Mechanisms Underlying the Neuroprotective Effects of Histone Deacetylase Inhibitors

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Submitted in accordance with the requirements for the degree of
Doctor of Philosophy

The University of Leeds
School of Biomedical Sciences
Faculty of Biological Sciences

September 2015

The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

Acknowledgments

I extend my thanks to my supervisor Dr Ian C Wood for giving me the opportunity to study for a PhD in his laboratory and for his support and excellent advice throughout. I would like to acknowledge Abdulelah Alshawli who joined the Wood lab last year. His arrival brought new perspectives and companionship. It has been a joy to work alongside him and I wish him every success in his PhD.

Ewa Jaworska, is a wonderful person and I would like to thank her for the support and friendship she provided. I would also like to thank Rachel Dods and Dr Clare Wilson who throughout these testing times have been good and supporting friends. Both are gifted scientists and I wish them the very best in their scientific careers.

Most importantly, without the constant support from my Mum, Dad and Sister, I wouldn't have been able to undertake this PhD, so this thesis is dedicated to them.

This PhD was funded by the Biotechnology and Biological Sciences Research Council (BBSRC).

Abstract

Histone deacetylase (HDAC) inhibitors prevent neuronal death in *in vivo* models of cerebral ischaemia, brain injuries and neurodegenerative disease. However the molecular mechanisms underlying this are not fully understood and neither the cell type nor the specific HDAC isoform responsible is known. To address these questions, I investigated the effects of selectively inhibiting HDAC isoforms in different neural cell models of the pathophysiological mechanisms implicated in these brain conditions. Initially, the efficacy of HDAC inhibitors to protect cerebellar granule neurones from glutamate excitotoxicity and oxygen glucose deprivation (OGD) was examined. It was found that inhibiting HDACs did not protect isolated neurones from these insults. To determine if HDAC inhibitors are neuroprotective through effects in other neural cells, I investigated if inhibiting HDACs in BV2 microglia could suppress the inflammatory response stimulated by lipopolysaccharide (LPS). Using this model, the data presented in this thesis shows for the first time that selective inhibition of class I HDAC isoforms, or knockdown of HDAC1 or HDAC2 in microglia, suppresses the expression of pro-inflammatory mediators interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α). Investigating the possible underlying mechanisms suggests an increase in protein expression is not important and HDAC inhibitors have an anti-inflammatory effect by increasing the acetylation state of pre-existing proteins. The data presented also suggests there is functional redundancy of HDAC1 & HDAC2 in regulating the inflammatory response. Therefore, selectively inhibiting either isoform may be a strategy to reduce neuroinflammation and by doing so protect neurones in cerebral ischaemia and other brain conditions, whilst minimising the side effects associated with pan-HDAC inhibition.

Contents

List of Tables

1. General Introduction

2. Experimental Procedures

4. Results II

List of Figures

1. General Introduction

3. Results I

4. Results II

Abbreviations

4-VO	Four-vessel occlusion
aa	Amino acid
AAV	Adeno-associated virus
Aβ	Amyloid beta
Ac	Acetylated
ac-DEVD-al	N-acetyl-Asp-Glu-Val-Asp-al
AcK	Acetylated lysine
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
APP	Amyloid precursor protein
APS	Ammonium persulfate
ARAC	Cytosine β-D-arabinofuranoside hydrochloride
ATP	Adenosine triphosphate
BAY11	BAY11-7082
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
BDNF	Brain derived neurotrophic factor
bp	Base pairs
BSA	Bovine serum albumin
CaMKIIα	Ca^{2+}/calmodulin-dependent protein kinase II alpha
CBP	CREB-binding protein
Cdk5	Cyclin-dependent kinase 5
cDNA	complementary DNA
CGN	Cerebellar granule neurone
CHX	Cycloheximide
CNS	Central nervous system
CoA	Coenzyme A
CoREST	Co-repressor for element-1-silencing transcription factor
COX-2	Cyclooxygenase-2
CPLM	Compendium of protein lysine modifications
CREB	cAMP response element binding-protein

CY3	Cyanine 3
DAPI	4', 6-diamidino-2-phenylindole
DI	De-ionized
DIV	Days *in vitro*
DL-TBOA	DL-*threo*-β-Benzyloxyaspartic acid
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
DPI	Dots per inch
DTT	Dithiothreitol
EAAT	Excitatory amino acid transporter
EAE	Experimental autoimmune encephalomyelitis
EAN	Experimental autoimmune neuritis
EBSS	Earle's balanced salt solution
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
Egr#	Early growth response protein #
ELISA	Enzyme linked immunosorbent assay
ELM2	Egg-laying defective protein 27 and metastasis-associated protein 1 homology 2
FBS	Foetal bovine serum
FDA	United States Food & Drug Administration
FUS	Fused-in-Sarcoma
GABA	γ-Aminobutyric acid
Gadd45α	Growth arrest and DNA damage-inducible 45 alpha
Gcn5	General control nonrepressed 5
GFAP	Glial fibrillary acidic protein
GNAT	GCn5 *N*-acetyltransferase
GSK3β	Glycogen synthase kinase 3 beta
HAT	Histone acetyltransferase
HBSS	Hanks balanced salt solution
HDA1	Yeast histone deacetylase 1

HDAC	Histone deacetylase
HDAC#KD	HDAC# knockdown
HDAC#KO	HDAC# knockout
HDAC#OE	HDAC# overexpression
HEK	Human embryonic kidney
HK	High potassium
HMG-1	High-mobility group protein 1
HRP	Horseradish peroxidase
HSP70	Heat-shock protein 70
IC_{50}	Half maximal inhibitory concentration
IFNβ	Interferon beta
IFNγ	Interferon gamma
IκBα	Nuclear factor kappa-light-chain-enhancer of activated B-cells inhibitor alpha
IL-10	Interleukin-10
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
K	Lysine
K_i	Inhibition constant
LK	Low potassium
LPS	Lipopolysaccharide
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MCAO	Middle cerebral artery occlusion
MCF-7	Michigan cancer foundation-7
Mdm2	Mouse double minute 2 homolog
MEF-2	Myocyte enhancer-binding factor-2
miRNA	microRNA
MKP-1	Mitogen-activated protein kinase phosphatase 1
MMP-9	Matrix metalloproteinase-9
Morf	Monocytic leukemia zinc finger protein-related factor
MPP+	1-methyl-4-phenylpyridinium

MPTP+	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger RNA
MTA#	Metastasis-associated protein #
MTT	Metylthiazolyldiphenyl-tetrazolium bromide
MW	Molecular weight
NA	Nucleic acid
NAD^+	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCoR	Nuclear receptor co-repressor
NCX	Na^+/Ca^{2+} exchanger
NES	Nuclear export signal
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
$NH3^+$	Amino group
NLS	Nuclear localisation signal
NMDA	*N*-Methyl-D-aspartic acid
NO	Nitric oxide
Nrf2	Nuclear factor-like 2
NuRD	Nucleosome remodelling and deacetylation
OGD	Oxygen & glucose deprivation
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCAF	p300/CBP-associated factor
PCR	Polymerase chain reaction
PDC	L-*trans*-Pyrrolidine-2,4-dicarboxylic acid
PFA	Paraformaldehyde
PMSF	Phenylmethylsulfonyl fluoride
PPARγ	Peroxisome proliferator-activated receptor gamma
PS1/PSEN1	Presenilin 1
PTM	Post-translational modification
PUMA	p53 upregulated modulator of apoptosis
PVDF	Polyvinylidene fluoride membrane
qPCR	quantitative PCR
REST	Repressor element-1 silencing transcription factor

RIPA	Radioimmunoprecipitation
RNA	Ribonucleic acid
RNase	Ribonuclease
RPD3	Reduced potassium dependency 3
SAHA	Suberoylanilide hydroxamic acid
Sas2	Something about silencing protein 2
SB	Sodium butyrate
Scr	Scrambled
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
shRNA	short hair-pin RNA
Sin3	Switch independent 3
siRNA	small interfering RNA
SIRT#	Sirtuin #
SMRT	Silencing mediator of retinoid and thyroid receptors
Sp1	Specificity protein 1
SPB	Sodium phenylbutyrate
STAT1	Signal transducer and activator of transcription 1
TAF(II)250	TATA box-binding protein associated factor 250
TBI	Traumatic brain injury
TBS	Tris buffered saline
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor-beta
Tip60	60 kDa Tat-interactive protein
TNF-α	Tumour necrosis factor-alpha
TSA	Trichostatin A
TSS	Transcription start site
VPA	Valproate
XPO1	Exportin 1 receptor

Chapter 1

General Introduction

1.1. Preface

This thesis is an investigation into the mechanisms underlying the neuroprotection observed when using histone deacetylase (HDAC) inhibitors in experimental models of brain insults, injuries and disease. There is an emphasis on their effects on the pathophysiological mechanisms involved in cerebral ischaemia. However, it is important to point out that some of these mechanisms are also involved in traumatic brain injury, Alzheimer's disease, Parkinson's disease, multiple sclerosis and motor neurone disease, and HDAC inhibitors are neuroprotective in *in vivo* models of these conditions as well.

In the general introduction, I will introduce lysine acetylation a protein post-translational modification regulated by HDACs. This will move on to a specific discussion of the function of protein acetylation and HDACs in the brain. Then, the pathophysiological components of cerebral ischaemia will be briefly examined, followed by an analysis of the current literature that demonstrates HDAC inhibitors, in appropriate experimental models of this and other brain conditions, are neuroprotective, neuro-restorative and improve neurological outcome.

1.2. Protein (lysine) acetylation

Post-translational modification (PTM) of proteins is a major regulatory mechanism of protein function through changing functional activity, subcellular localisation, interactions with other molecules and protein stability. Lysine acetylation is a reversible PTM and from a historical perspective was originally discovered as a modification to histone proteins (Phillips, 1963; Allfrey et al., 1964). Since this discovery, many other proteins have been identified, which are modified in this way. Early studies revealed that proteins such as α-tubulin (L'Hernault and Rosenbaum, 1985), p53 (Gu and Roeder, 1997) and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B-cells, Chen et al. (2001); Kiernan et al. (2003)) are regulated by acetylation (see reviews by Glozak et al. (2005); Spange et al. (2009); Yao and Yang (2011) for these and other examples). As technology has advanced, large-scale proteomic studies have expanded the library of known acetylated targets. Choudhary et al. (2009) have identified 1750 acetylated proteins in human cells and Zhao et al. (2010) have shown there are 1042 acetylated proteins in the human liver. The analysis of sixteen different rat tissues revealed a combined total of 4541 uniquely acetylated proteins (Lundby et al., 2012). On a similar scale, the Compendium of Protein Lysine Modifications (CPLM, Liu et al. (2014)) now reports a total of 4817 and 4024 acetylated proteins in humans and mice respectively. Combined, these acetylated proteins make-up the "acetylome" (Smith and Workman, 2009; Choudhary et al., 2014) a collection of proteins that come from many; protein classes, subcellular compartments and are involved in many cellular processes. The size and diversity of the acetylome suggests acetylation is a PTM of great importance. Having now identified thousands of proteins that make-up the acetylome, much work needs to be done in order to understand the precise role acetylation and deacetylation of each protein, has on the processes and pathways they are involved in and how this affects normal cell physiology and how this is affected in injury and disease.

Fundamentally, the acetylation of a lysine residue within a protein involves the transfer of an acetyl moiety from acetyl coenzyme A to the amino group ($NH3^+$) of a lysine residue. Conversely, deacetylation of a protein involves the removal of an acetyl moiety from an acetylated lysine. This process is controlled by the activity of two enzymes, histone acetyltransferases (HATs), which add acetyl groups and histone deacetylases (HDACs), which remove them (figure 1.1). These enzymes are named so because histone proteins were the first targets identified.

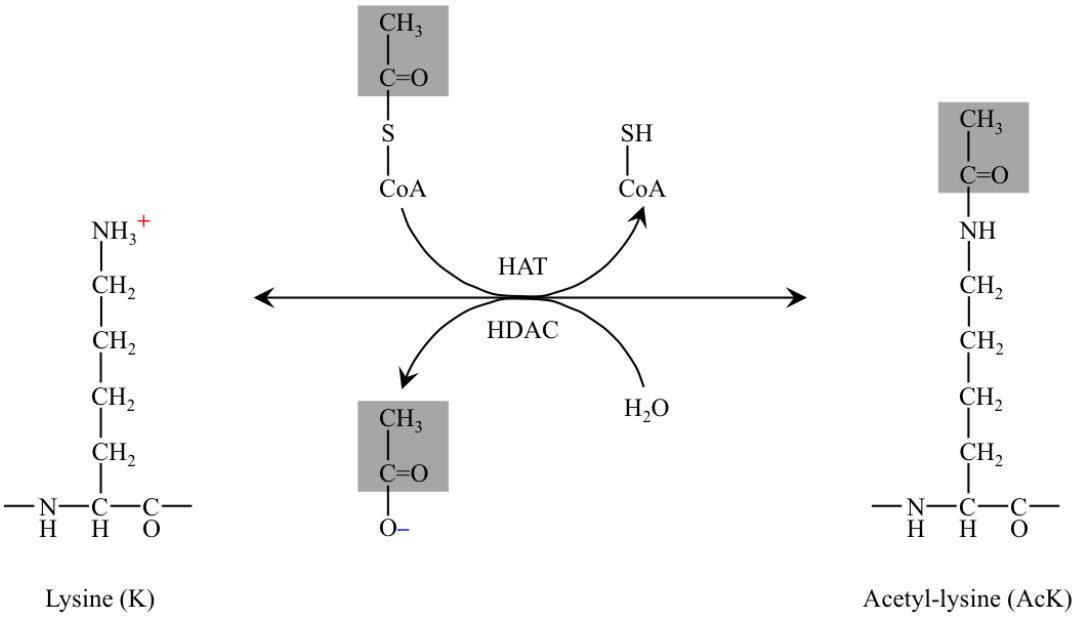


Figure 1.1. Reversible lysine acetylation. A histone acetyltransferase (HAT) transfers an acetyl moiety (shaded box) from acetyl coenzyme A (CoA) to the amino group ($NH3^+$) of a lysine (K) residue in a protein. A histone deacetylase (HDAC) removes an acetyl group from an acetylated lysine residue (AcK) in a protein.

Acetylation can regulate the function of a protein in many ways (see sections 1.2.1 to 1.2.3 for a discussion of some known examples). The addition of an acetyl group to a lysine neutralises the positive charge of the amino acid and the removal of an acetyl group (deacetylation) restores this positive charge. In brief, these alterations in the electrostatic properties of lysine are known to induce changes in the conformation of the protein and also affect how the protein interacts with other molecules. Furthermore, a lysine that is acetylated can act as a binding motif for other proteins and gaining or losing an interaction with another protein can have different functional outcomes.

1.2.1. Histone protein acetylation

Deoxyribonucleic acid (DNA) in the nuclei of eukaryotic cells is packaged into a highly organised but dynamic structure called chromatin. At the fundamental level, chromatin is made up of repeating units called nucleosomes. These comprise of ~200 base pairs (bp) of DNA of which ~146 bp is wrapped around an octamer structure of histone proteins; the remaining DNA serves as a linker between nucleosomes. In each nucleosome, there are two copies of the four histone proteins H2A, H2B, H3 and H4. Each histone protein in the nucleosome has a C-terminal domain, a globular domain of α-helices and protruding N-terminal tail which contains lysine residues. These are available for acetylation by HATs and deacetylation by HDACs (table 1.1). An additional histone protein, histone H1 is found outside of octamer, interacting with the linker DNA between nucleosomes. This histone has a role in linking the nucleosomes together and regulating the packaging of chromatin into higher order structures.

Table 1.1. Acetylated lysine residues found in N-terminal histone tails.

Histone	Tail length (aa's)	Acetylated lysine residues[a]
H2A	142	5, 9, 13, 15, 36, 95 & 118
H2B	120	5, 11, 12, 15, 16, 20, 23, 24, 46, 85, 108, 116 & 120
H3	129	4, 9, 14, 18, 23, 27, 36, 56, 64, 79, 115 & 122
H4	91	5, 8, 12, 16, 20, 77, 79 & 91

[a]Found to be acetylated in humans and/or mice. aa's: amino acids. Sources used: Wang et al. (2008); Tweedie-Cullen et al. (2012); Roadmap Epigenomics et al. (2015).

Histone acetylation has been correlated with active and the activation of gene expression and histone deacetylation with inactive and the inactivation of gene expression (Wang et al., 2008). Non-acetylated/deacetylated lysine residues are positively charged and in histone proteins these residues have electrostatic interactions with negatively charged DNA and other histone proteins. This has a compacting effect on chromatin where the DNA is wrapped around the histones more tightly and the nucleosomes are pulled closer together (figure 1.2). As a consequence, access to the underlying DNA for factors that drive gene transcription

is reduced. On acetylation by HATs (figure 1.2), the positive charge of the lysine in the histone tails is neutralised, preventing the electrostatic interactions, resulting in an opening up of the chromatin for gene transcription to occur (Struhl, 1998; Kornberg and Lorch, 1999; Strahl and Allis, 2000; Kouzarides, 2007). In addition to structural changes in chromatin, acetylation of lysine residues in histone proteins further regulates gene expression by acting as binding motifs for "reader" proteins (figure 1.2). These proteins contain a bromodomain, which has been shown to specifically recognise and interact with an acetylated lysine (Dhalluin et al., 1999; Hassan et al., 2007; Filippakopoulos et al., 2012). This domain is found in many positive gene regulatory proteins including chromatin remodelling proteins, helicases and transcription co-activators (Strahl and Allis, 2000; Marmorstein and Zhou, 2014).

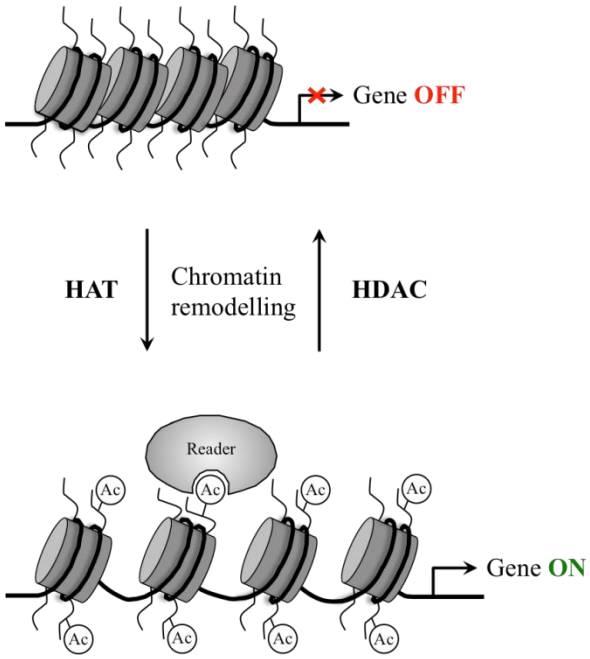


Figure 1.2. Simplified representation of the effect of histone acetylation on chromatin remodelling and gene expression. A string of nucleosomes is shown, each grey cylinder represents an octamer of histone proteins. Protruding histone N-terminal tails are acetylated and deacetylated by histone acetyltransferases (HATs) and histone deacetylases (HDACs) respectively; only four tails and one acetyl group per nucleosome are shown for simplicity. Histone acetylation promotes gene expression through chromatin decondensation and generation of binding sites for transcriptional co-activators termed "readers". Histone deacetylation results in the removal of binding sites for "reader" proteins, chromatin condensation and inhibition of gene expression.

Like many proteins, histones can undergo several other post-translational modifications in addition to acetylation including, methylation of lysine and arginine residues, phosphorylation of serine and threonine residues and ubiquitination and sumoylation of lysine residues (reviewed by Bannister and Kouzarides (2011); Kouzarides (2007)). Like acetylation, these modifications regulate chromatin structure and function, and therefore gene expression either through changing electrostatic interactions between histones and DNA (e.g. occurs following the addition of a negatively charged phosphate group), or by forming binding sites for "reader proteins" (e.g. occurs following methylation). In the case of ubiquitination, the large polyubiquitin molecule physically disrupts chromatin structure simply because of its size and bulkiness.

The effects of these post-translational modifications on histone function and gene expression vary depending on which site is modified (reviewed by Kouzarides (2007)). For example, methylation at lysine 4 in histone H3 is associated with active gene expression whereas methylation at the adjacent lysine 9 is associated with a repression of gene expression. These two lysine residues are also targets for acetylation (see table 1.1) and so competition exists between methylation and acetylation at these and other sites. The functional effect following the modification of a site depends on which modification is present. For example, methylation at lysine 9 in histone H3 is associated with repressing gene expression, whereas acetylation at the same site is associated with promoting gene expression (Kouzarides, 2007). There is also interplay between the different modifications, where a modification at one site can promote or prevent a different modification at another site. For example, acetylation of histone H3 lysine 14 prevents the methylation of the adjacent lysine 9 (Kouzarides, 2007), methylation of arginine 3 in histone H4 promotes the acetylation of adjacent lysine residues 8 and 12 (Kouzarides, 2007), and acetylation of lysine 18, 23 and 27 in histone H3 promotes the methylation of arginine 17 (Daujat et al., 2002; Kouzarides, 2007). For each of these three examples the patterns of histone modification are associated with active gene expression. In addition to methylation, histone phosphorylation can also regulate the acetylation of other sites. Phosphorylation of histone H3 serine 10 for example promotes the acetylation of the neighbouring lysine 14 (Walter et al., 2008).

The particular patterns generated by the interactions between histone modifications are thought to constitute a "histone code" and proteins, which regulate gene expression, are recruited to and bind to DNA by recognising specific sequences of this code (Strahl and Allis, 2000). Because of the crosstalk amongst protein post-translational modifications, one should consider that when one particular modification is altered, this may in-turn effect others and these other changes may also be contributing to the generation of the phenotype observed.

1.2.2. p53 acetylation

One of the well-studied examples of non-histone protein acetylation is that of p53 acetylation. This protein is both a transcription factor and a cell signalling molecule involved in stimulating cell-cycle arrest and apoptosis in response to cellular stresses, such as incorrect DNA replication and DNA damage (Prives and Hall, 1999). Normally, p53 exists in an inactive form and is present at low levels by the actions of its inhibitor protein, Mdm2 (mouse double minute 2 homolog) an ubiquitin ligase (Freedman et al., 1999). Upon an appropriate stimulus, p53 expression is increased and the protein undergoes post-translational modification. Then, p53 acts as a transcription factor to directly activate the expression of genes that control the cell cycle or apoptosis and a fraction of p53 also translocates to the mitochondria where it is involved in the release of the apoptosis inducing protein cytochrome *c* (Sykes et al., 2009).

The protein p53 can be acetylated at lysine residues 120, 164 and 292 in the DNA binding domain and 320, 351, 357, 370, 372, 373, 381, 382 and 386 in the C-terminal domain (Spange et al., 2009). Generally, acetylation of p53 is thought to promote its activity by inhibiting the interaction with the inhibitor protein Mdm2 (Tang et al., 2008). Acetylation of p53 has also been reported to affect its binding to p53 dependent genes; however varying effects have been reported. Gu and Roeder (1997) have shown that acetylation of p53 in the C-terminal domain is essential for binding to the gene promoter and promoting the expression of the p53-dependent Gadd45α (growth arrest and DNA damage-inducible 45 alpha) cell-cycle arrest gene. Gu and Roeder (1997) go on to hypothesise how acetylation regulates binding of p53

to DNA. The C-terminal and the DNA binding domains of p53 are known to interact and when the C-terminal domain is not acetylated, the positively charged lysine residues can interact with the DNA binding domain, which locks the protein into a conformation that is unable to bind to DNA. Upon lysine acetylation in the C-terminus and the neutralisation of the positively charged residues, the interaction between these and the DNA binding domain is disrupted and p53 undergoes a conformational change permissive for DNA binding (Gu and Roeder, 1997). In direct contradiction, Brochier et al. (2013) show specific acetylation of lysine 381 and 382 in the C-terminus negatively regulates the binding of p53 to the promoters of and the expression of the cell-cycle arrest gene p21 and the pro-apoptotic gene PUMA (p53 upregulated modulator of apoptosis).

In addition to the effects on DNA binding, acetylation also regulates the transcriptional activity of p53 by affecting interactions with transcriptional co-activators. HATs and p53 are known to interact with one another and p53 requires HATs to remodel chromatin structure in order to promote gene transcription (Gu et al., 1997). This interaction is thought to be meditated via the acetylated lysine 382 in the C-terminal domain and the bromodomain found in HATs such as the cAMP response element binding protein (CREB)-binding protein (CBP, Mujtaba et al. (2004)).

Acetylation at lysine 120 in the DNA binding domain, selects the activity of p53 towards mediating apoptosis rather than cell-cycle arrest. This is mediated through the selective expression of apoptosis inducing genes (Sykes et al., 2006) and by non-transcriptional effects on mitochondrial dependent apoptosis (Sykes et al., 2009). The mechanism underlying the selective expression of apoptosis genes and not cell-cycle regulatory genes is not understood. Sykes et al. (2006) have hypothesised that the acetylation of lysine 120 may act as a binding motif for a specific transcriptional cofactor, which targets p53 to apoptosis-inducing genes over others.

1.2.3. NF-κB *acetylation*

Another non-histone protein which is modified and whose function is regulated by acetylation is NF-κB, a transcription factor involved in regulating the inflammatory responses of innate immune cells. Prototypically, NF-κB is a heterodimer of the proteins p50 and RelA[p65]. In un-stimulated cells, NF-κB is sequestered in the cytoplasm by an association with an inhibitor protein IκBα nuclear factor kappa-light-chain-enhancer of activated B-cells inhibitor alpha. Upon an appropriate inflammatory stimulus, NF-κB is activated by a phosphorylation and ubiquitination dependent degradation of IκBα. Then, NF-κB rapidly translocates to the nucleus where it regulates inflammatory gene expression. NF-κB activity is stopped by its re-association with IκBα and export from the nucleus to the cytoplasm, where it remains sequestered until activated again (Baldwin, 1996; Ghosh et al., 1998).

The RelA[p65] subunit of NF-κB can be reversibly modified by acetylation. Seven lysine residues; 122, 123, 218, 221, 310, 314 and 315 are acetylated and deacetylated by HATs and HDACs in the nucleus (Chen et al., 2001; Chen et al., 2002; Kiernan et al., 2003; Ziesche et al., 2013). Acetylation and deacetylation of these different sites has specific effects on NF-κB activity such as modulating binding to DNA. When RelA[p65] binds to a target DNA sequence, lysine residues 122 and 123 are found to be in close proximity to DNA (Chen et al., 1998). Neutralisation of these positively charged amino acids upon acetylation, is thought to destabilise the electrostatic interactions and therefore reduce the binding of RelA[p65] to the negatively charged DNA. This facilitates NF-κB removal, promoting its re-association with the inhibitor protein IκBα and consequent export out of the nucleus (Kiernan et al., 2003). This ultimately reduces transcriptional activity (Kiernan et al., 2003; Ziesche et al., 2013). Acetylation of lysine 221 enhances DNA binding of RelA[p65] and together with the acetylation of lysine 218 impairs its association with IκBα (Chen et al., 2002). Furthermore, the acetylation of lysine residues 310, 314 and 315 in RelA[p65] can either inhibit or potentiate transcriptional activity of NF-κB at specific genes (Buerki et al., 2008; Rothgiesser et al., 2010b; Chen et al., 2002; Ziesche et al., 2013).

Acetylation of lysine 314 and 315 also has a role in terminating NF-κB dependent gene expression (Rothgiesser et al., 2010b).

The p50 subunit of NF-κB can also be regulated by acetylation, with lysine residues 431, 440 and 441 modified in this way (Furia et al., 2002). Acetylation of these residues increases the binding of p50 to DNA and enhances its transcriptional activity (Furia et al., 2002; Deng and Wu, 2003).

1.3. Histone acetyltransferases (HATs)

Histone acetyltransferases are enzymes that add acetyl groups to lysine residues in proteins. HATs are grouped on the basis of their catalytic domains (reviewed by Kimura et al. (2005); Lee and Workman (2007)). The GCn5 *N*-acetyltransferase (GNAT) family includes Gcn5 (general control nonrepressed 5, Brownell et al. (1996)) and p300/CBP-associated factor (PCAF, Yang et al. (1996)) amongst others. A second family of HATs the MYST family, is named after the founding members Morf (monocytic leukemia zinc finger protein-related factor, Champagne et al. (1999)), Ybf2 (Takechi and Nakayama, 1999), Sas2 (something about silencing protein 2, Kimura et al. (2002)) and Tip60 (60 kDa Tat-interactive protein, Yamamoto and Horikoshi (1997)). Other HATs include p300, CBP (Bannister and Kouzarides (1996); Ogryzko et al. (1996)) and TAF(II)250 (TATA box-binding protein associated factor 250) a subunit of transcription factor II D (TFIID), which is a major component of the transcription preinitiation complex (Mizzen et al., 1996).

1.3.1. HAT expression and distribution in the brain

To date, no comprehensive study looking at the expression of all the HATs throughout the brain has been performed, but an analysis of the literature supports the general opinion that HATs are ubiquitously expressed in the brain. Indeed the HATs, Gcn5, PCAF, Morf, Tip60, p300 and CBP are all expressed in the frontal lobe in humans (Pedre et al., 2011).

Additionally, some of these HATs have been observed in the brains and in various neural cell types of mice and rats. PCAF is expressed in the forebrain in mice (Maurice et al., 2008) and the hippocampus in both mice and rats (Maurice et al., 2008; Bousiges et al., 2010). PCAF is also expressed in neurones isolated from mouse cerebellum (Rouaux et al., 2003) and in isolated murine microglia (Park et al., 2013). The HAT p300 is expressed in mouse cortex and the amygdala (Oliveira et al., 2007), as well as the hippocampus of both mice (Ogawa et al., 2001; Oliveira et al., 2007) and rats (Bousiges et al., 2010). Consistent with this, neurones isolated from rat hippocampus (Hardingham et al., 1999), mouse cortex (Marinova et al., 2009) and cerebellum (Rouaux et al., 2003) express p300 and this HAT is expressed in astrocytes (Ogawa et al., 2001; Fonte et al., 2007; Marinova et al., 2011) and microglia (Tang et al., 2007; Kim et al., 2013) isolated from rat and mouse brains respectively. Another HAT, CBP is found to be expressed in the cortex, thalamus, hypothalamus, hippocampus and amygdala of rats (Stromberg et al., 1999) and hippocampus (Korzus et al., 2004), midbrain, brainstem and cerebellum of mice (Chung et al., 2003). Neurones isolated from the mouse cortex and cerebellum (Rouaux et al., 2003) and rat hippocampus (Hardingham et al., 1999; Impey et al., 2002) express CBP and so do astrocytes and microglia isolated from rat and mouse brains respectively (Delgado, 2002; Fonte et al., 2007).

