with Welch's correction to assess the differences between samples. Results were expressed as mean ± standard deviation (SD). A $p < 0.05$ represented statistically significant differences.

RESULTS

FA induced caspase activation in neuronal tissue

Several studies have reported the induction of apoptosis by FA *in vitro* and in plants [5,4,6]. Although acute activation of caspases in neuronal tissue contributes to development, prolonged activation can be detrimental and cause cellular degeneration. Therefore, to determine the toxicity of FA on neuronal tissue, active cleaved forms of caspases -8, -9 and -3/7 were measured in fresh whole brain tissue. In keeping with our previous findings [28], exposure to FA (1 day) significantly increased initiator (-8 and -9) and effector (-3/7) caspase activities whereas at 10-day exposure only caspase 8 activity was significantly increased ($p < 0.0026$) (Table 2).

Table 2: The effect of FA (1- and 10-days) on caspase activity in mice brain tissue

		Mean ± SD (RLUx10^4)		Fold change	*p* value
		Control	FA		
1 day	Caspase 8	1.1782 ± 0.0330	1.3400 ± 0.0995	1.14	0.0428*
	Caspase 9	2.2143 ± 0.0145	3.9024 ± 0.1985	1.76	0.0007***
	Caspase 3/7	0.6475 ± 0.0593	0.7380 ± 0.3123	1.14	0.0231*
10 days	Caspase 8	0.3826 ± 0.0892	0.6760 ± 0.0530	1.77	0.0026 ##
	Caspase 9	0.8004 ± 0.1941	8.4456 ± 6.9069	10.55	0.1952
	Caspase 3/7	0.1954 ± 0.0489	0.2736 ± 0.1385	1.40	0.35009

The data are represented as mean ± SD (n=3). RLU: relative light units. * $p < 0.05$ *** $p < 0.0005$ (FA relative to control 1 day); ## $p < 0.005$ (FA relative to control 10 days).

FA alters neuronal glial cell density

To determine the effect of caspase activation in neuronal tissue, sagittal sections of the cerebral cortex and hypothalamus were stained with H&E for the observation of histological abnormalities in brain tissue. While FA amplified caspase activation, neuro-histopathology of the cerebral cortex and hypothalamus appeared normal, with no apparent signs of histological lesions or apoptotic/necrotic features (1- and 10- days) (Fig. 1). However, FA-treated mice showed significant differences between glial cell density patterns when compared to control mice in both the cerebral cortex and hypothalamus (1- and 10- days). Reductions in glial cell (astrocytes and oligodendrocytes) density and enlarged neurons were observed in the cortical regions following 1-day FA exposure (Fig. 1a and 1b). In contrast, FA induced proliferation of glial cells in the cerebral cortex that was enhanced along with reduced neuron size at 10 days (Fig. 1e and 1f). Though heightened proliferation of glial cells is a characteristic of gliosis, no glial scars were observed. Furthermore, FA reduced hypothalamic glial density (1 day) (Fig. 1c and 1d) but significantly at longer exposure (10 day) (Fig. 1g and 1h), as were reductions in density and size of hypothalamic neurons.

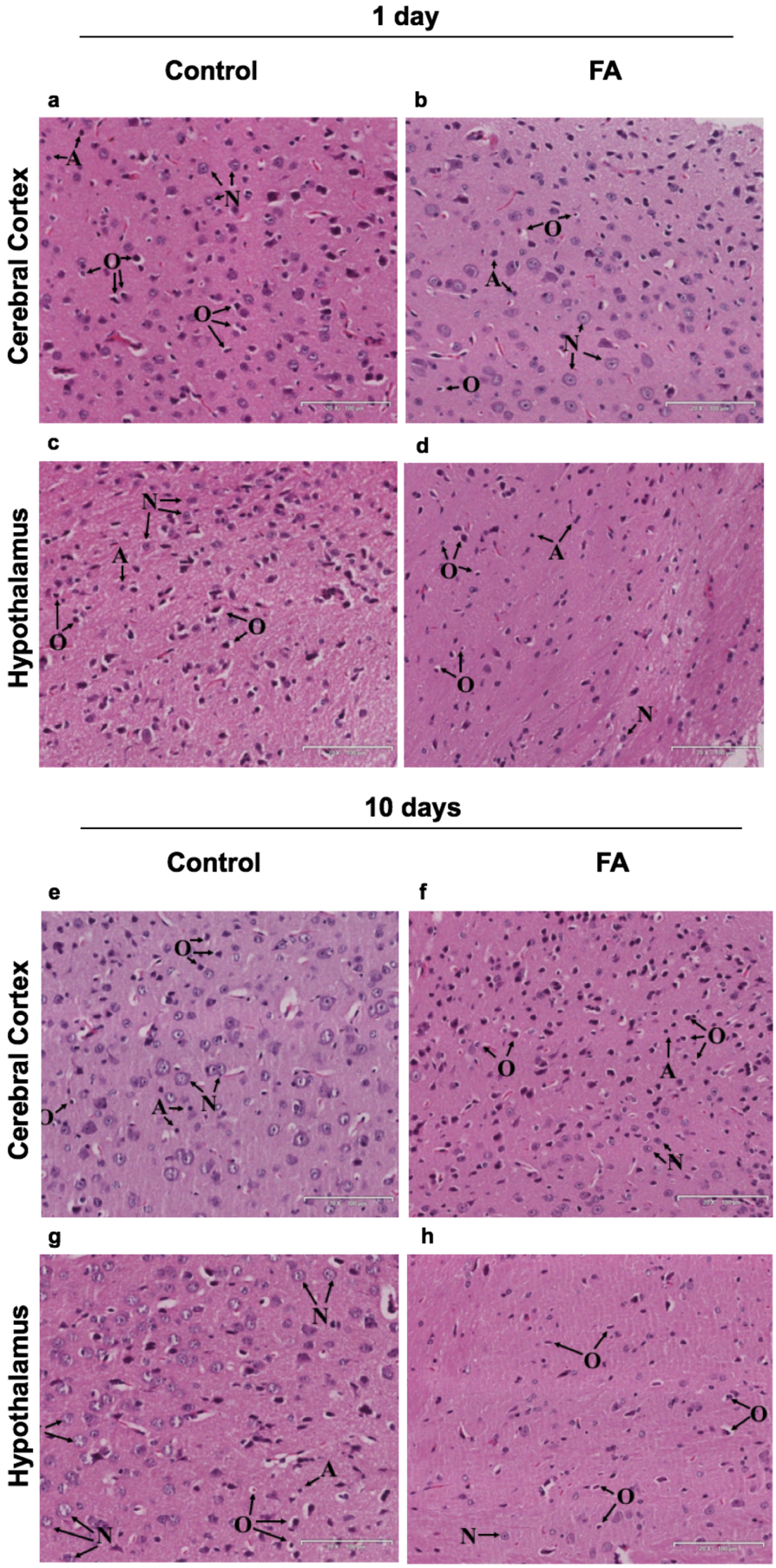

1 day
Control
FA
a
b
Cerebral Cortex
A
N
O
O
O
A
O
N
O
c
d
Hypothalamus
N
A
O
O
O
A
O
N
10 days
Control
FA
e
f
Cerebral Cortex
O
O
A
N
O
O
A
O
N
g
h
Hypothalamus
N
A
O
N
O
O
O
N

Fig. 1: Histopathological analysis of brain tissue sections from mice treated with FA for 1- and 10-days. Healthy (**1 day: a and c; 10 days: e and g**) and FA treated (**1 day: b and d; 10 days: f and h**) tissue sections of the cerebral cortex and hypothalamus showing no tissue disruptions but visible variations in cytoarchitectural densities. Neurons (**N**) were identified by their large nuclei and cytoplasm; whilst oligodendrocytes (**O**) were distinguished by their small, condensed nuclei and unstained cytoplasm and astrocytes (**A**) by their large oval nuclei. (Objective lens: 20x; Scale bar: 100 μm)

Ultrastructural analysis of the cerebral cortex and hypothalamus

While a relationship between FA and altered mitochondrial bioenergetics has been established, its impact on mitochondrial morphology in the brain, an important component that couples cellular metabolism and neuropathological processes, remains unknown [29]. To further understand the association and impact of increased caspase activity on tissue structure, micro-analysis of the cerebral cortex and hypothalamus brain regions were examined using TEM. At short and longer FA exposures (1- and 10-days, respectively), electron micrographs exhibited normal tissue characteristics with mitochondria that appeared intact and uniform in shape and size in both the cerebral cortex and hypothalamus (Fig. 2).

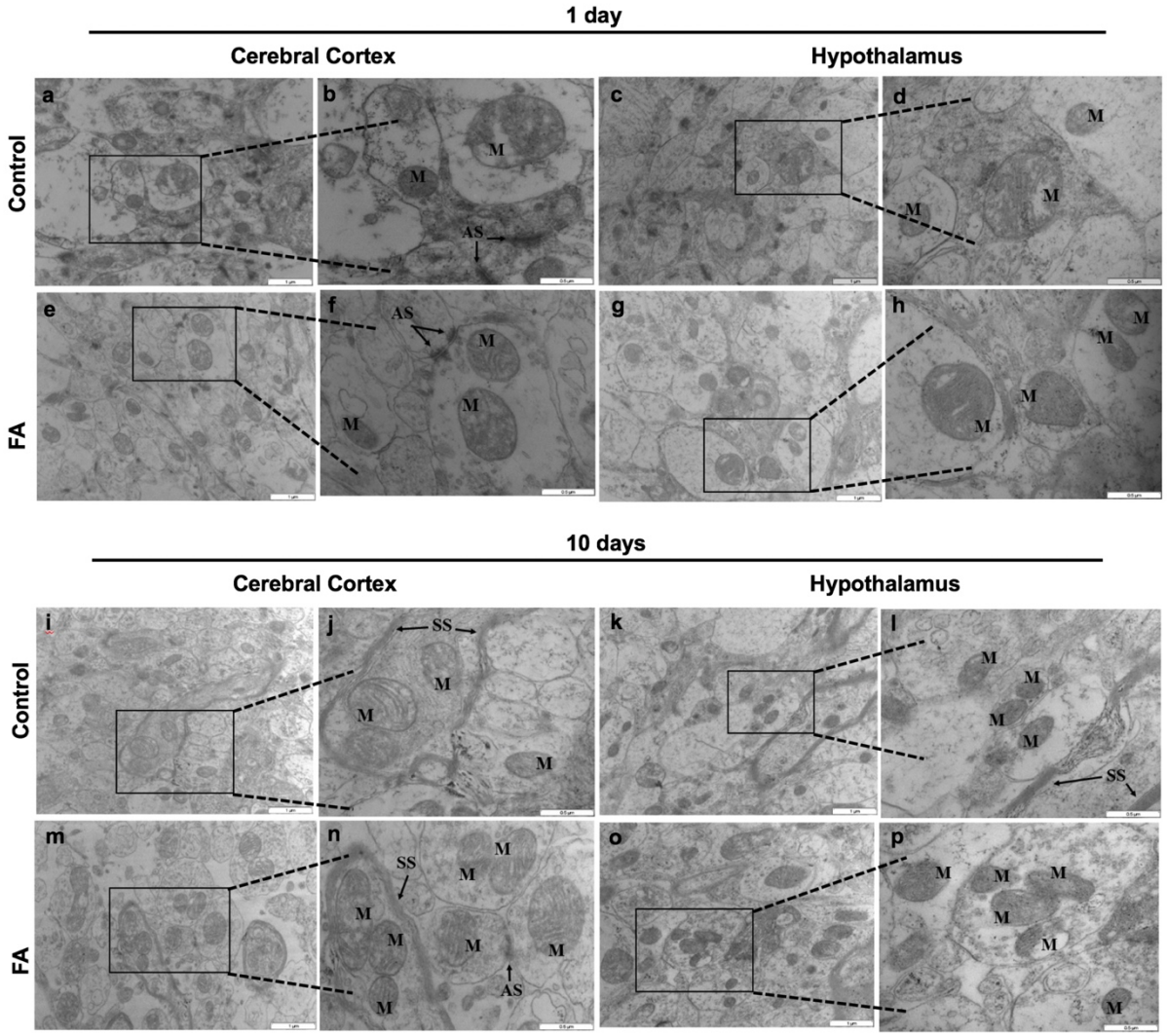


Fig. 2: Transmission electron micrographs of the cerebral cortex and hypothalamus from mice treated with FA for 1- and 10-days. Low and high magnification micrographs of healthy [**1 day: a and b (cerebral cortex), c and d (hypothalamus); 10 days: i and j (cerebral cortex), k and l (hypothalamus)**] and FA treated [**1 day: e and f (cerebral cortex), g and h (hypothalamus); 10 days: m and n (cerebral cortex), o and p (hypothalamus)**] tissue sections of the cerebral cortex and hypothalamus showed no destruction of tissue architecture. Correspondingly, higher magnification micrographs showed unobstructed morphology and uniform distribution of mitochondria in healthy and FA treated tissue regions (cerebral cortex and hypothalamus). Mitochondrion **(M)**, asymmetric synapse **(AS)**, symmetric synapse **(S)**. Scale bars were depicted as 1 µm (low magnification) and 0.5 µm (high magnification)

FA reduced cytochrome c protein levels

Cytochrome c is a well-established mitochondrial protein involved in maintaining mitochondrial membrane potential by participating in electron transport and ATP synthesis [30]. Cytochrome c also assists in caspase cleavage and execution of apoptosis [31]. Thus, as a result of increased caspase activity and unchanged

mitochondrial dynamics, we evaluated the expression of cytochrome c. Consistent with intact mitochondrial integrity, cytochrome c protein levels were reduced at both short (1 day) ($p < 0.0105$) and prolonged (10 days) ($p < 0.0125$) FA-exposures (Fig. 3a and 3b). Moreover, there were invariable differences in transcript cytochrome c expression levels (Fig. 3c) following FA administration (1- and 10 days, $p < 0.8447$ and $p < 0.0613$, respectively); suggesting that FA did not cause mitochondrial rupture and release of cytochrome c and that cleavage of caspases were independent of cytochrome c.

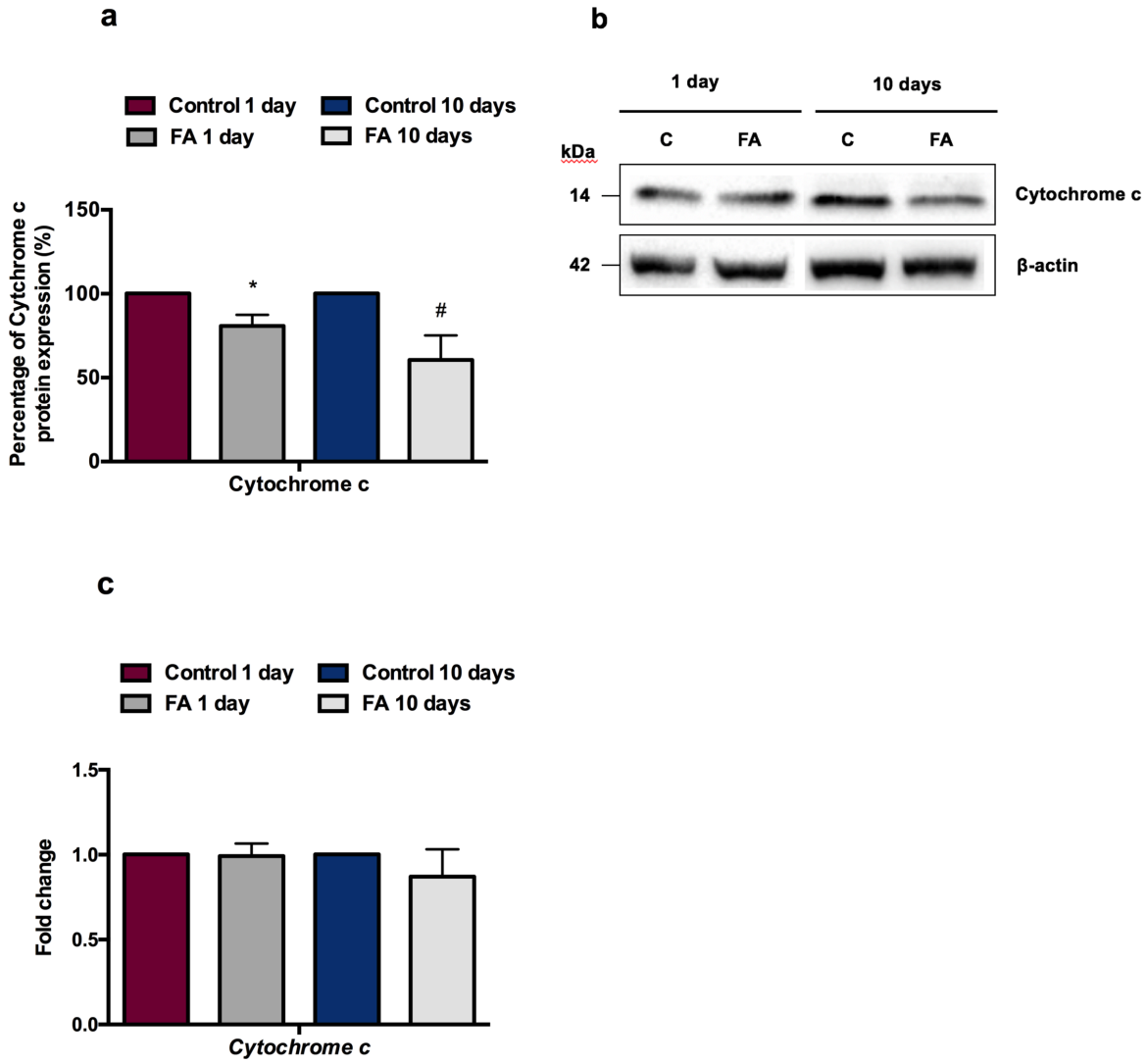


Fig. 3: Cytochrome c levels in mice brain tissue. Densiometric protein analysis (**a and b**) and transcript levels (**c**) of cytochrome c were measured by western blots and qPCR, respectively. Expression of β-actin was used as an internal control for western blots. The data are represented as mean ± SD (n=3). * $p < 0.05$ (FA relative to control 1 day); # $p < 0.05$ (FA relative to control 10 days)

FA exposure alters CREB/BDNF signalling

CREB activity in the brain has been implicated in several neuronal intracellular processes, including neuronal proliferation and differentiation, neurogenesis, survival and neuronal plasticity [15,16]. While activation of CREB commonly occurs in response to growth and survival stimuli, its activity can also be stimulated in stressful environments. CREB activity was first evaluated using a commercial phosphorylated-protein pathway array. In mice treated with FA, at 1-day CREB activity was amplified, as evidenced by increased S133 phosphorylation ($p < 0.0001$) from the array results (Fig. 4a). In contrast, prolonged exposure to FA (10 days) reduced S133 phosphorylation ($p < 0.0002$) thus dampening CREB activity (Fig. 4a) [protein phosphorylation array membrane (Online resource 1: Fig. S1)]. Differences in CREB phosphorylation were validated by immunoblots and revealed an equivalent increase ($p < 0.0170$) and decrease ($p < 0.7342$) at short and prolonged FA-exposures, respectively (Fig. 4b and 4d). Similarly, CREB transcript levels were slightly up- ($p < 0.3893$) and down-regulated ($p < 0.1210$) at 1- and 10 days post FA-treatment, respectively (Fig. 4e).