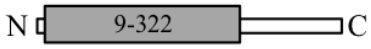
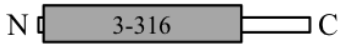
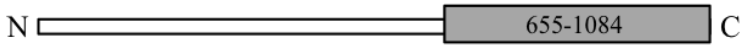
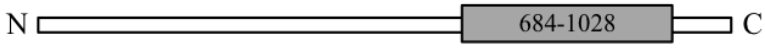
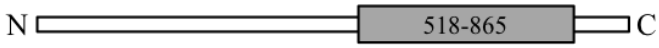
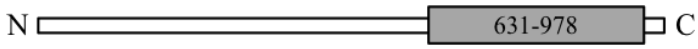
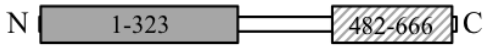
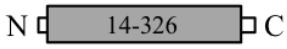
1.4. Histone deacetylases (HDACs)

1.4.1. HDAC isoforms and classes

There are eighteen different HDAC isoforms that deacetylate lysine residues and these are grouped into four classes according to their catalytic domains, sequence homology and similarity to Saccharomyces cerevisiae (yeast) deacetylases (de Ruijter et al., 2003; Gregoretti et al., 2004). The zinc-dependent HDACs (table 1.2) include class I HDACs (1, 2, 3 and 8), which are closely related to the yeast deacetylase RPD3 (reduced potassium dependency 3), and class IIa HDACs (4, 5, 7 and 9) and class IIb HDACs (6 & 10), which are similar to the yeast histone deacetylase 1 (HDA1). The fourth class comprises of HDAC11, which shares limited

sequence homology to any of the other HDACs. Unlike class I, II and IV HDACs which require Zn^{2+} as a cofactor for catalytic activity, the class III HDACs known as sirtuins, of which there are seven members (SIRT1-7), are nicotinamide adenine dinucleotide $(NAD)^{+}$-dependent enzymes. This thesis will focus on the role of and effects of inhibiting class I and II HDACs in the brain.

Table 1.2. Zinc-dependent histone deacetylase (HDAC) isoforms.

HDAC	Protein domains	Size
Class I		
HDAC1		482
HDAC2		488
HDAC3		428
HDAC8		377
Class IIa		
HDAC4		1084
HDAC5		1122
HDAC7		952
HDAC9		1011
Class IIb		
HDAC6		1215
HDAC10		669
Class IV		
HDAC11		347

Grey box and striped box: number of amino acids comprising the zinc-dependent catalytic domain and the catalytically inactive domain respectively. Size: number of amino acids, N: N-terminus, C: C-terminus. Data sourced from Micelli and Rastelli (2015).

1.4.2. HDAC structure and catalytic mechanism

The structures for all human class I HDACs isoforms, as well as HDAC4 and HDAC7 have been solved (reviewed by Micelli and Rastelli (2015)). Each zinc-dependent class I and IIa HDAC is comprised of a single domain protein composed of a central eight stranded parallel β-sheet flanked by numerous α-helices on either side. Access to the catalytic site is through a tube structure lined mainly with hydrophobic residues leading to a cavity lined with polar residues and the catalytic cofactor Zn^{2+} (Micelli and Rastelli, 2015). Aligning the amino acid sequences of the catalytic domains of class I and IIa HDACs, reveals that the catalytic sites amongst these HDAC isoforms are largely conserved (Lahm et al., 2007). Three catalytic mechanisms of deacetylation by HDACs have been proposed (Finnin et al. (1999); Vanommeslaeghe et al. (2005); Corminboeuf et al. (2006) and reviewed by Micelli and Rastelli (2015)) and the residues involved, include those that bind the Zn^{2+} cofactor (a histidine residue and two aspartic acid residues) and those that surround it and interact with the acetyl moiety of the substrate (a tyrosine and two histidine residues). In all three proposed catalytic mechanisms of deacetylation, the presence and reactivity of the tyrosine residue with its hydroxyl group (-OH) is important and class I HDAC isoforms lose deacetylase activity when this tyrosine is mutated to phenylalanine, or a histidine as found in class IIa HDACs (Lahm et al., 2007). Because class IIa HDACs lack the tyrosine residue, which is thought to be important for catalytic activity, it is not clear if these isoforms have intrinsic deacetylase activity. The pioneering studies that first identified HDACs 4, 5, 7 & 9 showed they were able to deacetylate histone proteins (Grozinger et al., 1999; Miska et al., 1999; Wang et al., 1999; Zhou et al., 2001). Furthermore, Bradner et al. (2010) as well as others who have studied the selectivity of HDAC inhibitors using commercially available recombinant full-length class IIa HDACs and acetylated substrates, measure deacetylase activity of these isoforms and this is subsequently inhibited with HDAC inhibitors (see section 1.8). However other groups have shown class IIa HDACs have no intrinsic deacetylase activity and any deacetylase activity associated with these isoforms is because they can interact with HDAC3 (Fischle et al., 2001; Fischle et al., 2002). However, Lahm et al. (2007) report that HDAC4 exhibits weak deacetylase activity against acetylated histones and commercially available

substrates in the absence of class I HDACs. This deacetylase activity is significantly increased when the histidine residue in the catalytic site of class IIa HDACs 4, 5 & 7 is mutated to tyrosine as found in class I HDACs (Lahm et al., 2007). If class IIa HDACs do not have intrinsic deacetylase activity, or if it is relatively weak compared to other isoforms, this raises an interesting question; what is the contribution of class IIa HDAC activity towards regulating the acetylome?

1.4.3. HDAC subcellular localisation

The localisation of HDAC isoforms within a cell will dictate if the HDAC can deacetylate a specific protein substrate and which substrates are deacetylated. Class I HDACs 1, 2 and 8 each contain a nuclear localisation signal (NLS) and have often been thought to be exclusively located in the nucleus (de Ruijter et al., 2003). However, subcellular fractionation analysis and immunocytochemistry of human cells has shown HDAC1 and 2 can also be present in the cytoplasm and in other organelles in addition to the nucleus (Kahali et al., 2012). In support of this, Kim et al. (2010) have identified a putative nuclear export signal (NES) motif in the amino acid sequence of HDAC1 expressed in mice and observed translocation of HDAC1 from the nucleus to the cytoplasm and axons of cultured murine neurones mediated by the nuclear pore Exportin 1 receptor (XPO1). Furthermore, Baltan et al. (2011a) have also observed HDAC1 in the cytoplasm and the nucleus of murine neurones and further show HDAC1 and 2 proteins are present in both the nucleus and cytoplasm of murine astrocytes as well.

HDAC3 and class II HDACs 4, 5, 9 & 10 all contain a NLS and a NES and shuttle between the nucleus and cytoplasm (de Ruijter et al., 2003) via importins and exportins found in pores of the nuclear membrane. HDACs 4, 5 & 7, can be sequestered in the cytoplasm by 14-3-3 anchor proteins in a phosphorylation dependent manner (Grozinger and Schreiber, 2000; Bertos et al., 2001; Kao et al., 2001). HDAC6 is predominantly a cytoplasmic protein but a fraction of this isoform can shuttle between the cytoplasm and nucleus (Verdel et al., 2000). Finally the class IV HDAC, HDAC11 is found to localise predominantly to the nucleus (Gao et al., 2002).

1.4.4. HDAC containing multi-protein complexes

In the nucleus, HDACs cannot bind to DNA directly and in order to deacetylate histones they must be recruited to genes as members of multi-protein complexes. These complexes bind to chromatin through DNA and histone-binding domains found within components of the complexes themselves and also through recruitment to genes by transcription factors. HDAC1 and 2 are components of three multi-protein complexes called Sin3 (switch independent 3, Laherty et al. (1997)), NuRD (nucleosome remodelling and deacetylation, Zhang et al. (1999)) and CoREST (co-repressor for element-1-silencing transcription factor, You et al. (2001)). The composition of these is reviewed by Kelly and Cowley (2013). These complexes contain a dimer of HDACs and are classically depicted with one molecule of both HDAC1 and 2 (Kelly and Cowley, 2013). In the case of human MCF-7 (Michigan cancer foundation-7) cells this is mostly true, where 80 to 90% of HDAC1 and 2 proteins are associated with each other (He et al., 2005). However, HDAC1 has also been found in complexes as a homodimer as well as a heterodimer with HDAC2 (Taplick et al., 2001). In mouse embryonic fibroblasts 40 and 60% of HDAC1 and HDAC2 respectively are found in the Sin3 and CoREST complexes independent of the other HDAC (Yamaguchi et al., 2010). In mouse embryonic stem cells and thymocytes, the Sin3 complex predominantly contains HDAC1 whereas the NuRD and CoREST complexes can contain HDAC1 and/or HDAC2 (Dovey et al., 2013; Dovey et al., 2010b). The dimerization of these two HDACs is indirect and is mediated via a HDAC binding domain found on a component of the multi-protein complex. For example, HDAC1 and HDAC2 can form homodimers or heterodimers through binding to the ELM2 (egg-laying defective protein 27 and metastasis-associated protein 1 homology 2) domain of MTA1 (metastasis-associated protein 1) found in the NuRD complex (Millard et al., 2013). Together, these findings suggest that HDAC1 & HDAC1, HDAC2 & HDAC2 and HDAC1 & HDAC2 configurations for each multi-protein complex (CoREST, Sin3 and NuRD) exist, but how these configurations come about and the functional relevance of each of these different complexes is currently not known (Kelly and Cowley, 2013).

HDAC3 is a member of the nuclear receptor co-repressor (NCoR)/silencing mediator of retinoid and thyroid receptors (SMRT) complex (Wen et al., 2000) and class IIa HDACs 4, 5, 7, 9 and the class IIb HDAC10 can interact with this complex as well (Wen et al., 2000; Fischer et al., 2002; de Ruijter et al., 2003). In addition, HDAC4 & 5 can also associate with the Sin3 complex (Nakagawa et al., 2006) and HDAC4, 5 & 7 also interact directly with and inhibit MEF-2 (myocyte enhancer-binding factor-2) a transcription factor that controls neuronal function and survival (Miska et al., 1999; Wang et al., 1999; Lemercier et al., 2000; Bertos et al., 2001; Chawla et al., 2003; de Ruijter et al., 2003; Linseman et al., 2003). HDAC6 and 11 do not interact with any of the aforementioned complexes but are co-immunoprecipitated together (Gao et al., 2002).

The acetylome is comprised of thousands of proteins from many classes and subcellular compartments. How HDACs are specifically recruited to, how they specifically interact with and deacetylate these diverse non-histone targets is not understood. It is likely that HDACs are recruited to and interact with these proteins as members of multi-protein complexes such as those already described or complexes that are yet to be identified. In support of this, Luo et al. (2000) show that deacetylation of p53 by HDAC1, does not occur through a direct interaction of the two proteins. Rather, HDAC1 is recruited to p53 as a member of the NuRD complex and it is MTA2 (metastasis-associated protein 2), another component of the NuRD complex, which is responsible for binding directly to p53 (Luo et al., 2000). These multi-protein complexes and the composition of them may also provide a mechanism to control the substrate specificity of the different HDAC isoforms. To identify novel HDAC containing multi-protein complexes, HDAC-protein "interactome" analysis can be performed. Joshi et al. (2013) have studied the protein interaction networks of HDAC isoforms in human T-cells and reveal a total of 180 HDAC-protein interactions across class I and II HDACs. For class I HDACs, the majority of these interactions are with components of the respective chromatin binding complexes mentioned earlier, but there are other interactions with proteins involved in cell signalling, protein folding, protein and ion transport and cellular metabolism. Whether these are substrates for HDACs or are co-members of multi-protein complexes and whether HDACs bind directly to these proteins or interact with them

via other proteins, is not clear. This study also confirmed that class IIa HDACs 4, 5 & 7 predominantly interact with the NCoR multi-protein complex and 14-3-3 proteins (Joshi et al., 2013).

1.5. HDAC expression and distribution in the brain

1.5.1. Human brain

All eleven HDAC isoforms are expressed at the messenger RNA (mRNA) level in the human cerebrum; HDAC9 is the most prevalent followed by HDAC10, 11, 4, 6, 5, 8, 3, 1, 2 and 7 (Lucio-Eterovic et al., 2008). An analysis of the expression of each HDAC in each brain region in the human brain is lacking. However, the distribution of each HDAC isoform has been mapped in the brains of mice and rats and the expression profile of each has also been determined for a variety of neural cell populations isolated from these brain regions.

1.5.2. Rat brain

Class I, II and IV HDACs are all expressed and widely distributed throughout the developed rat brain. Broide et al. (2007) have performed the most comprehensive study to date using *in situ* hybridisation techniques to compare the mRNA expression of each of the eleven HDAC isoforms for each brain region. Based on expression in the whole brain, HDAC11 is the most expressed followed by HDAC3, 5, 4, 2, 1, 6, 8, 7, 9 and 10.

Class I HDACs 3, 2 and 1 (in rank order of mRNA expression levels) are found in all brain regions studied including cerebral cortex, amygdala, striatum, hippocampus, hypothalamus, thalamus, midbrain, pons, the medulla oblongata and the granule cell layer of the cerebellum. HDAC3 is ubiquitously and equally expressed in all these regions whereas HDAC1 and HDAC2 are predominantly expressed in the cerebral cortex, amygdala and hippocampus. HDAC2 is expressed to a greater extent in the medulla oblongata compared to HDAC1. HDAC8 mRNA is found at very low levels

throughout the rat brain except in the hippocampus where it is expressed to a similar level as HDAC1 and in the granule cell layer of the cerebellum where it is expressed at a level half of that observed for HDAC1.

Class IIa HDACs, HDAC4 and 5 are also expressed in all brain regions studied including the cerebral cortex, amygdala, striatum, hippocampus, hypothalamus, thalamus, midbrain, pons, the medulla oblongata and the granule cell layer of the cerebellum. HDAC5 mRNA is expressed at higher levels in the striatum and midbrain compared to HDAC4. HDAC7 and 9 are expressed at very low levels or are near absent in the cerebral cortex, hypothalamus, thalamus, midbrain and pons. HDAC7 is predominantly expressed in the amygdala, hippocampus, substantia nigra pars compacta and the granule cell layer of the cerebellum. HDAC9 is mostly expressed in the hippocampus and substantia nigra pars compacta.

The class IIb HDAC6 is expressed in all brain regions studied with levels in the hippocampus being the greatest. HDAC10 is only found in the cortex and hippocampus, but even here HDAC10 mRNA expression is at relatively low levels compared to the other HDAC isoforms. The class IV HDAC11 is the most abundant HDAC in the rat brain and is universally present at high levels in all brain regions.

1.5.3. Mouse brain

HDAC1-11 mRNA is expressed in developed mouse cortex with HDAC2 and 5 being the most expressed followed by HDAC11 then 4, whereas HDAC9 and 10 have the lowest mRNA expression (Chen et al., 2012c). This trend in mRNA levels closely matches that in rat (Broide et al., 2007) but not in the human brain, where HDAC9 & 10 mRNA is found to be the most abundant and HDAC2 mRNA is expressed at relatively low levels compared to the other HDACs (Lucio-Eterovic et al. (2008) and see section 1.5.1).

The expression of HDACs 1-3 at the protein level in the developed mouse brain is widespread and particularly prominent in the cerebral cortex and hippocampus (Baltan et al., 2011a). Another study, focusing on HDAC2, observed ubiquitous

protein expression throughout the whole of the developed mouse brain including the cerebral cortex, hippocampus, amygdala, basal ganglia, hypothalamus, thalamus, brain stem and the granule cell layer of the cerebellum (Yao et al., 2013). Class IIa HDAC4 protein is also widely expressed, in regions such as the cerebral cortex, amygdala, striatum, hippocampus, thalamus and the substantia nigra (Darcy et al., 2010). Class IIb HDAC6 mRNA and protein is expressed in mouse cerebral cortex and hippocampus with lower levels observed in the cerebellum (Govindarajan et al., 2013).

1.5.4. Neural cells of mice and rat brains

In summary, the majority of the HDAC isoforms are ubiquitously expressed across different brain regions and the specific neural cell populations of these. Table 1.3 summarises the expression of mouse and rat HDACs 1-11 at the mRNA and/or protein level, in neurones from three important brain regions and also the three populations of glial cells in the brain; astrocytes, microglia and oligodendrocytes.

Table 1.3. Expression of HDACs in mouse and/or rat neurones and glial cells.

Neural cell type	**HDAC expressed**
Cortical neurones[3, 4, 5, 6, 12, 13, 15, 16, 19, 21 & 24]	1, 2, 3, 4, 5, 6, 7, 8, 9, 10 & 11
Hippocampal neurones[4, 5, 9, 10, 11, 15, 18 & 22]	1, 2, 3, 4, 5, 6, 7, 8, 9, 10 & 11
Cerebellar granule neurones[2, 3, 4, 5, 7, 16, 17 & 20]	1, 2, 3, 4, 5, 6, 7, 8 & 11
Astrocytes[5, 8, 9, 12, 14, 15 & 23]	1, 2, 3, 4, 5, 6, 7, 8, 9, 10 & 11
Microglia[1, 8, 14 & 23]	1, 2, 3, 4, 5, 6, 7, 8, 9, 10 & 11
Oligodendrocytes[4, 12, 14, 15 & 22]	1, 2, 3, 4, 5 & 11

References in superscript: [1]Ajamian et al. (2004), [2]Panteleeva et al. (2004), [3]Bolger and Yao (2005), [4]Broide et al. (2007), [5]MacDonald and Roskams (2008), [6]Kim et al. (2008), [7]Leng et al. (2008), [8]Faraco et al. (2009), [9]Guan et al. (2009), [10]Chen et al. (2010), [11]Darcy et al. (2010), [12]Kim et al. (2010), [13]Sugo et al. (2010), [14]Baltan et al. (2011b), [15]Baltan et al. (2011a), [16]Bardai and D'Mello (2011), [17]Ma and D'Mello (2011), [18]McQuown et al. (2011), [19]Soriano and Hardingham (2011), [20]Bardai et al. (2012), [21]Chen et al. (2012c), [22]Yao et al. (2013) [23]Kannan et al. (2013) and [24]Wei et al. (2015). Note: Reference 8 refers to HDAC expression in astrocyte and microglia co-cultures.

1.6. The acetylome of the brain

HATs and HDACs are expressed throughout the brain where they control the acetylation state of proteins. To date histone proteins are one of the most studied members of the acetylome in the brain. Experimental evidence suggests that the acetylation of histone proteins regulates the expression of genes important for cognitive processes such as learning and memory (reviewed by Graff and Tsai (2013); Lopez-Atalaya and Barco (2014); Penney and Tsai (2014)). However, histones are only a small fraction of the collection of proteins that make-up the acetylome and in the rat brain this comprises of 1653 acetylated proteins in total (Lundby et al., 2012). These are located in many of the subcellular compartments including the nucleus, cytoplasm, mitochondria, endoplasmic reticulum, Golgi apparatus and the cell membrane (Lundby et al., 2012). Characterising these acetylated proteins and their known interacting partners on the basis of their known functions, shows acetylation regulates major processes in neurones including synaptic vesicle formation, trafficking and exocytosis, neurotransmitter release, axon guidance and migration, synaptogenesis, ion and solute transport across membranes, calcium signalling, phosphorylation and ubiquitination (Lundby et al., 2012). Therefore HATs and HDACs, which regulate the acetylation status of these proteins, are clearly important for normal brain function.

1.7. Role of HDACs in the brain

In order to study the function of HDACs in the brain; conditional brain specific deletions have been used. Knockout of HDAC1 (HDAC1KO, Montgomery et al. (2009)) or knockout of HDAC2 (HDAC2KO, Montgomery et al. (2009); Guan et al. (2009)) in the embryonic brain in mice has no effect on mouse brain development, histoarchitecture or mouse survival. In contrast, knockout of both HDAC1 and 2 is lethal by postnatal day seven. In these double knockout mice, neuronal precursors in the brain are unable to differentiate into mature neurones and subsequently undergo cell death resulting in major abnormalities in cerebral cortex, hippocampus and cerebellum structure (Montgomery et al., 2009). This suggests HDAC1 and 2 have

redundant functions during neural development. However, as will be subsequently discussed, in the developed brain, HDAC1 and 2 have some specific and independent roles; therefore at some point during postnatal development, the individual roles of these two HDACs must become established.

Knockout of HDAC3 (HDAC3KO) in the mouse embryonic brain is lethal and pups die within 16 hours after birth. In the brains of these mice, there are fewer neural cells and major abnormalities in the development and cytoarchitecture of the cerebral cortex and cerebellum (Norwood et al., 2014). The loss of HDAC3 cannot be compensated for by other HDACs and this suggests that HDAC3 has a unique and important role in the development of the mouse brain.

1.7.1. HDAC1

Much of the research studying HDACs in the developed brain has focussed on their roles in cognition and behaviours associated with psychological disorders. Investigating these roles has often involved studying and manipulating the expression levels and activity of HDACs in *in vivo* and *in vitro* model systems.

In order to understand the role of HDAC1 in cognition, brain specific overexpression mouse models have been employed. Mice overexpressing HDAC1 (HDAC1OE) in the brain have no obvious changes in brain anatomy, cellular architecture or overall neurone numbers (Guan et al., 2009). HDAC1 has been found overexpressed in frontal cortex of patients with schizophrenia (Sharma et al., 2008). Specifically overexpressing HDAC1 in the hippocampus of mice by directly injecting HDAC1 expressing adeno-associated viral (AAV) vectors into the hippocampus, does not induce any quantifiable psychological effects such as anxiety or depression in these mice (Bahari-Javan et al., 2012). Furthermore, overexpression of HDAC1 in the brain and specifically in the hippocampus, does not affect learning and memory associated with object recognition (Bahari-Javan et al., 2012), fear conditioning or the Morris water maze (Guan et al., 2009; Bahari-Javan et al., 2012). Regarding the cellular correlates of learning and memory, HDAC1OE in hippocampal neurones has no effect on long-term potentiation (LTP) or synapse formation (Guan et al., 2009).

However, HDAC1 is important in the extinction of memories in particular those associated with fear. HDAC1 knockdown (HDAC1KD) in the hippocampus of mice prevents the extinction of fear memories whereas hippocampal HDAC1OE enhances extinction (Bahari-Javan et al., 2012). During fear extinction, HDAC1 is recruited to the promoters of memory genes c-Fos and early growth response protein 2 (Egr2). This is followed by the deacetylation of the histones of these genes, which correlates with a reduction in the expression of c-Fos and Egr2 and an extinction of the fear memory (Bahari-Javan et al., 2012).

1.7.2. HDAC2

HDAC2 also has a role in cognition. Mice overexpressing HDAC2 (HDAC2OE) but not HDAC1 in the brain, have impaired hippocampus-dependent learning and memory, as demonstrated by poor performances in the fear conditioning test and the Morris water maze compared to wild-type mice. HDAC2OE is accompanied with a global decrease in lysine acetylation as well as a decrease in histone acetylation at specific neuronal genes in the hippocampus. These mice also have a suppressed hippocampal LTP response and a reduction in the number of synapses on hippocampal neurones. In contrast, specific knockout of HDAC2 in the brains of mice improves learning and memory. Neurones of the hippocampus from HDAC2KO mice have an increased number of synapses and a more robust LTP response. HDAC2 but not HDAC1 is found enriched at the promoters of genes implicated in synaptic formation, synaptic plasticity, memory formation and genes regulated by neuronal activity such as synapsin, brain derived neurotrophic factor (BDNF), glutamate receptor subunits, cAMP response element binding-protein (CREB), CBP, c-Fos, Ca^{2+}/calmodulin-dependent protein kinase II alpha (CaMKIIα) and early growth response proteins 1 (Egr1). The cognitive enhancement in HDAC2KO mice is accompanied with an increase in the acetylation of histones found in the promoter of many of these genes and a subsequent increase in the expression of them. Collectively, these data suggest HDAC2 but not HDAC1, negatively regulates learning and memory formation in mice (Guan et al., 2009).

In support of this, the excitatory postsynaptic currents of isolated hippocampal neurones from HDAC2KO mice or HDAC2 knockdown (HDAC2KD) in neurones in rat hippocampal organotypic slice cultures are significantly increased compared to wild-type, HDAC1KO and HDAC1KD neurones (Nelson et al., 2011; Hanson et al., 2013). HDAC2KD but not HDAC1KD also lowers the stimulation threshold to induce LTP in neurones in hippocampal slices (Hanson et al., 2013). Like Guan et al. (2009), Hanson et al. (2013) show that the increased excitatory synaptic function by reducing HDAC2 levels is accompanied by an increase in the expression of synaptic function genes including the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor subunit GluR2. Furthermore, HDAC2KD reduces whereas HDAC2OE increases the amplitude of inhibitory postsynaptic currents in neurones in hippocampus slice culture. These changes are brought about by HDAC2 dependent modulation of the abundance of synaptic inhibitory GABA (γ-Aminobutyric acid)-ergic receptors. Together, these results suggest HDAC2 in the hippocampus suppresses neuronal excitation and enhances inhibitory synaptic transmission and both combined may contribute to the negative regulation of learning and memory by HDAC2 (Hanson et al., 2013).

Since an increase in HDAC2 activity has a negative effect on learning and memory, it is not surprising to find that HDAC2 but not HDAC1 or 3 expression is increased in the hippocampus and the entorhinal cortex in the brains of individuals with early stage Alzheimer's disease (Graff et al., 2012). It has been suggested that this increase in HDAC2 expression and activity, and consequent changes in gene expression, is an early pathological event (Graff et al., 2012). This specific increase in the expression of HDAC2 is recapitulated in isolated mouse hippocampal neurones exposed to amyloid beta (Aβ) and in two mouse models of Alzheimer's disease (the p25/Cyclin-dependent kinase 5 (Cdk5) model and the five familial Alzheimer's disease mutation model (5XFAD)), where HDAC2 levels are increased in the hippocampus and prefrontal cortex (Graff et al., 2012). Compared to wild-type mice, HDAC2 in the p25/Cdk5 mouse hippocampus is significantly enriched at the promoter regions of genes involved in synaptic function, synaptic plasticity and learning and memory, such as BDNF IV, Egr1, glutamate receptor subunits and synaptic proteins synaptophysin and synaptotagmin. This coincides with histone hypoacetylation in the

promoter region and a reduction in the expression of these genes. These findings suggest that in Alzheimer's disease, HDAC2 represses genes important for learning and memory and this may contribute to the cognitive decline associated with this disease (Graff et al., 2012). Reducing HDAC2 expression in hippocampal neurones of p25/Cdk5 mice to wild-type levels, increases both histone acetylation and expression of those genes important in learning and memory already described. Furthermore, at the cellular level, HDAC2KD in p25/CdK5 mice improves LTP and significantly increases the synaptic density and abundance of dendrites of hippocampal neurones. As a consequence, HDAC2 knockdown significantly improves learning and memory in p25/Cdk5 mice as measured by fear conditioning and the Morris water maze tests (Graff et al., 2012).

1.7.3. HDAC3

Similar to other class I HDACs, HDAC3 is expressed in the cortex and hippocampus therefore it is not surprising to find it involved in regulating cognitive processes. Similarly to HDAC1, HDAC3 activity acts as a restraint on learning and memory. Focal deletion of HDAC3 in the hippocampus of adult mice enhances hippocampal dependent learning and memory for object locations. This is accompanied by histone hyperacetylation and increased expression of learning and memory genes such as c-Fos (McQuown et al., 2011). In support of the role for HDAC3 containing complexes in the negative regulation of learning and memory, global knock-in mice that carry a mutation in the nuclear receptor co-repressor protein (NCoR is an essential component of the NCoR multi-protein complex) so it cannot interact with HDAC3, also have improved learning and memory associated with object recognition and locations (McQuown et al., 2011).

1.7.4. HDAC4

Mice with a conditional cerebrum specific deletion of HDAC4 have reduced anxiety-like behaviour but deficits in learning and memory examined using the Morris water maze test. This learning and memory deficit is associated with impairments in hippocampal LTP but not basal synaptic transmission, which suggests HDAC4 is a positive regulator of memory formation (Kim et al., 2012b).

1.7.5. HDAC5

Constitutive HDAC5 knockout mice are viable and display no abnormalities in behaviour, basal synaptic transmission or learning and memory associated with fear conditioning (Kim et al., 2012b). However, a second report showed there are deficits in learning and memory associated with fear conditioning and the Morris water maze test in old HDAC5 null mice (Agis-Balboa et al., 2013).

1.7.6. HDAC6

HDAC6 knockout (HDAC6KO) mice are viable, histological examination reveals there are no brain abnormalities and mice show no detectable motor deficits (Fukada et al., 2012; Taes et al., 2013). HDAC6 has been reported to be upregulated in the brains of Alzheimer's disease patients (Ding et al., 2008) and also in the hippocampi of a mouse Alzheimer's disease mouse model called APPPS1 (mutations in both amyloid precursor protein (APP) and presenilin 1 (PS1/PSEN1), Govindarajan et al. (2013)). Knocking out HDAC6 in this model reduces the deficit in learning and memory (Govindarajan et al., 2013). Changes in psychological behaviour following knockout of HDAC6 in healthy mice is currently disputed. Fukada et al. (2012) observe HDAC6KO mice to be emotionally aroused, hyperactive, have decreased anxiety and antidepressant-like behaviour, whereas Govindarajan et al. (2013) do not observe any of these changes when comparing HDAC6 knockout mice to wild-type animals.

HDAC6 has been implicated in regulating the microtubule transport system in neurones. Microtubules are a major component of the cytoskeleton and are important for anterograde and retrograde axonal transport of molecules and organelles. The efficient movement of materials along axons is critical for neuronal function. Inhibition of HDAC6 leads to the hyperacetylation of α-tubulin (a constituent of microtubules); this enhances the recruitment of molecular motors to microtubules and consequently augments the transport of vesicles (Dompierre et al., 2007) and trafficking of mitochondria (Chen et al., 2010; Kim et al., 2012a) in axons of neurones.

1.8. HDAC inhibitors

Inhibitors of the zinc-dependent class I and II HDACs are classified into four groups on the basis of common pharmacophores; 1) short chain fatty acids, 2) hydroxamates, 3) cyclic tetrapeptides and 4) benzamides. By solving crystal structures of HDACs with bound HDAC inhibitors (reviewed by Micelli and Rastelli (2015)) we have an understanding of how these drugs interact with and inhibit HDACs. Each inhibitor comprises of 1) a zinc-binding domain, which chelates the Zn^{2+} cofactor in the active site and this chelation is responsible for inhibiting the catalytic activity of the HDAC, 2) a surface binding domain/cap which interacts with the entrance of the channel leading to the active site, thus blocking access for the natural substrate, and 3) a linker which connects the two domains and occupies the hydrophobic channel leading to the catalytic site (figure 1.3). Each of these pharmacophores is thought to contribute to the selectivity of HDAC inhibitors for particular HDAC classes and isoforms.

The short-chain fatty acids sodium butyrate, sodium phenylbutyrate and valproate (VPA) are non-selective inhibitors of both class I and class II HDACs with effective concentrations in the millimolar range (Gurvich et al. (2004) and reviewed by Dokmanovic et al. (2007)). In addition to HDAC inhibition, these inhibitors have other targets. Valproate for example, is an approved drug to treat epilepsy and its mode of action is thought to be through inhibiting voltage-gated sodium channels

and potentiating GABAergic signalling. These combined produce a therapeutic effect by depressing the excitability of the brain (reviewed by Rosenberg (2007)).

Hydroxamate HDAC inhibitors such as ITF2357 (Givinostat), suberoylanilide hydroxamic acid (SAHA, Vorinostat) and trichostatin A (TSA), inhibit HDACs through chelation of the zinc ion in the catalytic site of the HDAC via the hydroxamic acid group of the inhibitor (figure 1.3). Hydroxamate HDAC inhibitors are predominantly non-selective and inhibit HDACs in classes I and II with effective concentrations in the nanomolar concentration range (table 1.4).

Cyclic tetrapeptide HDAC inhibitors are so named for their cyclic structure of four non-proteinogenic amino acids that forms the surface binding group/cap of the inhibitor (figure 1.3). One key member of this inhibitor class is apicidin, which is selective for class I HDACs (table 1.4).

The final group of inhibitors, the benzamides, are some of the most selective HDAC inhibitors for class I HDACs that have been developed to date. These inhibitors are classified on the basis of their zinc-binding domain, the amino-benzamide group. MS-275 (Entinostat) is a well-studied example of this class, which is selective for the class I HDACs, HDAC1, 2 and 3 with nanomolar potency (table 1.4). Another benzamide HDAC inhibitor MI192, is selective for HDAC2 and 3 (half maximal inhibitory concentration (IC_{50}) of 4.8 μM, 30 nM, 16 nM, 5 μM, >10 μM, 4.1 μM and >10 μM for HDAC1, 2, 3, 4, 6, 7 and 8 respectively, Boissinot et al. (2012)). Benzamide HDAC inhibitors inhibit class I HDACs by co-ordinating the zinc ion in the catalytic site of the HDAC. The chemical moieties of the amino-benzamide group important for this are the nitrogen of the aniline amide and the oxygen of the amide carbonyl group (figure 1.3).

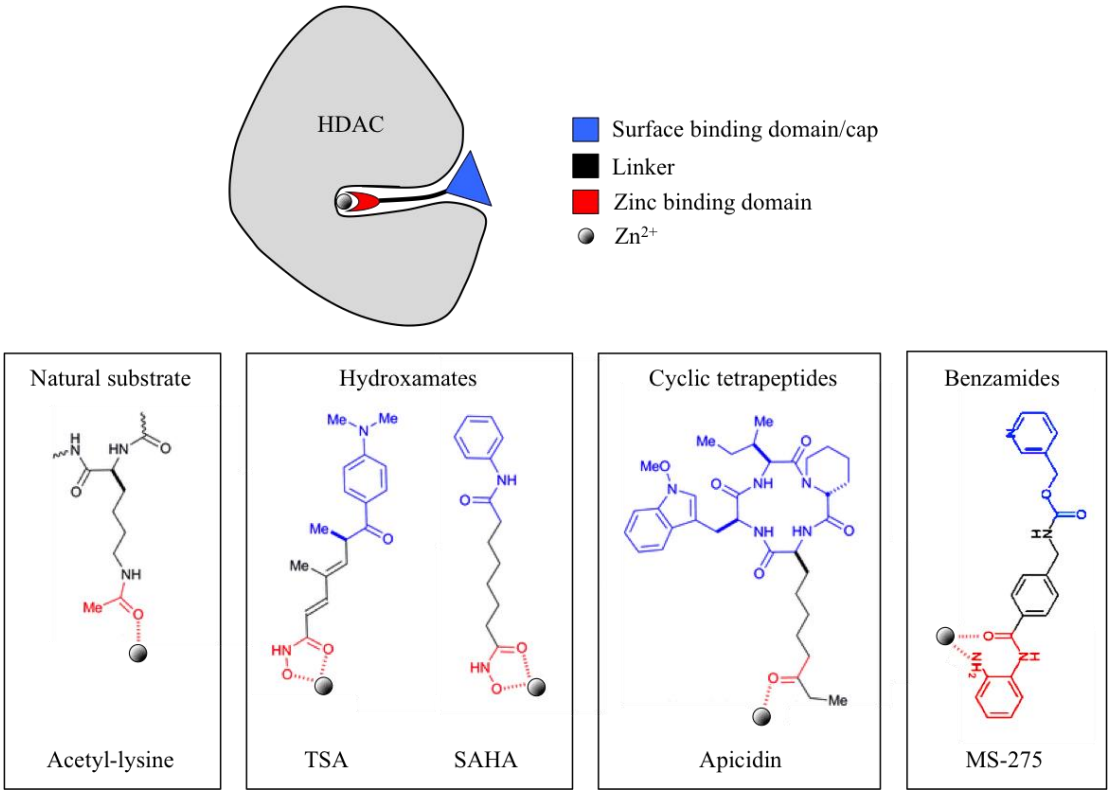


Figure 1.3. Structures of the representative histone deacetylase (HDAC) inhibitors and their pharmacophores. Adapted from Newkirk et al. (2009); Mottamal et al. (2015).

Table 1.4. Selectivity of HDAC inhibitors, (K_i ± SD, nM).

			H	**CT**	**B**
Class	**HDAC**	**TSA**	**SAHA**	**Apicidin**	**MS-275**
I	1	0.2 ± 0.045	1.3 ± 0.05	0.04 ± 4 × 10^{-3}	22 ± 2
	2	0.65 ± 0.05	1.6 ± 0.05	0.12 ± 3 × 10^{-3}	65 ± 5
	3	0.5 ± 0.1	5 ± 0.2	0.26 ± 5 × 10^{-3}	360 ± 15
	8	45 ± 15	480 ± 20	49 ± 20	>10,000
II	4	1400 ± 100	>10,000	>10,000	>10,000
	5	260 ± 35	3600 ± 380	>10,000	>10,000
	6	1 ± 0.1	1.6 ± 0.05	>10,000	>10,000
	7	195 ± 20	>10,000	>10,000	>10,000
	9	800 ± 100	>10,000	>10,000	>10,000

Abbreviations, H: Hydroxamate, CT: Cyclic tetrapeptide, B: Benzamide, SD: Standard deviation. Data taken from Bradner et al. (2010). No data for HDAC10 or 11.

The inhibitor profiles of HDAC inhibitors for HDACs discussed and shown in table 1.4 have been determined using individual recombinant HDACs and an acetylation reporter assay (Bradner et al., 2010). Bantscheff et al. (2011) have used a different approach looking at selectivity of HDAC inhibitors by performing chemoproteomic competition binding assays with human cell lysates. This technique first involved the development of a probe matrix based on hydroxamate HDAC inhibitors that would bind to and capture cellular HDACs and their associated complexes for subsequent identification and quantification by mass spectrometry. Using a competition binding approach with increasing concentrations of free-HDAC inhibitor, if the inhibitor is selective for the HDAC bound complex to the hydroxamate probe matrix, then the probe is outcompeted for binding and is displaced. This can be detected as a loss in signal for the HDAC and associated interacting proteins. This assay was performed for a range of HDAC inhibitors looking at the selectivity towards HDACs 1, 2, 3, 6, 8 and 10. For the majority of the HDAC inhibitors tested, the selectivity towards these particular isoforms is similar to that shown in other studies using reporter assays (table 1.4). But this study also revealed that HDAC inhibitors have selectivity towards HDACs in certain complexes they are members of (see section 1.4.4 for details on the different complexes). The non-selective class I and II HDAC inhibitor valproate, in order of selectivity for the HDAC containing complexes, preferentially binds to HDAC1 and 2 in the CoREST and NuRD containing complexes over HDAC3 in the complex NCoR, but does not bind to HDAC1 and 2 in the Sin3 complex. Whereas the non-selective HDAC inhibitors TSA and SAHA bind to HDACs in these four complexes equally. Likewise, the class I HDAC inhibitor apicidin does not preferentially bind to HDACs in either complex, but the class I HDAC1, 2 and 3 selective inhibitor MS-275, selectively binds to these isoforms in the NCoR, CoREST and NuRD (in order of selectivity) but not Sin3 complexes. How HDAC inhibitors, which selectively inhibit HDACs, have a preference for or can only bind to HDACs in certain HDAC-containing complexes is not understood. It has been suggested that the surface binding group/cap of the HDAC inhibitor confers selectivity to the specific complexes (Salisbury and Cravatt, 2007; Bantscheff et al., 2011). However, this hypothesis does not fit with the observations that SAHA and MS-275 despite having similar surface binding domains/caps (figure 1.3) have very different complex selectivities and also SAHA and apicidin which

have very different surface binding domains, have the same complex selectivities. This study has raised an interesting point, that when using HDAC inhibitors which inhibit HDAC1 and 2, the effects observed are not simply from inhibiting these isoforms, but are also from differentially inhibiting the HDAC1 and 2 containing complexes.

In addition to HDAC isoform selectivity, the binding kinetics of the different classes of HDAC inhibitors also differs, yet this is something that is often overlooked but may actually affect the physiological outcome of HDAC inhibition (Chou et al., 2008; Bressi et al., 2010; Lauffer et al., 2013). The association rate and dissociation rates of the benzamide HDAC inhibitor MS-275 are several orders of magnitude slower compared to the hydroxamate HDAC inhibitor SAHA and as a consequence the length of time it takes benzamide HDAC inhibitors to bind to and inhibit HDACs, as well as the length of time the inhibitors are bound to them is significantly longer (Lauffer et al., 2013). To explain the slow binding kinetics of benzamide HDAC inhibitors, it has been shown that an internal hydrogen bond in the zinc-binding domain of benzamide HDAC inhibitors, between the aniline amine and the amide carbonyl moieties, must be broken in order for the successful binding of benzamide inhibitors to a HDAC and coordination of the zinc ion in the active site (Bressi et al., 2010; Lauffer et al., 2013). This change in benzamide HDAC inhibitor structure must first take place in order for the inhibition of HDACs. The time it takes for this change to occur explains the slow binding kinetics of MS-275 and other benzamide HDAC inhibitors. The binding of hydroxamates such as SAHA to HDACs is much faster because structural rearrangements are not necessary for successful binding (Lauffer et al., 2013). A difference in a physiological response as a consequence of the different binding kinetics of hydroxamates and benzamides HDAC inhibitors is clearly demonstrated in the kinetics of acetylation of proteins over-time. The onset of histone acetylation and the time-dependent return of histone acetylation to the pre-HDAC inhibitor state are much slower with benzamide HDAC inhibitors compared to SAHA (Chou et al., 2008; Lauffer et al., 2013). These temporal differences in protein acetylation by these different classes of HDAC inhibitors, may affect the timings of functional effects, such as gene expression (Lauffer et al., 2013). Indeed, in cortical neurones, SAHA and MS-275 both increase

the expression of the neuroprotective and neurone-function enhancing protein BDNF, but do so at different times following treatment. SAHA rapidly induces the expression of BDNF during one hour, whereas BDNF upregulation following MS-275 treatment is significantly delayed, with expression increasing between three and six hours post-treatment (Koppel and Timmusk, 2013).

1.9. Effect of HDAC inhibition on gene expression in the brain

As mentioned in section 1.2.1, histone acetylation and deacetylation are traditionally associated with active/activation of gene expression and an inactive/inactivation of gene expression respectively. Lopez-Atalaya et al. (2013) have mapped the acetylation of histone H3 at lysine 9 and 14 (H3K9 & 14), histone H4 at lysine 12 (H4K12) and pan-acetylation of histone H2B (pan-H2B) for the whole-genome extracted from the mouse hippocampus in the basal state and in response to treatment of mice with the non-selective HDAC inhibitor TSA. In the basal state, all three acetylation marks are observed at the transcription start site (TSS), in introns and exons of genes (intragenic regions) and in non-transcribed DNA between genes (intergenic region). The abundance of these acetylated histone marks at TSSs and in the intragenic region of a gene correlates well with its level of expression. In the hippocampus of mice treated to TSA there was a significant but transient increase in global histone acetylation with an increase in the number of acetylation marks at H3K9 & 14, H4K12 and pan-H2B. Closer inspection of the topology of these changes reveals clear increases in histone acetylation at TSSs, intragenic regions and suspected gene expression enhancer regions but not intergenic regions. Genes in the hippocampus that are acetylated and actively expressed at the basal state undergo the greatest hyperacetylation in response to TSA, whereas poorly acetylated and low or non-expressed genes are largely unaffected. Furthermore, the overall impact of TSA on gene expression is actually very small, with changes in the expression of only 88 genes and of these, around equal numbers are upregulated and down-regulated. These observations are consistent with other studies looking at the effects of HDAC inhibitors on global histone acetylation in non-neuronal cells (Van Lint et al., 1996; Bernstein et al., 2000; Marks, 2004; Peart et al., 2005; Reid et al., 2005; Halsall et

al., 2012). Furthermore, of the 88 genes that are affected in the mouse hippocampus, TSA increases the acetylation of histones in the TSS of both the up and down-regulated genes. Together these findings contrast with the idea that histone acetylation is associated with active gene expression. The reasons behind these observations are poorly understood but a few possible explanations have been put forward, 1) HDAC inhibitors upregulate transcriptional repressor genes, which then down-regulate other genes, 2) HDAC inhibition causes the acetylation of non-histone substrates such as transcription factors, which positively or negatively regulate gene expression, 3) another explanation based on the fact that HDACs have been found to reside with HATs on active genes (Kurdistani et al., 2002; Wang et al., 2009b), is that HDACs deacetylate histones and reset the conformation of chromatin between rounds of transcriptional activation (Metivier et al., 2003; Kelly and Cowley, 2013; Dovey et al., 2010a). Therefore, preventing this resetting of chromatin structure with HDAC inhibitors will lead to the hyperacetylation of active genes and this may positively or negatively affect the transcription of those genes.

1.10. HDAC inhibitors as neuroprotective agents to treat cerebral ischaemia

There is a growing body of evidence to suggest a change in protein acetylation is part of the pathophysiology of neurodegenerative disease, brain insults and brain injuries. In the brains of rodents following ischaemia (Calderone et al., 2003; Ren et al., 2004; Faraco et al., 2006; Kim et al., 2007b; Wang et al., 2011a; Noh et al., 2012), in the spinal cord of a mouse model of motor neurone disease (Rouaux et al., 2003), in isolated cortical neurones following oxygen & glucose deprivation (OGD, Lanzillotta et al. (2013); Dmitriev and Papkovsky (2015)) or exposure to Aβ (Rouaux et al., 2003) and in cerebellar granule neurones (CGNs) following exposure to the apoptotic stimuli of low extracellular K^+ and glutamate excitotoxicity (Rouaux et al., 2003; Leng and Chuang, 2006), there is a consistent reduction in the acetylation of histones H3 and/or H4 (see figure 1.4 for an example). Potential causes of this deacetylation are changes in the expression of HDACs (see sections 3.1.1 to 3.1.7) and/or HATs (Rouaux et al., 2003; Rouaux et al., 2007; Yildirim et al., 2014).

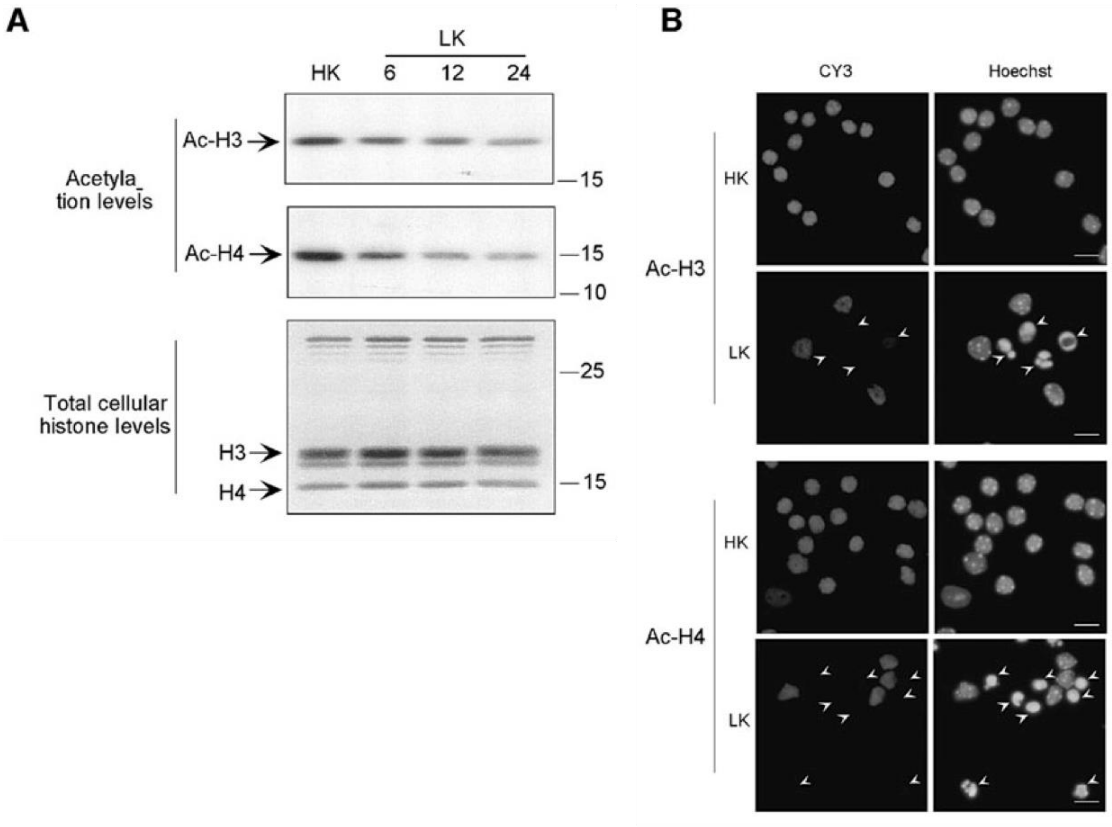


Figure 1.4. Histone acetylation levels decrease during neuronal apoptosis. Cerebellar granule neurones were maintained in survival conditions (high potassium, HK) or exposed to K^+ deprivation (low potassium, LK) in order to trigger apoptosis. **(A)** The acetylation state of histones H3 (Ac-H3) and H4 (Ac-H4) during 6, 12 and 24 hours exposure to the apoptosis stimulus was monitored by Western blot analysis. Total cellular histone protein expression levels was analysed by Coomassie staining and was found to be unaffected during neuronal apoptosis. Values quoted to the right of the blots & gel indicate the molecular weight in kDa. **(B)** Histone acetylation is specifically decreased in neurones undergoing apoptosis. Representative fluorescence images of Hoechst nuclei staining and immunostaining for Ac-H3 and Ac-H4 (indicated by cyanine 3 (CY3) labelling). Arrow heads indicate a reduction in histone acetylation in the condensed and fragmented nuclei of cells undergoing apoptosis. Scale bar 10 μm. Figure adapted from Rouaux et al. (2003) with permission and under license granted by the publisher John Wiley and Sons (license number: 3772490615009, issued on the 19th of December 2015). Copyright © 2003 European Molecular Biology Organization.

Cerebral ischaemia triggers the upregulation of the repressor element 1-silencing transcription factor (REST) in the rat hippocampus (Calderone et al., 2003; Noh et al., 2012) and rat cerebral cortex (Formisano et al., 2015). REST represses the expression of genes important for neuronal survival and function by recruiting the CoREST- and Sin3-HDAC1 & 2 containing repressor complexes to specific genes (Ooi and Wood, 2007). In cerebral ischaemia, REST and the multi-protein repressor complexes are found enriched at and inhibit the expression of many neuronal genes (Calderone et al., 2003; Formisano et al., 2015; Noh et al., 2012). Knockdown of REST, forced expression of a dominant negative competitor or combined knockdown of HDAC1 and 2, disinhibits REST-mediated repression of these genes and neurones are protected from ischaemia-induced death (Calderone et al., 2003; Formisano et al., 2015; Noh et al., 2012).

In neuronal models of apoptosis, ischaemia, Alzheimer's disease and motor neurone disease there is a consistent loss of CBP expression accompanied with histone deacetylation (Rouaux et al., 2003; Rouaux et al., 2007; Yildirim et al., 2014). Knocking down CBP in mouse cortical neurones exposed to OGD exacerbates neurone death (Yildirim et al., 2014) but overexpression of HATs CBP or p300 in neurones is neuroprotective (Rouaux et al., 2003). Moreover, CBP is actively upregulated in surviving neurones after ischaemia (Jin et al., 2001) and can be found enriched at the neuroprotective gene gelsolin (Yildirim et al., 2008; Yildirim et al., 2014). Taken together, these data suggest CBP activity is important for neurone survival and increasing the activity of HATs in conditions where it is reduced may be of therapeutic benefit. In support of the role of protein deacetylation in the pathogenesis of cerebral ischaemia and preventing deacetylation or augmenting acetylation is a viable neuroprotective strategy; HDAC inhibitors have been shown to be neuroprotective in a variety of models relevant to this brain insult and injury.

1.10.1. A brief overview of the pathophysiology of cerebral ischaemia

Cerebral ischaemia is caused by a reduction in blood flow to a region of the brain usually as a result of the occlusion of a cerebral artery. This leaves brain tissue unsupplied with oxygen and glucose, which causes cellular energy failure followed by a highly complex series of cellular, temporal and spatial events leading to neural cell and tissue dysfunction and death. This can be fatal to the individual, but those who survive the insult have permanent disabilities and neurological impairments reflective of the extent and location of the ischaemic damage in the brain. This ischaemic injury is temporal and spatial in nature. Within minutes there is rapid onset of tissue damage and death in the immediate region of the brain which the blocked blood vessel supplies. Here the cells that are damaged and are dying cannot be saved and this forms a lesion known as the ischaemic core. Over-time the pathogenesis spreads from the initial site to the surrounding tissue where energy metabolism is partially preserved. This forms a damaged and dying region called the penumbra (Barone and Feuerstein, 1999; Dirnagl et al., 1999; Lo et al., 2003). It is generally considered that there is a short therapeutic window to use pharmacological agents to prevent neural cell death and dysfunction in the penumbral region.

In the ischaemic core, cellular energy failure prevents the maintenance of the finely balanced membrane potential, crucial for proper functioning and survival in neural cells. The Na^+/K^+-ATPase and the Ca^{2+}-ATPase are energy-dependent pumps that maintain the membrane potential, but because of the reduction of adenosine triphosphate (ATP) synthesis during ischaemia, these pumps no longer function efficiently and neural cells depolarise due to Na^+ and Ca^{2+} accumulation in the cell. This triggers excessive glutamate release into the extracellular space by synaptic vesicle release from neurones as well as the reversal of the sodium-dependent, electrochemical gradient coupled, excitatory amino acid transporters (EAATs) found in the membranes of neurones and astrocytes. An increase in the levels of extracellular glutamate then causes a phenomenon called excitotoxicity (Dirnagl et al., 1999; Lo et al., 2003). This involves over- and chronic-activation of the AMPA, *N*-Methyl-D-aspartic acid (NMDA), Kainate and metabotropic glutamatergic receptors and collectively this facilitates a further influx of Na^+ and Ca^{2+} into the cell

and also the release of Ca^{2+} from intracellular stores. Described simply, this calcium overload causes neural cell death by activating proteases, lipases and endonucleases, which damage and break down the cell. Calcium overload also causes free radical production, which in turn leads to oxidative and nitrosative stress and this also damages DNA, proteins and lipids. As a consequence, the protective systems in neural cells within the ischaemic core are overwhelmed and cells die predominantly by necrosis and release their intracellular contents including toxic species into the extracellular space. These species then trigger the death of other neural cells, which leads to the exacerbation and spreading of injury and neural cell death (Dirnagl et al., 1999; Lo et al., 2003).

Neural cells in the surrounding penumbral region are confronted with propagating glutamate excitotoxicity, oxidative stress and toxic species from the ischaemic core, these damage cells and initiate delayed apoptotic cell death (Dirnagl et al., 1999; Lo et al., 2003). Furthermore, over the next few hours and days following the initial ischaemic attack, as the damage and death is spreading and accumulating, cellular damage, cells undergoing apoptosis, necrotic debris and spillage, and the production of inflammatory mediators by injured neural cells, all activate microglia which are the resident immune cells of the brain. In addition, ischaemic damage increases the permeability of the blood brain barrier, resulting in the infiltration of leukocytes, such as macrophages from the systemic circulatory immune system. Together, these innate immune cells accumulate in the ischaemic core and penumbra, and initiate an inflammatory response mediated by the NF-κB and MAPK (mitogen-activated protein kinase) signalling pathways. Upon activation of these pathways, these immune cells express and release of pro-inflammatory mediators including the cytokines; tumour necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6) and produce oxidative and nitrosative species amongst other things. These inflammatory mediators can be toxic to neurones and other neural cells such as oligodendrocytes, leading to secondary neural cell damage and death and further amplification and spreading of the injury (reviewed by Barone and Feuerstein (1999); Ceulemans et al. (2010); Dirnagl et al. (1999); Lakhan et al. (2009); Lo et al. (2003); Wang et al. (2007); Zheng and Yenari (2004)).

Over-time, there are also changes in the expression of many genes in neural cells of the penumbral region in response to cerebral ischaemia, including immediate early response genes, pro- and anti-apoptotic genes, growth factors, pro-and anti-inflammatory genes and many others (reviewed by Papadopoulos et al. (2000); Read et al. (2001)). These changes and the balance between pro-death and pro-survival gene expression contributes to the fate of neural cells in the penumbral region of the ischaemic brain. Therefore modulating these transcriptional programs may be a useful strategy to protect neural cells from ischaemia-induced damage and death.

In summary, cerebral ischaemia causes a complex cascade of spatial and temporal events including glutamate excitotoxicity, oxidative stress, necrosis, apoptosis and neuroinflammation. The ideal treatment regime using pharmacological agents would target all of these pathophysiological mechanisms in order to protect neural cells of the penumbra.

1.10.2. HDAC inhibitors are neuroprotective in in vivo models of cerebral ischaemia

The neuroprotective effects of HDAC inhibitors in *in vivo* mouse and rat models of cerebral ischaemia (middle cerebral artery occlusion (MCAO) and four-vessel occlusion (4-VO)) have been extensively reviewed (Baltan et al., 2013; Langley et al., 2009; Schweizer et al., 2013; Shein and Shohami, 2011; Ziemka-Nalecz and Zalewska, 2014). In general, when either administered immediately at the time of, or post-treated up to 7 hours after the onset of ischaemia, HDAC inhibitors have a plethora of therapeutic effects. HDAC inhibitors significantly protect neurones and oligodendrocytes, reduce brain damage and infarct volume (Faraco et al., 2006; Kim et al., 2009; Kim et al., 2007b; Qi et al., 2004; Ren et al., 2004; Wang et al., 2011a; Wang et al., 2011b; Kim and Chuang, 2014; Lanzillotta et al., 2013; Liu et al., 2012; Murphy et al., 2014; Noh et al., 2012; Xuan et al., 2012), improve functional recovery (Faraco et al., 2006; Kim et al., 2009; Kim et al., 2007b; Liu et al., 2012; Qi et al., 2004; Ren et al., 2004; Wang et al., 2012; Xuan et al., 2012; Yildirim et al., 2008), restore the integrity of the blood brain barrier (Wang et al., 2011b) and also improve blood flow in the ischaemic brain (Kim and Chuang, 2014; Wang et al., 2012). In the majority of these studies, HDAC inhibition prevents ischaemia-induced

loss of histone acetylation and in some instances increases it above normal levels. Overall, there is a reported decrease in neural cell apoptosis, which coincides with increases in anti-apoptotic mediators including heat-shock protein 70 (HSP70, Faraco et al. (2006); Kim et al. (2007b); Ren et al. (2004)), gelsolin (Yildirim et al., 2008) and B-cell lymphoma 2 (Bcl-2, Faraco et al. (2006); Kim et al. (2007b); Ren et al. (2004)), as well as a decrease in the expression of the apoptosis inducer p53 (Kim et al., 2007b) and a reduction in the activity of capsases (Kim and Chuang, 2014; Qi et al., 2004; Ren et al., 2004). There is also an anti-inflammatory effect, observed as a reduction in the expression of pro-inflammatory genes TNF-α (Xuan et al., 2012), IL-1β (Xuan et al., 2012), inducible nitric oxide synthase (iNOS, Kim et al. (2007b)) and cyclooxygenase-2 (COX-2, Kim et al. (2007b)), and also a suppression of microglial activation and accumulation (Kim et al., 2007b; Kim and Chuang, 2014; Xuan et al., 2012).

Despite these observations, the molecular mechanisms underlying them are not fully understood. Furthermore, an analysis of the global changes in the acetylome in response to cerebral ischaemia and HDAC inhibitor treatment has not been performed. It is important that this is not overlooked, because many of the reported neuroprotective effects of HDAC inhibitors could arise from an increase in the acetylation of non-histone proteins. The neuroprotective transcription factor, nuclear factor-like 2 (Nrf2) is an example. TSA is known to increase the acetylation of Nrf2, enhancing its transcriptional activity, which leads to an increase in the expression of neuroprotective antioxidant genes (Wang et al., 2011a).

Because HDAC inhibitors in *in vivo* models of cerebral ischaemia target multiple pathophysiological components, the use of these compounds as therapeutic agents looks promising. Unfortunately, the majority of the *in vivo* studies performed to date have used the non-selective HDAC inhibitors SAHA, sodium butyrate, sodium phenylbutyrate, TSA and VPA. This means we do not know which HDAC or HDACs when inhibited are responsible for the neuroprotection observed and this also makes it difficult to isolate and determine the molecular mechanisms underlying this neuroprotection. Furthermore, clinically available non-selective HDAC inhibitors are associated with adverse side effects (see Schweizer et al. (2013) for a

review and see section 5.1), which also emphasises the need to identify the appropriate HDAC isoform(s) to inhibit. Lanzillotta et al. (2013) and Murphy et al. (2014) have made some progress with respect to this problem and show that the class I HDAC1, 2 and 3 inhibitor MS-275, is neuroprotective in the mouse MCAO model of cerebral ischaemia. This finding warrants further work to investigate the mechanisms underlying the neuroprotection provided by inhibiting HDACs 1, 2 and 3, and to also determine which of these HDACs, upon inhibition, is responsible for this neuroprotective effect.