Importantly, CREB mediates its actions through transcriptional control of target genes including cytochrome c and BDNF (a crucial neurotrophin associated with neuronal survival and plasticity) [18]. Consistent with changes in CREB phosphorylation, BDNF expression was amplified by FA at 1-day exposure ($p < 0.0811$), and significantly decreased after a prolonged exposure (10 days) ($p < 0.0004$) (Fig. 4c and 4d). Whilst no significant differences in transcript levels were noted at both exposure periods (1-day, $p < 0.9370$ and 10 days, $p < 0.4151$) (Fig. 4e). In addition, cytochrome c transcript levels were inconsistent with CREB activity.

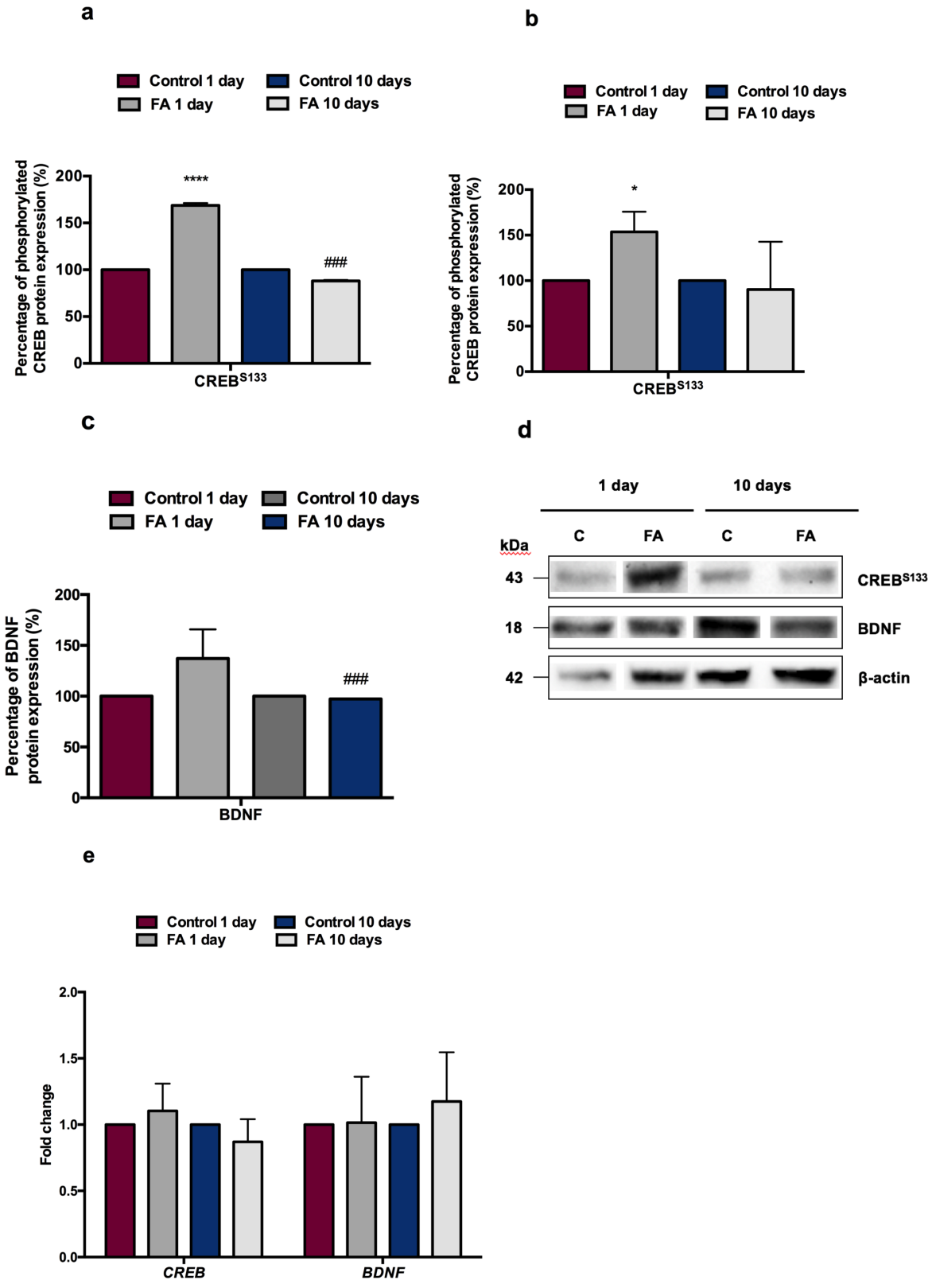


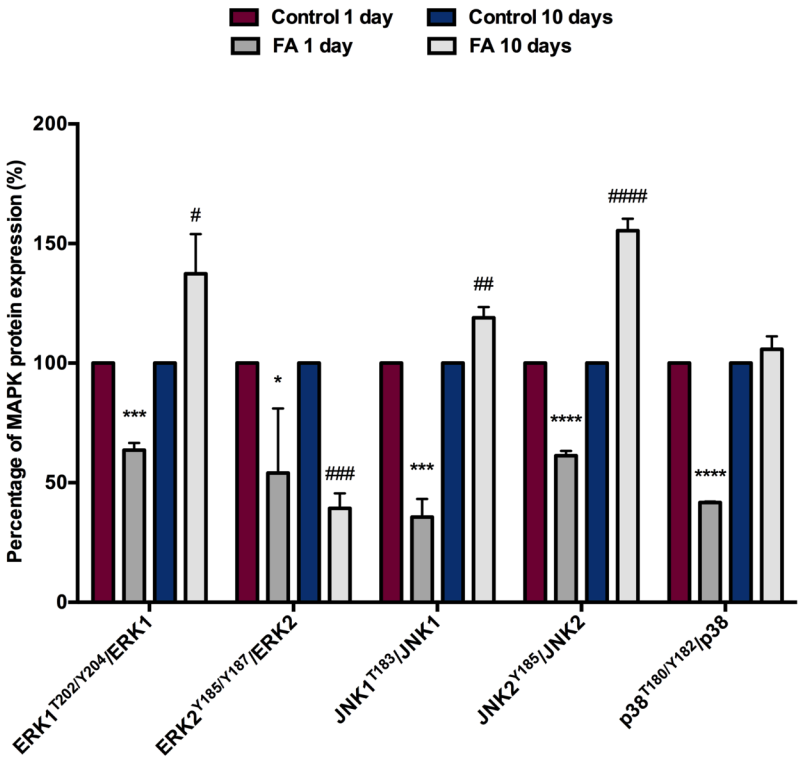
Fig. 4: FA alters CREB phosphorylation in mice brain tissue. Densiometric expression of the transcription factor CREB was determined using a MAPK phosphorylation protein array (**a**) and was validated by

immunoblots (**b and d**). BDNF protein expression was determined using immunblots (**c and d**). Expression of β-actin was used as an internal control. CREB and BDNF transcript levels were quantified by qPCR (**e**). GAPDH was used as the housekeeping gene. The data is represented as mean ± SD (n=3). * $p < 0.05$, **** $p < 0.0001$ (FA relative to control 1 day); ### $p < 0.0005$ (FA relative to control 10 days)

Short exposure to FA suppresses MAPK activation

An extensive body of literature confirms the profound influence of MAPK signalling on CREB activity in multiple neuronal processes [24,23]. Therefore, as a result of differential CREB activation by FA, we investigated the activity of MAPK proteins. Mice treated with FA for 1 day revealed significant reductions in epitope phosphorylation's of ERK1 (T202/Y204) ($p < 0.0001$), ERK2 (Y185/Y187) ($p < 0.0423$), JNK1(T183) ($p < 0.0005$), JNK2 (Y185) ($p < 0.0001$) and p38 (T180/Y182) ($p < 0.0001$) isoforms (1 day) (Fig. 5a and 5b). Whilst a prolonged exposure to FA (10 days) amplified activities of ERK1 (T202/Y204) ($p < 0.0202$), JNK1 (T183) ($p < 0.0035$) and JNK2 (Y185) ($p < 0.0002$) (Fig. 5a and 5b). In contrast, prolonged exposure to FA suppressed ERK2 (Y185/Y187) ($p < 0.0003$) activity and invariably affected p38 (T180/Y182) ($p < 0.1223$) activation (Fig. 5a and 5b). Viewed together, the data demonstrates that acute administration of FA inhibited ERK, JNK, and p38 MAPK activities suggesting that CREB activity was regulated independent of MAPK signalling (1 day). Inversely, FA augmented ERK and JNK activities and decreased CREB activation at 10 days.

a


Control 1 day
FA 1 day
Control 10 days
FA 10 days
Percentage of MAPK protein expression (%)
200
150
100
50
0
***
#
*
###
***
##
****
####
****
ERK1T202/Y204/ERK1
ERK2Y185/Y187/ERK2
JNK1T183/JNK1
JNK2Y185/JNK2
p38T180/Y182/p38

b

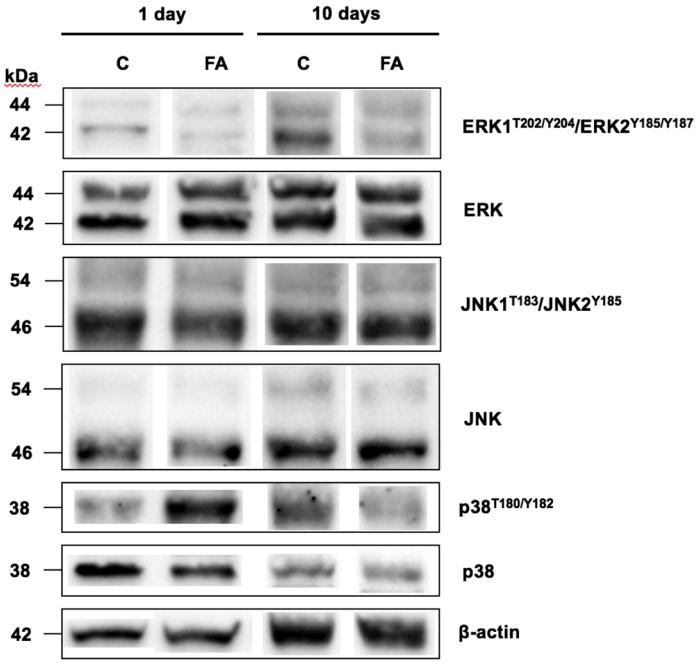

1 day
10 days
C
FA
C
FA
kDa
44
42
ERK1T202/Y204/ERK2Y185/Y187
44
42
ERK
54
46
JNK1T183/JNK2Y185
54
46
JNK
38
p38T180/Y182
38
p38
42
β-actin

Fig. 5: Effect of FA on MAPK activity. Densiometric analysis (**a**) and protein expressions (**b**) of phosphorylated and total MAPK were determined by immunoblots. Expression of β-actin was used as an internal control. The data are represented as mean ± SD (n=3). * $p < 0.05$, *** $p < 0.0005$, **** $p < 0.0001$ (FA relative to control 1 day); # $p < 0.05$, ## $p < 0.005$, ### $p < 0.0005$, #### $p < 0.0001$ (FA relative to control 10 days)

DISCUSSION

Several studies over the past few decades have demonstrated the toxicity of FA through the induction of apoptosis and impaired mitochondrial respiration [5,4,6]. Similarly, our previous investigations have shown the immunotoxicity and bioenergetic deficits caused by FA in immune cells [28] and murine brain tissue (unpublished data), respectively. The latter is of significant importance as metabolic alterations in the central nervous system (CNS) have shown to influence various neuropathological processes. Therefore, the present study aimed at investigating the relationship between FA-metabolic neurotoxicity and its translations in CREB signalling.

Mitochondrial dysfunction is widely believed to be a likely trigger for neuronal cell death and a contributing factor in the neuropathology of diseases such as Alzheimer's-, Parkinson's-disease and diabetes [12,32,13,14]. In apoptosis, rearrangements in mitochondrial ultrastructure is accompanied by elevated cytosolic cytochrome c levels which precedes caspase cleavage [31,33]. Notably, the variability in caspase amongst the control groups (at 1 and 10 days) could have been caused by several factors such as stress, nutritional status, temperature, acclimatisation etc. While these factors were controlled as much as possible across treatment periods (1 and 10 days), during moderate stress caspase activity is increased in a cell death-independent manner. Therefore, mice groups at day 1 may have experienced more stress due to a new environment, handling and being dosed; as compared to the prolonged mice groups that may have acclimatised to these factors by the 10th day decreasing stress and caspase activity levels.

Despite caspase activity being a hallmark of apoptosis, recent evidence shows that effector caspases have prominent non-apoptotic functions in several neuronal processes, such as synaptic plasticity, ageing and learning and memory [34,35]. Similar to histological observations by Li *et al*., [36], who showed a protective mechanism of FA (at 50 mg/kg and 100 mg/kg) against cardiac fibrosis and hypertrophy in C57BL/6 mice, microanalysis of neuronal tissue revealed no loss of tissue integrity, mitochondrial structure or signs of tissue damage and cellular death despite heightened caspase activity (1- and 10- days); suggesting possible non-apoptotic actions of FA-induced caspase activity. However, the precise non-apoptotic role of caspase activation was undetermined.

Accumulating evidence has demonstrated the importance of CREB activity in brain physiology and on brain-body communication [15,16]. In particular, CREB has been shown to regulate neurotrophin-induced transcriptional programs including metabolism, survival and behavioural responses in the central and peripheral

nervous systems [15,16]. In addition, a number of studies have demonstrated the neuroprotective role of CREB-mediated BDNF signalling against several neurotoxic agents. Notably, in this study CREB activity was enhanced followed by an increase in BDNF expression at acute exposure to FA (1 day). In contrast, FA suppressed CREB/BDNF signalling after a 10-day treatment.

MAPKs, particularly ERK and p38, are the main protein kinases known to phosphorylate and activate CREB [15,25]. Of interest, ERK, JNK and p38 activities were down-regulated by acute exposure to FA (1 day), indicating that MAPK signalling was not responsible for CREB phosphorylation. However, in addition to MAPKs, the phosphoinositide 3-kinase (PI3K)/Akt signalling pathway, a second major serine-threonine kinase signalling cascade, has recently been documented as an important regulator of CREB activity [21,22]. MAPK and Akt pathways converge at several points including glycogen synthase kinase 3 (GSK3), ribosomal protein S6 kinase (p70S6K) and p53 [15,17]. Consistent with this, our previous data reported up-regulation of Akt signalling in response to FA exposure (1 day), which may be attributed to increased CREB phosphorylation and activation. A study by Yoo *et al*., [37] described neuroprotective signalling of serotonin-induced tropomyosin receptor kinase B (TrkB)/CREB/BDNF and Akt/Nrf2 (nuclear factor erythroid 2-related factor 2) pathways in neuronal cells. Additionally, Mahgoub and colleagues [38] reported increased CREB phosphorylation and gene expression by serotonin in hippocampal neurons. Multiple studies have also shown dopamine-induced phosphorylation of CREB in the brains of schizophrenia patients, in human airway epithelial cells and during substance withdrawal from neuro-stimulants in rat striatum [39,17,40]. Likewise, previous investigations have shown enhanced neuronal dopamine and serotonin levels by FA which may have likely been a contributing factor to changes in Akt/CREB/BDNF signalling at acute exposure. In addition to MAPK activity, recent studies have shown post-translational regulation of caspases through inhibitory phosphorylation by MAPKs [41-43]. Correlating with FA-modulated MAPK activity (reduced MAPK activity at 1 day; increased MAPK activity at 10 days), caspase activities were enhanced (1 day) and suppressed (10 day) respectively, inferring that MAPKs may be associated with non-destructive neuronal caspase responses.