1.10.3. HDAC inhibitors are neuroprotective in models of oxygen & glucose deprivation (OGD)

During cerebral ischaemia, the fundamental pathological event that occurs is the starvation of neural cells of oxygen and glucose. This is often mimicked to simulate ischaemia-induced neural cell death in *ex vivo* nervous tissue and *in vitro* neural cell culture model systems. Using these systems, in comparison to *in vivo* models, allows for the studying of the responses of specific cell types, cell populations and their interactions during ischaemia, and upon pharmacological intervention in a more controlled and easy to manipulate environment.

Histone deacetylase inhibitors exhibit neuroprotective effects in several *ex vivo* and *in vitro* models of OGD. When TSA is applied immediately after inducing OGD in organotypic slice cultures of rat hippocampus, this significantly reduces the amount of ischaemia-induced neurone cell death (Noh et al., 2012). HDAC inhibition also protects isolated neurone cultures from OGD. Pre-treating mouse or rat cortical neurones with TSA for one to twelve hours prior to exposing to OGD is neuroprotective (Meisel et al., 2006; Wang et al., 2011a). Here, TSA increases the acetylation of histone proteins and there is a positive correlation between surviving neurones and histone acetylation state (Meisel et al., 2006). On closer inspection, the acetylation of histones in the gene promoter for the neuroprotective protein gelsolin is enhanced by TSA treatment and this coincides with an increase in gelsolin expression (Meisel et al., 2006). In order to determine which specific HDAC isoforms must be inhibited to have neuroprotective effects, Lanzillotta et al. (2013)

and Formisano et al. (2015) have examined the effects of treating cortical neurones after OGD with the HDAC1, 2 and 3 selective inhibitor MS-275. Here, post-treatment of MS-275 is neuroprotective and recovers an observed OGD-induced reduction in histone acetylation (Lanzillotta et al., 2013). From a neuroprotective mechanism standpoint, MS-275 or combined HDAC1 and 2 knockdown, increases the expression of the Na^{+}/Ca^{2+} exchanger (NCX, Formisano et al. (2015)) and this may lead to neuroprotection by enhancing the extrusion of toxic intracellular calcium from neurones.

Neurological recovery following cerebral ischaemia is dependent on the maintenance and repair of neurone axons and dendrites, connectivity between neurones and white matter integrity in the brain. Depriving mouse optic nerve cultures of oxygen and glucose is a useful model of ischaemia-induced white matter damage that allows for the studying of axon integrity and function following ischaemia. Pre-treating with HDAC inhibitors (TSA or MS-275) before inducing OGD, or administering them immediately after OGD, has a number of neuroprotective effects in this model of white matter. These include reducing glutamate release by neural cells, preventing ATP depletion and preserving mitochondrial function. These combined, protect oligodendrocytes, preserve axon integrity, maintain white matter cellular architecture and promote functional recovery of neurones (Baltan et al., 2011b; Murphy et al., 2014). Similarly, Kim et al. (2010) show MS-275 improves mitochondrial transport and prevents neurite beading in the axons of cortical neurones exposed to the neurotoxins glutamate and TNF-α. Furthermore, treating rat cortical neurones with VPA or TSA immediately after exposing to OGD, prevents neurone apoptosis and also partially recovers the number and extent of neurites over a period of seven days post-insult (Hasan et al., 2013). This coincides with an increase in histone acetylation, expression of genes involved in synaptic function (e.g. synaptophysin) and an increase in the expression of the neurotrophin BDNF (Hasan et al., 2013). Other studies have also shown VPA, SAHA, MS-275 or knockdown of HDAC1 increases BDNF expression in rat cortical neurones (Koppel and Timmusk, 2013; Yasuda et al., 2009).

Together, these studies show that non-selectively inhibiting HDACs or selectively inhibiting class I HDACs 1, 2 and 3, prevents neurones and oligodendrocytes from undergoing OGD-induced death and also preserves and enhances neurone connectivity and function.

1.10.4. HDAC inhibitors are neuroprotective in models of glutamate excitotoxicity, oxidative stress and apoptosis

In cerebral ischaemia and in other brain conditions such as traumatic brain injury, Alzheimer's disease, motor neurone disease, multiple sclerosis and Parkinson's disease, neural cells die by apoptosis caused by processes such as glutamate excitotoxicity and oxidative stress (reviewed by Dong et al. (2009); Lau and Tymianski (2010)). *In vitro* and *ex vivo* models that mimic these pathophysiological events have also been used to investigate the neuroprotective effects of HDAC inhibitors.

One such model of motor neurone disease involves treating rat spinal cord slice cultures to L-*trans*-Pyrrolidine-2,4-dicarboxylic acid (PDC) an inhibitor of EAATs 1-5. Following this, there is a loss of motor neurones caused by glutamate excitotoxicity. In this model, pre-treating slices with VPA for seven days before exposing to PDC significantly protects motor neurones in the slice (Sugai et al., 2004). HDAC inhibitors are also neuroprotective in isolated neuronal models of glutamate excitotoxicity. Pre-treating rat cortical neurones to HDAC inhibitors for three days protects from glutamate-induced excitotoxicity. Here, VPA increases histone acetylation at the promoter of and subsequently increases the expression of the neuroprotective protein HSP70 (Marinova et al., 2009). Other non-selective HDAC inhibitors sodium butyrate and TSA, as well as the selective class I HDAC inhibitors apicidin and MS-275, also increase HSP70 expression in cortical neurones (Marinova et al., 2009). Co-treatment of TSA, sodium butyrate, SAHA or apicidin at the same time as homocysteate (a glutamate analogue), protects cortical neurones from homocysteate-induced oxidative stress and apoptosis (Ryu et al., 2003; Sleiman et al., 2014). This neuroprotection is partly dependent on a HDAC inhibition induced increase in the acetylation and activity of the transcription factor Specificity protein 1

(Sp1, Ryu et al. (2003)). CGNs also die following exposure to high levels of glutamate or an inhibitor of the glutamate re-uptake transporters (SYM 2081). Pre-treating CGNs for two to seven days with the non-selective HDAC inhibitors VPA, sodium butyrate, sodium phenylbutyrate or TSA, prior to the induction of glutamate excitotoxicity, prevents these neurones from undergoing cell death (Kanai et al., 2004; Leng and Chuang, 2006). Glutamate excitotoxicity in CGNs results in a decrease in histone acetylation and HDAC inhibition restores this (Leng and Chuang, 2006). More specifically, neuroprotection coincides with the hyperacetylation of histones in the promoter of the α-synuclein gene and this correlates with an increase in the expression of this neuroprotective protein (Leng and Chuang, 2006).

HDAC inhibition also protects isolated neurones from classical inducers of apoptosis. Non-selective HDAC inhibitors including TSA, SAHA and sodium butyrate when administered at the same time as the p53-dependent apoptosis inducing, DNA damaging agent camptothecin, prevents the apoptosis of isolated cortical neurones (Brochier et al., 2013; Uo et al., 2009). Furthermore, selective inhibition of HDACs 1, 2 & 3 with MS-275, prevents the accumulation of activated p53 and caspase 3 in cortical neurones exposed to camptothecin (Murphy et al., 2014). Non-selective HDAC inhibitors also prevent the apoptosis of cortical neurones following exposure to p53-independent apoptosis inducing agents such as the protein kinase inhibitor staurosporine (Uo et al., 2009).

1.10.5. HDAC inhibition reduces neuroinflammation

There is strong evidence to implicate microglia-mediated neuroinflammation in the pathogenesis of cerebral ischaemia (see section 1.10.1), traumatic brain injury, Alzheimer's disease, motor neurone disease, multiple sclerosis and Parkinson's disease (reviewed by Block and Hong (2005); Block et al. (2007); Glass et al. (2010); Smith et al. (2012)). It is generally considered that limiting the pro-inflammatory phenotype of microglia will be beneficial in treating these brain insults and diseases. HDAC inhibitors have been shown to reduce neuroinflammation in rodent models of cerebral ischaemia (see section 1.10.2) and in other *in vivo* models where they are neuroprotective including traumatic brain injury, Alzheimer's disease (amyloidosis model), multiple sclerosis and Parkinson's disease (table 1.5).

In a mouse model of traumatic brain injury, post-treatment of the non-selective HDAC inhibitor ITF2357 reduces microglia and macrophage accumulation in the hippocampus of damaged brains (Shein et al., 2009). Analysing the different microglial phenotypes in the corpus callosum following traumatic brain injury, the non-selective hydroxamate HDAC inhibitor Scriptaid, reduces the number of the neurotoxic pro-inflammatory M1-phenotype microglia but increases the number of anti-inflammatory neuroregenerative M2-phenotype microglia (Wang et al., 2015). This coincides with a reduction in the expression of the pro-inflammatory mediators TNF-α, iNOS, nitric oxide (NO) and IL-6, and an increase in the expression of the anti-inflammatory cytokine interleukin-10 (IL-10) (Wang et al., 2015). This polarisation of inflammatory cells to an anti-inflammatory M2-phenotype following a reduction in the activity of HDACs has also been observed in macrophages. In these cells, deletion of HDAC3 leads to a gene expression program characteristic of the M2 phenotype (Mullican et al., 2011). Experimental autoimmune encephalomyelitis (EAE) and neuritis (EAN) are *in vivo* models of the chronic neuroinflammatory condition multiple sclerosis. In the encephalomyelitis model, TSA suppresses the infiltration of inflammatory cells in demyelinating spinal cords (Camelo et al., 2005). Also, in demyelinating sciatic nerves, a model of EAN, MS-275 attenuates inflammatory cell infiltration and expression of pro-inflammatory

mediators IL-1β, interferon gamma (IFNγ) and iNOS, and also the extracellular matrix degrading protein; matrix metalloproteinase-9 (MMP-9, Zhang et al. (2010)).

Looking at these *in vivo* models in isolation, it is difficult to interpret whether HDAC inhibitors reduce the inflammatory response because they protect neural cells and therefore reduce the stimulus for microglial activation, or if HDAC inhibitors directly affect the intracellular inflammatory pathways in microglia, or suppress neuroinflammation through both mechanisms. Many studies have now shown that HDAC inhibitors affect the inflammatory response of isolated microglia (table 1.5). In models physiologically relevant to cerebral ischaemia, pre-treatment of the HDAC inhibitors sodium phenylbutyrate or TSA has been shown to suppress pro-inflammatory mediator expression, including TNF-α, IL-1β, IL-6 and iNOS in isolated microglia and astrocytes when exposed to OGD (Qi et al., 2004) or hypoxia (Niu et al., 2009). HDAC inhibitors also reduce the activation and inflammatory response of microglia isolated from several species in response to the generic inflammatory stimulus lipopolysaccharide (LPS). Kannan et al. (2013) show pre-treatment of isolated mouse microglia cultures with SAHA or TSA for two hours prior to stimulating with LPS, increases histone acetylation and reduces the expression of LPS-induced pro-inflammatory mediators IL-6, TNF-α, IL-1β, iNOS and transforming growth factor-beta (TGF-β). TSA pre-treatment also suppresses the activation of microglia, measured as a reduction in pro-inflammatory cell surface marker expression (Kannan et al., 2013). Likewise, in isolated human microglia cultures, pre-treatment of TSA for one hour prior to LPS stimulation also reduces IL-1β, TNF-α and IL-6 expression (Suh et al., 2010). Also, pre-treatment of isolated rat microglia with sodium butyrate or TSA for one day prior to LPS, attenuates the expression of IL-6, TNF-α and NO (Huuskonen et al., 2004). Co-treatment of mouse microglia and astrocyte co-cultures with LPS and the non-selective HDAC inhibitors ITF2357 or SAHA, increases histone acetylation and reduces the inflammatory response as indicated by a reduction in iNOS, COX-2, IL-1β and TNF-α expression (Faraco et al., 2009). Furthermore, post-treatment of ITF2357 reduces the expression of iNOS and IL-1β in the striatum brain region of mice injected with LPS (Faraco et al., 2009).

Chronic neuroinflammation in cerebral ischaemia and other insults and neurodegenerative disease is thought be toxic to neurones. Therefore by inhibiting the inflammatory response of microglia, HDAC inhibitors may prevent neuronal death caused by neuroinflammation. In mesencephalic/midbrain neurone-glia cultures, LPS treatment induces the activation of microglia, expression of pro-inflammatory mediators TNF-α and nitrite and this causes the death of dopaminergic neurones (Peng et al., 2005). Not surprisingly, pre-treatment of these cultures with TSA or sodium butyrate for one day or VPA for two days prior to the LPS insult, suppresses the subsequent LPS-induced microglial activation and pro-inflammatory mediator expression resulting in the protection of dopaminergic neurones (Chen et al., 2007; Peng et al., 2005).

To summarise so far, HDAC inhibitors are anti-inflammatory in *in vivo* models of brain insults, injuries and neurodegenerative disease, and several studies have shown non-selective HDAC inhibitors also suppress the inflammatory response of isolated microglia. However, there are other reports that show HDAC inhibitors enhance the inflammatory response of isolated microglia (table 1.5). Pre-treatment of sodium butyrate or TSA as mentioned, suppresses the inflammatory response of isolated rat microglia exposed to LPS, however surprisingly in the same study, pre-treatment of sodium butyrate prior to LPS exposure in mouse N9 immortalised microglia, augments pro-inflammatory mediator expression (Suuronen et al., 2003). The same laboratory also show that the LPS-induced expression of inflammatory mediators IL-6 and NO in mouse N9 immortalised microglia cells is potentiated when either sodium butyrate (Huuskonen et al., 2004), TSA or VPA (Suuronen et al., 2003) are administered at the same time as LPS. Moreover, in isolated rat microglia exposed to LPS, the expression of IL-6 and the production of nitrite are increased by TSA or sodium butyrate treatment (Huuskonen et al., 2004; Suuronen et al., 2003). Furthermore, co-treatment of LPS and non-selective HDAC inhibitors TSA, SAHA and sodium butyrate potentiate the LPS evoked inflammatory response in rat hippocampal slice cultures (Huuskonen et al., 2004; Suuronen et al., 2003). Another study that has investigated the effects of HDAC inhibitors on the inflammatory response of isolated microglia from rats, shows that pre-treatment of VPA or sodium butyrate for thirty minutes prior to LPS exposure, increases histone acetylation and

potentiates the LPS-induced expression of pro-inflammatory prostaglandins and the enzymes that produce them (e.g. COX-2). The expression of other pro-inflammatory mediators such as IL-6, IL-1β and TNF-α is also potentiated (Singh et al., 2014). These HDAC inhibitors also decrease the expression of the anti-inflammatory cytokine IL-10 (Singh et al., 2014).

To date, all investigations of the effects of HDAC inhibitors on the inflammatory response of isolated microglia have used inhibitors that lack selectivity towards particular HDAC isoforms (table 1.4 and section 1.8). Therefore, the contrasting observations of anti- and pro-inflammatory effects upon treatment with these HDAC inhibitors may be a result of non-selective HDAC inhibition and variability in the balance between inhibiting HDACs that promote inflammation and inhibiting those that suppress it. It is important then, that we identify which HDAC isoforms are the appropriate ones to inhibit in microglia, in order to isolate the therapeutically beneficial anti-inflammatory and neuroprotective effect from a pro-inflammatory one, which could exacerbate neuronal injury.

Table 1.5. The effects of histone deacetylase inhibitors in experimental models of neuroinflammation.

Model		Stimulus	HDAC inhibitors	Treatment	Anti or Pro-inflammatory?	Ref
In vitro	Microglia	LPS	TSA	Co-	Pro	1
		LPS	SB, VPA	Pre-, Co-	Anti & Pro	2
		Hypoxia	SPB	Pre-	Anti	3
		LPS	VPA	Pre-	Anti	5
		LPS	SB, TSA, VPA	Pre-	Anti	6
		LPS	ITF2357, SAHA	Co-	Anti	8
		LPS	TSA, VPA	Pre-	Anti	10
		LPS or MPP+	SPB	Pre-	Anti	12
		LPS	SAHA, TSA	Pre-	Anti	14
		LPS	SB, VPA	Pre-, Post-	Pro	17
In vivo	Mouse	EAE	TSA	Post-	Anti	4
	Rat	MCAO	SB, VPA	Post-	Anti	7
	Mouse	TBI	ITF2357	Post-	Anti	9
	Rat	EAN	MS-275	Post-	Anti	11
	Mouse	MPTP	SPB	Pre-	Anti	12
	Rat	4-VO	VPA	Post-	Anti	13
	Mouse	Amyloidosis	MS-275	Post-	Anti	15
	Rat	MCAO	SB	Post-	Anti	16
	Mouse	TBI	SAHA, VPA, Scriptaid	Post-	Anti	18

Abbreviations, 4-VO: Four-vessel occlusion model of cerebral ischaemia, EAE & EAN: Experimental autoimmune encephalomyelitis & neuritis respectively, LPS: Lipopolysaccharide, MCAO: Middle cerebral artery occlusion model of cerebral ischaemia, MPP+: 1-methyl-4-phenylpyridinium & MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (neurotoxin models of Parkinson's disease), OGD: Oxygen & glucose deprivation, SAHA: Suberoylanilide hydroxamic acid, SB: Sodium butyrate, SPB: Sodium phenylbutyrate, TBI: Traumatic brain injury, TSA: Trichostatin A & VPA: Valproate. References: [1]Suuronen et al. (2003), [2]Huuskonen et al. (2004), [3]Qi et al. (2004), [4]Camelo et al. (2005), [5]Peng et al. (2005), [6]Chen et al. (2007), [7]Kim et al. (2007b), [8]Faraco et al. (2009), [9]Shein et al. (2009), [10]Suh et al. (2010), [11]Zhang et al. (2010), [12]Roy et al. (2012), [13]Xuan et al. (2012), [14]Kannan et al. (2013), [15]Zhang and Schluesener (2013), [16]Kim and Chuang (2014), [17]Singh et al. (2014) & [18]Wang et al. (2015).

1.11. Aim of this study

Non-selective and class I selective HDAC inhibitors are neuroprotective in *in vivo* models of cerebral ischaemia, other brain insults, brain injuries and neurodegenerative disease. However, the specific HDAC isoform(s) which when inhibited is/are responsible for this effect and the exact cellular and molecular mechanisms involved are not fully understood. The aim of this thesis is to use *in vitro* neural cell model systems and pharmacological and genetic tools to 1) determine the specific HDAC isoform(s) that should be selectively inhibited in order to have a neuroprotective effect and 2) to investigate the mechanisms underlying neuroprotection following this selective HDAC inhibition.

Chapter 2

Experimental Procedures

2.1. Primary rat cerebellar granule neurone (CGN) culture

All work with animals was carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. Seven-day old Wistar rats were sacrificed by cervical dislocation followed by decapitation as outlined in the Schedule 1 guidelines. The brain was rapidly removed and placed into ice-cold 0.22 μm filter-sterilized (Millipore) Dissection Solution [calcium, magnesium and phenol red-free Hanks Balanced Salt Solution (HBSS, Sigma), 50U penicillin/50 μg streptomycin (Sigma) and 10 mM HEPES (Sigma)]. From this point all work was performed in a Class II tissue culture hood.

The cerebellum was dissected away from the cerebrum and transferred into fresh ice-cold, filter-sterilized Dissection Solution. The meninges and vasculature were carefully removed using fine forceps before washing the cerebellum in fresh Dissection Solution. The cerebellum was subsequently mechanically and enzymatically dissociated, first using a sterile razor blade followed by incubation for 15 minutes at 37°C in 10 mL of pre-warmed Trypsin Solution [HBSS, 50U penicillin/50 μg streptomycin, 10 mM HEPES and 250 μg/mL trypsin from bovine pancreas (Sigma)] with gentle agitation every 3 minutes.

To terminate trypsin digestion, 10 mL of filter-sterilized and pre-warmed to 37°C Trypsin Inhibitor Solution [Dulbecco's Modified Eagle's Medium (DMEM, variant: high glucose (4500 mg/L), with sodium pyruvate and sodium bicarbonate but without L-glutamine, Sigma), 50U penicillin/50 μg streptomycin, 10 mM HEPES, 10% v/v horse serum (Sigma), 50 μg/mL deoxyribonuclease I from bovine pancreas (DNase I, Sigma) and 1.5 mM $MgSO_4$ (Sigma)] was added to the cell suspension which was then centrifuged at 1600 RPM/400 × *g* for 5 minutes at room temperature (Eppendorf Centrifuge 5702 with a A-4-38 rotor).

The supernatant was discarded and using a P1000 pipette and tip, the cell pellet was re-suspended by triturating 15 times into 1 mL of filter-sterilized, pre-warmed to 37°C DNase Solution [DMEM, 50U penicillin/50 μg streptomycin, 10 mM HEPES, 10% v/v horse serum, 50 μg/mL DNase I, 1.5 mM $MgSO_4$, 2 mM L-glutamine

(Sigma) and 19 mM KCl (Sigma)]. Another 1 mL of DNase Solution was added and the suspension was triturated 15 times using a sterile glass 230 mm Pasteur pipette (SLS). A further 3 mL of DNase Solution was added 3 times in 1 mL additions, with each addition followed by triturating 15 times with a glass Pasteur pipette. The cell suspension was filtered through a 40 μm cell strainer (BD Bioscience) and the volume made up to 20 mL and then centrifuged at 1600 RPM/400 × *g* for 5 minutes at room temperature. The cell pellet was re-suspended in 1 mL of filter-sterilized, pre-warmed to 37°C Culture Medium A [DMEM, 50U penicillin/50 μg streptomycin, 10 mM HEPES, 10% v/v horse serum, 2 mM L-glutamine and 19 mM KCl]. The number of cells was counted using a haemocytometer (Hawksley) and the cell suspension was diluted accordingly with Culture Medium A.

For experiments using the YOYO®-1 & SYBR® Green I viability assay (described in section 2.5), the cell suspension was diluted to give 1.2×10^6 cells/mL and 120,000 cells/well (100 μL was taken) were seeded into poly-d-lysine coated, black-walled, clear-bottomed 96-well plates (BD Bioscience). For immunocytochemistry assays, prior to CGN isolation, 13 mm glass coverslips (VWR) were autoclaved, placed into 24-well plates (Greiner) and incubated with 100 μg/mL poly-d-lysine hydrobromide (Sigma, dissolved in sterile de-ionized H_2O) overnight at 37°C in a humid atmosphere with 5% CO_2. Each well was then washed 2 times with sterile de-ionized H_2O and air-dried before seeding cells. For 24-well plates, the cell suspension was diluted to give 1.44×10^6 cells/mL and 720,000 cells/well were seeded (500 μL was taken). For oxygen glucose deprivation (OGD) experiments, prior to CGN isolation, wells of 4-well plates (Nunc) were coated with 100 μg/mL poly-d-lysine overnight, then washed and air-dried as described before. For 4-well plates, the cell suspension was diluted to give 1.8×10^6 cells/mL and 720,000 cells/well were seeded (400 μL was taken). For histone protein extractions, prior to CGN isolation, 6-well plates (Greiner) were also coated with 100 μg/mL poly-d-lysine overnight then washed and air-dried before seeding. For 6-well plates, the cell suspension was diluted to give 2.4×10^6 cells/mL and 3.6×10^6 cells/well were seeded (1.5 mL was taken).

After seeding the appropriate cell numbers in the appropriate volumes for each cell culture vessel, the cultures were incubated at 37°C in a humid atmosphere with 5% CO_2. Twenty-four hours after seeding, the culture medium was topped up to double the volume in each well with fresh filter-sterilized, pre-warmed to 37°C Culture Medium A supplemented with 10 μM cytosine β-D-arabinofuranoside hydrochloride (ARAC, Sigma). After 2 days *in vitro* (DIV), 60% of the culture medium was replaced with fresh Culture Medium A supplemented with 10 μM ARAC. On 3 and 4 DIV, the cell cultures were supplemented with ARAC to maintain the concentration at 10 μM. At 5 and 8 DIV, 60% of the culture medium was replaced with filter-sterilized, pre-warmed to 37°C Culture Medium B [DMEM, 50U penicillin/50 μg streptomycin, 10 mM HEPES, 2% v/v B27 supplement (Gibco, Life Technologies), 2 mM L-glutamine and 19 mM KCl]. Cultures were kept for a maximum of 13 DIV. To monitor culture development, phase-contrast images were taken every 24 hours using an IncuCyte™ FLR microscope (10× objective lens) at 37°C, in a humid atmosphere with 5% CO_2.

2.2. Immunocytochemistry of CGN cultures

CGNs cultured on coverslips in 24-well plates were washed with 1 mL of sterile phosphate buffered saline (PBS, Oxoid) and fixed for 10 minutes with 350 μL 4% w/v paraformaldehyde (PFA, dissolved in autoclaved PBS, Sigma) at room temperature. The PFA was removed and the fixed cells were washed 3 times with 1 mL autoclaved PBS for 5 minutes each time. After the final wash, the PBS was discarded and the cells were permeabilised with 350 μL of 0.25% v/v Triton X-100 (Sigma, dissolved in autoclaved PBS) for 10 minutes at room temperature. The Triton solution was removed and the cells were washed again 3 times with PBS for 5 minutes each time.

The cells were then blocked with 350 μL of 5% v/v goat serum (Sigma, dissolved in autoclaved PBS) for 30 minutes at room temperature. Each coverslip was removed from each well, placed face-up onto glass microscopy slides and the cells were incubated with 150 μL of either of the following primary antibodies; Anti-NeuN

[1:100, mouse monoclonal (Millipore), dissolved in autoclaved PBS] or Anti-Glial Fibrillary Acidic Protein (GFAP) [1:400, mouse monoclonal (Sigma), dissolved in autoclaved PBS] overnight at 4°C. The primary antibody solution was then discarded and the cells were washed 3 times with autoclaved PBS for 5 minutes each time at room temperature. After the final wash, excess PBS was removed before incubating with 150 μL of either of the following secondary antibodies Alexa Fluor® 633 Goat-Anti-Mouse IgG [1:1000, Invitrogen, Life Technologies, dissolved in autoclaved PBS] or Alexa Fluor® 488 Goat-Anti-Mouse IgG [1:1000, Invitrogen, Life Technologies, dissolved in autoclaved PBS] for 1 hour, in the dark, at room temperature. The secondary antibody solution was then discarded and the cells were washed 3 times with PBS for 5 minutes each time at room temperature.

Maintaining light exposure to a minimum, excess PBS was removed and a drop of 4', 6-diamidino-2-phenylindole (DAPI) with Vectashield (Vector Laboratories) was applied to each coverslip, the excess was removed and a larger coverslip was mounted on top and glued in place with UHU glue. Samples were stored at 4°C in the dark until imaging by confocal fluorescence microscopy. Four coverslips of CGNs were prepared per animal and this was repeated 3 times using 3 independent animals (n=3).

Imaging of cells was performed using an upright-configured Zeiss LSM 510 confocal microscope with a 20× objective lens. Images were taken using auto-exposure/gain, a 2 μm pinhole and at 405, 488 and 633 nm wavelengths to visualise DAPI+ (blue), GFAP+ (green) and NeuN+ (red) cells respectively. Four randomly selected non-overlapping images were taken from each coverslip, at 8-bit quality and 1024 × 1024 resolution.

To quantify the purity of CGN cultures, a 4 × 4 grid system (8000 μm^2 per grid square) was used and the number of DAPI+ cells and NeuN+ cells were counted within the grid for each image using the Cell Counter plugin in ImageJ. The number of NeuN+ cells (CGNs) was expressed as a percentage of the total number of cells (DAPI+) and results are presented as mean % CGNs ± SEM.

2.3. Induction of glutamate excitotoxicity and drug treatments in CGNs

On 10 DIV, CGNs cultured in 96-well plates were treated to various conditions before measuring cell viability using the YOYO®-1 & SYBR® Green I viability assay as described in section 2.5.1, four wells were used per experimental condition per animal and this was repeated with 3 independent animals (*n*=3). CGNs cultured in 6-well plates were treated to various conditions described below, before extracting histone proteins as described in section 2.9, one well was used per experimental condition and this was repeated with 3 independent animals (*n*=3).

2.3.1. Co-treatment experiments

The culture medium was replaced with 75% fresh pre-warmed to 37°C Culture Medium B, 25% of the old Culture Medium B taken from the wells and the following conditions: vehicle control 0.1% v/v sterile dimethyl sulfoxide (DMSO), 1 μM SAHA (Cayman Chemicals, dissolved in DMSO) or 1 μM MI192 (Professor Ronald Grigg, School of Chemistry, University of Leeds, UK, Boissinot et al. (2012), dissolved in DMSO). Glutamate excitotoxicity was induced with 100 μM L-glutamate (Sigma, dissolved in sterile de-ionized H_2O) or with 500 μM DL-*threo*-β-Benzyloxyaspartic acid (DL-TBOA, Tocris Bioscience, dissolved in DMSO). At the same time as exposing to either of these neurotoxic insults, CGNs were treated with either vehicle control, both 30 μM NBQX (Sigma, dissolved in sterile de-ionized H_2O) and 10 μM MK-801 (Sigma, dissolved in DMSO) an AMPA & Kainate and a NMDA glutamate receptor antagonist respectively, 200 μM N-acetyl-Asp-Glu-Val-Asp-al (ac-DEVD-al) a caspase 3 and 7 inhibitor (Sigma, dissolved in sterile de-ionized H_2O), 1 or 3 μM SAHA, 1 mM valproate (VPA, Sigma, dissolved in DMSO) or 1 or 3 μM MI192. CGNs were cultured at 37°C in a humid atmosphere with 5% CO_2 for a further 24 or 72 hours after treatment.

Another co-treatment paradigm used involved exposing CGNs to a short pulse of glutamate with or without HDAC inhibitors, followed by the removal of this and further treatment with HDAC inhibitors in the absence of glutamate. To do this, the culture medium was replaced with 75% fresh pre-warmed to 37°C Culture Medium

B, 25% of the old Culture Medium B taken from the wells and either vehicle control, or 100 μM L-glutamate with either vehicle control, 1 μM SAHA or 1 μM MI192 for 30 minutes. This was then removed and CGNs were treated with either vehicle control, 1 μM SAHA or 1 μM MI192 for a further 72 hours.

2.3.2. Pre-treatment experiments

The culture medium was replaced with 75% fresh pre-warmed to 37°C Culture Medium B, 25% of the old Culture Medium B taken from the wells and the following conditions: vehicle control 0.1% v/v DMSO, 1 μM SAHA or 1 μM MI192. CGNs were then cultured for 24 hours at 37°C in a humid atmosphere with 5% CO_2. After 24 hours of pre-treatment, the culture medium was replaced with 75% fresh pre-warmed to 37°C Culture Medium B, 25% of the old Culture Medium B taken from the wells and the following conditions: vehicle control 0.1% v/v sterile DMSO, or 100 μM L-glutamate with vehicle control, 1 μM SAHA or 1 μM MI192. CGNs were cultured for a further 24 hours at 37°C in a humid atmosphere with 5% CO_2.

2.4. Induction of oxygen glucose deprivation (OGD) and drug treatments in CGNs

CGNs cultured in 4-well plates for 10 DIV were exposed to oxygen glucose deprivation (OGD) and then treated to experimental conditions. One well was used per experimental condition, per animal and this was repeated using 3 independent animals (n=3).

Immediately prior to OGD induction, 0.22 μm filter-sterilized, modified glucose-free Earle's Balanced Salt Solution (EBSS, [116 mM NaCl, 25 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4 and 26.2 mM $NaHCO_3$ (all from Sigma)]) was bubbled with 0.22 μm filter-sterilized 95% N_2 and 5% CO_2 gas mixture (BOC) for 60 minutes, with the pressure and flow regulators set at 1 bar pressure and at a 0.4 L/min flow rate. After this time, the modified glucose-free EBSS was stored in an airtight tube and warmed to 37°C for 30 minutes. The OGD chamber (School of

Engineering, University of Leeds, UK) was also warmed to and maintained at 37°C throughout the experiment.

OGD was performed under sterile conditions in a Class II tissue culture hood. The culture medium from CGNs cultured in 4-well plates was quickly removed, kept and stored at 37°C until later use. Then 800 μL of oxygen depleted, pre-warmed to 37°C modified glucose-free EBSS was quickly added to each well. The plate was quickly transferred to the OGD chamber. The OGD chamber was sealed and flushed with 0.22 μm sterile filtered 95% N_2 and 5% CO_2 gas mixture for 2 minutes to expel atmospheric air, the pressure and flow regulators were set at 1 bar pressure and at a 1 L/min flow rate. The atmosphere in the chamber was then maintained at 95% N_2 and 5% CO_2 with the pressure and flow regulators set at 1 bar pressure and at a 0.4 L/min flow rate. A positive pressure was maintained so the total volume of 95% N_2 and 5% CO_2 inside the chamber was replaced approximately every minute. CGNs were exposed to OGD for 40 minutes.

After exposing to OGD, the 4-well plate was removed from the chamber and the deoxygenated, modified glucose-free EBSS was replaced with 75% fresh pre-warmed to 37°C Culture Medium B, 25% of the old Culture Medium B taken from the wells earlier and either vehicle control 0.1% v/v sterile DMSO or 1 μM SAHA. In parallel to exposing CGN 4-well plate cultures to OGD, other cultures were not exposed to OGD, but were treated to 0.1% v/v sterile DMSO to serve as a no OGD control.

After treatments, CGNs were cultured for a further 24 hours at 37°C in a humid atmosphere with 5% CO_2. Following this, cell viability was measured using the MTT assay as described in section 2.5.2.

2.5. Assessment of CGN viability

2.5.1. YOYO®-1 & SYBR® Green I assay

To assess cell viability of CGNs, YOYO®-1 (Invitrogen, Life Technologies, dissolved in DMSO) a cell-impermeant nucleic acid fluorescent stain that labels the nuclei of dying and dead cells (Becker et al., 1994), was added to the existing culture medium in each well to give a final concentration of 200 nM. The cells were then incubated for 1 hour at 37°C in a humid atmosphere with 5% CO_2.

Imaging of stained cells was carried out using an IncuCyte™ FLR microscope (10 × objective lens) at 37°C, in a humid atmosphere with 5% CO_2. Images were taken in phase-contrast and green-fluorescence in the wavelength range of 450-490 nm to visualise YOYO®-1+ cells (green). Two non-overlapping images at 1280 × 1024 resolution were taken per well. For a total cell count, all CGN nuclei were labelled by incubation with a 1:50,000 dilution of SYBR® Green I (Invitrogen, Life Technologies) a cell-permeant nucleic acid fluorescent stain for 30 minutes at 37°C in a humid atmosphere with 5% CO_2. The cells were imaged at the same position in phase-contrast and green-fluorescence to visualise SYBR® Green I+ cells.

The number of YOYO®-1+ cells and SYBR® Green I+ cells/well was quantified using an automated counting algorithm (Object Counting v2.0 and Edge Split v2.0, Essen BioScience) with a user defined segmentation fixed threshold of 150 AU and edge sensitivity of 1.00. Percentage cell viability was calculated by subtracting the number of YOYO-1+ (dying/dead cells) cells from the total number of cells (SYBR® Green I+). This value representing the number of viable cells (YOYO-1 negative) was expressed as a percentage of total number of cells (SYBR® Green I+). Results are presented as mean % cell viability ± SEM. Statistical analysis was performed comparing the percentage cell viability for each condition using a one-way ANOVA followed by the Bonferroni *post hoc* test at the 5% significance level (OriginPro, OriginLab) or using an unpaired Student's t-test assuming equal variance at the 5% significance level (Microsoft Excel).

2.5.2. MTT assay

To assess the viability of CGNs cultured in 4-well plates and treated as described in section 2.4, metylthiazolyldiphenyl-tetrazolium bromide (MTT) was added to the culture medium in each well to give a final concentration of 500 ng/mL. The cells were then incubated for 1 hour in the dark, at 37°C in a humid atmosphere with 5% CO_2. The culture medium was then removed and the intracellular formazan crystals were dissolved with 100 µL acidified isopropanol [90% isopropanol (Fisher Scientific), 40 mM HCl (Acros Organics) and 1% sodium dodecyl sulphate (SDS, Sigma)] by orbital shaking on rotating platform for 1 hour at room temperature. Once dissolved, the solution was triturated several times before transferring to a clear flat-bottomed 96-well assay plate (Greiner). The absorbance of each well was measured at 570 nm (formazan) and 720 nm (background) wavelengths using a spectrophotometric plate reader (FLUOstar Omega, BMG Labtech). The background absorbance reading at 720 nm was subtracted from that at 570 nm and these values were expressed as a mean ± SEM % cell viability of the no OGD +vehicle control. Statistical analysis was performed with Microsoft Excel using an unpaired Student's t-test assuming equal variance at the 5% significance level.

2.6. BV2 microglia cell culture

BV2 murine microglia were routinely cultured in 78.5 cm^2 cell culture treated Petri dishes (Greiner), with 10 mL Dulbecco's Modified Eagle's Medium (DMEM) high glucose AQmedia™ (Sigma) supplemented with 10% v/v foetal bovine serum (FBS, PAA Cell Culture Company) and 100U penicillin/100 μg streptomycin (Sigma) at 37°C in a humid atmosphere with 5% CO_2. Cells were passaged at ~80% confluence, first, the cells were washed with 10 mL sterile PBS (room temperature,) then incubated with 1 mL of Trypsin-Ethylenediaminetetraacetic acid (EDTA) solution (pre-warmed to 37°C, 0.5 g/L porcine trypsin and 0.2 g/L EDTA, Sigma) for 3 minutes at 37°C in a humid atmosphere with 5% CO_2. The cells were resuspended in 9 mL fresh culture medium (pre-warmed to 37°C) and this 10 mL was split appropriately into fresh culture medium in new Petri dishes. The Petri dishes were then topped-up with fresh culture medium to maintain a total volume of 10 mL. The cells were then cultured further until ~80% confluence and then were passaged again. BV2 microglia were cultured for a maximum of 25 passages.

2.7. Induction of the inflammatory response and drug treatments in BV2 microglia

BV2 microglia, were seeded into cell culture treated 24-well plates (Greiner) at a cell density of 175,000 cells/well in 1 mL of culture medium and into 6-well plates (Greiner) at a cell density of 350,000 cells/well or 500,000 cells/well with 3 mL culture medium. The cells were allowed to culture at 37°C in a humid atmosphere with 5% CO_2 for 24 hours before changing the culture medium (pre-warmed to 37°C) and exposing to various treatments.

2.7.1. Co-treatment experiments

BV2 microglia seeded into 6-well plates at a cell density of 500,000 cells/well or 24-well plates at a cell density of 175,000 cells/well, were treated to vehicle control 0.1% v/v sterile DMSO, 500 nM apicidin (Sigma, dissolved in DMSO), 1 μM MI192, 5 μM MS-275 (Cayman Chemicals, dissolved in DMSO) and 1 μM SAHA

with or without 500 ng/mL lipopolysaccharide (LPS, from Escherichia coli 0111:B4, LPS, Sigma, dissolved in autoclaved PBS) an inflammatory stimulant. BV2's were also treated to 500 ng/mL LPS with either vehicle control, 5 μM BAY 11-7082 (BAY11, Sigma, dissolved in DMSO), 5 μM Droxinostat (Sigma, dissolved in DMSO), 5 μM Droxinostat & 1 μM MI192 combined, or 5 mM VPA. The cells were then cultured for 6 or 24 hours after treatment at 37°C in a humid atmosphere with 5% CO_2. One well was used per experimental condition per passage number and this was repeated with 3 independent passage numbers (*n*=3).

2.7.2. Pre-treatment experiments

BV2 microglia seeded into 6-well plates at a cell density of 350,000 cells/well were treated to vehicle control 0.1% v/v sterile DMSO, 500 nM apicidin, 1 μM MI192 or 5 μM MS-275 then cultured at 37°C in a humid atmosphere with 5% CO_2 for 24 hours. After 24 hours of pre-treatment, the culture medium (pre-warmed to 37°C) was replaced and the BV2's were treated to vehicle control 0.1% v/v sterile DMSO alone or 500 ng/mL LPS with and without vehicle control, 500 nM apicidin, 1 μM MI192 or 5 μM MS-275 for a further 6 hours. One well was used per experimental condition per passage number and this was repeated with 3 independent passage numbers (*n*=3).

2.8. Transfecting BV2 microglia with small interfering RNA (siRNA)

BV2 microglia, were seeded into cell culture treated 6-well plates at a cell density of 350,000 cells/well. The cells were allowed to culture for 24 hours then washed with 1 mL sterile PBS (at room temperature) and then maintained in 1 mL Opti-MEM® (pre-warmed to 37°C, Gibco, Life Technologies) at 37°C in a humid atmosphere with 5% CO_2.

Cells were transfected with 50 pmoles of Silencer® Select Negative Control siRNA (Ambion, Scrambled (Scr) siRNA) or Silencer® Select Pre-designed siRNA targeted against HDAC1 (id: s119557, Ambion) or HDAC2 (id: s67417, Ambion) as follows. Per well of a 6-well plate, 3 μL of Lipofectamine™ 2000 (Invitrogen, Life Technologies) was added to 100 μL of pre-warmed to 37°C Opti-MEM®. Also, 1 μL of 50 μM siRNA was added to 100 μL of pre-warmed to 37°C Opti-Mem®. These mixtures were then gently mixed and then incubated for 5 minutes at room temperature before combining, gently mixing and incubating for 20 minutes at room temperature. Afterwards, the 200 μL of siRNA and Lipofectamine™ 2000 mix was added to the 1 mL of Opti-MEM® in the well followed by gentle agitation and incubated at 37°C in a humid atmosphere with 5% CO_2 for 4 hours. Similarly, BV2 microglia were also transfected with 100 pmoles of Scr siRNA or 50 pmoles HDAC1 siRNA with HDAC2 siRNA (a range of amounts 3, 5, 10, 30 & 50 pmoles) using Lipofectamine™ 2000 at a ratio of 3 μL Lipofectamine™ per 50 pmoles of siRNA.

After 4 hours incubation with the DNA-Lipofectamine™ 2000 complexes, this mix was removed and the cells were then cultured in 3 mL DMEM high glucose AQmedia™ supplemented with 1% v/v FBS and 100U penicillin/100 μg streptomycin at 37°C in a humid atmosphere with 5% CO_2 for 48 hours. After this time, some transfected cells were treated to 500 ng/mL LPS followed by culturing for a further 6 hours at 37°C in a humid atmosphere with 5% CO_2. One well was used per transfection, per treatment and per passage number and this was repeated with 3 independent passage numbers (n=3).

2.9. Histone protein extraction

For histone protein extraction, cells cultured in 6-well plates were washed with 1 mL sterile PBS which was then discarded and the cells were then scraped on ice into 1 mL ice-cold sterile PBS and pelleted by centrifugation at 13,300 RPM/16,300 × *g* (GenFuge 24D Centrifuge, Progen) for 30 seconds at room temperature. The supernatant was discarded and the cell pellet was resuspended in a volume of ice-cold Triton Lysis Buffer [0.5% v/v Triton X-100 (Sigma), 2 mM phenylmethylsulfonyl (PMSF, Apollo Scientific, dissolved in isopropanol), 0.02% w/v NaN_3 (Sigma, dissolved in autoclaved de-ionized H_2O) and autoclaved PBS] dependent on cell density (1 mL of lysis buffer was used per 1 × 10^7 cells). The suspension was incubated on ice for 10 minutes with vortexing every 3 minutes. Lysed cells were centrifuged at 8400 RPM/6600 × *g* for 10 minutes at 4°C (Eppendorf Centrifuge 5415R with a FA-45-24-11 rotor) to pellet the nuclei. The supernatant was discarded and the white nuclei pellet was resuspended in half the volume of ice-cold Triton Lysis Buffer used earlier then centrifuged once more at 8400 RPM/6600 × *g* for 10 minutes at 4°C. The supernatant was once again discarded and the pellet was resuspended in 1 mL of ice-cold 200 mM HCl (Acros Organics, dissolved in autoclaved de-ionized H_2O) per 4 × 10^7 cells. Histone proteins were extracted overnight at 4°C, followed by centrifugation at 8400 RPM/6600 × *g* for 10 minutes at 4°C then the supernatant containing histone proteins was taken and stored at -20°C until further analysis.

The protein concentration of each sample was determined using the Bradford protein assay where 5 μL of autoclaved de-ionized H_2O (blank 1), bovine serum albumin (BSA, Sigma, dissolved in autoclaved de-ionized H_2O) standards of a known concentration (0.25 to 2 μg/μL range), 2.5 μL of 200 mM HCl plus 2.5 μL of autoclaved de-ionized H_2O (blank 2) or 2.5 μL histone protein acid extract sample plus 2.5 μL of autoclaved de-ionized H_2O (dilution factor of 2) was added to 250 μL of Bradford reagent (pre-incubated to room temperature, Sigma) and incubated for 10 minutes at room temperature. These solutions were mixed gently and transferred to a clear flat-bottomed 96-well assay plate (Greiner) and absorbance was measured at 595 nm wavelength using a spectrophotometric plate reader (FLUOstar Omega,

BMG Labtech). A standard curve was produced of absorbance versus BSA concentration and the concentration of the histone protein acid extracts was determined using this curve.

2.10. Whole cell protein extraction

For whole cell protein extraction of BV2 microglia cells cultured and treated in 6-well plates, cells were first washed with 1 mL sterile PBS and then scraped on ice into 250 μL ice-cold Radioimmunoprecipitation (RIPA) Buffer [sterile 50 mM Tris-HCL pH 8.0 (Sigma), sterile 1 mM EDTA pH 8.0 (Sigma), 1% Triton X-100, 0.1% sodium deoxycholate (Sigma), sterile 0.1% sodium dodecyl sulphate (SDS, Sigma), sterile 150 mM NaCl (Sigma), 0.02% w/v NaN_3, 1 mM PMSF and autoclaved de-ionized H_2O]. The cell suspension was triturated 10 times using a pipette then incubated for 30 minutes on ice with vortexing every 10 minutes. The lysate was clarified by centrifugation at 12,000 RPM/13,400 × *g* for 20 minutes at 4°C (Eppendorf Centrifuge 5415R with a FA-45-24-11 rotor) and the supernatant containing proteins was collected and stored at -20°C until further analysis.

The protein concentration of each sample was determined using the Bicinchoninic acid (BCA) protein assay (Sigma), 10 μL of autoclaved de-ionized H_2O (blank 1), 10 μL of BSA standard (0.25 to 1 μg/μL range), 3 μL of RIPA Buffer (blank 2) plus 7 μL of autoclaved de-ionized H_2O or 3 μL of protein sample plus 7 μL of autoclaved de-ionized H_2O (dilution factor of 3.33) was added to wells of a clear flat-bottomed 96-well assay plate followed by 200 μL of BCA working reagent (50 : 1, BCA solution : 4% $CuSO_4$, Sigma, pre-incubated to room temperature). The contents of the 96-well plate were mixed by trituration and the plate was covered and then incubated for 30 minutes at 37°C. The plate was gently agitated and then the absorbance of each well was measured at 562 nm wavelength using a spectrophotometric plate reader. A standard curve was produced of absorbance versus BSA concentration and the concentration of the whole cell protein extracts was determined using this curve and adjusted using the dilution factor.

2.11. Western blotting

A volume of each histone protein acid extract sample or of each whole cell protein extract sample was take to give 10 μg of protein. An appropriate volume of autoclaved de-ionized H_2O and 5× Laemmli sample buffer [250 mM Tris-HCl pH 6.8 (Sigma), 10% w/v SDS, 50% glycerol (Sigma), 500 mM dithiothreitol (DTT, Melford) and 0.2% w/v bromophenol blue (Sigma)] or 2× Laemmli sample buffer [100 mM Tris-HCl pH 6.8, 4% w/v SDS, 20% glycerol, 200 mM DTT, and 0.2% w/v bromophenol blue] was added to each 10 μg sample to give 1× and a total volume of ≤20 μL. These were boiled at 95°C for 5 minutes and then chilled on ice for 5 minutes.

Samples underwent sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, Mini-PROTEAN® Tetra Cell, Bio-Rad) alongside 10 μL of Pre-stained Protein Marker, Broad Range (with reference bands covering 7 to 175 kDa, New England Biolabs) or 10 μL PageRuler™ Pre-stained Protein Ladder (with reference bands covering 10 to 170 kDa, Thermo Scientific). SDS-PAGE gels of 1.5 mm thickness comprised of a 5% stacking gel and a 6, 10% or 15% resolving gel for whole cell protein extract samples, or a 15% resolving gel for histone protein acid extracts were used. Gel recipes are listed in table 2.1. Electrophoresis was performed at 150V in electrophoresis buffer [25 mM Tris (Sigma), 250 mM glycine (Fisher Scientific), 0.1% w/v SDS and de-ionized H_2O]. Gels were run until the dye front had just migrated off the gel, the resolving gels were separated from the stacking gel and then underwent Western blotting.

Gels were wet-transferred (XCell II™ Blot Module, Invitrogen, Life Technologies) onto Hybond-C Extra nitrocellulose membrane (Amersham) or Hybond-P polyvinylidene fluoride membrane (PVDF, Amersham). Before transferring, the nitrocellulose membranes were rinsed in de-ionized H_2O then soaked in transfer buffer [48 mM Tris, 39 mM glycine and 20% methanol (Fisher Scientific)] for 30 minutes, PVDF membranes were activated with 100% methanol for 1 minute, rinsed with de-ionized H_2O and soaked in transfer buffer for 30 minutes. Wet-transfer was performed at 30V for 60 minutes in transfer buffer.

Table 2.1. SDS-PAGE Recipes.

Constituent	5% Stacking gel	Resolving gel		
		6%	10%	15%
Autoclaved DI-H_2O	3.3 mL	4 mL	2.67 mL	1 mL
30% Acrylamide[1]	1.02 mL	2 mL	3.33 mL	5 mL
0.5 M Tris pH 6.8	1.5 mL	-	-	-
1 M Tris pH 8.8	-	3.8 mL	3.8 mL	3.8 mL
10% SDS	60 μL	100 μL	100 μL	100 μL
10% APS	60 μL	100 μL	100 μL	100 μL
TEMED	6 μL	8 μL	8 μL	8 μL

[1]30% Acrylamide:Bis-acrylamide 29:1, Severn Biotech. All other constituents are from Sigma. Abbreviations, APS: Ammonium persulfate, DI: De-ionized, SDS: Sodium dodecyl sulphate, TEMED: Tetramethylethylenediamine.

To confirm successful transfer of proteins, membranes were stained with Ponceau S solution (Sigma) for 5 minutes at room temperature. Protein bands were visualised and the membrane was rinsed with de-ionized H_2O, destained with 100 mM NaOH (dissolved in autoclaved de-ionized H_2O) for 60 seconds and washed in de-ionized H_2O for 3 minutes on a rotating platform at room temperature. The membranes were then blocked with blocking solution [5% w/v non-fat dried milk powder (Oxoid), 0.1% v/v Tween® 20 (Sigma) and autoclaved PBS] for 2 hours at room temperature or overnight at 4°C on a spiramix.

Membranes were incubated with either of the following primary antibodies; Anti-Histone H3 [1:1000, rabbit polyclonal (Millipore), dissolved in blocking solution +0.02% NaN_3,], Anti-acetyl Histone H3 lysine 9 [1:1000, rabbit polyclonal (Millipore), dissolved in blocking solution +0.02% NaN_3], Anti-acetyl Histone H4 pan-lysine [1:10,000, rabbit polyclonal (Millipore), dissolved in blocking solution +0.02 NaN_3], Anti-acetyl Tubulin [1:2000, mouse monoclonal (Sigma), dissolved in blocking solution +0.02% NaN_3], Anti-HDAC1 [1:2000, rabbit polyclonal (Abcam), dissolved in blocking solution +0.02% NaN_3], Anti-HDAC2 [1:2000, rabbit polyclonal (Abcam), dissolved in blocking solution +0.02% NaN_3], Anti-HDAC3 [1:2000, rabbit polyclonal (Abcam), dissolved in blocking solution +0.02% NaN_3],

Anti-acetylated lysine (Ac-K^2-100) [1:1000, rabbit monoclonal (Cell Signaling), dissolved in 5% w/v BSA, Tris Buffered Saline (137 mM NaCl and 20 mM Tris, pH 7.6, TBS) and 0.1% v/v Tween® 20], or Anti-β-actin [1:10,000, mouse monoclonal (Sigma), dissolved in blocking solution +0.02% NaN_3] for 1 hour at room temperature or overnight at 4°C on a spiramix.

Membranes were then washed 3 times with PBS-tween [0.1% v/v Tween® 20 dissolved in PBS] or with TBS-tween [0.1% v/v Tween® 20 dissolved in TBS] on a spiramix for 15 minutes each time. Membranes were then incubated with the appropriate secondary antibody; Goat-Anti-Rabbit IgG-horseradish peroxidase (HRP) linked [1:2000, dissolved in blocking solution, (Cell Signaling)] or Goat-Anti-Mouse IgG-HRP linked [1:2000, dissolved in blocking solution, (Cell Signaling)] for 1 hour at room temperature on a spiramix followed by washing 3 times with PBS-tween or TBS-tween as before. Membranes were then incubated for 5 minutes at room temperature with an enhanced chemiluminescence (ECL) substrate (Amersham) and were either exposed to photographic film which was subsequently developed and fixed in the dark using a Xograph Imaging System Compact X4, or the membranes were directly photographed using a Fujifilm LAS-3000 imaging system (Exposure Type: Precision, Sensitivity: High) or scanned with a LI-COR cDigit® Scanner (Sensitivity: High).

Following visualisation, membranes were washed once with PBS-tween or TBS-tween for 15 minutes and stripped of bound primary and secondary antibodies if necessary. During stripping, the membranes were rolled in a hybridisation tube and oven with pre-warmed to 50°C stripping buffer [62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 0.4% v/v β-mercaptoethanol (Sigma) and de-ionized H_2O] for 30 minutes at 50°C. The membranes were then washed 3 times with PBS-tween or TBS-tween for 15 minutes each time and blocked with blocking solution. Membranes were then re-probed with appropriate primary and secondary antibodies and visualised as described earlier.

Photographic films were digitally scanned in grey-scale at 1200 dots per inch (DPI) and the intensity of bands was determined using ImageJ (Miller, 2010; Rasband,

1997-2014). Images acquired using the Fujifilm LAS-3000 were saved in grey-scale at 72 DPI and the intensity of bands was determined using the Aida Image Analyzer v4.15 software (Raytest) with background correction. Membranes scanned with the cDigit® were quantified using Image Studio Lite v4.0 (LI-COR) with background correction. All bands were normalised to the β-actin or total Histone H3 loading control as appropriate.

2.12. Ribonucleic acid (RNA) extraction, reverse transcription and quantitative PCR

RNA was extracted from BV2 microglia cells cultured and treated in 6-well plates. The culture medium was removed and the cells were washed with 1 mL sterile PBS before scraping into 500 μL of ice-cold TRI Reagent® (Sigma). Each sample was triturated 10 times using a pipette and incubated for 5 minutes at room temperature followed by the addition of 100 μL of chloroform (Arcos Organics). The samples were vortexed for 15 seconds, incubated for a further 5 minutes at room temperature then centrifuged at 13,000 RPM/16,000 × *g* for 10 minutes at 4°C (Eppendorf Centrifuge 5415R with a FA-45-24-11 rotor). The clear aqueous layer containing RNA was carefully removed (250 μL) into a new 1.5 mL microcentrifuge tube and then 250 μL of ice-cold isopropanol was added and the samples were then briefly vortexed and incubated overnight at -80°C to precipitate the RNA.

To pellet the precipitated RNA, the frozen samples were centrifuged at 13,000 RPM/16,000 × *g* for 30 minutes at 4°C. The supernatant was removed and the pellets were washed with 500 μL ice-cold 75% ethanol (Sigma), vortexed and centrifuged at 13,000 RPM/16,000 × *g* for 10 minutes at 4°C. The supernatant was again removed, the pellets were centrifuged dry at 13,000 RPM/16,000 × *g* for 5 minutes at 4°C, residual ethanol was removed and the pellets were air-dried for 5 minutes at room temperature then resuspended in ice-cold 50 μL Tris-EDTA (TE, pH 7.5, Invitrogen, Life Technologies). To dissolve the RNA pellet in the TE, the samples were gently mixed, incubated at 55°C for 5 minutes, chilled on ice for 5 minutes followed by gentle mixing again. Samples were stored at -20°C until further analysis.

The concentration of each RNA sample was determined in duplicate using a NanoDrop 2000c (Thermo Scientific). The absorbance at 260 nm wavelength was measured of the TE (blank) and each RNA sample. Using the formula, 1 A_{260} = 40 μg/mL, the average RNA concentration of each sample was determined. The absorbance at 280 nm wavelength (maximum absorbance of protein, A_{280}) was also measured and the A_{260}/A_{280} value was calculated as a measure of the RNA purity of the sample. Samples with a ratio between 2.0 and 2.2 were then reverse transcribed to produce cDNA.

An appropriate volume of RNA to give 2.5 μg was primed for reverse transcription at 65°C for 5 minutes with 1.25 μL of Oligo(dT)15 primers (0.5 μg/μL, Promega), 1.25 μL of Random primers (0.5 μg/μL, Promega) and autoclaved de-ionized H_2O to make the reaction volume 32.5 μL. The samples were then chilled on ice for 1 minute before adding 17.5 μL of reverse transcription mastermix [10 μL of 5× M-MLV Reverse Transcriptase 5× reaction buffer (Promega), 5 μL of 20 mM deoxynucleoside triphosphate (dNTP) mix containing 5 mM of each dNTP (deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP) and deoxythymidine triphosphate (dTTP), Bioline), 1.25 μL of RNasin® Plus ribonuclease (RNase) Inhibitor (40 U/μL, Promega) and 1.25 μL of M-MLV Reverse Transcriptase, RNase H Minus (200 U/μL, Promega)] to make the final reaction volume 50 μL. These were then incubated at 37°C for 60 minutes, chilled on ice for 1 minute and stored at -20°C until further analysis.

Quantitative PCR (qPCR) reactions were carried out in duplicate using a Rotor Gene 6000 PCR Analyser (Corbett). Each reaction comprised of 50 or 100 ng of sample cDNA (1 and 2 μL of reverse transcribed sample respectively), 300 nM of forward/sense primer (0.6 μL of 100 mM stock), 300 nM of reverse/anti-sense primer (0.6 μL of 100 mM stock), 10 μL 2× SensiMix™ SYBR® & Fluorescein (to give 1×, Bioline) and autoclaved de-ionized H_2O to make the total reaction volume to 20 μL. All sequences of primers used in this study are listed in table 2.2. A RNA control (no reverse transcription) and an autoclaved de-ionized H_2O (no template)

control was run for each primer set. The hot-start DNA polymerase was activated by holding the samples at 95°C for 10 minutes and the PCR was run for 45 cycles of 95°C for 10 seconds, 60°C for 15 seconds and 72°C for 30 seconds. This was followed by a melt curve to check for specific amplification by each primer set and the absence of primer dimers. To generate the melt curve the temperature was ramped from 55°C to 95°C rising by 1°C per step and waiting 5 seconds between each step.

Table 2.2. List of qPCR primers.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
IL-6	CCCAACTTCCAATGCTCTCC	ACAGTGAGGAATGTCCACAAAC
iNOS	CAGCTGGGCTGTACAAACCTT	CATTGGAAGTGAAGCGTTTCG
TNF-α	TGAACTTCGGGGTGATCG	GGGCTTGTCACTCGAGTTTT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

Transcript levels were analysed by real time analysis of SYBR® Green fluorescent reporter dye intercalation into amplified double stranded DNA. Qualitative analysis of transcript levels was performed using the $2^{-\Delta\Delta Ct}$ method ($\Delta\Delta Ct = \Delta C_{t,sample} - \Delta C_{t,house\text{-}keeping\ gene,}$ Livak and Schmittgen (2001)) with the house-keeping gene U6. Results are presented as mean relative expression or as a relative mean percentage of an appropriate experimental condition ± standard error of the mean (SEM). Statistical analysis was performed as described by Lew (2007). The absolute C_t values for each experimental condition vs. an appropriate condition were compared using a one-way repeated measures analysis of variance (ANOVA) followed by the Dunnet *post hoc* test at the 5% significance level (performed using OriginPro, OriginLab) or a paired Student's t-test at the 5% significance level (performed using Microsoft Excel).

2.13. Enzyme linked immunosorbent assays (ELISAs)

Cell culture supernatants were taken from BV2 microglia cells cultured and treated in 24-well plates. The 1 mL of culture medium was carefully removed from each well, transferred to a 1.5 mL microcentrifuge tube, centrifuged for 30 seconds at 13,300 RPM/16,300 × *g* (GenFuge 24D Centrifuge, Progen) to pellet any detached cells then 950 μL was carefully removed and placed into a new 1.5 mL microcentrifuge tube. Supernatants were stored at -20°C until analysis.