Importantly, while the structural integrity of neuronal tissue remained unchanged, FA altered cerebral and hypothalamic glial cell densities (1- and 10-days). Glial cells, in particular astrocytes, form an integral part of normal neuronal functioning including metabolic control, inflammation and neurotransmission with various studies implicating reductions in hypothalamic astrocytes with a suppressed appetite in murine models [44]. Unsurprisingly, reduced hypothalamic astrocyte numbers, in response to FA, could explain behavioural changes (lethargy, loss of appetite and vomiting) as previously reported in animals fed FA-contaminated diets [45]. Additionally, studies by Li *et al.*, [46] and Quinlan *et al*., [47] highlighted the importance of astrocytes in pathophysiological processes and have demonstrated the loss of normal astrocyte function as a primary contributor to neurodegenerative disorders such as Alexander's disease, which is associated with the destruction of the myelin sheath. Whilst others have shown neuroprotective activity against ischaemia and neuroinflammation through biochemical- and structural- astrocyte modifications [48,49]. While a significant number of studies confirm the influence of glial-density alterations in major diseases, the precise mechanisms underlying altered glial cell density by FA in neuronal tissue remains unclear.

CONCLUSION

In conclusion, this study demonstrates the differential regulation of CREB/BDNF signalling in neuronal tissue, independent of MAPK activation; and importantly, significant modifications of FA-induced glial cell densities presenting a major concern on the interplay between neuroprotective and neurotoxic effects of FA in- or as a risk factor for cognitive and neurodegenerative disorders upon long term exposure to FA.

COMPLIANCE WITH ETHICAL STANDARDS

COMPETING INTERESTS

The authors declare that they have no competing interests.

ETHICAL APPROVAL

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. [Animal Research Ethics Committee of the University of KwaZulu-Natal (AREC/079/016)].

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CHAPTER FIVE

5.1. Synthesis and conclusion

In an ever-expanding global population, especially in developing countries on the African continent, with shrinking percentages of arable land and a rapidly changing climate, food security is an inevitable challenge. Throughout Africa, maize-based diets are an essential staple food for many, however they come with a significant risk of contamination with naturally occurring mycotoxins (toxins produced by fungi) and presents a major concern for food safety and nutrition. This not only, significantly impacts human and animal health but affects worldwide food trade and economic growth. Since many infected crops are either destroyed or are diverted into animal feeds, there is potential for the bioaccumulation of toxic or bio-transformed residues via meat and dairy/poultry products destined for human consumption. Although agricultural and manufacturing practices have improved in many areas, mycotoxins are formed "naturally" at both the pre- and post-harvest stages, and often survive manufacturing processes (into food components such as flour), making mycotoxin exposure inevitable.

Despite the high incidence of mycotoxins in Africa, smallholder farms and poor communities, who struggle with malnutrition, and follow traditional agricultural methods are largely unaware of mycotoxins and their adverse health outcomes, which often leads to the trade and consumption of contaminated foods.

Aspergillus, Penicillium, Fusarium and *Alternaria* are the main protagonists of mycotoxin feed contamination and have been associated with the development of neurological disorders, malignancies and birth defects amongst its other toxigenic properties. Among many genera that produce mycotoxins, *Fusarium* species are the most widespread in cereal-growing regions worldwide as they have adapted to moderate climates. Some of the predominant mycotoxins produced by the *Fusarium* genus include trichothecenes, deoxynivalenol, T2-toxin, zearalenone, fumonisins and FA; however, exposure to FA is often overlooked. First discovered as a plant pathogen, FA demonstrated pharmacological activities against hypertension and neurotransmitter shifts; and more recently, anti-tumorigenic activity. There is limited data on FA prevalence in food stuffs, however independent studies in Canada and Austria have reported FA concentrations greatly exceeding those of deoxynivalenol, T2-toxin and zearalenone in contaminated grains (Smith and Sousadias 1993; Streit et al. 2013; Swamy et al. 2002); highlighting the significance of FA as a risk factor to human and animal health, either individually or synergistically with other co-contaminating mycotoxins.

The important characteristics of FA is its ability to inhibit dopamine-ß-hydroxylase and compete for the binding of albumin with tryptophan, altering neuronal neurotransmitter and catecholamine levels, that play an important role in the regulation of energy balance, thermoregulation, cardiovascular tone, digestion, coagulation, behaviour, and stress response. These neurotransmitters and catecholamines are modulators of immune function, with alterations potentially resulting in impaired immune responses. Its relationship with the CNS influences physiological homeostasis, immune disorders and immune-mediated neurological syndromes. Elucidation of the molecular mechanisms of toxicity ultimately aid in improving our understanding of its functional and behavioural effects and are important factors in the determination of the toxicological and pharmacological relevance of FA.

In addition to modulating immune interaction with other organ systems such as the CNS, the immune system is primarily responsible for the host defence against foreign organisms. Consequently, impaired immune responses (via ingestion of contaminated food) may lead to an increased susceptibility to infectious diseases, reactivation of chronic infections and decreased vaccine efficacy; traits commonly observed in malnourished individuals. Therefore, this study aimed to evaluate the immunotoxicity of FA *in vitro* on normal human PBMCs and Thp-1 cells and the neurotoxicity of FA *in vivo* on C57BL/6 mice brain tissue following acute and prolonged exposures.

FA demonstrated cytotoxicity to both healthy PBMCs and Thp-1 cells, although at a significantly higher concentration in PBMCs. Interestingly, FA-induced cytotoxicity in PBMCs did not show patterns of the classical hallmarks of apoptotic or necrotic cell death pathways; and instead demonstrated a caspase-independent form of cell death that was possibly mediated by ERK and JNK MAPKs in response to stress-induced stimuli (increased oxidative status and ATP reduction). Similarly, in Thp-1 cells, cellular ATP levels were significantly depleted, accompanied by increased oxidative levels and enhanced ERK, a central kinase implicated in transmitting apoptotic signals, activity. Subsequent assessment of cell death effector molecules in FA-treated Thp-1 cells, revealed the activation of intrinsic apoptotic proteases (caspases) and mitochondrial apoptotic regulator proteins (Bax and Bcl-2) responsible for executing intrinsic apoptosis. Although MAPK-dependency of FA immunotoxicity was not substantiated with the use of inhibitors, the MAPK family play a central role in the mediation of almost all cellular functions including survival, inflammation and differentiation, therefore alterations to MAPK activity by FA may not only impact immune survival but simultaneously affect immune responses.

For decades the brain has been recognized as an important target for FA. Notably, being the most metabolically active organ, the brain relies heavily on ATP, most of which are generated through the aerobic cellular respiration of glucose. As such, the magnitude of FA's ability to substantially reduce

cellular ATP levels in immune cells, has significant implications on brain function, activation and disease resistance. Consistent with ATP deficits in immune cells, FA depleted ATP levels in murine brain tissue, more significantly at a prolonged exposure. However, despite reduced energy levels, AMPK, an essential kinase involved in promoting glucose uptake, suppressed the expression of glucose receptors (GLUT-1 and -4) following acute exposure to FA. Whilst, concurrent activation of PI3K-Akt metabolic signalling failed to improve ATP availability (1 day). On the contrary, activation of AMPK and PI3K-Akt pathways and its inhibitory effect on glucose receptors were reversed at a prolonged exposure; promoting the expression of GLUT receptors for glucose entry, possibly in an attempt to increase cellular ATP levels. Although FA alters glucose-mediated entry by counter-regulatory Akt and AMPK signalling cascades, these observations suggest that FA depletes ATP, independent of metabolic signalling.

FA-induced neurometabolic restrictions demonstrate profound influences on normal brain development and neuronal function, with particular importance in new-borns and infants. While some neurometabolically compromised juveniles appear healthy, others present with acute symptoms of poor feeding, vomiting, lethargy, seizures and respiratory distress; features commonly seen in animals exposed to a FA-contaminated diet. In adulthood, progressive neurometabolic dysfunction results in the dysfunctional mental, motor and perceptual functions with patients presenting with mental decline, aggressiveness and, mood and sleep disorders that manifest into behavioural and neurological conditions such as psychosis, dementia, Alzheimer's and Parkinson's disease.

At a cellular level, transcriptional dysregulation, oxidative stress, mitochondrial toxicity, and synaptic dysfunction have all been implicated in neuronal dysfunction and cell death. While FA slightly elevated caspase activities at acute and prolonged exposures, no signs of neurotoxicity in brain tissue was evident by histological and ultrastructural tissue analysis (evaluation of tissue sections showed intact structural and mitochondrion ultrastructure). Recent research has been centred around the transcription factor, CREB, for its overlapping roles in dietary restriction, energy homeostasis and survival signalling. FA modulated CREB/BDNF (a CREB-associated neurotrophin linked with facilitating metabolic adaptation and survival) activity in a manner independent of MAPK activity (1 day: increased CREB/BDNF activity and decreased ERK, JNK and p38 activities; 10 days: decreased CREB/BDNF activity and increased ERK, JNK and p38 activities). In contrast, changes in CREB activity correlated with the differential regulation of Akt induced by FA at acute and prolonged exposures.

Collectively, this study demonstrated low to moderate FA toxicity in healthy human peripheral immune cells (acute) and murine neuronal cells (acute and prolonged). This was accompanied by significant reductions to cellular energy levels and alterations to central signalling pathways that are essential for

behavioural changes (appetite, depression, dementia), normal brain and immune development, and immune susceptibility to infectious diseases such as human immunodeficiency virus (HIV) and tuberculosis (TB) that are predominant in developing countries. The consequent outcome of neurochemical disturbances, impaired cellular respiration and immune dysfunction induced by FA, are pathophysiological changes often expressed clinically as neurobehavioral and metabolic disorders.

On the other hand, various studies have proven FA's cytotoxic activity against cancer cells and viral infections at low concentrations (Elaasser and El Kassas 2013; Fernandez-Pol 1998; Stack Jr et al. 2004). While Thp-1 cells represent suitable models to characterize monocytic structure and function, it is an immortalized acute monocytic leukaemia cell line known to effectively withstand apoptosis; and therefore, serves as an ideal model to identify effective oncological therapies in resistant malignant cells. Of note, in this study Thp-1 cells were found to be much more susceptible to FA exposure, at a concentration significantly less than that of PBMCs, and may potentiate FA's pharmacological activity at low doses; however, keeping in mind it's toxicity to healthy cells.

Importantly, while this study demonstrated immunological exposure to FA, *in vitro* systems are unicellular models which limits our interpretations of interactions between various cell types found in a multicellular organism. Additionally, long term exposures in primary cells are difficult to maintain away from the normal environment and several cellular factors (metabolism, proteins, regulation of genes, etc.) may be altered. Also, adhering to ethical guidelines (the 3Rs: replacement, reduction and refinement), immune validation in *in vivo* systems are challenging as they often require large sample numbers. Therefore, for a holistic assessment of human health risks associated with mycotoxin exposure and its biological effects, several models have to be combined in order to compensate for their respective limitations. Notably, findings from this study provides awareness on the potential risks of exposure to FA and greatly enhances our understanding of FA's effect on cellular function and biological responses. Nevertheless, further research on the immunotoxicity of FA *in vivo* and the consequences of the altered neuronal cellular architecture, are required to better understand the risks of FA-contaminated food consumption to human and animal health. In addition, different vulnerability factors such as age, nutritional status and disease susceptibility, and the co-occurrence of other mycotoxins (content on food and routes of exposure), need to be taken to account to provide more effective risk assessment studies.

In conclusion, exposure to mycotoxins is a growing health concern, especially in developing countries where the effects of poverty and malnutrition exacerbate the adverse effects of these food-borne toxins. Moreover, in developed countries, where the risks of human consumption of mouldy grains are recognised, contaminated grains are diverted as feed to livestock, impacting on animal productivity and food supply, further increasing the probability of poverty and human exposure. Additionally, even when

mycotoxins are detected, it is difficult to associate a specific mycotoxin with a given health effect. However, there is adequate data from *in vitro* human-, *in vivo* animal-models and human epidemiological studies to conclude on the importance of mycotoxin exposure in human and animal health. Therefore, exposure to these toxins is a global food safety issue and needs to be addressed in order to improve human health in developing countries. In addition, public awareness of the possible health risks posed by mycotoxigenic fungi and their mycotoxins has to be developed. This coupled with the implementation of practical and affordable mycotoxin control methods, especially at the household level, is equally important and should be prioritized together with further toxicological studies on ubiquitous mycotoxins, such as FA and other co-occurring fungal metabolites, to identify overlapping and synergistic toxigenic activities.

5.2. References

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APPENDIX A

Manuscript presented as published by Scientific Reports (Chapter 2)

SCIENTIFIC REPORTS

OPEN

Fusaric Acid immunotoxicity and MAPK activation in normal peripheral blood mononuclear cells and Thp-1 cells

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Fusaric acid (FA), a food-borne mycotoxin, is a potent divalent metal chelator. The human immune system is complex and susceptible to environmental insult however, the immunotoxity of FA remains unknown. We investigated the immunotoxicity of FA on human peripheral blood mononuclear cells (PBMCs) and Thp-1 cells. FA was cytotoxic to PBMCs (IC_{50}-240.8 μg/ml) and Thp-1 (IC_{50}-107.7 μg/ml) cells at 24 h. FA induced early apoptosis but significantly decreased caspase activity in PBMCs, a characteristic of paraptosis. In Thp-1 cells, FA induced apoptosis and increased caspase −9 and −3/7 activities. In PBMCs, FA maintained mitochondrial membrane potential and decreased protein expression of Bax whilst increasing expression of p-Bcl-2; FA induced oxidative stress and depleted ATP levels in both cell types. In Thp-1 cells, FA increased mitochondrial membrane depolarization and decreased p-Bcl-2 expression. In PBMCs, FA significantly up-regulated the MAPK protein expression of p-ERK and p-JNK but down-regulated p-p38 expression. In Thp-1 cells, FA up-regulated MAPK protein expression of p-ERK whilst p-JNK and p-p38 expression were down-regulated. In conclusion FA induced programmed cell death and altered MAPK signaling in healthy PBMCs and Thp-1 cells strongly suggesting a possible mechanism of FA induced immunotoxicity *in vitro*.

Endophytic fungi produce mycotoxins that are toxic to animals and humans[1]. Fusaric acid (FA, 5-butylpicolinic acid), is a picolinic acid derivative produced by several strains of *Fusarium* species[2,3]. These fungal strains are ubiquitous in soil and are known to parasitize maize and many other cereal grains[4,5].

FA contains a pyridine ring with a butyl side chain that allows it to easily permeate cell membranes[6]. The toxicity of FA is also due to its ability to chelate divalent ions such as magnesium, calcium, zinc and iron[2,7]. The nitrogen in the pyridine ring and the deprotonated, negatively charged oxygen on the carboxylic acid group are responsible for FA's divalent metal chelating ability[8,9].

The human immune system functions in host defense against environmental exposure to bacteria, viruses, parasites, fungi and other perturbations, and in acquiring immunity against invading pathogens[10,11]. In response to foreign particle or pathogen, several signaling pathways are activated in immune cells[12]. Foremost of these pathways, is the activation of mitogen-activated protein kinases (MAPKs)[12]. MAPK activity directs diverse immune responses ranging from stress, cell death/survival and immune defense[12–14].

Optimal cellular mitochondrial function increases ATP synthesis and reactive oxygen species (ROS) that mediate cell signaling pathways[8]. The amount of intracellular ROS will significantly influence the MAPK pathway[6]. The MAPK family comprises of three universal serine/threonine protein kinases; these include the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 kinase[15,16]; each group of MAPK is activated via a series of phosphorylation events[16]. The first event involves the phosphorylation and activation of a MAPK kinase kinase (MAPKKK), which in turn, phosphorylates and activates a MAPK kinase (MAPKK). MAPKKs activate MAPKs through dual phosphorylation on both threonine and tyrosine residues located within the tri-peptide motif of the MAPK[14,15,17,18]. Once activated, MAPKs phosphorylate several transcription factors, thereby regulating gene expression and cellular functions[13,14].

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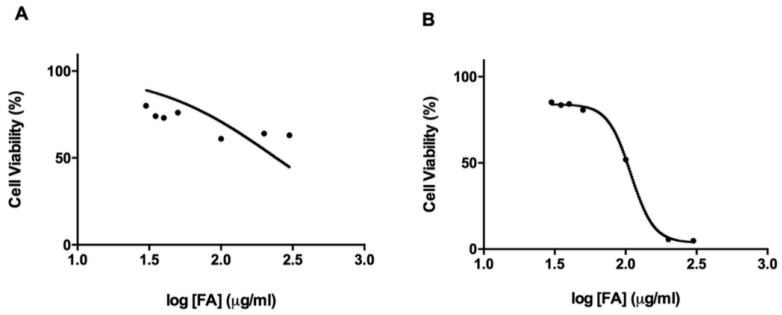

Figure 1. Cytotoxicity of FA on PBMCs and Thp-1 cells. FA induced a dose dependent decrease in PBMC (**A**) and Thp-1 (**B**) cell viability.

Apoptosis is executed by immune cells to maintain homeostasis of the immune system[19–21]. Apoptosis occurs via two main pathways, the intrinsic and extrinsic apoptotic pathways[19, 22, 23]. Both the intrinsic and extrinsic pathways are activated by caspases; the initiator caspases (−8 and −9) are involved in the intrinsic pathway, whilst the executioner caspases (−3/7) are integral to the extrinsic pathway[19, 24]. Paraptosis is distinct from necrotic and apoptotic cell death and its features are defined by the lack of apoptotic morphology and independent of caspase activation[19, 22, 23, 25–27].

The phytotoxicity of FA is well documented and includes altered mitochondrial membrane potential and inhibition of ATP synthesis[28, 29]. In animals, FA inhibits the activity of dopamine-β-hydroxylase, synthesis of nucleic acids (zinc finger proteins involved in DNA repair) and impairs protein synthesis[30]. In young swine, FA showed moderate toxicity, induced vomiting and increased concentration levels of tryptophan and serotonin in the brain[31]. Elevated levels of serotonin results from its impaired regulation and consequently amplifies behaviors distinctive of the firing of serotonergic neurons such as loss of appetite and lethargy[32]. In zebrafish, FA induced teratogenic effects by inhibition of lysyl oxidase (a copper-dependent enzyme)[33]. FA also decreased norepinephrine levels in the brain, heart, spleen and adrenal gland of rats[34].

To date, no study has investigated the effect of FA on the mammalian immune system. In this study, we assessed the immunotoxicity of FA associated with MAPK activity in healthy human peripheral blood mononuclear cells (PBMCs) and the acute monocytic leukemic (Thp-1) cell line. It was hypothesized that FA altered MAPK signaling was immunotoxic in both cell types. This study shows that FA, a common food borne mycotoxin, is toxic to the human immune system. This data may help develop a better understanding of the immune risks associated with FA consumption. This is of importance in South Africa, the epicenter of infectious diseases, where the majority population relies on maize as a food staple.

Results

Cell viability of PBMCs and Thp-1 cells. The WST-1 assay showed that FA induced a dose dependent decrease in PBMC and Thp-1 cell viability over 24 h (Supplementary Tables: S3-PBMC, S4-Thp-1). Thp-1 cells were more susceptible than PBMCs to FA toxicity. An IC_{50} of 240.8 μg/ml (Fig. 1A) and 107.7 μg/ml (Fig. 1B) determined for PBMCs and Thp-1 cells respectively; and was used in all subsequent assays.

FA activates caspase-independent cell death in PBMCs and intrinsic apoptosis in Thp-1 cells. To confirm the toxicity of FA, we assessed the externalization of phosphatidylserine (PS) on the plasma membranes of PBMCs and Thp-1 cells. FA significantly increased the externalization of PS in PBMCs and Thp-1 cells by 1.42 (18.43 + 0.006% vs. 26.16 + 0.003%; $p = 0.0003$) and 2.27 (8.03 + 0.004% vs.18.19 + 0.002%; $p < 0.0001$) fold, respectively (Fig. 2). Propidium iodide (PI) staining showed a decreased percentage of necrotic cells by FA as compared to the controls in both PBMCs and Thp-1 cells (Supplementary Fig. S1). This was confirmed by quantifying the release of lactate dehydrogenase (LDH). FA did not induce membrane leakage in both PBMCs and Thp-1 cells (Supplementary Fig. S2), therefore, necrotic cell death was excluded.

Next, we measured caspase −8, −9 and −3/7 activities to determine the type of programmed cell death induced by FA in PBMCs and Thp-1 cells. Interestingly, despite the increase in PS externalization, FA significantly decreased caspase −8 activity by 0.81 fold ($1.09 \pm 0.001 \times 10^4$ RLU vs. $0.88 \pm 0.042 \times 10^4$ RLU; $p = 0.0022$), caspase −9 by 0.73 fold ($10.89 \pm 0.609 \times 10^4$ RLU vs. $7.92 \pm 0.241 \times 10^4$ RLU; $p = 0.0070$) and caspase −3/7 activities by 0.10 fold ($1.19 \pm 0.258 \times 10^4$ RLU vs. $0.12 \pm 0.026 \times 10^4$ RLU; $p = 0.0035$) in PBMCs, relative to the control (Table 1). This result suggests that FA induced caspase-independent cell death in PBMCs. In Thp-1 cells, however, FA significantly decreased caspase −8 activity by 0.74 fold ($4.52 \pm 0.306 \times 10^4$ RLU vs. $3.3265 \pm 0.021 \times 10^4$ RLU; $p = 0.0211$), but significantly increased both caspase −9 activity (1.43 fold; $62.67 \pm 3.701 \times 10^4$ RLU vs. $89.37 \pm 0.590 \times 10^4$ RLU; $p = 0.0065$) and caspase −3/7 activity (5.33 fold; $0.82 \pm 0.482 \times 10^4$ RLU vs. $4.38 \pm 0.604 \times 10^4$ RLU; $p = 0.0041$) when compared to the control (Table 2); an indicator of activation of intrinsic apoptosis.

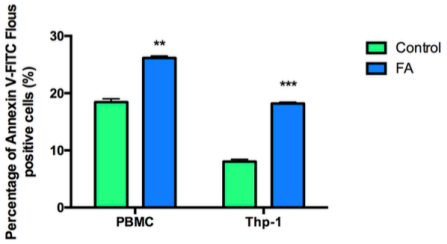

Figure 2. Translocation of PS in PBMCs and Thp-1 cells. FA induced PS externalization in both PBMCs and Thp-1 cells. Data are expressed as mean ± SD. $**p < 0.005$ relative to respective control; $***p < 0.0001$ relative to respective control.

	Mean ± SD (RLU × 10^4)		Fold change	*p* value
	PBMC			
	Control	FA		
Caspase −8	1.0918 ± 0.0007	0.8831 ± 0.0419	0.81	0.0022**
Caspase −9	10.8855 ± 0.6094	7.9185 ± 0.2409	0.73	0.0070*
Caspase −3/7	1.1858 ± 0.2581	0.1218 ± 0.0261	0.10	0.0035**

Table 1. Effect of FA on caspase (−8, −9, −3/7) activity in healthy PBMCs. SD: standard deviation; RLU: relative light units; $*p < 0.05$; $**p < 0.005$.

	Mean ± SD (RLU × 10^4)		Fold change	*p* value
	Thp-1			
	Control	FA		
Caspase −8	4.5235 ± 0.3055	3.3265 ± 0.0206	0.74	0.0211*
Caspase −9	62,6683 ± 3.7013	89.3652 ± 0.5900	1.43	0.0065*
Caspase −3/7	0.8210 ± 0.4816	4.3758 ± 0.6041	5.33	0.0041**

Table 2. Effect of FA on caspase (−8, −9, −3/7) activity in Thp-1 cells. SD: standard deviation; RLU: relative light units; $*p < 0.05$; $**p < 0.005$.

FA induces oxidative stress in PBMCs and Thp-1 cells. Increased intracellular ROS is known to promote oxidative stress, that is not only injurious to cells but may also regulate cell signaling pathways[8]. Lipid peroxidation, a marker for oxidative stress, was measured by quantifying malondialdehyde (MDA) using the Thiobarbituric acid (TBARS) assay. FA significantly elevated MDA levels in PBMCs (7.59 fold; 0.02 ± 0.010 μM vs. 0.16 ± 0.016 μM; $p = 0.0006$) and Thp-1 cells (1.59 fold; 0.18 ± 0.020 μM vs. 0.28 ± 0.010 μM; $p = 0.0039$) (Fig. 3A). FA induced oxidative stress in both normal human PBMCs and Thp-1 cells.

Mitochondria are important in maintaining cellular redox homeostasis and activation of the intrinsic apoptotic pathway. The evaluation of FA induced mitochondrial membrane integrity by flow cytometry in PBMCs showed that despite increased MDA levels, there was no effect on the mitochondrial membrane potential; however, in Thp-1 cells, mitochondrial membrane depolarization was significantly increased (1.95 fold; 33.58 ± 1.425% vs. 65.48 ± 0.329%; $p = 0.0007$) when compared to the controls (Fig. 3B).

Further, FA significantly depleted ATP levels in both PBMCs (0.16 fold; 38.97 ± 1.183 × 10^4 RLU vs. 6.11 ± 0.266 × 10^4 RLU; $p = 0.0002$) and in Thp-1 cells (0.52 fold; 225.21 ± 8.014 × 10^4 RLU vs. 117.26 ± 10.017 × 10^4 RLU; $p = 0.0007$) relative to controls (Fig. 3C).

Effect of FA on Bax and p-Bcl-2 protein expression in PBMCs and Thp-1 cells. To validate caspase-dependent and –independent cell death, protein expressions of pro-apoptotic Bax and anti-apoptotic p-Bcl-2 was determined. FA significantly decreased Bax protein expression (0.71 fold; 100 ± 10.33% vs. 70.90 ± 7.34%; $p = 0.0201$) and increased p-Bcl-2 protein expression (1.18 fold; 100 ± 6.40% vs. 118.30 ± 2.83%; $p = 0.0455$) in PBMCs compared to the control (Fig. 4A and B), whilst it only significantly decreased the protein expression of p-Bcl-2 (0.78 fold; 99.39 ± 0.89% vs. 77.40 ± 2.82%; $p = 0.0007$) in Thp-1 cells (Fig. 4C and D).

Effect of FA on MAPKs expression in PBMCs and Thp-1 cells. Finally, to evaluate the effect of FA on MAPK signaling, phosphorylation of MAPK protein expressions was determined using western blotting. In PBMCs, FA significantly increased expression of p-ERK (42 kDa fragment and 44 kDa fragment; 1.94 fold; 100 ± 5.73% vs. 194.19 ± 26.83%; $p = 0.0271$ and 1.36 fold; 99.80 ± 0.68% vs. 136.20 ± 5.29%; $p = 0.0006$,

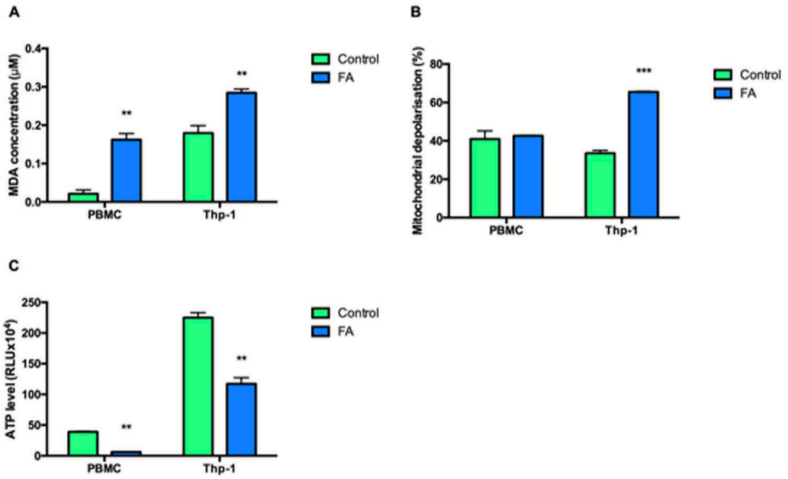

Figure 3. Effect of FA on the oxidative status and mitochondrial function in PBMCs and Thp-1 cells. FA increased MDA levels in both PBMCs and Thp-1 cells (**A**) had no effect on the mitochondrial membrane potential in PBMCs but increased depolarization of the mitochondrial membrane in Thp-1 cells (**B**). FA depleted ATP levels in PBMCs and Thp-1 cells (**C**). Data are expressed as mean ± SD. $^{**}p < 0.005$ relative to respective control; $^{***}p < 0.0001$ relative to respective control.

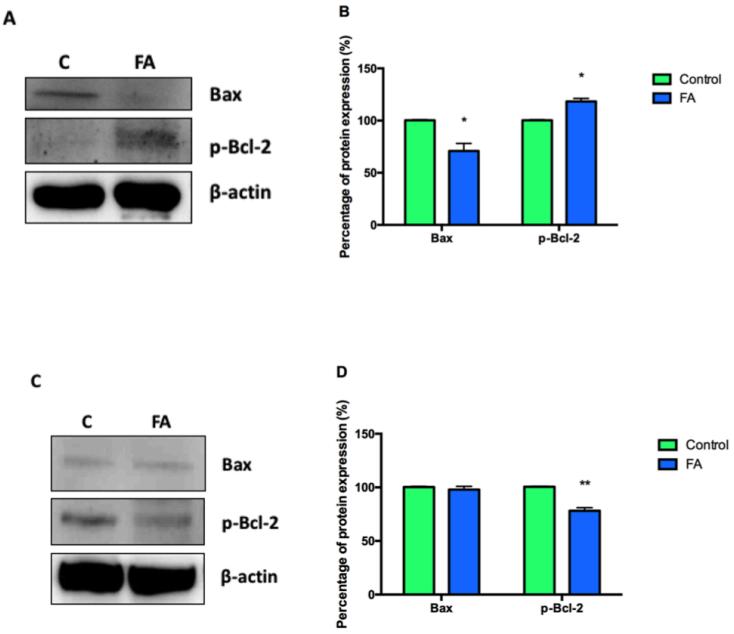

Figure 4. Effect of FA on apoptotic regulator proteins Bax and p-Bcl-2 in PBMCs and Thp-1 cells. Protein expressions of Bax and p-Bcl-2 in healthy PBMCs (**A**) and Thp-1 cells (**C**). Percentage of Bax and p-Bcl-2 protein expression in healthy PBMCs (**B**) and Thp-1 cells (**D**). Percentage of protein expressions were represented as mean ± SD. $^{*}p < 0.05$ relative to respective control; $^{**}p < 0.005$ relative to respective control.

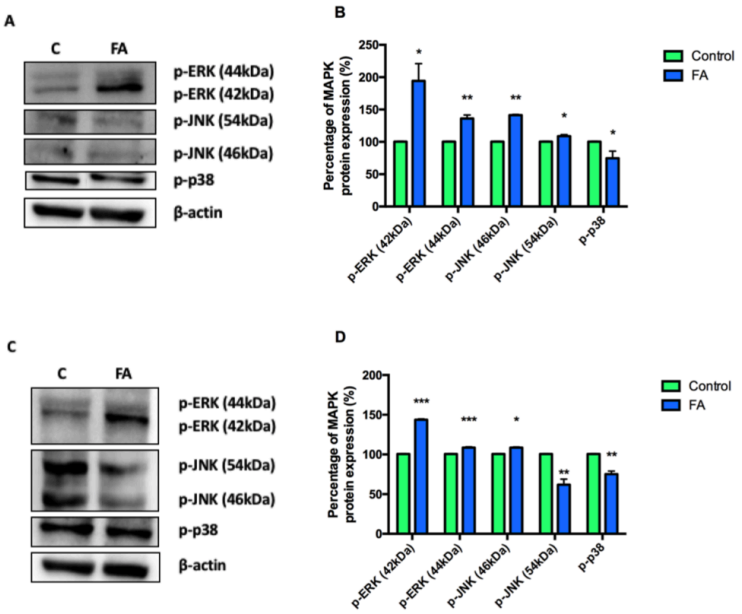

Figure 5. Effect of FA on MAPK protein expression. MAPK protein expression in healthy PBMCs (**A**) and Thp-1 cells (**C**). Percentage of MAPK protein expression in healthy PBMCs (**B**) and Thp-1 cells (**D**). Percentage of protein expressions were represented as mean ± SD. *$p < 0.05$ relative to respective control; **$p < 0.005$ relative to respective control; ***$p < 0.0001$ relative to respective control.

respectively) and p-JNK (46 kDa fragment and 54 kDa fragment; 1.46 fold; 97.10 ± 10.06% vs. 141.39 ± 0.92%; $p = 0.0035$ and 1.13 fold; 96.56 ± 11.92% vs. 108.77 ± 2.45%; $p = 0.0454$, respectively) (Fig. 5A and B). In Thp-1 cells, FA also significantly increased p-ERK (42 kDa and 44 kDa fragments; 1.35 fold; 100 ± 3.54% vs. 135.13 ± 15.06%; $p < 0.0001$ and 1.05 fold; 100 ± 5.15% vs. 104.68 ± 6.93%; $p = 0.0006$, respectively) (Fig. 5C and D), whilst significantly decreasing p-JNK (46 kDa and 54 kDa fragments; 1.02 fold; 105.85 ± 20.25% vs. 108.37 ± 0.75%; $p = 0.0461$ and 0.62 fold; 103.48 ± 12.05% vs. 63.98 ± 7.03%; $p = 0.0055$, respectively) (Fig. 5C and D). Furthermore, FA significantly decreased the expression of p-p38 in PBMCs and Thp-1 cells (0.75 fold; 100 ± 9.17% vs. 74.73 ± 11.08%; $p = 0.0401$ and 0.70 fold; 100 ± 17.40% vs. 69.86 ± 9.89%; $p = 0.0033$, respectively) (Fig. 5A–D).