The concentration of secreted mouse IL-6 protein was determined by a 96-well plate format ELISA following the manufacturer's instructions (Invitrogen, Life Technologies). The ELISA consisted of a chromogen blank, standards of known IL-6 concentrations (0 to 500 pg/mL range, Invitrogen, Life Technologies) and low and high controls of IL-6 known to be within the measurable range of the assay (Invitrogen, Life Technologies). The cell culture supernatant samples were diluted 10-fold (in provided diluent buffer, Invitrogen, Life Technologies). All controls, standards and samples were run in duplicate.

The assay procedure was as follows, 100 μL of the controls, standards and diluted samples were added to appropriate wells pre-coated with a monoclonal antibody specific for mouse IL-6. The plate was incubated for 2 hours at room temperature. The plate was then decanted and washed as follows, 400 μL of 1 × Wash Buffer was added to each well, incubated for 30 seconds, decanted once more then blotted dry onto tissue paper 10 times. This washing step was performed a total of 4 times. Then, 100 μL of mouse IL-6 Biotin Conjugate solution (Invitrogen, Life Technologies) was added to each well (excluding the chromogen blank), followed by incubation for 30 minutes at room temperature. The wells were subsequently washed 4 times as described before, followed by incubation with 100 μL of Streptavadin-HRP (Invitrogen, Life Technologies) for 30 minutes at room temperature. The plates were again washed 4 times before incubating with 100 μL of Stabilised Chromogen (Invitrogen, Life Technologies) for 30 minutes at room temperature. Stop solution (100 μL, Invitrogen, Life Technologies) was added directly to the Stabilised Chromogen in each well, the plate was gently agitated and the absorbance at 450 nm

wavelength was measured using a spectrophotometric plate reader (FLUOstar Omega, BMG Labtech).

A standard curve was produced of absorbance versus IL-6 concentration and the concentration of IL-6 in the cell culture supernatant samples was determined using this curve and adjusted using the dilution factor of 10. Results are presented as a mean IL-6 protein (pg/mL) ± SEM. Statistical analysis comparing the absolute absorbance values for each experimental condition was performed using an unpaired Student's t-test assuming equal variance at the 5% significance level.

2.14. Measuring protein synthesis in BV2 microglia

BV2 microglia cultured for 24 hours in 6-well plates at a cell density of 500,000 cells/well and 24-well plates at a cell density of 175,000 cells/well, were treated to 1) 500 ng/mL LPS and either vehicle control 0.1% v/v sterile DMSO, 1 μM SAHA or 500 nM apicidin, or 2) 500 ng/mL LPS with 1 μg/mL cycloheximide (CHX) and either vehicle control, 1 μM SAHA or 500 nM apicidin for 3 hours in total.

Protein synthesis was assessed in the treated 24-well plates using a Click-iT® Plus O-propargyl-puromycin Protein Synthesis Assay Kit (Molecular Probes, Life Technologies). Briefly, after 2.5 hours of the aforementioned drug treatments, Click-iT® O-propargyl-puromycin was added directly to each well to give a final concentration of 20 μM. The BV2 microglia were cultured at 37°C in a humid atmosphere with 5% CO_2 for the remaining 30 minutes (3 hours drug treatment in total). The culture medium was then removed, the cells were washed with 1 mL sterile PBS, followed by fixation with 100 μL of 4% w/v paraformaldehyde (PFA, dissolved in autoclaved PBS) for 15 minutes at room temperature. The cells were then permeabilised with 100 μL of 0.5% v/v Triton X-100 (dissolved in autoclaved PBS) for 15 minutes at room temperature. The cells were washed 2 times with 1 mL autoclaved PBS and processed further following the manufacturer's instructions. Imaging of labelled cells was carried out using the IncuCyte™ FLR with a 10× objective lens taken in phase-contrast and green-fluorescence to visualise the OPP+

cells/the cells that are undergoing protein synthesis. Nine non-overlapping images at 1280 × 1024 resolution were taken per well. The settings for each image, per well, per condition were kept constant.

In parallel to the protein synthesis assay, RNA was extracted from BV2 microglia cultured in 6-well plates and treated to the aforementioned conditions. The RNA was reverse transcribed and the cDNA generated underwent qPCR analysis as described in section 2.12.

Chapter 3

Results I

Investigating the efficacy of HDAC inhibitors to prevent neuronal death in in vitro models of cerebral ischaemia

3.1. Introduction

The mechanisms that induce neuronal death in brain insults such as cerebral ischaemia, as well as injuries such as traumatic brain injury and neurodegenerative diseases such as Alzheimer's, multiple sclerosis, motor neurone disease and Parkinson's disease, lead to neuronal death through either necrosis or apoptosis. In *in vivo* models of cerebral ischaemia, histone deacetylase (HDAC) inhibitors are neuroprotective, neuro-restorative and improve neurological outcome (see section 1.10.2). However, the molecular mechanisms underlying these effects are not well understood. One way to investigate the mechanisms underlying neuroprotection seen *in vivo,* is to study the effects of inhibiting HDACs in isolated neurones challenged with neurotoxic insults. As discussed earlier in sections 1.10.3 and 1.10.4, non-selective and class I selective HDAC inhibitors protect cortical neurones and CGNs from a variety of neurotoxic insults including OGD, glutamate excitotoxicity, oxidative stress and classical apoptosis inducers such as low extracellular K^+, DNA damaging agents and protein kinase inhibitors. However, in contrast to these observations, other studies have reported that applying these HDAC inhibitors to neurones independent of an insult, or at the same time as exposing to the aforementioned insults, can also cause neurotoxicity and exacerbate neuronal death (Bollino et al., 2015; Boutillier et al., 2003; Gaub et al., 2010; Rouaux et al., 2004; Salminen et al., 1998; Vashishta and Hetman, 2014). It is generally considered that selectively inhibiting the HDACs responsible for producing a neuroprotective effect and not inhibiting HDACs, which are important for neurone function and survival, will prevent this non-selective HDAC inhibitor mediated neurotoxicity. Furthermore, selectively inhibiting particular HDACs will also allow for more focused experiments to be performed in order to understand the molecular mechanisms involved in the protection of neurones upon inhibition of the appropriate HDAC(s). However, as will now be presented, choosing the most appropriate HDACs to inhibit is not that simple, because the role of each of the HDAC isoforms in neuronal death and neurone survival processes is not clear.

3.1.1. The role of HDAC1 in neurone survival and death

The role HDAC1 in neurone survival and death is disputed. As already mentioned in section 1.7.1, mice overexpressing HDAC1 have no abnormalities in brain structure, cellular architecture or neurone numbers (Guan et al., 2009). Despite this, other studies show that HDAC1OE causes neurotoxicity in isolated rat cortical and cerebellar granule neurone (CGN) cultures (Bardai et al., 2012). In this study, the neurotoxic effect of HDAC1 activity is dependent on an interaction with HDAC3 (Bardai et al., 2012). Consistent with an increase in HDAC1 expression being neurotoxic, the same study observed HDAC1 to be upregulated in both the cortex and hippocampus of the p25/Cdk5 mouse model of Alzheimer's disease (Bardai et al., 2012). These two brain areas are known to degenerate in this condition. HDAC1 has also been found to be upregulated in experimental models relevant to cerebral ischaemia including in the mouse brain following MCAO (Baltan et al., 2011a) and in white matter tracts deprived of oxygen and glucose (mouse optic nerve OGD model, Baltan et al. (2011a)). Furthermore, in the corpus callosum of mice following cuprizone-induced axonal demyelination, in the brains of patients with multiple sclerosis and in a mouse cerebellar organotypic slice culture model of inflammatory demyelination, there is a consistent abnormal localisation and accumulation of HDAC1 in the cytoplasm and axons of neurones (Kim et al., 2010). Using cortical neurones to investigate this observation further, it was shown that neuronal HDAC1, in response to neurotoxic stimuli such as glutamate and TNF-α, undergoes translocation to the cytoplasm and axons, where it accumulates and inhibits axonal transport (Kim et al., 2010). This leads to the formation of protein aggregates, which cause neurite beading, swelling and irreversible axon transections (Kim et al., 2010). This axonal damage can be prevented by inhibiting HDACs 1, 2 & 3 with MS-275, or knocking down HDAC1 but not HDAC2, 3, 4, 6 or 8 (Kim et al., 2010).

Contrary to the work by Montgomery et al. (2009), which shows HDAC1KO mice have no obvious brain abnormalities, and the aforementioned studies that suggest an increase in HDAC1 activity promotes neurotoxicity, other studies have shown HDAC1 activity is important for neurone survival and increasing it has neuroprotective effects. Unlike Bardai et al. (2012), Kim et al. (2008) found HDAC1

to not be upregulated in the brains of p25/Cdk5 mice, rather HDAC1 activity is reduced (Kim et al., 2008). The same group have also shown HDAC1 levels are not affected in the brains of patients with Alzheimer's disease either (Graff et al., 2012). One study shows HDAC1 expression and therefore activity is also reduced in the mouse brain following MCAO-induced cerebral ischaemia (Chen et al., 2012c). Kim et al. (2008) show that a reduction in HDAC1 expression in cortical neurones causes neurotoxicity. Here, HDAC1KD causes double-strand DNA breaks, aberrant expression of cell-cycle genes and these ultimately trigger neuronal death. Consistent with this, HDAC1OE is neuroprotective in the p25/Cdk5 mouse model of Alzheimer's disease and in the rat bilateral common carotid artery occlusion (BCCAO) model of cerebral ischaemia (Kim et al., 2008). More recently, the same laboratory show HDAC1 is required for successful DNA repair in mouse cortical neurones (Wang et al., 2013b). Here, knockdown of HDAC1 prevents the repair of double-strand DNA breaks whereas HDAC1OE enhances it. During DNA repair signalling, HDAC1 is found to interact with the Fused-in-Sarcoma (FUS) protein which is recruited to sites of DNA damage in neurones and is important in detecting and activating DNA repair processes (Wang et al., 2013b).

3.1.2. The role of HDAC2 in neurone survival and death

HDAC2 is found to be upregulated in the post-mortem brain of patients with Alzheimer's disease and in the p25/Cdk5 mouse model of this neurodegenerative condition. This increase is thought to contribute to the cognitive decline associated with Alzheimer's disease (Graff et al., 2012). An upregulation of HDAC2 has also been observed in other neurodegenerative and neurotoxic conditions. HDAC2 expression is increased in the motor cortex of the post-mortem brain of patients with motor neurone disease (Janssen et al., 2010) and in the ischaemic mouse brain following MCAO (Baltan et al., 2011a). It is not known if the upregulation of HDAC2 causes the neuronal loss in these conditions, but HDAC2 expression is also increased in isolated mouse hippocampal neurones exposed to oxidative stress conditions (Graff et al., 2012) and a recent study shows that knocking down HDAC2 protects CGNs from this insult (Peng et al., 2015). Taken together, these studies imply that HDAC2 may also be involved in regulating neuronal apoptosis.

3.1.3. The role of HDAC3 in neurone survival and death

HDAC3 is also involved in regulating neurone survival and death. One of the earliest studies suggested HDAC3 was important in neurone survival and the active process of HDAC3 degradation by caspase enzymes was important during low K^+ induced CGN apoptosis. Mechanistically, the loss of HDAC3 during neurone death relieves the repression on the apoptosis-inducing gene E2F-1 (Boutillier et al., 2003; Panteleeva et al., 2004). More recently, another study revealed that knockout of HDAC3 in mouse Purkinje neurones of the cerebellum, has deleterious effects resulting dendritic pruning, neurone death and motor impairments in mice (Venkatraman et al., 2014).

Contrary to the aforementioned studies, there are others that show HDAC3 activity, is involved in neuronal death. Bardai and D'Mello (2011) found that knocking down HDAC3 in isolated rat cortical neurones or CGNs protects against apoptosis induced by either homocysteate or low extracellular K^+ respectively. Also, overexpressing HDAC3 in healthy neurones causes neurotoxicity (Bardai and D'Mello, 2011). HDAC3 mediated neurotoxicity is dependent on HDAC3 interacting with HDAC1 and also its phosphorylation by glycogen synthase kinase 3 beta (GSK3β), a kinase which itself is implicated in neurodegenerative diseases (Bardai et al., 2012). HDAC3 is found to be upregulated in neurones that are dying following nerve crush. Here, increased HDAC3 expression followed by nuclear translocation is correlated with widespread deacetylation of histones and presumably aberrant gene expression in dying neurones following axon damage (Pelzel et al., 2010). HDAC3 is not upregulated in the cortex of the post-mortem brain of Alzheimer's disease patients or in the cortex or hippocampus of p25/Cdk5 mice (Graff et al., 2012) but its expression is increased in the brains of mice following cerebral ischaemia (Baltan et al., 2011a; Chen et al., 2012c) and in cortical neurones exposed to OGD (Chen et al., 2012c). Consistent with the role of HDAC3 in causing neuronal death, knocking down HDAC3 in cortical neurones is neuroprotective against OGD (Chen et al., 2012c).

3.1.4. The role of HDAC4 in neurone survival and death

Like HDAC1 and HDAC3 discussed earlier, the role of HDAC4 in neurone survival and death is disputed. Bolger and Yao (2005) report that during low K^+ induced apoptosis or glutamate excitotoxicity, HDAC4 in isolated mouse CGNs, translocates to the nucleus and represses the activity of the pro-survival transcription factors MEF-2 and CREB. Knocking down HDAC4 is neuroprotective in this context, whereas overexpression of HDAC4 in the nucleus of healthy neurones is neurotoxic. HDAC4 also translocates to the nucleus of isolated rat cortical neurones following the induction of cell death due to oxidative stress (Yang et al., 2011). Once in the nucleus, HDAC4 interacts with and inhibits another pro-survival transcription factor peroxisome proliferator-activated receptor gamma (PPARγ, Yang et al. (2011)). In this study, knocking down HDAC4 also protects cortical neurones from oxidative stress induced neuronal death (Yang et al., 2011).

However, in disagreement with the evidence suggesting HDAC4 activity causes neurone cell death, other studies suggest HDAC4 activity is important for neurone survival and is neuroprotective. In contradiction to the work by Bolger and Yao (2005), Majdzadeh et al. (2008) report that overexpression of HDAC4 (HDAC4OE) is neuroprotective and protects isolated rat CGNs from low K^+ induced apoptosis, rat cortical neurone cultures from 6-hydroxy dopamine-induced apoptosis and mouse hippocampal neurone-like cells (HT22 neuroblastoma cell-line) from homocysteate-induced oxidative stress. Additionally, forced expression of HDAC4 in rat neurone-like cells (differentiated pheochromocytoma PC-12 cell-line) is neuroprotective against OGD and this is correlated with a decrease in expression of high-mobility group protein 1 (HMG-1), a mediator of tissue damage following acute injury (He et al., 2013). Consistent with HDAC4 being important for neurone survival, HDAC4 mRNA and protein expression levels are significantly reduced in these rat neurone-like cells following OGD and also in the ischaemic brains of rats during MCAO (He et al., 2013). Surprisingly, in the latter model, there is no change in the levels of HDAC1, 2 or 3 as reported by others (see sections 3.1.1, 3.1.2 and 3.1.3).

3.1.5. The role of HDAC5 in neurone survival and death

Knockout of HDAC5 in healthy mice does not affect hippocampal integrity or survival as measured by immunoreactivity for neurones, axons and synapses (Agis-Balboa et al., 2013). However, in models relevant to cerebral ischaemia, HDAC5 levels are significantly reduced in the ischaemic brains of mice and rats following MCAO (He et al., 2013; Chen et al., 2012c) and in rat neurone-like cells (differentiated pheochromocytoma PC-12 cell-line) after OGD (He et al., 2013). The underlying cause of this reduction and the consequence of this change is not clear. HDAC5OE in rat neurone-like cells exposed to OGD is neuroprotective (He et al., 2013) and HDAC5OE in the nucleus of isolated rat cortical neurones protects against glutamate excitotoxicity (Wei et al., 2015). Wei et al. (2015) also report that HDAC5 is phosphorylated in neurones undergoing apoptosis and is exported out of the nucleus. This loss of nuclear HDAC5 may lead to neuronal death through a de-repression of apoptosis-inducing genes. Together these studies show a reduction in HDAC5 levels and activity in the nucleus is detrimental to neurones and enhancing its activity through overexpression is neuroprotective.

In a different study, HDAC5OE in isolated rat CGNs is neurotoxic in the absence of a neurotoxic stimulus (Linseman et al., 2003). Furthermore, unlike cortical neurones, when CGNs are undergoing apoptosis, HDAC5 is dephosphorylated and undergoes nuclear translocation, where it inhibits the pro-survival transcription factor MEF-2 (Linseman et al., 2003).

3.1.6. The role of HDAC6 in neurone survival and death

In isolated rat cortical neurones, an upregulation of HDAC6 coincides with neuronal commitment to apoptosis upon homocysteate-induced oxidative stress (Rivieccio et al., 2009) and OGD (Yuan et al., 2015; Chen et al., 2012c). Knocking down or inhibiting HDAC6 is neuroprotective in both neurotoxic insults. The molecular mechanisms underlying neuroprotection are not known. The neuroprotective effect following HDAC6 inhibition comes as a surprise because HDAC6 in addition to regulating intracellular transport mechanisms, is important for the clearance of

misfolded proteins (Boyault et al., 2007; Iwata et al., 2005; Kawaguchi et al., 2003). These proteins are known to accumulate and aggregate as a result of damage by oxidative stress, which then causes neuronal dysfunction and neurotoxicity in conditions such as Alzheimer's disease. In addition to protecting neurones, knocking down HDAC6 also facilitates neurite outgrowth, which in-turn helps re-establish neuronal networks that have been damaged and lost following an insult (Rivieccio et al., 2009).

3.1.7. The role of HDAC7 in neurone survival and death

Little is known about the function of HDAC7 in the developed brain, but one report shows HDAC7 is important for neurone survival (Ma and D'Mello, 2011). Knocking down HDAC7 is neurotoxic in isolated rat CGNs and HDAC7 protein is dramatically reduced during low K^+ induced apoptosis of CGNs and glutamate excitotoxicity in hippocampal neurone-like cells (HT22 neuroblastoma cell-line). Consistent with the role of HDAC7 in neurone survival, overexpressing this isoform in CGNs and cortical neurones prevents apoptosis induced by low K^+ and homocysteate respectively. HDAC7 promotes neurone survival by repressing the expression of c-Jun a transcription factor implicated in neuronal death. Together, these findings suggest under normal circumstances HDAC7 activity is important for neurone survival, but in neurones primed to die, HDAC7 levels are reduced and the associated neurone survival activities are lost (Ma and D'Mello, 2011).

3.1.8. Chapter aim

Several studies have shown selectively inhibiting class I HDACs 1, 2 & 3 protects isolated neurones from neuronal death caused by OGD (Formisano et al., 2015; Lanzillotta et al., 2013) and oxidative stress (Sleiman et al., 2014), as well as preventing axon damage when neurones are exposed to glutamate & TNF-α (Kim et al., 2010). The inhibition of HDAC2 may underlie the neuroprotective effects seen with class I HDAC inhibitors in neurones. Therefore, this isoform is a plausible candidate to selectively inhibit with pharmacological agents, to promote neuroprotection whilst minimising the risk of causing neurotoxicity from inhibiting other HDACs. Currently available HDAC inhibitors do not exclusively inhibit HDAC2 and inhibit HDAC1 and/or HDAC3 with similar pharmacological properties (table 1.4). The most selective HDAC inhibitor for HDAC2 is MI192, which has selectivity towards HDAC2 & 3 over HDAC1 (see section 1.8). In this chapter, the efficacy of selective HDAC2 & 3 inhibition to protect neurones from neurotoxicity was examined. To do this, CGNs were isolated and cultured, then neuronal cell death was induced by exposing them to 1) glutamate excitotoxicity, which is a major pathophysiological event in cerebral ischaemia, as well as traumatic brain injury, multiple sclerosis, Alzheimer's disease and motor neurone disease (reviewed by Dong et al. (2009); Lau and Tymianski (2010)), or 2) oxygen glucose deprivation (OGD), the initiating pathophysiological event in cerebral ischaemia.

3.2. HDAC inhibitors do not protect CGNs from glutamate excitotoxicity

CGNs were isolated from seven-day-old rats and cultured for ten days *in vitro* (DIV) before experimentation. After 10 DIV, the cultures had developed a stable morphology and neuronal network (figure 3.1) and of this cell population 91.7 ± 0.315% of the cells were identified as being CGNs (figure 3.2A & C). The remaining 8.3% of the cell population included astrocytes, as indicated by the presence of cells which were highly branched and positive for the astrocyte marker GFAP (figure 3.2B).

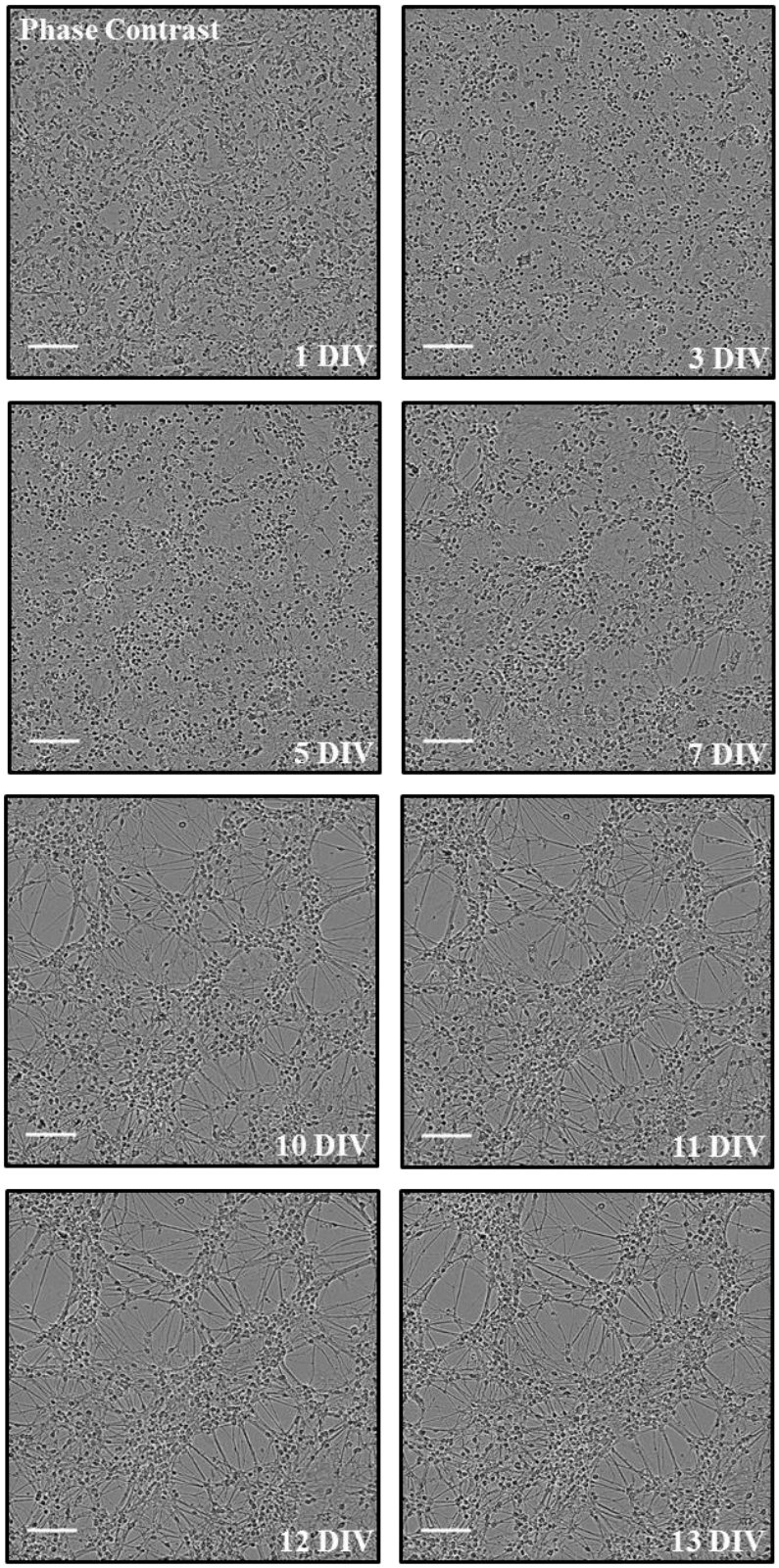


Figure 3.1. Development of cerebellar granule neurones (CGNs) during culture. Representative phase-contrast images show the morphological changes of CGNs every 24 hours over a period of 13 days *in vitro* (DIV). Scale bar 100 μm.

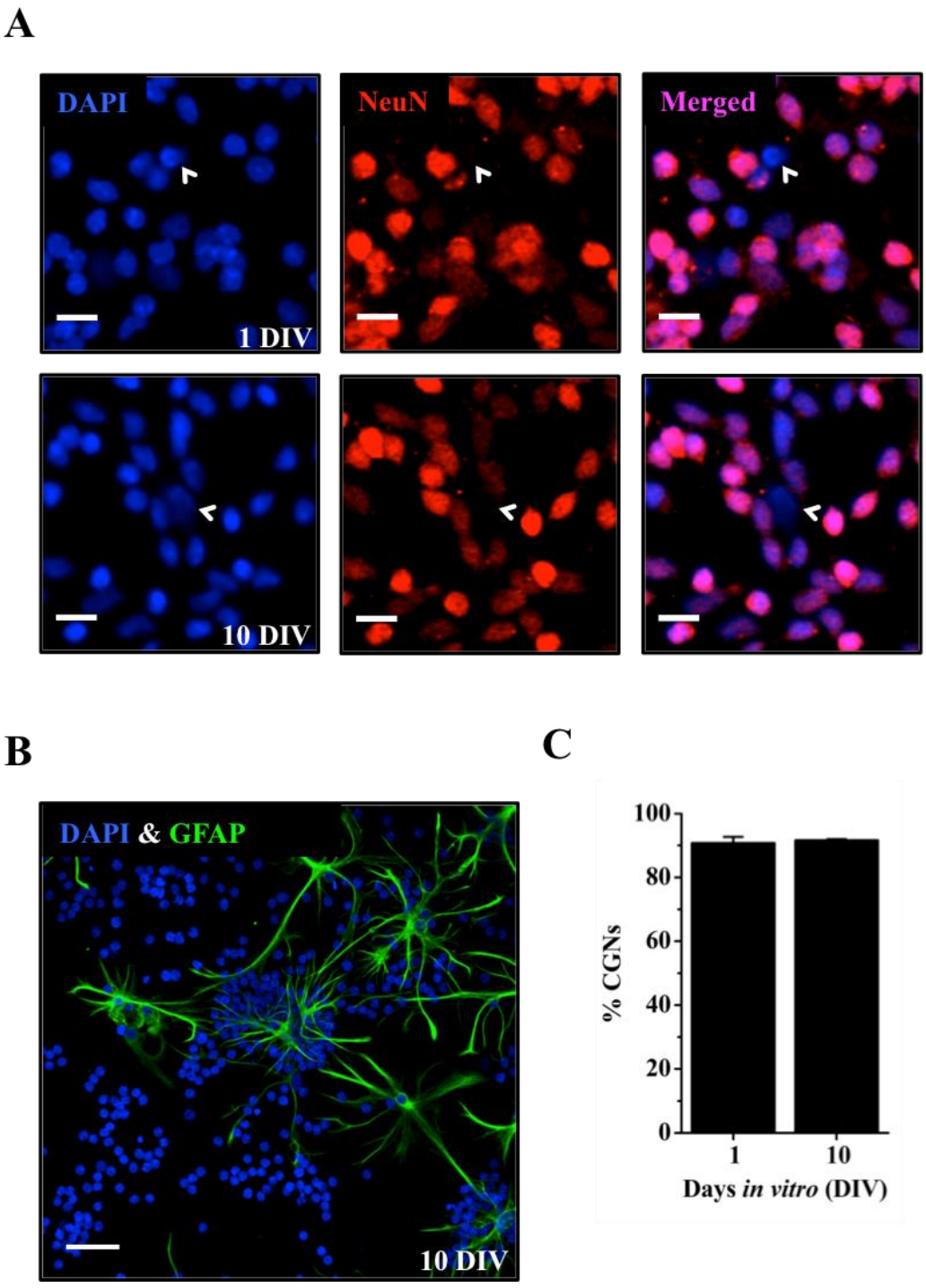


Figure 3.2. Purity of cerebellar granule neurone (CGN) cultures. Representative fluorescence immunocytochemistry images showing; **(A)** all cells (DAPI+, blue), CGNs (NeuN+, red) and a merged image of the two (magenta) at 1 day *in vitro* (DIV) and 10 DIV, arrow heads indicate non-neurone cells (NeuN-), and **(B)** all cells (DAPI+, blue) and astrocytes (GFAP+, green) at 10 DIV. Scale bar 15 & 50 μm for (A) & (B) respectively. **(C)** Quantification of (A), purity of CGN cultures at 1 and 10 DIV is expressed as mean percentage CGNs (NeuN+ cells) ± SEM, *n*=3.

To determine neurone viability, CGNs were incubated with the cell death stain YOYO®-1, which is a nucleic acid dye that works on the basis that dying and dead cells have increased membrane permeability so accumulate the stain, whereas intact-viable cells are impermeable to the stain (Becker et al., 1994). Images were taken of the cells by fluorescence microscopy and then all the cells were stained with the cell-permeable nucleic acid dye SYBR Green I and imaged once more. The number of YOYO®-1+ cells and SYBR® Green I+ cells were quantified from the fluorescent images and percentage cell viability was calculated.

Glutamate excitotoxicity was induced in CGNs by administering 100 μM L-glutamate (as used by Ankarcrona et al. (1995); Du et al. (1997); Leng and Chuang (2006)). After 24 hours of treatment, there was a significant reduction in cell viability of 44.69 ± 2.56% ($P=8.44 \times 10^{-11}$ compared to the vehicle control, figure 3.3A & 3.3B). This cell death was significantly suppressed by co-administering both 30 μM NBQX an AMPA & Kainate glutamate receptor antagonist, and 10 μM MK-801 a NMDA glutamate receptor antagonist. These drugs restored cell viability to 90.96 ± 1.46% ($P=2.88 \times 10^{-10}$ compared to glutamate +vehicle, figure 3.3A & 3.3B). Glutamate excitotoxicity causes neurone death through necrosis and apoptosis (Ankarcrona et al., 1995; Du et al., 1997). Necrotic cell death is an uncontrolled event resulting from an intense stimulus, which causes overwhelming cell stress and damage. Apoptosis on the other-hand is a controlled intracellular signalling cascade, which is triggered by the cell in response to detecting damage or when the cell is unable to perform its normal function. Apoptosis involves a complex intracellular pathway involving many proteins but importantly the proteolytic enzymes caspases 3 & 7. In CGNs, administering 200 μM N-acetyl-Asp-Glu-Val-Asp-al (ac-DEVD-al) a caspase 3 & 7 inhibitor at the same time as L-glutamate did not protect CGNs from glutamate excitotoxicity ($P=1.74 \times 10^{-10}$ compared to the vehicle control, figure 3.3B). It was assumed the caspase inhibitor was working because, when used in a parallel experiment (see figure 3.5), caspase inhibition was able to protect CGNs from neurotoxicity induced by another stimulus. Because caspases, which are essential for apoptosis were not involved in glutamate-induced neuronal death during 24 hours, this implied that these neurones were not dying from apoptosis and were likely to be undergoing necrotic cell death.

To examine the effects of HDAC inhibition on this glutamate-induced cell death, first the effect of HDAC inhibitors in healthy CGN cultures was examined. CGNs were treated to 1 μM of the non-selective HDAC inhibitor SAHA or 1 μM of the HDAC2 & 3 selective inhibitor MI192 and after 24 hours cell viability of neurones exposed to these HDAC inhibitors was 89.6 ± 0.687% and 90.34 ± 0.339% respectively (figure 3.3B). These viabilities were not significantly different from the vehicle control, therefore non-selective HDAC inhibition and selective inhibition of HDAC2 & 3 did not induce neurotoxicity in this time frame. Treatment of CGNs with either HDAC inhibitor for 24 hours robustly increased the acetylation of histone H3 at lysine 9 (acH3K9, figure 3.3C) and pan-acetylation of histone H4 (acH4, figure 3.3C). Despite HDAC inhibition having a positive effect on histone acetylation, treatment of 1 μM SAHA, 1 mM VPA (a non-selective HDAC inhibitor) or 1 μM MI192 at the same time as glutamate, did not protect CGNs from glutamate-induced cell death after 24 hours, with cell viabilities of 48.6 ± 1.72% ($P=8.18 \times 10^{-11}$ compared to the vehicle control), 45.03 ± 2.55% ($P=2.89 \times 10^{-11}$ compared to the vehicle control) and 51.01 ± 3.047% ($P=1.41 \times 10^{-10}$ compared to the vehicle control) respectively (figure 3.3B). Administering 3 μM SAHA or 3 μM MI192 at the same as glutamate did not protect CGNs after 24 hours either (figure 3.3B). Also, SAHA or MI192 did not protect CGNs from glutamate-induced cell death after treating for 72 hours, with cell viabilities of 53.5 ± 3.58% ($P=3.71 \times 10^{-4}$ compared to the vehicle control) and 46.9 ± 6.43% ($P=1.24 \times 10^{-4}$ compared to the vehicle control) respectively (figure 3.4A).

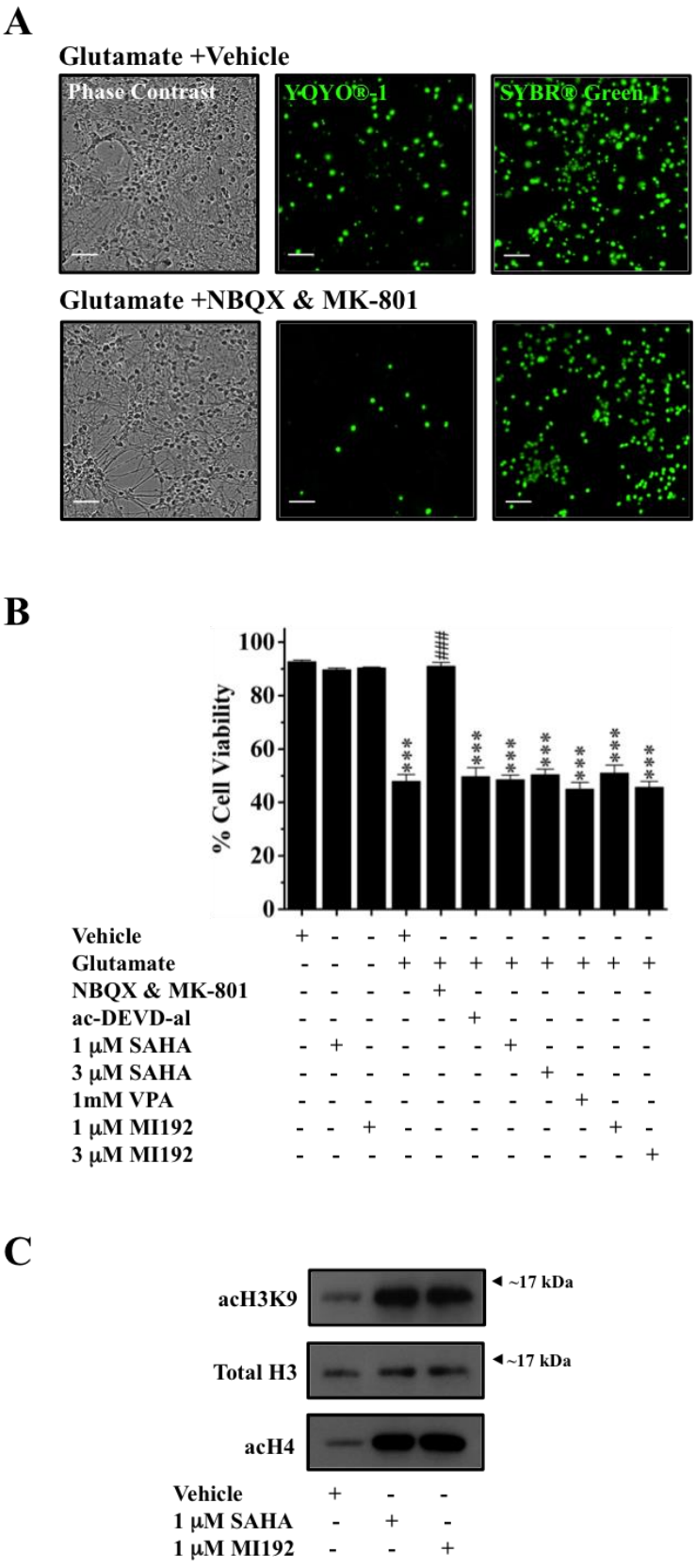


Figure 3.3. Histone deacetylase inhibitors do not protect rat cerebellar granule neurones (CGNs) from excitotoxicity when administered at the same time as glutamate for 24 hours.

Figure 3.3 ***continued…*** CGNs after 10 days *in vitro* (DIV) were treated to vehicle (0.3% v/v DMSO), 1 μM SAHA or 1 μM MI192. To induce glutamate excitotoxicity and examine the effects of pharmacological interventions on this, CGNs were exposed to 100 μM L-glutamate and simultaneously treated with either vehicle, both 30 μM NBQX and 10 μM MK-801 (glutamate receptor antagonists), 200 μM ac-DEVD-al (a caspase inhibitor), 1 or 3 μM SAHA, 1 mM VPA or 1 or 3 μM MI192 for 24 hours. Cell viability was then determined by fluorescence microscopy. **(A)** Representative images of phase contrast and fluorescently labelled dying/dead CGNs (YOYO®-1+, green) and all CGNs (SYBR® Green I+) after 24 hours of excitotoxicity induced by glutamate with either vehicle or glutamate receptor antagonists. Scale bar 50 μm. **(B)** The number of YOYO®-1 and SYBR® Green I positive CGNs were quantified from all images across all conditions and data shown are mean percentage cell viability ± SEM. All experiments were performed n=3. ***P<0.001 vs. vehicle, ###P<0.001 vs. glutamate +vehicle. **(C)** CGNs were treated to vehicle, 1 μM SAHA or 1 μM MI192 for 24 hours followed by histone protein extraction and Western blot to analyse acetylated histone H3 at lysine 9 (acH3K9) and pan-acetylated histone H4 (acH4). Total histone H3 was used as the loading control.

One of the reasons why HDAC inhibitors do not protect CGNs from glutamate-induced excitotoxicity, when administered at the same time as glutamate, may be because the neurotoxic insult is continually present. When glutamate is added to CGN cultures, if this is in excess and is not transported into and then metabolised by neurones and contaminating astrocytes, and/or there is no glutamate receptor desensitisation, then toxic levels of glutamate in the media will persist. This will chronically stimulate neuronal glutamate receptors, which would maintain a constant state of depolarisation and calcium overload; processes that trigger cellular damage and neuronal cell death. This could mean that any protective mechanisms induced by HDAC inhibitors may be antagonised or are always overwhelmed by the insult. Also, because there is no point in time where CGNs are not being challenged by glutamate, there is no opportunity for the intracellular environment to return to normal and for HDAC inhibitor induced repair mechanisms to repair damage and have a neuroprotective effect. To examine if HDAC inhibitors are neuroprotective against a brief pulse of glutamate, CGNs were exposed to 100 μM L-glutamate for 30 minutes in the presence of either the vehicle control, 1 μM SAHA or 1 μM MI192. These treatments were then removed and CGNs were treated to either the vehicle control, 1

μM SAHA or 1 μM MI192 for a further 72 hours. It was noticed that the viability of CGNs in the control condition (79.9 ± 0.170%) was reduced compared to previous experiments. This was presumably because of the treatment paradigm itself, which involved more handling and manipulating of the neurone cultures outside of optimal culture conditions compared to other treatment regimes. Thirty minutes of glutamate exposure followed by 72 hours of culture in the absence of glutamate, induced 42.1 ± 0.892% cell death ($P=2.86 \times 10^{-14}$ compared to the vehicle control, figure 3.4B). SAHA or MI192 when given at the same time as the glutamate pulse and for 72 hours after this, did not protect CGNs from the initial insult or induce repair mechanisms that promoted neurone survival (figure 3.4B). Cell viabilities following SAHA and MI192 treatment were 36.8 ± 0.478% ($P=2.16 \times 10^{-14}$ compared to the vehicle control) and 38.9 ± 0.713% ($P=3.95 \times 10^{-14}$ compared to the vehicle control) respectively (figure 3.4B).

To protect against glutamate-induced neuronal death, HDAC inhibitors may need to be pre-treated before an insult to allow time for the neuroprotective molecular changes to take place. To test this hypothesis, CGNs were pre-treated with either the vehicle control, 1 μM SAHA or 1 μM MI192 for 24 hours before exposing to L-glutamate for a further 24 hours (figure 3.4C). Pre-treating with the vehicle control followed by glutamate exposure induced, 46.7 ± 2.72% cell death ($P=5.8 \times 10^{-8}$ compared to the vehicle control). Both HDAC inhibitors when administered before the insult, failed to protect CGNs from glutamate-induced cell death with cell viabilities of 24.5 ± 1.802% and 41.9 ± 3.401% respectively ($P=6.5 \times 10^{-10}$ and $P=1.87 \times 10^{-8}$ compared to the vehicle control respectively). Pre-treatment of SAHA for 24 hours significantly exacerbated glutamate-induced cell death ($P=1.54 \times 10^{-4}$ compared to glutamate +vehicle). This effect was not due to exposure to SAHA on its own for 24 hours because this had no effect on CGN viability (figure 3.3B), nor was it due to the co-treatment paradigm or the length of time CGNs were exposed to SAHA because when these neurones were treated to this HDAC inhibitor alongside L-glutamate for 24 or 72 hours, the cell death induced (44.07 ± 1.72% and 53.5 ± 3.58% for 24 and 72 hours respectively) was not significantly different to that caused by glutamate +vehicle (44.7 ± 2.56% and 53.8 ± 2.55% for 24 and 72 hours

respectively). This suggests SAHA pre-treatment causes CGNs to be more susceptible to neurotoxicity by glutamate.

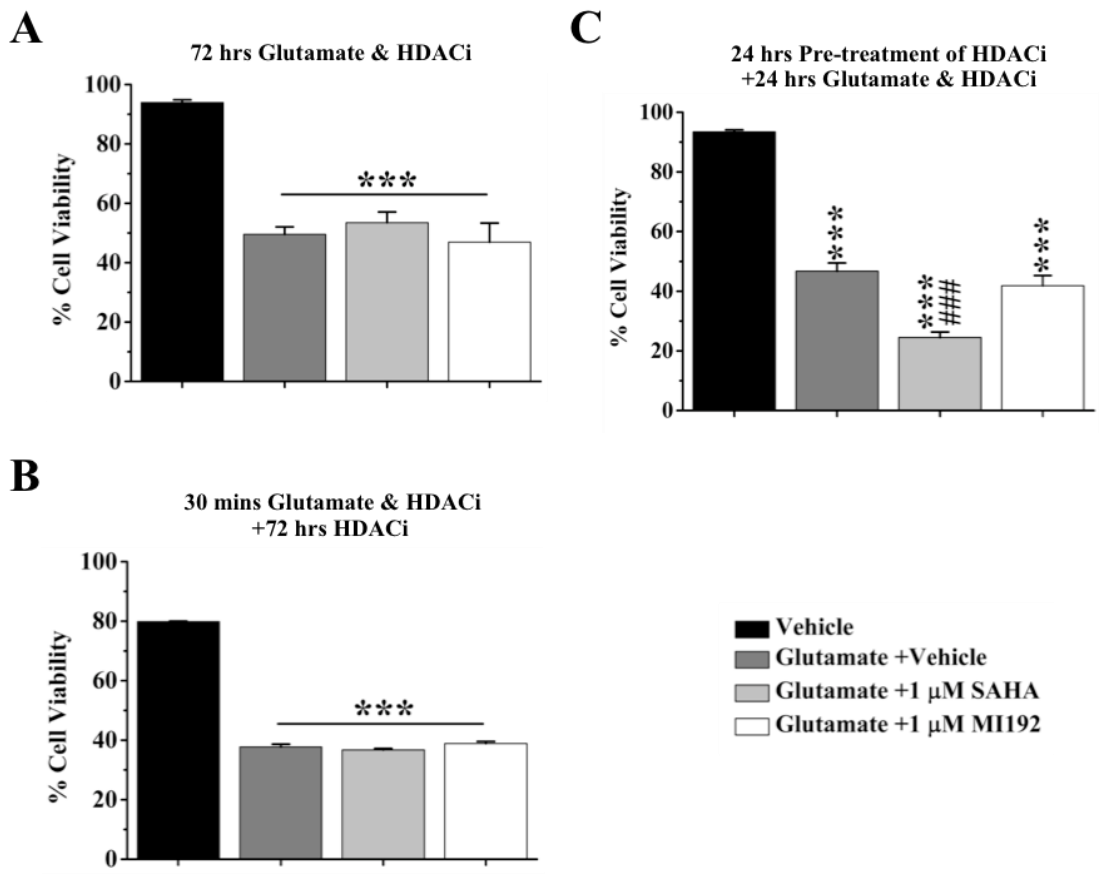


Figure 3.4. Histone deacetylase inhibitors (HDACi) do not protect rat cerebellar granule neurones (CGNs) from glutamate excitotoxicity when treated for longer, treated after a short pulse of glutamate or when pre-treated before glutamate. **(A)** CGNs after 10 days *in vitro* (DIV) were exposed to 100 μM L-glutamate and simultaneously treated with either vehicle (0.1% v/v DMSO), 1 μM SAHA or 1 μM MI192 for 72 hours. **(B)** CGNs were treated to 100 μM L-glutamate with either vehicle, 1 μM SAHA, 1 μM MI192 for 30 minutes. This was then removed and CGNs were treated to either vehicle, 1 μM SAHA, 1 μM MI192 for a further 72 hours. **(C)** CGNs were treated to vehicle, 1 μM SAHA, 1 μM MI192 for 24 hours followed by exposure to 100 μM L-glutamate with either the vehicle control or the HDAC inhibitors for a further 24 hours. For each panel, cell viability was determined by fluorescence microscopy and data shown are mean percentage cell viability ± SEM. All experiments were performed *n*=3. ***P<0.001 vs. vehicle, ###P<0.001 vs. glutamate +vehicle.

Rather than a sudden and overwhelming neurotoxic stimulus as modelled when applying glutamate directly to CGNs, it may be that HDAC inhibitors are only neuroprotective when the induction of cell death is slower and/or is through the apoptotic cell death pathway. To model a slower induction of glutamate excitotoxicity and one with an apoptotic component which HDACs such as HDAC2 may positively regulate and/or HDAC inhibitors can protect against, CGNs were treated for 24 hours with 500 μM DL-*threo*-β-Benzyloxyaspartic acid (DL-TBOA) a blocker of excitatory amino acid re-uptake transporters (EAATs 1-5, Shigeri et al. (2001); Shimamoto et al. (1998)). It was expected that during this time, glutamate would gradually accumulate in the culture medium as CGNs released this neurotransmitter but were unable to re-uptake it. This glutamate, after a period of time, would reach levels sufficient to induce neurotoxicity and so the cell would have the time to detect the cellular damage caused by excitotoxicity and initiate appropriate repair mechanisms or apoptosis. This temporally dependent insult would better mimic the cell death induced by glutamate excitotoxicity in the penumbral brain region in *in vivo* models of cerebral ischaemia (Dirnagl et al., 1999). Furthermore, as the induction of apoptosis would be relatively slow, there may be a therapeutic window where HDAC inhibitors may act to have a neuroprotective effect by preventing the damage, which would trigger apoptosis and/or by inhibiting the apoptotic-signalling cascade itself.

After 24 hours exposure to DL-TBOA, cell viability was significantly reduced to 58.2 ± 3.12% ($P=1.17 \times 10^{-7}$ compared to the vehicle control, figure 3.5A & 3.5B). This DL-TBOA induced cell death could be significantly suppressed by co-administering both 30 μM NBQX and 10 μM MK-801 (glutamate receptor antagonists) with cell viability restored to 84.2 ± 2.17% ($P=3.051 \times 10^{-6}$ compared to DL-TBOA, figure 3.5A & 3.5B), which was not significantly different to vehicle control (P=0.845). Neuronal death induced by DL-TBOA in CGNs was through an apoptotic pathway as predicted; with treatment of a caspase inhibitor (ac-DEVD-al) at the same time as DL-TBOA suppressing neuronal death (viability of CGNs was restored to 73.3 ± 2.47%, $P=5.72 \times 10^{-4}$ compared to DL-TBOA +vehicle, $P=1.42 \times 10^{-3}$ compared to the vehicle control, figure 3.5A & 3.5B). However, when isolated CGNs were exposed to DL-TBOA and at the same time either 1 μM SAHA, 1 mM

VPA or 1 μM MI192, these HDAC inhibitors did not protect CGNs from DL-TBOA induced apoptosis with cell viabilities of 53.2 ± 2.41% ($P=1.66 \times 10^{-7}$ compared to the vehicle control), 56.5 ± 4.19% ($P=5.76 \times 10^{-7}$ compared to the vehicle control) and 57.9 ± 2.503% ($P= 9.704 \times 10^{-7}$ compared to the vehicle control) respectively (figure 3.5B).

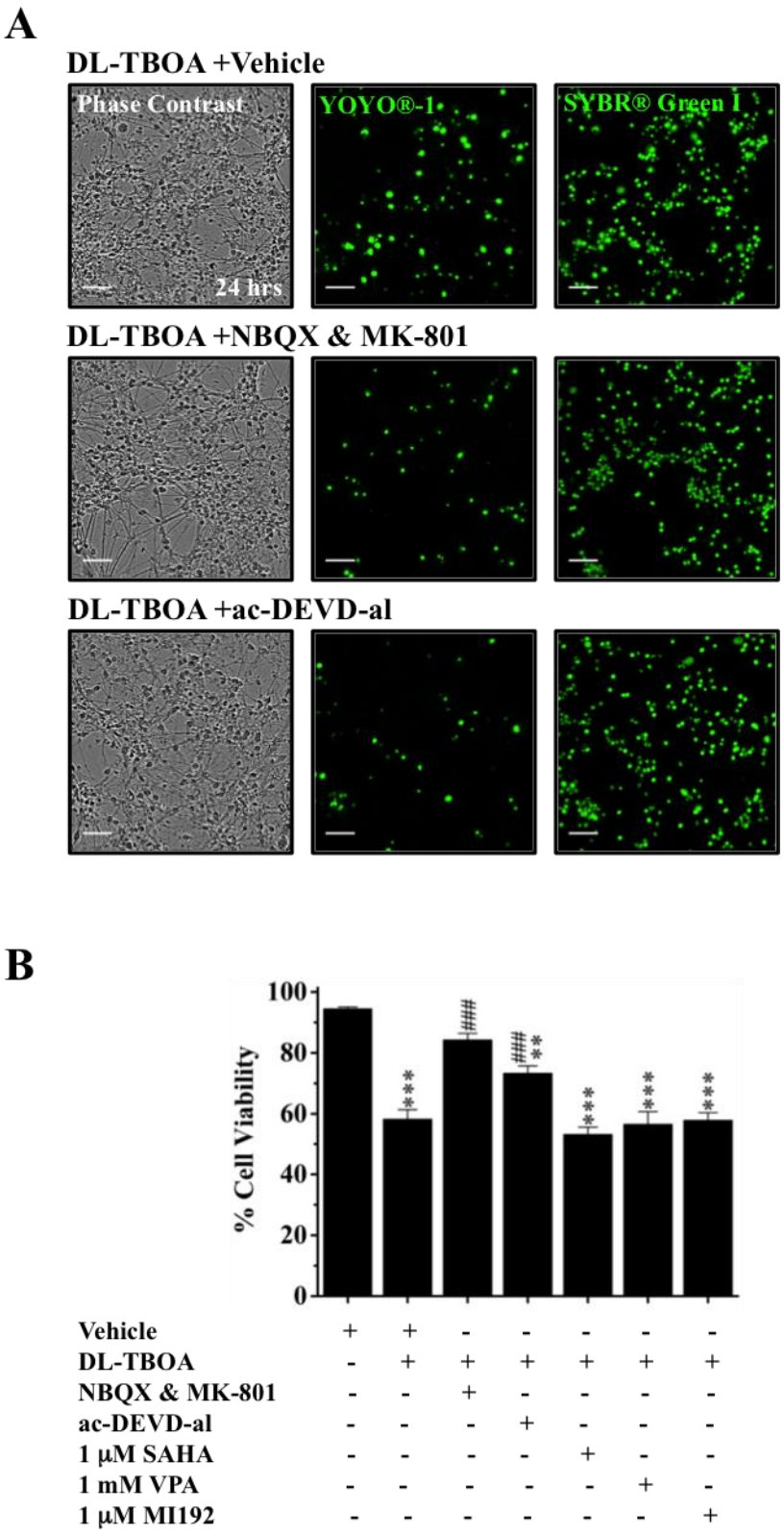


Figure 3.5. Histone deacetylase inhibitors do not protect rat cerebellar granule neurones (CGNs) from glutamate excitotoxicity induced by inhibiting glutamate re-uptake transporters.

Figure 3.5 ***continued…*** CGNs after 10 days *in vitro* (DIV) were treated for 24 hours to vehicle (0.6% v/v DMSO) or 500 μM DL-TBOA (a blocker of excitatory amino acid re-uptake transporters, EAATs) with either vehicle, both 30 μM NBQX and 10 μM MK-801 (glutamate receptor antagonists), 200 μM ac-DEVD-al (a caspase inhibitor) or the HDAC inhibitors SAHA, VPA, or MI192. Cell viability was then determined by fluorescence microscopy. **(A)** Representative images of phase contrast and fluorescently labelled dying/dead CGNs (YOYO®-1+, green) and all CGNs (SYBR® Green I+) after 24 hours of treatment with DL-TBOA and vehicle, glutamate receptor antagonists or a caspase inhibitor. Scale bar 50 μm. **(B)** The number of YOYO®-1 and SYBR® Green I positive CGNs were quantified from all images across all conditions and data shown are mean percentage cell viability ± SEM. All experiments were performed *n*=3. **P<0.01 & ***P<0.001 vs. vehicle, ###P<0.001 vs. DL-TBOA +vehicle.

3.3. HDAC inhibitors do not protect CGNs from oxygen glucose deprivation (OGD)

In the models of glutamate excitotoxicity used in this chapter, treatment of HDAC inhibitors did not prevent necrotic or apoptotic cell death caused by this insult. Therefore, the effect of HDAC inhibitors on neurone survival was investigated using another model system of cerebral ischaemia; oxygen and glucose deprivation (OGD). A reduction in the supply of oxygen and glucose to a region of the brain is the fundamental pathophysiological event that causes neuronal death in cerebral ischaemia. To induce OGD, CGNs cultured for 10 DIV were exposed to deoxygenated culture medium and incubated at 37°C in an atmosphere composed of 95% N_2 and 5% CO_2 for 40 minutes (exposure time was based on the findings reported by Kalda et al. (1998) and preliminary work looking at a time series of OGD exposures (30 to 90 mins), *n*=1 data not shown). After this, the deoxygenated medium was changed to normal culture medium with either the vehicle control or 1 μM SAHA and then the CGNs were cultured for a further 24 hours, a period of time termed the reperfusion period (as performed by Kalda et al. (1998); Scorziello et al. (2001); Scorziello et al. (2004)). Then, cell viability was determined using the MTT assay. Depriving CGNs of oxygen and glucose significantly reduced cell viability by 44.73 ± 12.5% (P=0.00937 compared to the no OGD control, figure 3.6). Administering SAHA immediately after exposing to OGD for 40 minutes, did not

prevent neuronal death after 24 hours of reperfusion (cell viability was 59.6 ± 8.33%, P= 0.00313 compared to the no OGD control, figure 3.6).

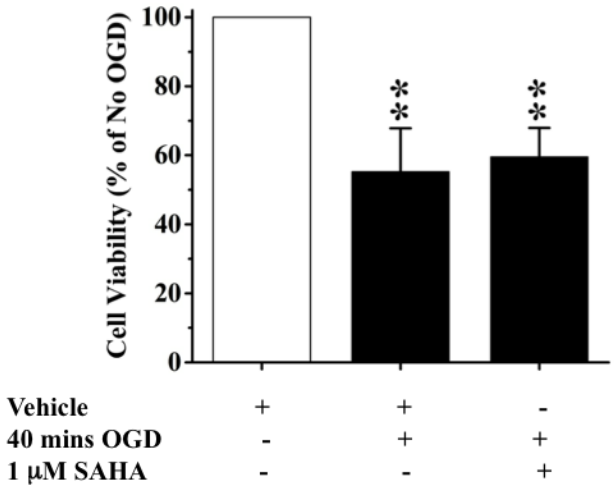


Figure 3.6. Inhibiting histone deacetylases does not protect rat cerebellar granule neurones (CGNs) from oxygen glucose deprivation (OGD)-induced death. CGNs after 10 days *in vitro* (DIV) were exposed OGD for 40 minutes followed by reperfusion in normal culture conditions with either the vehicle control (0.1% v/v DMSO) or 1 μM SAHA for a further 24 hours. Cell viability was determined by MTT assay and data shown are mean cell viability expressed as a percentage of the no OGD control ± SEM. All experiments were performed *n*=3. **P<0.01 vs. no OGD control.

3.4. Discussion

3.4.1. HDAC inhibitors do not protect neurones from glutamate-induced death

Glutamate excitotoxicity causes neuronal death through necrosis or apoptosis (Ankarcrona et al., 1995; Du et al., 1997). In this study, inhibiting caspases 3 & 7, which are enzymes essential for apoptosis, did not protect CGNs from glutamate-induced neuronal death (figure 3.3B). This suggests the predominant mode of cell death induced by glutamate exposure was not apoptotic but necrotic. It may be possible, through the use of HDAC inhibitors, to change the expression of specific genes thus proteins, in order to reduce the responsiveness of neurones to glutamate, reduce the susceptibility of neurones to excitotoxicity, or induce mechanisms that repair neurones from damage and therefore prevent the commitment to necrosis. However, administering HDAC inhibitors SAHA, VPA or MI192 at the same time as exposing CGNs to glutamate, did not protect these neurones (figure 3.3B, 3.4A & 3.4B). If changes in the acetylation of proteins such as histones and transcription factors, then changes in the transcription, translation and expression of genes upon HDAC inhibition are important for neuroprotection, it is likely that a certain length of time is required for these changes to take place following treatment with a HDAC inhibitor. This may be longer than the time it takes for the toxic effects of glutamate to cause neuronal damage and evoke necrotic cell death. Indeed, the amount of cell death induced when CGNs were exposed to just 30 minutes of glutamate followed by further culture without it (figure 3.4B) was not significantly different to the amount of cell death induced when CGNs were constantly exposed to glutamate for 24 and 72 hours (figure 3.3B & 3.4A). This suggests glutamate rapidly activates cell death processes during the first 30 minutes of exposure. Taking this fast kinetics into consideration, it is not too surprising that treatment of HDAC inhibitors at the same time as exposing to glutamate did not prevent CGNs undergoing glutamate-induced necrosis.

Based on the molecular mechanisms known to be involved in glutamate excitotoxicity (see section 1.10.1), some of the changes in gene expression that have been reported to occur in neurones and the central nervous system (CNS) following

HDAC inhibition, which might protect neurones from this insult include an increase in the expression of the glutamate re-uptake transporter EAAT2 (Baltan et al., 2011b; Yoo and Ko, 2011). This may enhance the uptake of glutamate from the extracellular space and reduce glutamate-induced activation of glutamate receptors. HDAC inhibition can also trigger an upregulation of calpastatin an inhibitor of the proteolytic calcium-dependent enzyme calpain 1 (Seo et al., 2013) and an increase in the expression of the Na^{+}/Ca^{2+} exchanger (Formisano et al., 2015), which may prevent the rise in intracellular calcium to the levels that are toxic to neurones. HDAC inhibition has also been shown to increase the expression of antioxidant enzymes in neurones and the brain including Glutathione peroxidase 1 (Camelo et al., 2005) and NADPH (nicotinamide adenine dinucleotide phosphate) dehydrogenase (Wang et al., 2011a). Together these changes in gene expression may prevent and reduce cellular damage caused by excitotoxicity. In order to change the expression of these genes, CGNs may need to be pre-treated with HDAC inhibitors to allow for the expression levels of these and other neuroprotective proteins to reach or surpass a threshold in order to protect from excitotoxicity. In support of this idea, pre-treatment of non-selective HDAC inhibitors VPA, TSA or sodium butyrate for two to seven days before exposing to glutamate, has been shown to protect CGNs from excitotoxicity (Leng and Chuang, 2006). Based on this, to investigate if selective HDAC2 & 3 inhibition prior to glutamate exposure was neuroprotective, CGNs were pre-treated with the SAHA or MI192 for 24 hours and then exposed to glutamate in the presence of the HDAC inhibitor. Following this, it was found that neither HDAC inhibitor protected CGNs from this insult. In fact, pre-treatment of the non-selective HDAC inhibitor SAHA, exacerbated/increased the susceptibility of CGNs to glutamate-induced CGN death (figure 3.4C).