Discussion

The promising role of divalent ion chelators in proliferative and virulent diseases has led to growing interest[35–37]. FA, a picolinic acid analogue and potent divalent metal chelator, has shown potential as an anti-cancer, anti-microbial and anti-viral agent[35–37]. Recently, the role of divalent ion chelators in proliferative and virulent diseases has been extensively studied. However, the toxicity of FA, a food-borne mycotoxin, on the immune system containing a diverse population of cells, has to date not been studied.

In this study, we showed the immunotoxic potential of FA to both healthy PBMCs (diverse population of immune cells) and the distinct immune Thp-1 cells. Interestingly, FA inhibited Thp-1 cell proliferation at an IC_{50} value less than half that of PBMCs (Fig. 1A and B). This result is in agreement with other studies that showed cytotoxic effects of FA on WI-38 cells (fibroblastic cells), LoVo cells (colorectal adenocarcinoma cells) and MDA-468 cells (human breast adenocarcinoma cells) in which FA had preferentially inhibited the proliferation of cancerous cells (LoVo and MDA-468) when compared to the normal cells (WI-38)[35]. Our data suggests that FA may exert selective toxicity to distinct immune cell types as evidenced by the Thp-1 response, albeit a leukemic cell line. Additionally, in comparison to the anti-neoplastic drug, ellipticine failed to inhibit PBMC and Thp-1 cell viability; which further exemplifies the potency of FA.

Contrary to the study by Fernandez-Pol (1998), FA significantly increased the externalization of PS in both PBMCs and Thp-1 cells by 26.16% and 18.19%, respectively (Fig. 2). Given that the externalization of PS occurs during both apoptosis and paraptosis, activities of caspases −8, −9 and −3/7 were assayed to determine type of cell death induced by FA in both cell lines; apoptosis requires caspase activation whilst paraptosis is independent of caspase activation. FA substantially decreased caspase activities in PBMCs (Table 1), strongly suggesting that paraptosis was the preferred mode of cell death. In Thp-1 cells, however, FA significantly increased caspase −9 and −3/7 activities were (Table 2), indicating the induction of intrinsic apoptosis in Thp-1 cells.

Immune cells respond to stimuli by activating MAPK signaling to amplify other signals to elicit an appropriate physiological response for programmed cell death[14,38]. Previous studies showed that prolonged activation of MAPK signaling induced cell death via ROS-activation of MAPK signaling pathways[16]. ROS are continuously generated by cellular processes, with the mitochondrion being the major source[6]. Excessive ROS generated during oxidative phosphorylation can cause oxidative damage to proteins, DNA and phospholipids[16]; oxidative degradation of lipids results in the formation of lipid peroxides such as MDA[39]. FA significantly increased MDA levels in both PBMCs and Thp-1 cells (Fig. 3A), indicative of a prolonged oxidative stress environment. Furthermore, FA disrupted mitochondrial membrane potential by increasing mitochondrial depolarization in Thp-1 cells (Fig. 3B). This could be due to the weak acidic nature of the carboxylic acid group of FA[40]. Weak acids act as proton carriers across lipid membranes, thereby disrupting the proton gradient along the electron transport chain (ETC)[41,42]. Interestingly, FA did not disrupt the mitochondrial membrane potential in normal PBMCs (Fig. 3B) despite the significant depletion of ATP levels in both normal PBMCs and Thp-1 cells (Fig. 3C). In Thp-1 cells, this is substantiated by the decreased cell viability (decreased redox potential) and increased mitochondrial membrane depolarization. Also, the activation of ATP dependent caspases −9 and −3/7 may further deplete ATP levels[43]. In PBMCs, however, the decreased ATP levels may be due to increased activation and prolonged activation of ATP dependent protein kinases.

Intracellular ROS not only alters cellular integrity but is also important to MAPK signaling cascades[6]; FA induced increased ROS production and up-regulated protein expression of ERK in Thp-1 cells (Fig. 5C and D). Although ERK signaling pathways are well known for their role in promoting cell survival, recent studies have demonstrated their ability to potentiate apoptosis[44]. Prolonged activation of ERK may be due to the inhibition of tyrosine phosphatases, a group of enzymes responsible for the removal of phosphate groups on phosphorylated tyrosine residues, hence inactivating the protein[3]. However, tyrosine phosphatases are sensitive to increased ROS and become oxidized, thereby inhibiting their activity and prolonging ERK activation[3,45].

JNK and p38 MAPK signaling pathways are generally directed towards initiating cell death upon activation by stress signals. Recently, however, these signaling pathways have been associated in both cell death and survival[38]. In Thp-1 cells, FA significantly decreased p-JNK activation and p-p38 protein expressions (Fig. 5C and D). A study by Pedram *et al.* (1998) documented the cross-talk between the ERK and JNK MAPKs where the activation of JNK by ERK MAPK was followed by the activation of ERK by vascular endothelial growth factor (VEGF) whilst JNK stimulated ERKs proliferative signaling. Therefore, it can be inferred that a decrease in JNK activity hinders the cross-talk between JNK and ERK MAPKs, preventing survival signaling by ERK[46].

Additionally, JNK and ERK MAPKs regulate the expression of Bcl-2 family proteins that are central in regulating the mitochondrial apoptotic death pathway[3,47]. Bcl-2 inhibits apoptosis by forming a complex with pro-apoptotic proteins such as Bax[47,48]. Phosphorylation of Bcl-2 compromises it's protein stability and affects dimerization with Bax[49]. Thus, dissociation from the complex at the mitochondrial membrane leads to the formation of mitochondrial permeability transition pore (mPTP) and subsequent caspase activation[50]. In support of the increased caspase −9 and −3/7 activities in Thp-1 cells, FA decreased p-Bcl-2 expression resulting in apoptotic cell death (Fig. 4C and D). JNK signaling regulates the expression of Bcl-2 and is up-regulated in response to JNK activation. FA activated ERK death signaling, decreased p-Bcl-2 expression and induced apoptosis in Thp-1 cells, but had no significant effect on Bax expression (Fig. 4C and D). This may be due to the deletion of the p53 gene in the Thp-1 cell line as p53 acts as a transcription factor for Bax expression and recruitment to the mitochondrial membrane[24,51–55]. Additionally, increased ERK activity regulates mitochondrial membrane potential[56] and corresponds with the increased caspase −9 and −3/7 activities, and the subsequent activation of cell death in Thp-1 cells by FA. In PBMCs, FA increased p-Bcl-2 expression (Fig. 4A and B) and decreased Bax expression (Fig. 4A and B), with a corresponding decrease in caspase −8, −9 and −3/7 activities. Increased p-Bcl-2 expression helps maintain the mitochondrial membrane integrity and subsequent mitochondrial membrane potential by preventing the release of cytochrome c, activation of caspase −9 and the initiation of intrinsic apoptosis, further validating the induction of paraptosis in normal PBMCs by FA.

Although the molecular activation of paraptosis remains unknown, studies have suggested the involvement of MAPK signaling in the induction of cell death. In PBMCs, FA significantly increased the expression of ERK and JNK whilst decreasing p38 expression (Fig. 5A and B). Sperandio *et al.*, (2000) reported that ERK and JNK activity mediated paraptosis stimulation by insulin-like growth factor 1 receptor, and that inhibition of these MAPKs prevented the induction of paraptosis in 293 T cells[57]. Another study by Yumnam *et al.*, (2014) showed the involvement of ERK MAPK in hesperidin-induced paraptosis of human hepatocellular carcinoma (HepG2) cells[58]. Sugimori and colleagues (2015) recently showed that activated JNK induced paraptosis induction in HL-60 and NB4 human promyelocytic leukemic cell lines and in bone marrow blasts treated with benfotiamine[59]. Contrary to the studies by Sperandio *et al.*, (2000) and Yumnam *et al.*, (2014), benfotiamine inhibited the activity of ERK in bone marrow blasts and had no effect on ERK activity in HL-60 and NB4 cell lines[57,58]. This suggests that the involvement of MAPK in the induction of paraptosis may be dependent on the cell line and type of activation. Additionally, caspase −9 was reported to be a direct target of ERK MAPK, and that phosphorylation at threonine 125 on caspase −9 inhibits its pro-apoptotic activity[60]. These findings support the activation of MAPK signaling pathways in the induction of paraptosis in PBMCs treated by FA.

Conclusion

FA is immunotoxic to both healthy PBMCs and Thp-1 cells, albeit at a higher concentration in PBMCs. The cancerous Thp-1 cells are highly susceptible to FA toxicity. Collectively, the results show that the host response to FA exposure augmented MAPK signaling and induction of apoptosis in Thp-1 cells (via the mitochondrial apoptotic pathway) and paraptosis in PBMCs. This study shows that FA, a common food borne mycotoxin, is toxic to the human immune system. This data may help develop a better understanding of the immune risks associated with

FA consumption. This has great importance in socio-economically challenged countries where the majority population relies on corn as a food staple.

Material and Methods

Materials. Cell culture reagents for PBMC maintenance and FA (*Gibberella fujikuroi*) were purchased from Sigma Aldrich (Johannesburg, SA). The Thp-1 cells and media were purchased from ATCC (University Boulevard Manassas, USA) and Scientific group (Johannesburg, SA), respectively. Luminometry reagents were obtained from Promega (Madison, USA).

Cell culture. PBMCs were isolated from whole blood using Histopaque 1077 (Sigma Aldrich) and gradient centrifugation from young healthy males following institutional (University of KwaZulu-Natal) ethical approval (BE057/15) and written informed consent. Collection and use of blood was in compliance with relevant institutional guidelines and procedures. Isolated PBMCs were maintained at 37 °C with 5% CO_2 in RPMI 1640 medium (supplemented with 10% FCS, 1% L-glutamine and 1% pencillin-streptomycin). Thp-1 cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 1% L-glutamine, 1% pencillin-streptomycin, 1 mM sodium pyruvate and 0.05 mM β-mecaptoethanol. Thp-1 cells were maintained at 3×10^5 cells/ml in 75 cm^3 ventilated flasks at (37 °C, 5% CO_2) and were split at a cell count of 8×10^5 cells/ml. Viability of cells was assessed using trypan blue exclusion.

Cell viability. The cytotoxicity of FA on PBMCs and Thp-1 cells was analyzed using the WST-1 reagent. Briefly, PBMC and Thp-1 cells (20,000 cells/well) were seeded into a 96-well microtitre plate. The cells were incubated with varying FA concentrations (30–300 µg/ml) in triplicate (200 µl/well) for 24 h (37 °C, 5% CO_2). Ellipticine, anti-neoplastic agent, was used as a positive control to FA toxicity (Data shown in Supplementary Tables: S1 PBMC; S2 Thp-1). A positive control of cells with RPMI only and a negative control with RPMI/WST-1 reagent solution was also seeded. Following incubation, the plate was centrifuged at 24 °C, 400*xg* for 10 min. The supernatant was then aspirated and 110 µl/well of a RPMI/WST-1 reagent solution (1:10) was added and incubated for 3 h (37 °C, 5% CO_2). The optical density of the colorimetric reaction was measured at a wavelength of 450 nm and reference wavelength of 620 nm using a spectrophotometer (Bio-Tek uQuant, Winooski, VT, USA). The percentage cell viability was calculated by standardizing untreated (control) cells to 100% and then comparing FA treated cells to the control cells (Detailed calculation shown as Supplementary Information). The concentration of half maximum inhibition (IC_{50}) was determined using GraphPad Prism v5.0 software. All assays were performed in triplicate, twice independently.

Cell death parameters. *PS Externalization.* Flow cytometry was performed to determine the externalization of PS. Following treatment, 100 µl of an Annexin V-FITC Fluos solution (1:1:50; annexin V-FITC: PI: staining buffer) was added to each sample (200,000 cells in 100 µl PBS) and incubated in the dark at room temperature (RT, 15 min). Thereafter, the samples (20,000 events) were analyzed for apoptosis on the AccuriTM C6 flow cytometer. The cells were gated to exclude cellular debris using the Fl-1 channel (525 nm) (BD Biosciences, Johannesburg, SA). The results were expressed as a percentage.

LDH activity. The LDH cytotoxicity detection kit (Roche, Mannheim, Germany) was used to confirm damaged/necrotic cells. In brief, cell homogenates (100 µl) were seeded into a 96-well opaque polystyrene microtitre plate in triplicate. Subsequently, 100 µl of a substrate mixture containing a catalyst (diaphorase/NAD^+) and dye solution (INT/sodium lactate) was added to each homogenate and incubated in the dark for 25 min (RT). The optical density was measured (500 nm) using a spectrophotometer (Bio-Tek uQuant, Winooski, VT, USA). The results were reported as mean optical density.

Caspase activity. Caspase activities of −8, −9 and −3/7 were determined using luminometry. Cells (20,000 cells/well) were seeded into a 96-well opaque polystyrene microtitre plate in triplicate. 20 µl/well of the reagent (Caspase-Glo® 3/7, Caspase-Glo® 8 and Caspase-Glo® 9 Assays) was added to each sample and incubated in the dark for 30 min (RT). Thereafter, the luminescence was measured on a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA). All data was expressed as relative light units (RLU).

Evaluation of oxidative stress. *Lipid peroxidation.* The TBARS was used to determine FA generation of ROS[61]. Briefly, cell homogenates (400 µl) were added to a 7% phosphoric acid (400 µl) and a thiobarbituric acid (1%W w/v)/butylated hydroxytoluene (0.1 mM) (TBA/BHT) solution. A positive control containing MDA (1 µl) and a negative control containing 3 mM hydrogen chloride were prepared. All samples were heated in a water bath (100 °C, 15 min) and allowed to cool (RT). Thereafter, lipids were extracted with butanol (1.5 ml) and were measured on a spectrophotometer (Bio-Tek uQuant, Winooski, VT, USA) at 532 nm with reference wavelength of 600 nm. The mean optical density for each sample was calculated and divided by the absorption coefficient ($156\ mM^{-1}$). The results were expressed in µM.

Mitochondrial membrane potential. Mitochondrial membrane potential was measured using the JC-1 Mitoscreen kit (BD Biosciences, Johannesburg, SA) and flow cytometry. Briefly, 100 µl of a JC-1 working solution was added to each sample (200,000 cells in 100 µl PBS) and incubated in the dark for 30 min (RT). Following incubation, 100 µl flow cytometry sheath fluid was added to each sample and were analyzed on the AccuriTM C6 flow cytometer. A total of 20 000 events were gated using AccuriTM C6 flow cytometer Fl-1 channel (525 nm) (BD Biosciences, Johannesburg, SA). The results were expressed as a percentage.

ATP levels. Intracellular ATP levels were measured using the ATP CellTitre Glo reagent (Promega, Madison, USA). Following treatment, 20,000 cells/well were seeded into a 96-well opaque polystyrene microtitre plate in triplicate. The reagent (20 µl/well) was added to each sample and incubated in the dark for 30 min (RT). Thereafter, the luminescence was measured on a ModulusTM microplate luminometer (Turner Biosystems, Sunnyvale, USA). The data was expressed as relative light units (RLU).

Western blotting. Western blots were performed to analyze the protein expressions of p-ERK, p-JNK, p-p38, Bax and p-Bcl-2. Briefly, total protein was isolated using Cytobuster™ reagent (Novagen, San Diego, CA, USA). Cells were re-suspended in 200 µl Cytobuster and incubated on ice for 30 min. Following incubation, the cells were centrifuged for 10 min (180xg, 4 °C). Protein samples were quantified by the bicinchoninic acid (BCA) assay and standardized to 0.2 mg/ml (PBMCs) and 1.0 mg/ml (Thp-1). The samples were then boiled in Laemmli Sample buffer (dH_20, 0.5 M Tris-HCl (pH 6.8), glycerol, 10% SDS, β-mercaptoethanol, 1% bromophenol blue) for 5 min (100 °C). Thereafter, the samples (25 µl- Thp-1; 40 µl- PBMC) were electrophoresed in sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gels (4% stacking gel, 7.5% resolving gel) for 1.5 h at 150 V (Bio-Rad compact power supply). The separated proteins were electro-transferred onto nitrocellulose membranes for 30 min (400 mA) using the Trans-Blot Turbo Transfer system (Bio-Rad). The membranes were then blocked for 1 h (RT) with 5% bovine serum albumin (BSA) in Tris Buffer Saline with tween 20 (TTBS- NaCl, KCl, Tris, dH_2O, 0.5% tween 20, pH 7.5) or 5% Non-Fat Dry Milk (NFDM) in TTBS for phospho- and non-phospho-antibodies, respectively. Thereafter, the membranes were incubated with primary antibody [mouse anti-p-ERK (9106), mouse anti-p-JNK (9255), rabbit anti-Bax (5023), rabbit anti-p-Bcl-2 (2827), Cell Signalling, 1: 1000; mouse anti-p-p38 (M8177), β-actin (A3854), Sigma Aldrich, 1: 5000] for 1 h (RT) and then overnight at 4 °C. The membranes were washed 5 times with TTBS (10 min intervals) and incubated (RT) with horseradish peroxidase (HRP)- conjugated secondary antibody [goat anti-rabbit (ab6112), anti-mouse (ab97046), Abcam, 1: 5000] for 1 h. Once more, the membranes were washed 5 times with TTBS (10 min intervals). Protein band images were detected using Clarity Western ECL Substrate (Bio-Rad) and captured using Alliance 2.7 Image documentation system (UViTech, Cambridge, UK). The expression of protein bands was analyzed using UViBand Advanced Image Analysis software v12.14 (UViTech, Cambridge, UK). All proteins were normalized to β-actin before comparison (i.e. control vs. FA treatment. The data was expressed as relative band intensity (RBI).

Statistical analysis. Statistical analysis was performed using GraphPad Prism v5.0 software (GraphPad Software Inc., La Jolla, USA). GraphPad Prism Software was used for the unpaired t-test with Welch's correction to assess the differences between samples. Level of significance (p) was established at a $p < 0.05$.

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Author Contributions
S.D. performed all experiments and analyzed data. S.D., S.N. and A.A.C. wrote main manuscript text. A.A.C. designed the study. D.B.N. assisted in cell culture and analyzed data. All authors reviewed the manuscript.

Additional Information
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Supplementary information accompanying the manuscript published by Scientific Reports (Chapter 2)

Fusaric Acid immunotoxicity and MAPK activation in normal peripheral blood mononuclear cells and Thp-1 cells

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Supplementary information

The percentage cell viability was calculated as follows:

$$\% \text{ cell viability} = \frac{(\text{mean absorbance of treatment})}{(\text{mean absorbance of control})} \times 100$$

Supplementary tables

Table S1: Cell viability of PBMCs treated with ellipticine for 24 hours.

Ellipticine concentration (µg/ml)	Average optical density (OD) ± Standard deviation (SD)	Cell viability (%)
0	0,290 ± 0,016	-
Vehicle control (0,1% DMSO)	0,309 ± 0,004	100
0,01	0,301 ± 0,032	97,41
0,025	0,315 ± 0,034	102,05
0,05	0,314 ± 0,015	101,62
0,1	0,319 ± 0,003	103,13
0,25	0,299 ± 0,015	96,87
0,6	0,322 ± 0,000	104,21
0,8	0,310 ± 0,008	100,22

Table S2: Cell viability of Thp-1 cells treated with ellipticine for 24 hours.

Ellipticine concentration (µg/ml)	Average optical density (OD) ± Standard deviation (SD)	Cell viability (%)
0	0,258 ± 0,019	-
Vehicle control (0,1% DMSO)	0,271 ± 0,012	100
0,01	0,272 ± 0,024	100,62
0,025	0,265 ± 0,010	97,78
0,05	0,269 ± 0,006	99,51
0,1	0,290 ± 0,030	107,27
0,25	0,269 ± 0,011	99,26
0,6	0,250 ± 0,004	92,49
0,8	0,274 ± 0,010	101,23

Table S3: Cell viability of PBMCs treated with FA for 24 hours.

FA concentration (µg/ml)	Log [FA] concentration	Average optical density (OD) ± Standard deviation (SD)	Cell viability (%)
0		0,340 ± 0,048	100
30	1,477121255	0,274 ± 0,014	80,41
35	1,544068044	0,252 ± 0,037	74,05
40	1,602059991	0,250 ± 0,036	73,36
50	1,698970004	0,262 ± 0,013	76,89
10	2	0,210 ± 0,003	61,80
200	2,301029996	0,221 ± 0,009	64,94
300	2,477121255	0,218 ± 0,004	63,96

Table S4: Cell viability of Thp-1 cells treated with FA for 24 hours.

FA concentration (µg/ml)	Log [FA] concentration	Average optical density (OD)	Cell viability (%)
0		2,492 ± 0,105	100
30	1,477121255	2,1223 ± 0,117	85,19
35	1,544068044	2,082 ± 0,192	83,57
40	1,602059991	2,099 ± 0,059	84,23
50	1,698970004	2,010 ± 0,205	80,68
10	2	1,294 ± 0,165	51,93
200	2,301029996	0,140 ± 0,010	5,63
300	2,477121255	0,120 ± 0,006	4,83

Supplementary figures

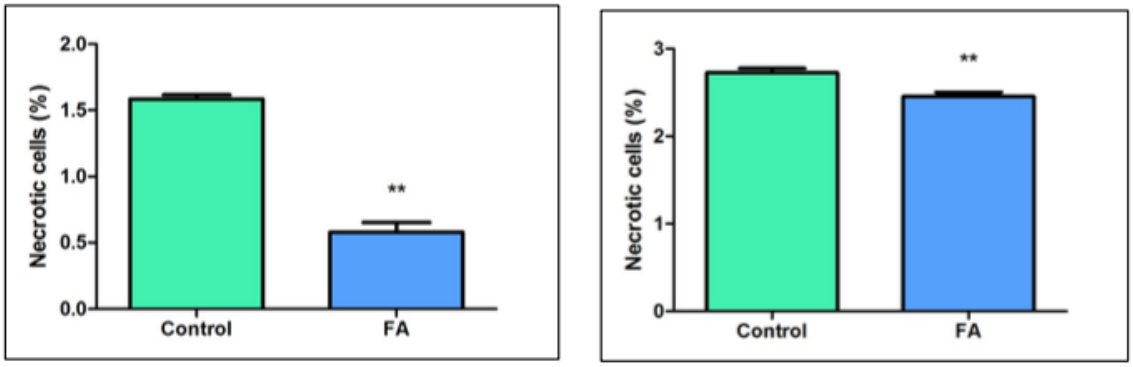


Figure S1: Effect of FA on necrotic cell death in PBMCs (left) and Thp-1 cells (right). **p<0,005.

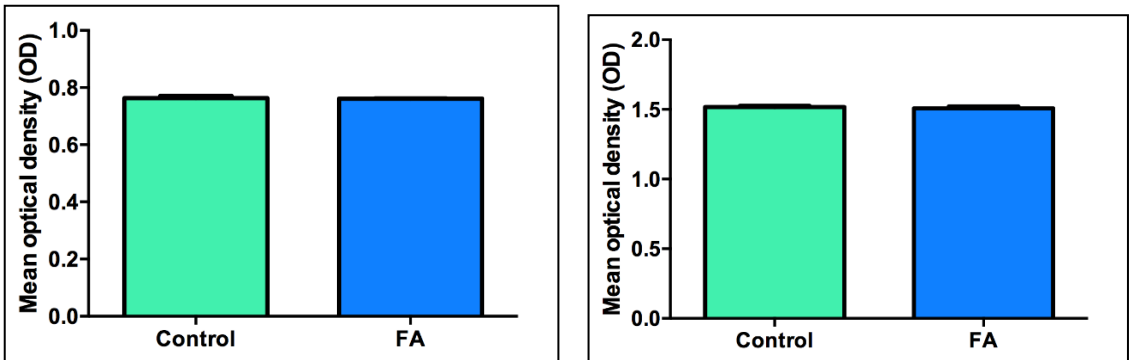


Figure S2: Quantification of LDH in PBMCs (left) and Thp-1 cells (right) treated with FA for 24 hours.

APPENDIX B

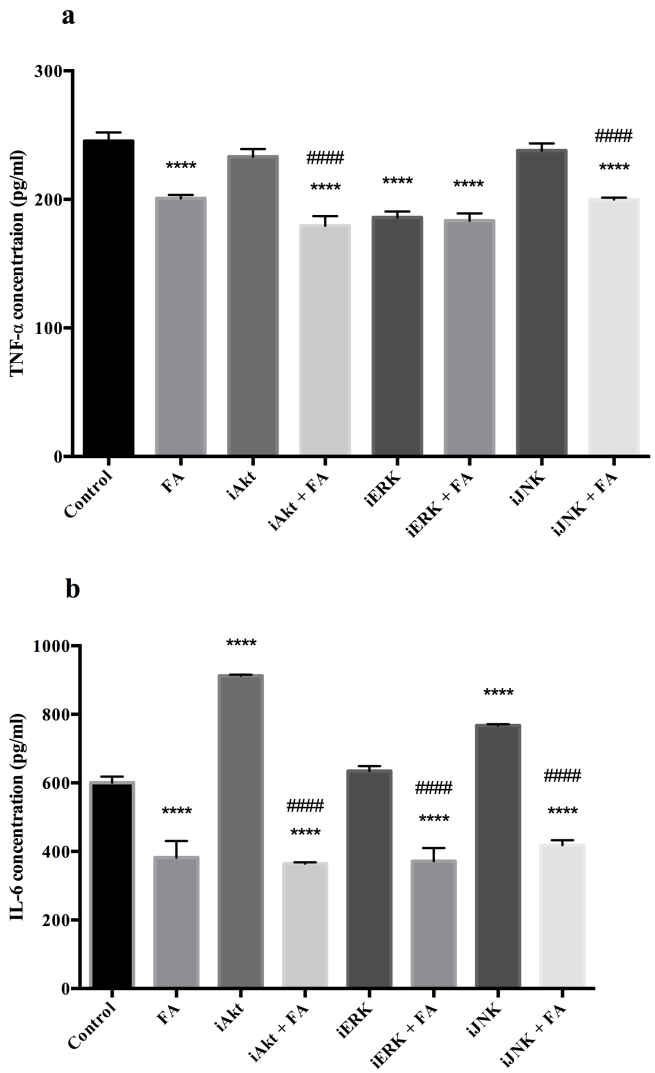
Involvement of MAPK and Akt signalling proteins on the release of inflammatory cytokines in healthy human PBMCs following acute (1 day) FA exposure

Kinase inhibition

Following density separation of whole blood, isolated PBMCs (2×10^5 cells/ml) were maintained at 37 °C with 5% CO_2 in RPMI 1640 medium (supplemented with 10% FCS, 1% L-glutamine and 1% pencillin-streptomycin). The PBMCs were treated for 1 hr with the respective kinase inhibitors [Akt (LY294002; 20 μM), ERK (PD98059; 20 μM), JNK (SP600125; 20 μM), p38 (SB203580; 10 μM)] before incubation with FA (240.8 μg/ml) for 24 hrs. Following treatment with FA, the supernatants were aspirated and used for cytokine detection, whilst the cell homogenate was used for the luminometric detection of caspas-1 activity.

Cytokine detection

To evaluate the effect of FA on the inflammatory response in lymphoid cells, pro- [interleikin-6 (IL-6), IL-1ß and tumour necrosis factor-α (TNF-α) and anti-inflammatory [interleukin-10 (IL-10)] cytokine levels were determined by enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences). Briefly, microwells (96-well plate) were coated with 100 μl/well capture antibody and incubated overnight at 4°C. Thereafter, the plate was washed three times with 300 μl/well wash buffer (1X) and blocked with 200 μl/well assay diluent (1 hr, RT). Subsequently, the plate was once more (three times with 300 μl/well wash buffer) before incubation with 100 μl/well of each cytokine standard (eight standards of the respective cytokine were prepared as per manufactures instructions) and sample (2 hrs, RT). Afterwards, the plate was washed five times with wash buffer (300 μl/well) and incubated with 100 μl/well of working detector (prepared as per manufactures instructions) for 1 hr (RT). Following incubation, the plate was wash seven times with 300 μl/well of wash buffer and incubated with 100 μl/well of substrate solution for 30 min in the dark (RT). Thereafter, 50 μl/well of stop solution was added to each well. The optical density was measured on a spectrophotometer (Bio Tek uQuant) at 450nm with reference wavelength of 570nm. The obtained data was assessed using one-way ANOVA (Bonferroni's multiple comparison test) with GraphPad prism v6 software.


a
TNF-α concentrtaion (pg/ml)
300
200
100
0
****
####
****
****
****
####
****
Control
FA
iAkt
iAkt + FA
iERK
iERK + FA
iJNK
iJNK + FA
b
IL-6 concentration (pg/ml)
1000
800
600
400
200
0
****
****
****
####
****
####
****
####
****
Control
FA
iAkt
iAkt + FA
iERK
iERK + FA
iJNK
iJNK + FA

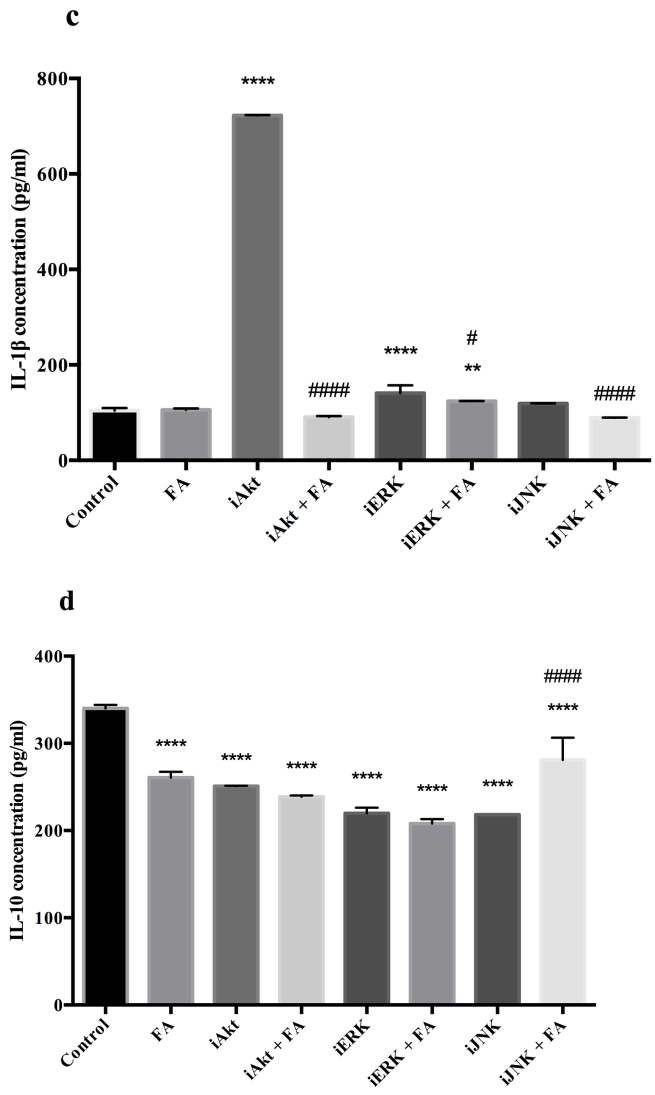


Figure 1: Effect of FA on MAPK (ERK and JNK) and Akt signalling on cytokine production in healthy human PBMCs following a 24-hr exposure. FA decreased pro- [TNF-α **(a)**, IL-6 **(b)** and IL-1ß **(c)**] and anti-inflammatory [IL-10 **(d)**] cytokines independent of MAPK (ERK and JNK) and Akt proteins when compared to healthy control PBMCs. The data are represented as mean ± SD. ** $p < 0.005$, **** $p < 0.0001$ (FA relative to control); # $p < 0.05$, #### $p < 0.0001$ [i(MAPK/Akt) + FA relative to the respective i(MAPK/Akt)]

Caspase-1 activity

Caspase-1 is a cysteine protease that converts the inactive pro-form of IL-1β to the active inflammatory cytokine and hence mediates the effects of IL-1β. Additionally, caspase-1 activation induces pyroptosis, an immunogenic form of cell death. Therefore, caspase-1 activity was assessed by

the Caspase-Glo® 1 Inflammasome Assay kit (Promega, Madison, USA; as per manufactures instruction). Briefly, 2×10^4 cells/well were seeded in a 96-well opaque polystyrene microtitre plate (in triplicate) and incubated in the dark with 20µl of the reagent (30 min, RT). The luminescent signal was detected on a Modulus^{TM} microplate luminometer (Turner Biosystems, Sunnyvale, USA). The obtained data was assessed using one-way ANOVA (Bonferroni's multiple comparison test) with GraphPad prism v6 software were expressed as relative light units (RLU).

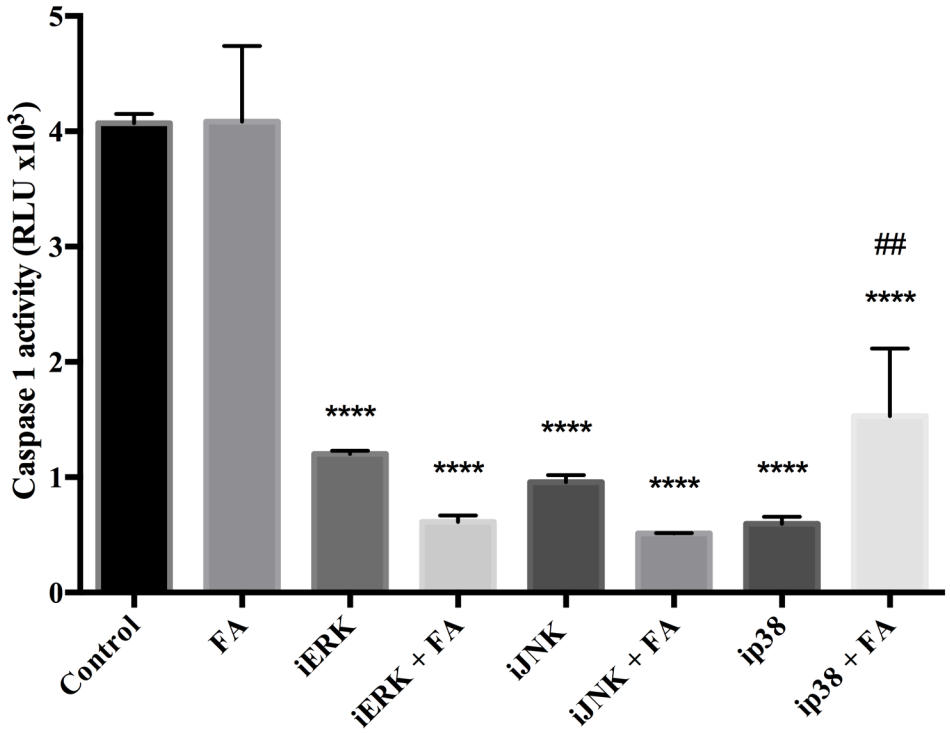


Figure 2: Effect of FA on MAPK-mediated caspase-1 activity in healthy human PBMCs following a 24-hr exposure. FA decreased caspase-1 activity in healthy control PBMCs. However, caspase-1 activity was increased upon p38 inhibition, when compared to ip38 control. The data are represented as mean $\pm$ SD. **** $p < 0.0001$ (FA relative to control); ## $p < 0.005$ [i(MAPK) + FA relative to the respective i(MAPK)]

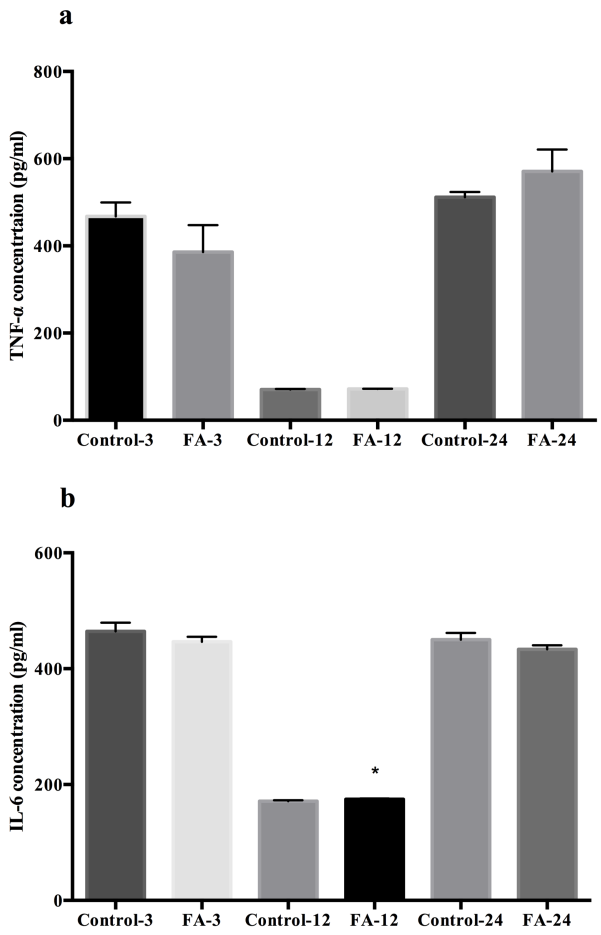
APPENDIX C

Effect of FA on the inflammatory cytokine profile in human monocytes (Thp-1) and macrophages

Monocytes and macrophages are vital components of the innate immune system with distinct roles in tissue homeostasis and immunity. Monocytes are central players during inflammation and pathogen challenge; whereas, macrophages (monocytes differentiate into macrophages and dendritic cells) are important in development, tissue homeostasis, regeneration and inflammation. Because the innate immune response is rapid (becomes active immediately-hours), the effect of FA on monocytes and monocytic-differentiated macrophages were assessed at 3-, 12- and 24-hr intervals.

Protocol for differentiating Thp-1 cells into macrophages

Briefly, Thp-1 cells (3×10^6 cells/ml) were maintained in RPMI 1640 medium supplemented with 10% FCS, 1% L-glutamine, 1% pencillin-streptomycin, 1 mM sodium pyruvate and 0.05 mM β-mecaptoethanol, and were differentiated into macrophages by incubation (4 days) with 200 nM phorbol 12-myristiate-12 acetate (PMA, Sigma-Aldrich) in 25 cm^3 ventilated flasks (37 °C, 5% CO_2). Following incubation, the PMA was aspirated and the cells were washed once with PBS to remove undifferentiated cells. The differentiated macrophages were incubated (24 hrs) with RPMI to reach M0 polarization. Thereafter, macrophage M1 polarization (pro-inflammatory macrophages) was obtained by incubation with lipopolysaccharide (LPS, 100 ng/ml) for 24 hrs; after which the macrophages were treated with FA (107.7 µg/ml) at varying time periods (3-, 12- and 24 hrs).


a
800
600
400
200
0
TNF-α concentrtaion (pg/ml)
Control-3
FA-3
Control-12
FA-12
Control-24
FA-24
b
600
400
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IL-6 concentration (pg/ml)
*
Control-3
FA-3
Control-12
FA-12
Control-24
FA-24

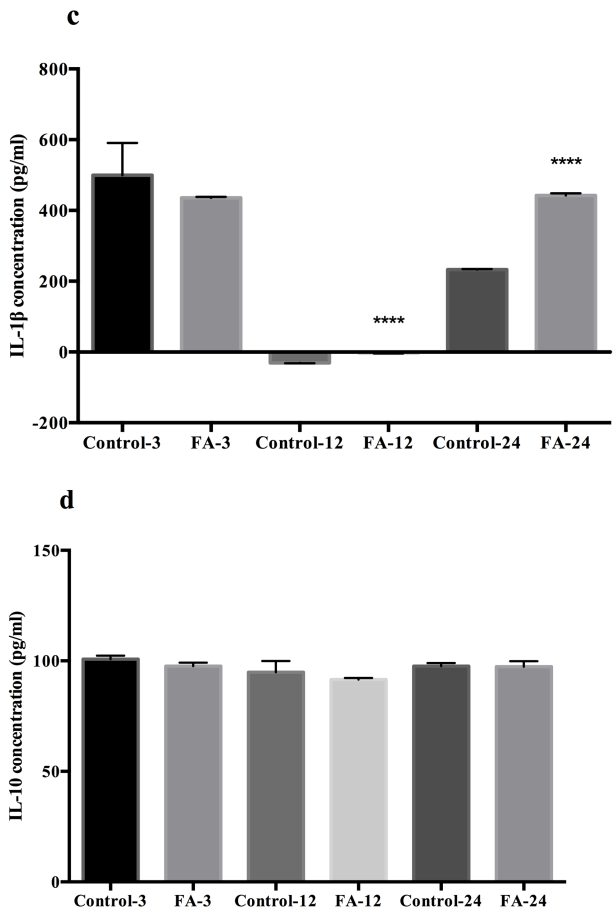
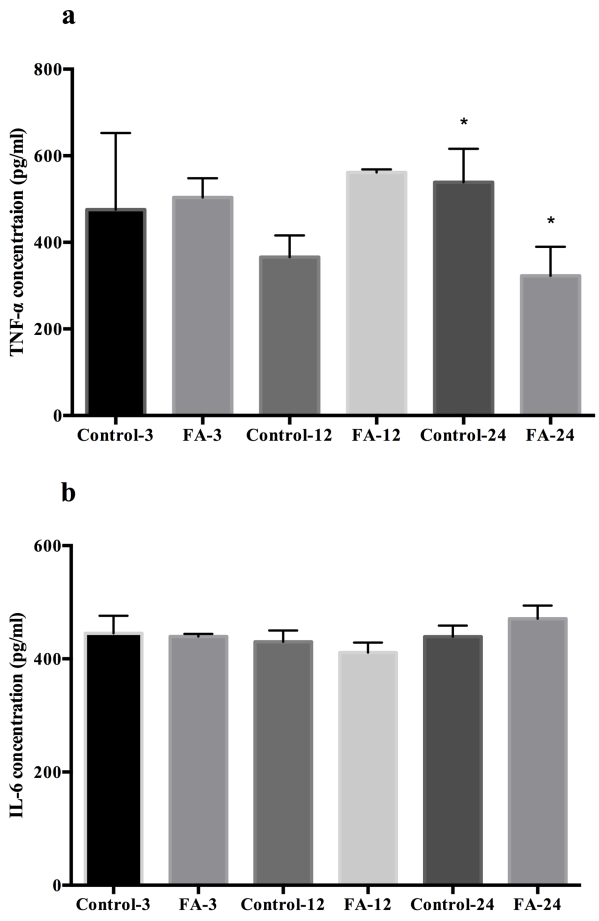


Figure 1: Effect of FA on inflammatory cytokine levels in human monocytes at 3-, 12-, and 24 hrs. FA had little effect on the release of pro- [TNF-α **(a)** and IL-6 **(b)**] and anti-inflammatory [IL-10 **(d)**] cytokines at 3- 12- and 24 hrs relative to each control. However, at 12- and 24 hrs, FA significantly increased IL-1ß **(c)** levels in Thp-1 cells. The data are represented as mean $\pm$ SD. * $p <$ 0.05, **** $p < 0.0001$ (FA relative to control for the respective treatment period)


a
800
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TNF-a concentrtaion (pg/ml)
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*
Control-3
FA-3
Control-12
FA-12
Control-24
FA-24
b
600
400
200
0
IL-6 concentration (pg/ml)
Control-3
FA-3
Control-12
FA-12
Control-24
FA-24

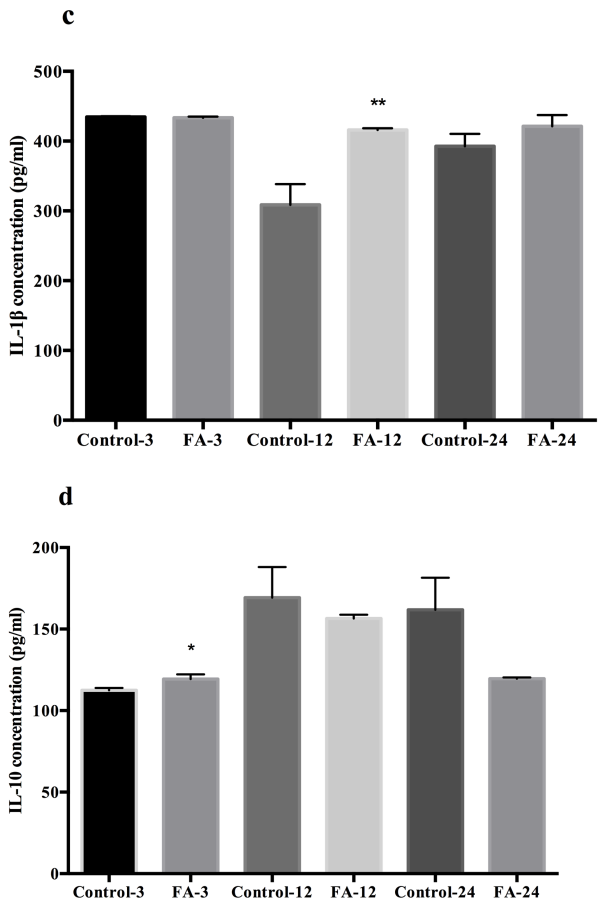

Figure 2: Effect of FA on inflammatory cytokine levels in human macrophages at 3-, 12-, and 24 hrs. FA decreased TNF-α **(a)** levels at 24 hrs despite increases at 3- and 12 hr periods. While FA had no effect of IL-6 levels **(b)**, IL-1ß **(c)** were significantly increased at 12 hrs. Whilst anti-inflammatory, IL-10 **(d)** levels were elevated at 3 hrs and subsequently decreased (12- and 24 hrs) in the pro-inflammatory macrophages primed cells. The data are represented as mean ± SD. * $p < 0.05$, ** $p < 0.005$ (FA relative to control for the respective treatment period)

APPENDIX D

Supplementary information submitted with the manuscript "Differential regulation of CREB/BDNF signalling by Fusaric acid in C57BL/6 mice brain tissue" (Chapter 4)

Differential regulation of CREB/BDNF signalling by Fusaric acid in C57BL/6 mice brain tissue

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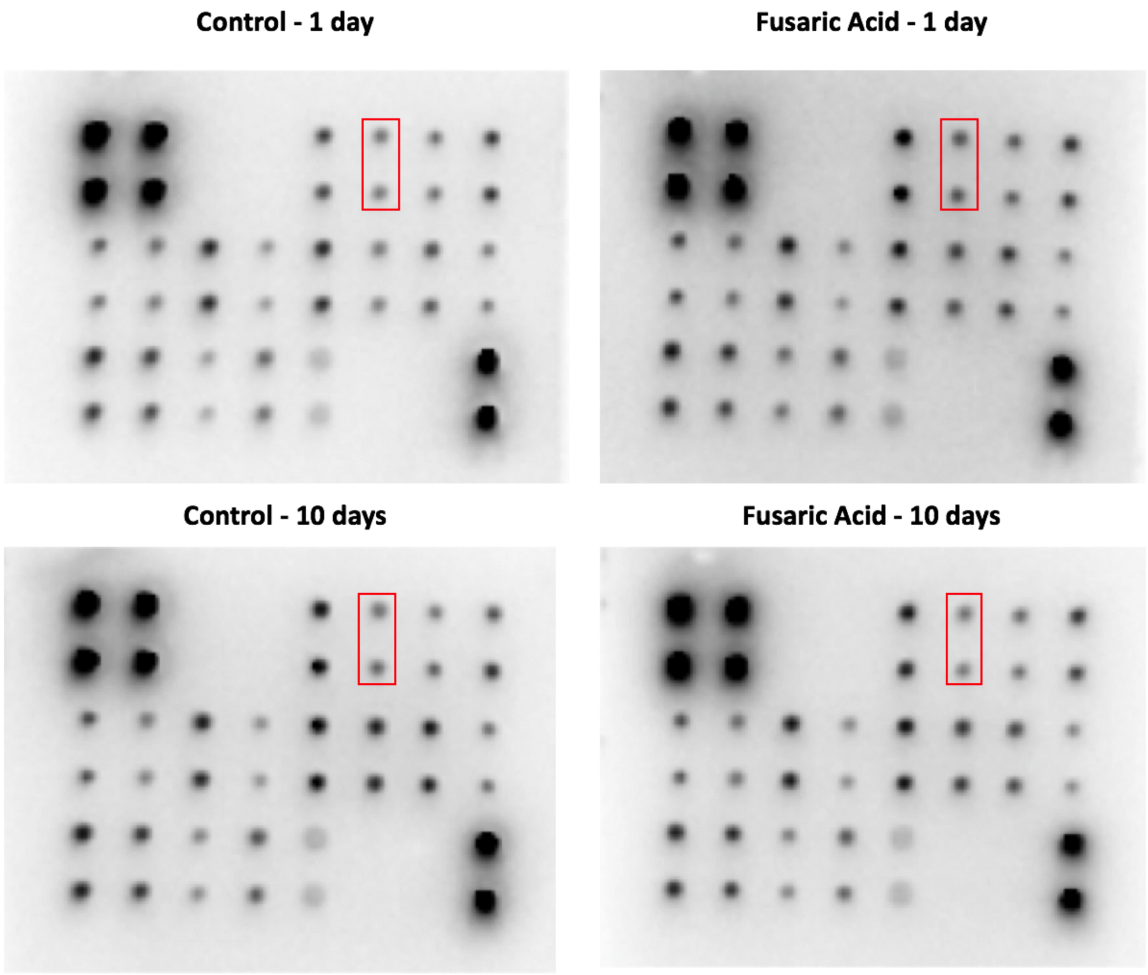
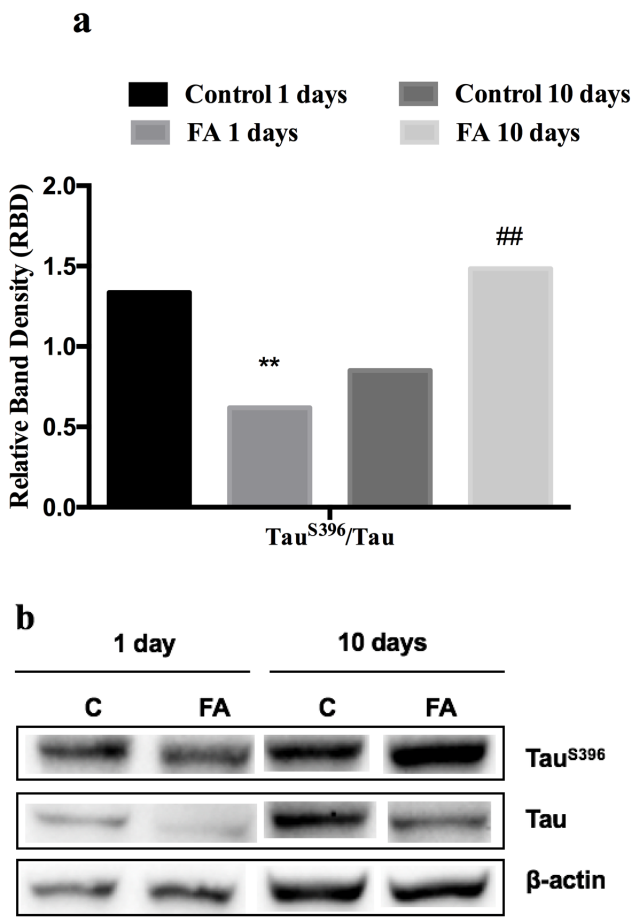


Fig. S1: Effect of FA exposure on the phosphorylation of CREB. Representative densiometric expression of p-CREB^{S133} **(1)** phosphorylation using a MAPK protein phosphorylation microarray.

APPENDIX E

Effect of FA on Tau in mice brain tissue following acute (1 day) and prolonged (10 days) exposures

Tau, a microtubule-associated protein, is important for microtubule stabilization, neurite outgrowth, and axonal transport. Microtubules are involved in the maintenance of neuronal morphology and the formation of axonal and dendritic processes. Tau's affinity for microtubules is strongly affected by its phosphorylation status. The kinases that phosphorylate tau include GSK-3α and β, cyclin dependent kinase 5 (cdk5), protein kinase A (PKA), MAPK and the microtubule affinity regulating kinase (MARK) family. In particular, GSK3-β is a prominent tau kinase and directly phosphorylates tau at its serine-396 (S396) residue, inhibiting tau's ability to stabilise microtubules and leading to the polymerization of tau. In addition, unbound tau may be hyperphosphorylated by other kinases- an early event in neurodegenerative diseases. Furthermore, several studies suggest that impaired glucose metabolism contributes to tau hyperphosphorylation. As a result of decreased ATP availability and altered metabolic signalling (Chapter 2), the protein and transcript expressions of tau were evaluated.



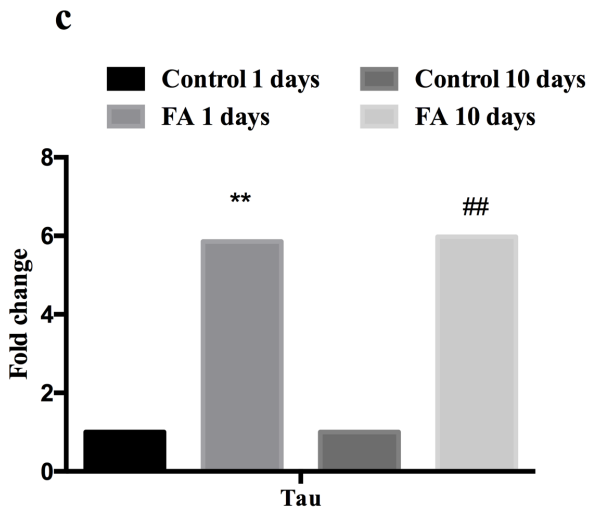


Figure 1: Tau levels in mice brain tissue. Densiometric protein analysis **(a and b)** and transcript levels **(c)** of Tau were measured by western blots and qPCR, respectively. While tau transcript levels were significantly elevated at both acute and prolonged exposures **(c)**, post-translational phosphorylation at its S396 residue was decreased at 1 day and increased following prolonged FA treatment **(a and b)**. The phosphorylation of tau was consistent with GSK3 activity (Chapter 2). Expression of β-actin was used as an internal control for western blots. The data are represented as mean + SD. ** $p < 0.005$ (FA relative to control 1 day); ## $p < 0.005$ (FA relative to control 10 days)

Effect of FA on MAPK phosphorylated-activation sites in mice brain tissue following acute (1 day) and prolonged (10 days) exposures

To assess the effect of FA on the phosphorylation of the active sites on MAPK proteins and substrate targets in brain tissue, a commercial MAPK protein phosphorylation array was used as per manufactures instructions.

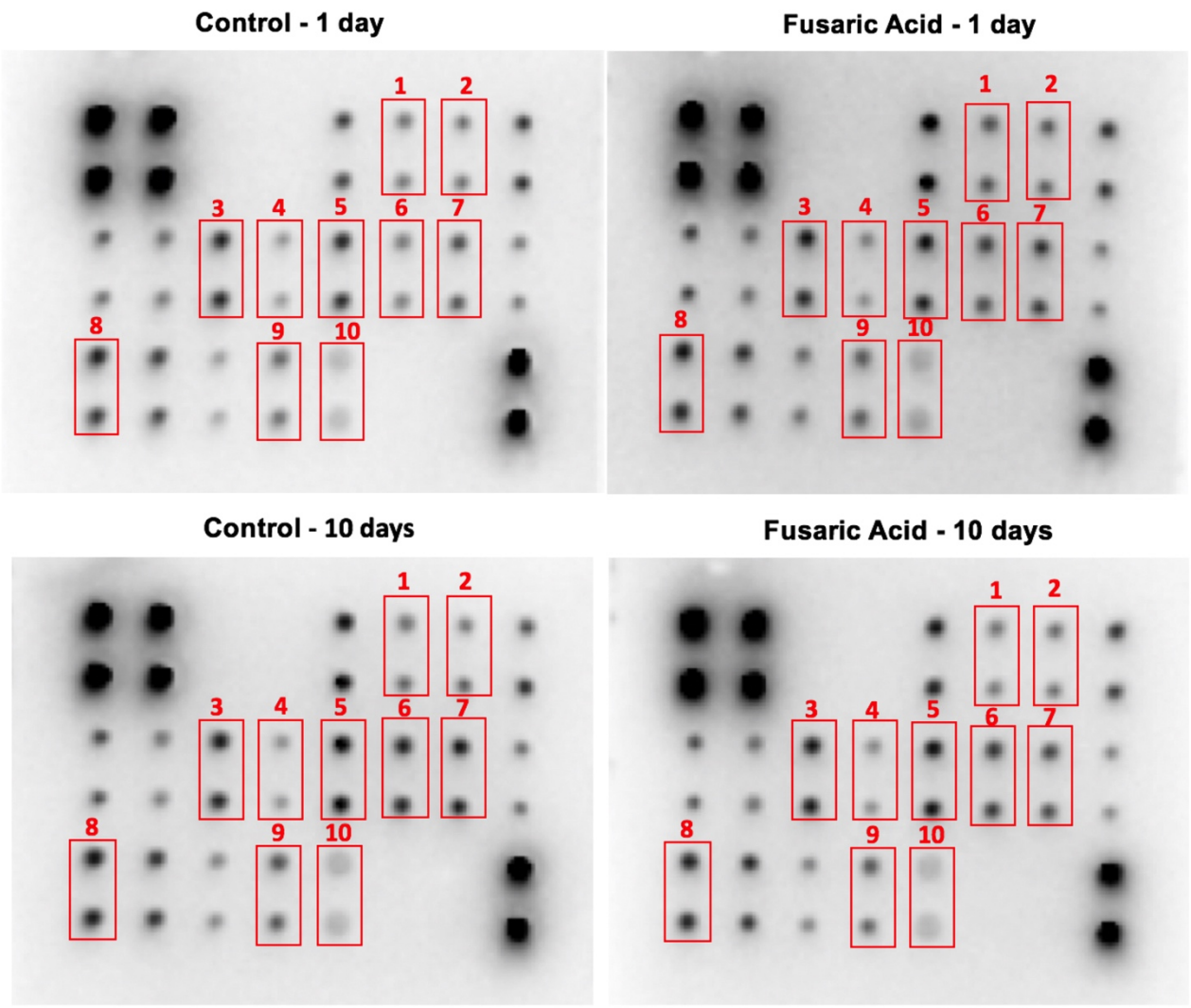


Figure 2: Effect of FA exposure on the phosphorylation of MAPK signalling proteins.
Representative densiometric expression of MAPK phosphorylated proteins using a MAPK protein phosphorylation microarray for p-CREB^{S133} **(1)**, p-ERK1$^{T202/Y204}$/p-ERK2$^{Y185/Y187}$ **(2)**, p-JNK^{T183} **(3)**, p-MEK$^{S217/221}$ **(4)**, p-MKK3^{S189} **(5)**, p-MKK6^{S207} **(6)**, p-MSK2^{S360} **(7)**, p-p38$^{T180/Y182}$ **(8)**, p-RSK1^{S380} **(9)** and p-RSK2^{S386} **(10)**

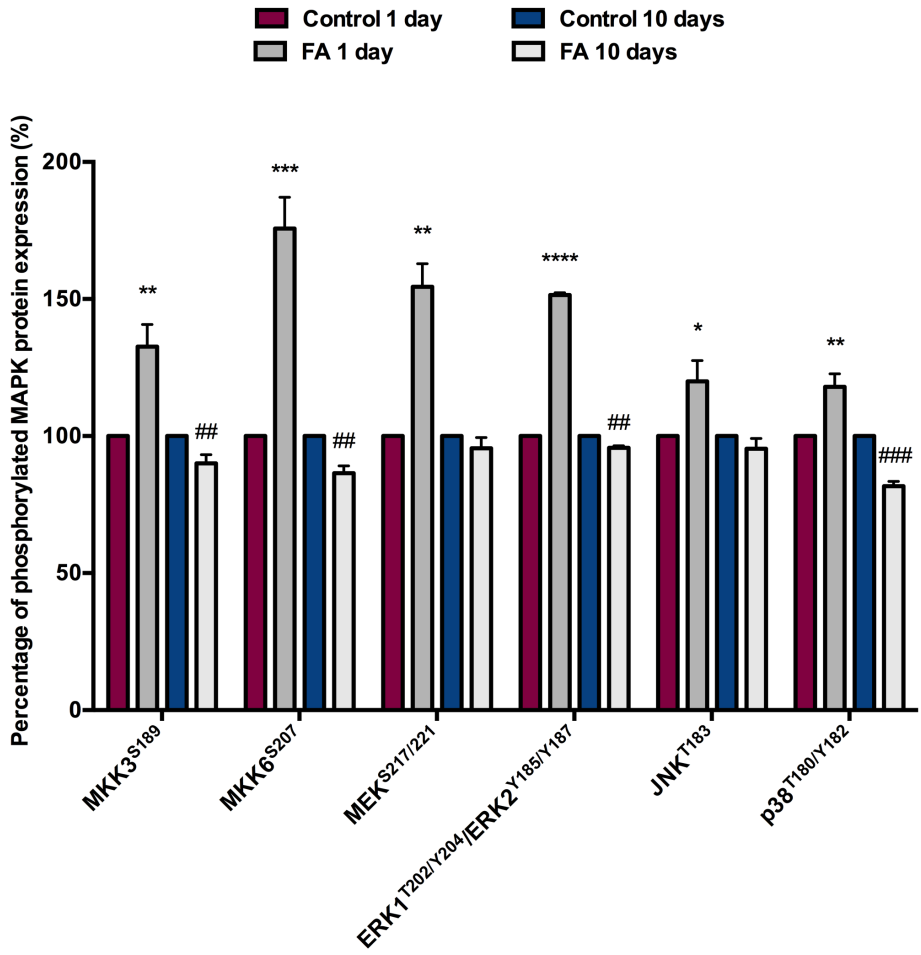


Figure 3: Effect of FA exposure on phosphorylation MAPK signalling proteins in mice brain tissue. Densitometric protein measurements of phosphorylated MAPK signalling proteins were determined using a MAPK phosphorylation protein array for MAPKKs and MAPK expressions. The data are represented as mean ± SD. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$ (FA relative to control 1 day); ## $p < 0.005$, ### $p < 0.0005$ (FA relative to control 10 days)

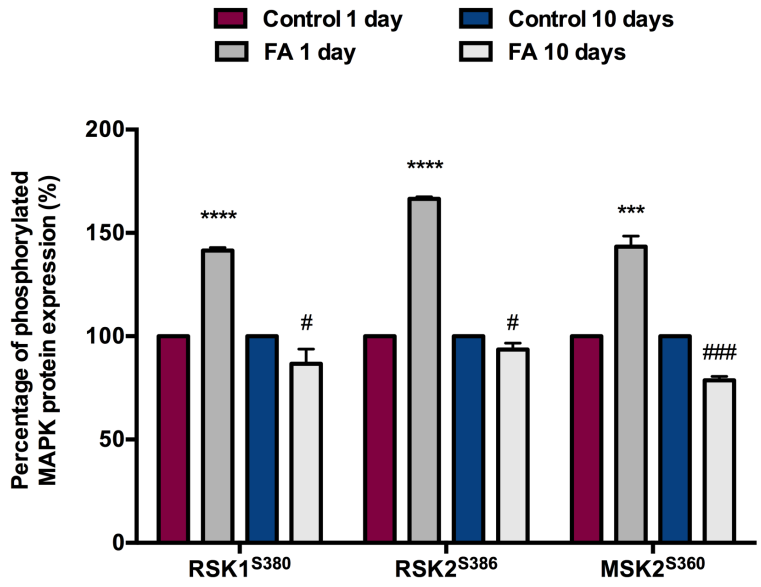


Figure 4: Effect of FA on the phosphorylation of MAPK substrates in mice brain tissue. Densitometric protein quantification of RSK1, RSK2 and MSK2 were analysed using a MAPK protein phosphorylation array. The data are represented as mean ± SD. *** $p < 0.0005$ **** $p < 0.0001$ (FA relative to control 1 day); # $p < 0.05$, ### $p < 0.0005$ (FA relative to control 10 days)

Effect of FA on miR-132 in mice brain tissue following acute (1 day) and prolonged (10 days) exposures

MicroRNA (miR)-132 is an endogenous small RNA responsible for the post-transcriptional regulation of gene expression via controlled degradation of mRNA or transcription inhibition. In the nervous system, miR-132 is significant for regulating neuronal differentiation, maturation and functioning, and widely participates in axon growth, neural migration, and plasticity. It has been shown that the expression of miR-132 is induced by BDNF in an ERK1/2-MSK1/2-CREB-dependent manner in neurons and may represent a mechanism of fine-tuning protein expressions for neuronal function and survival. Therefore, the expression of miR-132 was assessed.

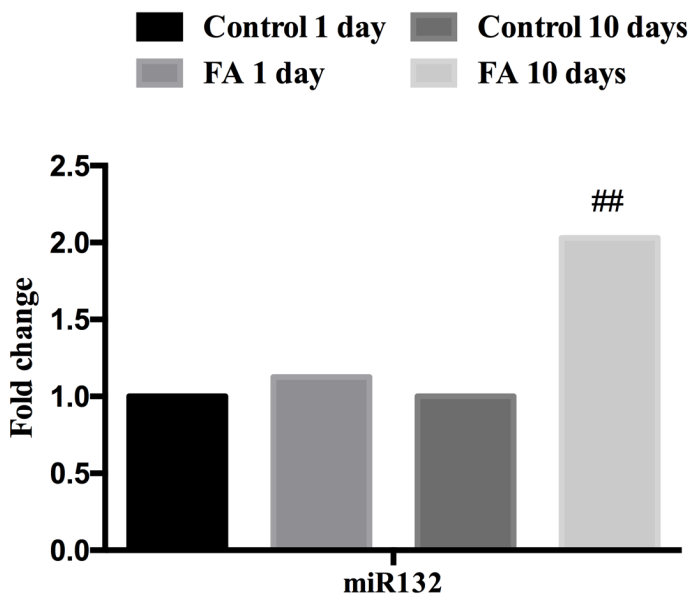


Figure 5: Effect of FA on miR-132 expression in mice brain tissue. Despite elevated CREB and BDNF levels at 1 day (Chapter 3), miR-132 expression was only slightly elevated in brain tissue. On the contrary, miR-132 expression was significantly elevated impartial of the decreased CREB and BDNF expressions (Chapter 3) at a prolonged exposure to FA. The data are represented as mean + SD. ## $p < 0.005$ (FA relative to control 10 days)

Effect of FA on c-myc expression in mice brain tissue following acute (1 day) and prolonged (10 days) exposures

c-Myc is an important oncoprotein that has been extensively studied for its role in proliferation and growth of normal and neoplastic cells. In addition, c-myc cooperates with additional transcriptional factors (such as p53 and CREB) at target promoters to regulate gene expression. In the brain, c-myc is essential for normal brain development. More recently, inhibition of c-myc was shown to be neuroprotective whereby elevated c-myc levels rendered neurons vulnerable to cell death. Therefore, transcript levels of c-myc were quantified.

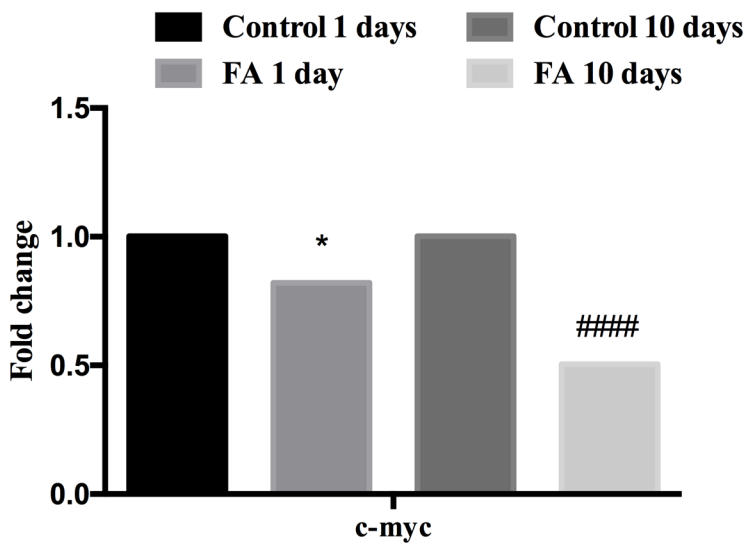


Figure 6: Effect of FA on c-myc transcript levels in mice brain tissue. In keeping with the lack of tissue degeneration observed in Chapter 3, transcript levels of c-myc were significantly reduced at both acute and prolonged exposures to FA. The data are represented as mean + SD. * $p < 0.05$ (FA relative to control 1 day); #### $p < 0.0001$ (FA relative to control 10 days)