This toxicity as-well as that seen by others when using non-selective HDAC inhibitors (Bollino et al., 2015; Boutillier et al., 2003; Gaub et al., 2010; Rouaux et al., 2004; Salminen et al., 1998; Vashishta and Hetman, 2014), could be caused by inappropriately inhibiting HDAC isoforms, which are important for neurone function and survival, or HDACs that when inhibited increase the susceptibility of neurones to toxicity. Therefore, it is important to selectively inhibit the appropriate HDAC isoforms to prevent the neurotoxicity associated with non-selective HDAC

inhibition. Consistent with this idea, pre-treatment of CGNs to MI192 did not exacerbate glutamate-induced CGN death. This suggests that the neurotoxic effect of SAHA is caused by an inappropriate inhibition of HDAC isoforms other than HDAC2 & 3 such as HDAC1, 5 and 8 (see table 1.4 and sections 3.1.1 and 3.1.5).

The absence of a neuroprotective effect following pre-treatment with MI192 could be for several reasons. The first is that HDAC2 & 3 activity is not involved in the induction and progression of necrotic cell death, therefore HDAC inhibitors do not prevent and protect from such activity. The second reason is that pre-treatment of MI192 for 24 hours is not a long enough period of time for appropriate neuroprotective changes to become established. The third could be that selective inhibition of HDAC2 & 3 before the insult is insufficient to cause necessary molecular changes to protect CGNs, therefore inhibiting other isoforms on their own or together with HDAC2 & 3 is required for a neuroprotective effect. However this is unlikely because specific knockdown of either HDAC2 (Peng et al., 2015) or HDAC3 (Bardai and D'Mello, 2011) before an insult has been shown to prevent CGNs undergoing apoptosis.

In search of a more appropriate model of excitotoxicity to use to examine possible neuroprotective effects of selective HDAC2 & 3 inhibition in neurones, it was important to bear in mind that glutamate excitotoxicity in the brain, following a cerebral ischaemic attack consists of two phases. The first is an overwhelming insult to neurones in the brain region starved of blood and this area of damage is known as the ischaemic core and here neurones die by necrosis (discussed in section 1.10.1, Dirnagl et al. (1999)). This first phase was modelled when CGNs were exposed to glutamate. The second phase of glutamate excitotoxicity occurs within several hours as the pathogenesis evolves and is triggered by the slow propagation of glutamate into the surrounding brain tissue known as the penumbral region. Here neuronal death is predominantly apoptotic (discussed in section 1.10.1, Dirnagl et al. (1999)). It is this apoptotic cell death and brain region, which is considered preventable and salvageable by using neuroprotective agents respectively. Therefore an *in vitro* model system that closely represents the death of penumbral neurones when exposed to glutamate excitotoxicity *in vivo*, is not a model where neurones get a single and

overwhelming "hit" of toxic glutamate, but rather a model where neurones are exposed to a progressive accumulation of glutamate which causes cellular damage and triggers apoptotic cell death.

3.4.2. HDAC inhibitors do not protect CGNs from apoptosis caused by glutamate excitotoxicity when administered at the same time as exposing to the insult

One way to induce progressive glutamate accumulation *in vitro* is to treat neurones to an inhibitor of glutamate re-uptake transporters such as DL-TBOA or SYM 2081 (Kanai et al., 2004). In this study, DL-TBOA triggered glutamate excitotoxicity and induced apoptotic cell death of CGNs. This was indicated by significant neuroprotection with the treatment of glutamate receptor antagonists and ac-DEVD-al an inhibitor of the apoptosis-inducing enzymes caspase 3 & 7 (figure 3.5A & 3.5B). However, treating CGNs to either SAHA or MI192 at the same time as exposing to DL-TBOA did not prevent this neuronal apoptosis. This rules out a mechanism whereby HDACs are mediators of the apoptotic cascade triggered by glutamate excitotoxicity.

Other studies have shown that treating CGNs with non-selective HDAC inhibitors initiates anti-apoptotic mechanisms such as an increase in the expression of anti-apoptotic genes Bcl-2 (Leng and Chuang, 2006) and HSP70 (Marinova et al., 2009). For non-selective HDAC inhibitors and possibly HDAC2 & 3 selective inhibitors to evoke these and other mechanisms to prevent DL-TBOA induced apoptosis, it is likely that CGNs must be exposed to these inhibitors for an appropriate length of time prior to exposure of the insult. With respect to the length of time required to have this neuroprotective effect, Kanai et al. (2004) report that three days is the minimum but seven days is the optimum amount of time to pre-treat with VPA, sodium butyrate & TSA in order to protect CGNs from apoptosis induced by inhibiting glutamate re-uptake transporters. Also, non-selective HDAC inhibitors need to be pre-treated for two or more days to protect CGNs and cortical neurones from subsequent glutamate-induced excitotoxicity (Leng and Chuang, 2006; Marinova et al., 2009). Pre-treating with HDAC inhibitors may prevent the apoptosis of CGNs exposed to DL-TBOA, but this hypothesis was not investigated

experimentally. Nevertheless, it is likely that pre-treatment of non-selective HDAC inhibitors will not prevent DL-TBOA induced glutamate excitotoxicity anyway, but will exacerbate it as seen in figure 3.4C.

The requirement for a prolonged pre-treatment of several days to protect neurones from glutamate excitotoxicity, is unusual given that the same HDAC inhibitors can protect neurones from other apoptosis inducing insults such as DNA damaging agents, oxidative stress, OGD and low extracellular K^+ without requiring days of pre-treatment to have a neuroprotective effect (see section 1.10.3 & 1.10.4). This suggests HDAC inhibitors may be having different effects to protect neurones from these other apoptosis-inducing insults compared to glutamate excitotoxicity and these effects may be time-dependent. For example, unlike glutamate excitotoxicity, HDACs may be actively involved in causing neuronal apoptosis in these other neurotoxic insults (see sections 3.1.1 to 3.1.7). Therefore inhibiting them would prevent this directly, without requiring other slower molecular changes to take place. Because the literature suggests that a prolonged pre-treatment is only required to protect neurones from neuronal death caused by excitotoxicity and not other stimuli, this raises doubts over the validity of this model system. It is unclear if the neuroprotective mechanisms that occur during this prolonged pre-treatment time frame represent the mechanisms involved when HDAC inhibitors are neuroprotective in other *in vitro* models and more importantly, neuroprotective in *ex vivo* and *in vivo* models when administered either immediately before or sometime after the onset of the neurotoxic insult (see sections 1.10.2, 1.10.3 and 1.10.5 for examples).

3.4.3. HDAC inhibitors do not protect CGNs from oxygen glucose deprivation (OGD)-induced death when administered immediately after OGD

One model of neurotoxicity where non-selective HDAC inhibitors have been shown to be neuroprotective, when either given shortly before or immediately after a neurotoxic insult, is when cortical neurones are exposed to OGD (Hasan et al., 2013; Lanzillotta et al., 2013; Meisel et al., 2006; Wang et al., 2011a). Recently the class I selective HDAC inhibitor MS-275 has also been shown to protect cortical neurones from OGD-induced death when administered immediately after OGD (Formisano et al., 2015).

The ability of HDAC inhibitors to protect CGNs from OGD-induced death has not been investigated before. Using these neurones, the aim was to establish an OGD model system where as the literature suggests, non-selective HDAC inhibitors are neuroprotective against OGD. Then this system would be used to determine which HDAC isoforms when inhibited are important for this effect and to investigate the molecular mechanisms involved. However, having established a paradigm to cause a similar amount of OGD-induced death of CGNs compared to the studies with cortical neurones, it was found that despite these other studies showing non-selective HDAC inhibitors are neuroprotective when administered immediately after exposing cortical neurones to OGD, the same treatment regime did not protect CGNs from this insult (figure 3.6). As hypothesised earlier when discussing the ineffectiveness of HDAC inhibitors to protect CGNs from glutamate excitotoxicity, the inability of HDAC inhibitors to protect from OGD may also be because of the mode of cell death induced. If OGD predominantly caused necrosis, then for HDAC inhibitors to induce mechanisms that protect from or repair the initiating damaging, it is likely that they will need to be administered for an appropriate amount of time before the insult. If OGD caused apoptosis, then HDACs were not involved in the induction and progression of the apoptotic cell death pathway in CGNs. However, having not examined the effectiveness of caspase inhibitors or glutamate receptor antagonists to prevent OGD-induced CGN death, it is impossible to confirm at this time what mode of cell death was induced and what pathophysiological molecular mechanisms were involved.

3.5. Conclusion

In conclusion, treatment of CGNs with either non-selective HDAC inhibitors or a selective HDAC2 & 3 inhibitor did not protect from glutamate-induced necrotic cell death when given at the same time as glutamate, or pre-treated for 24 hours beforehand. Also, these HDAC inhibitors did not prevent the apoptosis of CGNs when administered at the same time as inhibiting glutamate re-uptake transporters. Furthermore, non-selective HDAC inhibitors when given immediately after OGD did not prevent CGNs undergoing OGD-induced neuronal death. It is possible that pre-treating CGNs with HDAC inhibitors for an extended period may prevent neuronal death caused by glutamate excitotoxicity and OGD, but this remains to be determined. However, it is questionable whether the effects of HDAC inhibitors during a prolonged pre-treatment regime, represents the neuroprotective mechanisms involved when HDAC inhibitors are neuroprotective post-insult in *ex vivo* and *in vivo* models.

Chapter 4

Results II

Investigating the effects of inhibiting HDACs on the inflammatory response of microglia

4.1. Introduction

Histone deacetylase inhibitors are neuroprotective, neuro-restorative and improve neurological outcome in *in vivo* models of cerebral ischaemia and other brain insults and neurodegenerative diseases. HDAC inhibitors may do this through a number of mechanisms including direct effects in neurones, but as discussed in the previous chapter, the effect of HDAC inhibitors in isolated neurones is controversial. HDAC inhibitors could have neuroprotective effects *in vivo* by acting in other neural cells such as suppressing the inflammatory response of microglia, the innate immune cells of the brain, and as a consequence inhibit the neurotoxicity associated with neuroinflammation. Based on this, the effect of selective HDAC inhibition on the inflammatory response of microglia has been examined in this chapter. To date, every study that has looked at the effects of HDAC inhibition in microglia has used non-selective HDAC inhibitors (table 1.5). Therefore we do not know which HDAC isoforms are the important ones to inhibit and how this suppresses neuroinflammation. In order to identify which HDAC isoforms may be suitable to selectively inhibit to suppress the inflammatory response in microglia, I turned to studies that have investigated the effects of selective HDAC inhibitors in innate immune cells, which normally reside outside of the CNS (table 4.1). This analysis strongly suggested examining the effects of selectively inhibiting class I HDACs or HDAC6 in microglia. In isolated macrophages from mice, MS-275 a selective inhibitor of class I HDACs 1, 2 & 3 (table 1.4), significantly reduces the LPS-induced expression of iNOS and production of nitrite (Jeong et al., 2014) and expression of the pro-inflammatory cytokines TNF-α and IL-1β (Zhang and Schluesener, 2013). Consistent with this, combined knockdown of HDACs 1, 2 & 3 has the same anti-inflammatory effect (Jeong et al., 2014). One study found that MI192 a HDAC2 & 3 selective inhibitor (see section 1.8) also inhibits the expression of pro-inflammatory mediators such as TNF-α, IL-6 and IFNγ in human peripheral blood mononuclear cells (PBMCs) exposed to LPS (Gillespie et al., 2011). More specifically, knocking down HDAC3 in murine macrophages suppresses LPS-induced interferon beta (IFNβ) and IL-6 expression (Chen et al., 2012a) and following a more comprehensive analysis, knocking out HDAC3 in macrophages, has been shown to reduce the expression of approximately 45% of the genes that are

increased in response to LPS (Chen et al., 2012a). Very little is known about the cellular functions of the class I HDAC, HDAC8, but recent data suggests this isoform may be involved in regulating the inflammatory response of innate immune cells. Selective inhibition of this isoform with a novel hydroxamate HDAC8 inhibitor (ITF3056, IC_{50} of 285 nM for HDAC8, 2 μM for HDAC1, >3 μM for HDAC2, 3, 6, 10 & 11 and >10 μM for HDAC4, 5, 7 & 9, Li et al. (2015)) attenuates LPS-induced IL-1β, TNF-α and IL-6 expression in human PBMCs (Li et al., 2015). Selective inhibition of HDAC8 and HDAC6 with Tubastatin (IC_{50} of 15 nM for HDAC6, 854 nM for HDAC8, 16.4 μM for HDAC1 and >30 μM for HDAC2-5, 7 & 9-11, Butler et al. (2010)) also suppresses pro-inflammatory mediator expression in mononuclear cells and macrophages (Vishwakarma et al., 2013).

Taken together, these data show that selective inhibition of class I HDACs or HDAC6 inhibits the inflammatory response of cells of the innate immune system outside of the CNS. On the basis of these findings, it is possible that selective inhibition or specific knockdown of these HDAC isoforms has the same anti-inflammatory effect in activated microglia. This hypothesis is supported by studies which have shown the selective class I HDAC inhibitor MS-275 is neuroprotective in rodent models of cerebral ischaemia (Lanzillotta et al., 2013; Murphy et al., 2014), experimental autoimmune neuritis (Zhang et al., 2010) and amyloidosis (Zhang and Schluesener, 2013) all of which have a neuroinflammatory component. To investigate if selectively inhibiting class I HDACs or HDAC6 suppresses the inflammatory response of microglia, the immortalised murine BV2 microglia cell-line was used and the inflammatory response of these microglial cells was activated with LPS.

Table 4.1. The effect of HDAC inhibitors on the inflammatory response of monocytes and macrophages.

HDAC inhibitors	Treatment	Anti or Pro-inflammatory?	Ref
ITF2357, SAHA	Pre	Anti-	1 & 2
TSA	Co	Anti- & Pro-	3
SAHA, TSA	Pre, Co	Anti-	4
MS-275, SAHA	Pre	Anti-	5
TSA, SPB	Co	Anti-	6
MS-275, SAHA, SB, TSA	Co	Anti- & Pro-	7
MI192, TSA	Co	Anti-	8
HDAC3KD	Pre	Anti-	9
SAHA, Tubastatin	Pre	Anti-	10
MS-275	Post	Anti-	11
MS-275, HDAC1, 2 & 3 cKD	Pre	Anti-	12
ITF2357, ITF3056	Pre	Anti-	13

In all studies, the inflammatory response of monocytes and macrophages was activated with lipopolysaccharide (LPS). Abbreviations, cKD: Combined knockdown, HDAC3KD: HDAC3 knockdown, SAHA: Suberoylanilide hydroxamic acid, SB: Sodium butyrate, SPB: Sodium phenylbutyrate. References: [1]Leoni et al. (2002), [2]Leoni et al. (2005), [3]Aung et al. (2006), [4]Bode et al. (2007), [5]Choo et al. (2010), [6]Grabiec et al. (2010), [7]Halili et al. (2010), [8]Gillespie et al. (2011), [9]Chen et al. (2012a), [10]Vishwakarma et al. (2013), [11]Zhang and Schluesener (2013), [12]Jeong et al. (2014), [13]Li et al. (2015).

The BV2 microglia cell-line is a suitable model system because like isolated primary microglia or during neuroinflammation *in vivo,* when BV2 microglia are activated by physiologically relevant inflammatory stimuli such as LPS (Blasi et al., 1990; Bocchini et al., 1992; Gresa-Arribas et al., 2012; Henn et al., 2009; Horvath et al., 2008; Lund et al., 2005), IFNγ (Gresa-Arribas et al., 2012; Henn et al., 2009), hypoxia (Zhou et al., 2013), OGD (Li et al., 2013), Aβ (He et al., 2011) and MPP+ (1-methyl-4-phenylpyridinium, a neurotoxin model of Parkinson's disease, Roy et al. (2012)), these microglia cells produce cytokines including IL-1β, IL-6 and TNF-α, as well as other pro-inflammatory substances such as COX-2, iNOS, nitrite and others. Furthermore, similar to the effects of neuroinflammation *in vivo* and in co-

culture models of isolated primary microglia and neurones, activation of the inflammatory response of BV2 microglia when co-cultured with neurones, causes the production of pro-inflammatory mediators, which induce neuronal death (Gresa-Arribas et al., 2012; Yu et al., 2008). The magnitude of the inflammatory response of BV2 microglia in response to inflammatory stimuli has been compared with that of primary isolated microglia. Horvath et al. (2008) report that the amount of each pro-inflammatory mediator expressed by BV2 cells in response to LPS is less compared to primary microglia; however this study has a major caveat in that it compared mouse BV2 cells with rat primary microglia and so these differences may be because of a difference in species. A later study comparing mouse BV2 microglia with mouse primary microglia, showed that in response to LPS +IFNγ, the amount of TNF-α expression is equal between the two cell types, whereas primary microglia express significantly more IL-6 and COX-2 than BV2's, but BV2 microglia express a greater amount of iNOS and thus produce more nitrosative stress-inducing species in response to LPS +IFNγ compared to primary microglia (Gresa-Arribas et al., 2012). Although there are differences in the amount expressed for each pro-inflammatory mediator, the underlying mechanisms of microglial activation and downstream signalling which induce pro-inflammatory expression in BV2 microglia and primary microglia is most likely the same. Consistent with this, treating BV2 microglia with a HDAC inhibitor (sodium phenylbutyrate) known to suppress the inflammatory response of primary microglia (see section 1.10.5), also suppresses the inflammatory response of BV2 microglial cells (Roy et al., 2012). This suggests the anti-inflammatory mechanisms involved and therefore the inflammatory signalling pathways in BV2 microglia and primary microglia are the same. In addition to expressing pro-inflammatory mediators, BV2 microglia also retain the ability to migrate towards stimuli (Horvath et al., 2008) and phagocytose physiologically relevant materials such as microorganisms (Blasi et al., 1990; Bocchini et al., 1992), necrotic and apoptotic cells (Hirt and Leist, 2003) and Aβ (Fleisher-Berkovich et al., 2010).

4.1.1. Chapter aim

Using the BV2 murine microglia model system, which shares many characteristics of primary microglia, the effect of selectively inhibiting class I HDACs and HDAC6 on the inflammatory response of microglia and the mechanisms underlying this effect were investigated.

4.2. Non-selective HDAC inhibitors suppress pro-inflammatory cytokine expression in microglia

The cytokines IL-6 and TNF-α are classical markers and participants of the inflammatory response of isolated microglia and neuroinflammation *in vivo.* Measuring the changes in the levels of these cytokines has often been used as an indicator of the effects of HDAC inhibitors on the inflammatory response (see sections 1.10.5 and 4.1). The expression of these two cytokines in BV2 microglia following stimulation with LPS (500 ng/mL, as used by Gibson et al. (2012)) for 6 hours was determined by qPCR. After LPS exposure, IL-6 mRNA expression was robustly increased by 50990 ± 5190% and TNF-α mRNA by 3380 ± 271% compared to un-stimulated microglia (figure 4.1A). After 24 hours of LPS stimulation, the amount of IL-6 protein secreted by BV2 microglia into the cell culture supernatant was examined and was found to have increased to a concentration of 5406 ± 439 pg/mL (figure 4.1C, measured by an ELISA). The amount of IL-6 protein secreted by un-stimulated microglia was too low to be detected.

Activation of the transcription factor NF-κB is one of the major signalling pathways involved in generating the inflammatory response of innate immune cells upon exposure to LPS or other inflammatory stimuli. In part, NF-κB is responsible for inducing the expression of many pro-inflammatory mediators including IL-6 and TNF-α. To serve as an anti-inflammatory positive control, the activation of NF-κB was inhibited using the drug BAY11-7082 (BAY11) and the effects of this on LPS-induced IL-6 and TNF-α mRNA was measured. When BV2 microglia were stimulated with LPS for 6 hours, but in the presence of 5 μM BAY11, the expression

of both IL-6 and TNF-α mRNA was significantly reduced by 75.4 ± 6.032% (P=0.00686) and 34.01 ± 2.44% (P=0.0118) compared to the expression in LPS +vehicle respectively (figure 4.1B).

Non-selective HDAC inhibitors such as VPA of the short-chain fatty acid class and SAHA of the hydroxamate class (see section 1.8) have been shown to either attenuate or potentiate the inflammatory response of isolated primary microglia (see section 1.10.5 and table 1.5) and other innate immune cells (see table 4.1). The effect of these two HDAC inhibitors on the inflammatory response in BV2 microglia was investigated (figure 4.1B). The expression of both pro-inflammatory cytokines IL-6 and TNF-α in BV2 microglia in response to LPS stimulation for 6 hours were significantly reduced, not increased, in the presence of either 1 μM SAHA (IL-6 mRNA by 84.1 ± 1.63% (P=0.00375) and TNF-α by 59.7 ± 3.19% (P=0.00143)), or 5 mM VPA (IL-6 mRNA by 89.7 ± 1.59% (P=0.0272) and TNF-α by 77.9 ± 2.50% (P=0.0214)). Furthermore, the amount of IL-6 protein secreted by BV2 microglia during 24 hours when stimulated with LPS in the presence of SAHA was also significantly reduced by 85.6% ($P=1.50 \times 10^{-6}$) compared to LPS +vehicle (figure 4.1C).

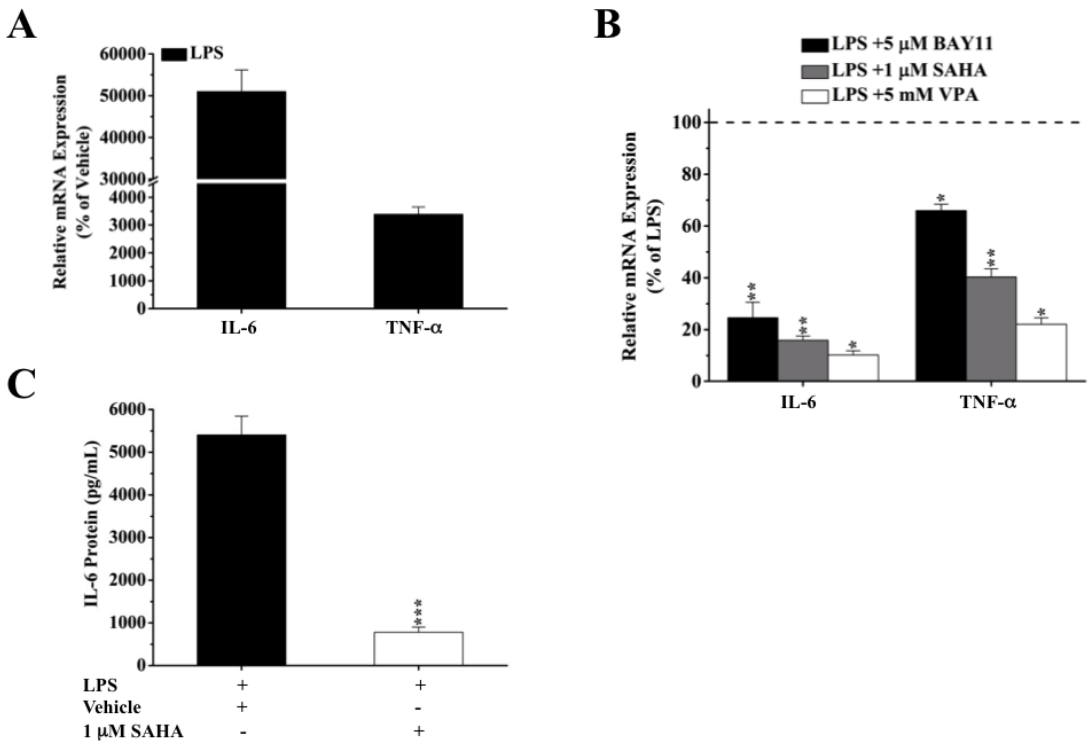


Figure 4.1. Non-selective histone deacetylase (HDAC) inhibitors suppress pro-inflammatory cytokine expression in microglia. **(A)** BV2 microglia were stimulated with 500 ng/mL lipopolysaccharide (LPS) for 6 hours and the mRNA expression of interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) was measured by quantitative PCR. Transcript levels were normalised to U6 and data shown are mean mRNA expression levels expressed as a percentage of the expression in the vehicle control ± SEM. **(B)** Effects of the NF-κB inhibitor BAY11-7082 (5 μM, BAY11) and the non-selective HDAC inhibitors SAHA (1 μM) and valproate (5 mM, VPA) on LPS-induced cytokine expression in (A). LPS and vehicle (0.1% v/v DMSO) or drugs were administered at the same time and RNA was extracted after 6 hours. Transcript levels for each treatment were normalised to U6 and data shown are mean mRNA expression levels expressed as a percentage of the expression in LPS +vehicle ± SEM. **(C)** BV2 microglia were treated with 500 ng/mL LPS and either vehicle or 1 μM SAHA for 24 hours followed by measurement of IL-6 secretion into the cell culture supernatant using an ELISA. Data shown are mean IL-6 protein (pg/mL) ± SEM. In all experiments, *n*=3. *P<0.05, **P<0.01 and ***P<0.001 vs. LPS +vehicle.

4.3. Selective inhibition of HDAC1, 2 & 3 or HDAC2 & 3, but not HDAC6 & 8 suppresses pro-inflammatory cytokine expression in microglia

When BV2 microglia were stimulated with 500 ng/mL LPS in the presence of the class I selective HDAC inhibitor apicidin (500 nM), the inflammatory response was significantly suppressed. This was indicated by a significant reduction in the expression of IL-6 mRNA (by 82.7 ± 2.26%, P=0.00405) and TNF-α mRNA (by 50.26 ± 4.48%, P=0.00355) compared to that in LPS +vehicle (figure 4.2A). Furthermore, apicidin significantly suppressed LPS-induced IL-6 protein secretion during 24 hours of treatment (89.75% reduction, $P=6.96 \times 10^{-7}$, figure 4.2B). This finding showed that inhibiting at least one of the class I HDACs suppresses the inflammatory response of microglia.

HDAC inhibitors with selectivity towards the class I HDAC8 and the class IIb HDAC6 are anti-inflammatory in LPS activated mononuclear cells from the peripheral innate immune system (table 4.1). To examine if this is also the case in microglia, BV2 microglia were stimulated with LPS in the presence of Droxinostat, a hydroxamate HDAC inhibitor selective for HDAC6 & 8 (IC_{50} of 2.47 μM for HDAC6, 1.46 μM for HDAC8, 16.9 μM for HDAC3 and >20 μM for HDAC1 & 2, Wood et al. (2010)). Then, the inflammatory response was measured by looking at changes in the expression of IL-6 and TNF-α. It was found that when BV2 microglia were co-treated to LPS and 5 μM Droxinostat for 6 hours, the LPS-induced expression of the pro-inflammatory cytokines IL-6 and TNF-α was unaffected (103.4 ± 10.6% and 99.5 ± 4.605% compared to LPS with vehicle respectively, figure 4.2A). Despite having no effect on the inflammatory response, Droxinostat was functional in BV2 microglia and robustly increased the acetylation of α-tubulin, a protein, which is uniquely targeted for deacetylation by HDAC6 (Hubbert et al. (2002), figure 4.2F). This suggested inhibition of HDAC6 & 8 does not suppress the inflammatory response of microglia.

Based on this, to determine which of the remaining class I HDACs, HDAC1, 2 & 3 should be inhibited to suppress pro-inflammatory mediator expression in microglia; the effect of MI192 a HDAC2 & 3 selective inhibitor (see section 1.8) was

investigated. Treatment of BV2 microglia with 1 μM MI192 at the same time as LPS for 6 hours, had no effect on the LPS-induced expression of IL-6 and TNF-α mRNA (figure 4.2A). Furthermore, treatment of either SAHA or apicidin at the same time as LPS for 6 hours significantly reduced the LPS-induced expression of iNOS mRNA (46 ± 1.41%, P=0.0394 and 52.9 ± 3.34%, P=0.0366 of LPS with vehicle respectively, figure not shown), however treatment of MI192 did not (93.7 ± 6.59% of LPS with vehicle, figure not shown). SAHA and VPA may reduce the expression of pro-inflammatory cytokines by inhibiting a combination of HDACs, which MI192 and Droxinostat on their own do not. This hypothesis was examined by stimulating BV2 microglia for 6 hours with LPS in the presence of both 1 μM MI192 and 5 μM Droxinostat together. Theoretically this would lead to the selective inhibition of HDAC2, 3, 6 and 8. Nevertheless, combining these two HDAC inhibitors did not significantly affect the inflammatory response in BV2 microglia (figure 4.2A).

Interestingly, when looking at the morphology of BV2 microglia following LPS treatment for 6 hours with or without the HDAC inhibitors SAHA, apicidin and MI192, there were clear changes in cell morphology when the microglia were treated with a HDAC inhibitor that suppressed the expression of pro-inflammatory cytokines (SAHA and apicidin) compared to microglia treated to LPS with vehicle or MI192. In the LPS and vehicle condition, BV2 microglia were mostly spherical, but following SAHA or apicidin treatment, the majority of the cells had flattened and had become bipolar in shape (figure 4.2C). This is indicative of a reduction in proliferation and/or change to a resting state/non-pro-inflammatory phenotype (Pottler et al., 2006; Zierler and Kerschbaum, 2005). Consistent with the absence of any changes in inflammatory mediator expression, MI192 treatment had no discernible effect on BV2 microglia morphology (figure 4.2C).

When the acetylation of histone proteins was assessed, both SAHA and apicidin within 6 hours, robustly increased histone H3 acetylation at lysine 9 (acH3K9) and also histone H4 acetylation (acH4) to a similar extent (figure 4.2D). Treatment with MI192 increased histone acetylation by 6 hours compared to the vehicle control, but did not increase it to the same extent as the other two HDAC inhibitors (figure 4.2D). An analysis of the timings of histone hyperacetylation by these HDAC inhibitors

over a period of 24 hours revealed that SAHA and apicidin increased the acetylation of histone H4 within the first hour of treatment and this was maintained over 24 hours (figure 4.3A & 4.3B). However, MI192 and MS-275, gradually increased histone acetylation over time (figure 4.3C & 4.3D). Consistent with other studies (see section 1.8), the data presented here suggests that the onset of HDAC inhibition and therefore protein acetylation by inhibiting HDACs with benzamide HDAC inhibitors such as MI192 and MS-275 is much slower compared to the hydroxamate HDAC inhibitor SAHA and the cyclic tetrapeptide HDAC inhibitor apicidin. As a consequence, specific proteins that become acetylated following HDAC inhibition, which are responsible for the suppression of pro-inflammatory mediator expression, may not become acetylated during 6 hours of MI192 treatment. To allow time for binding to and inhibition of HDACs and the acetylation of the necessary proteins, the effect on the inflammatory response after pre-treating with benzamide HDAC inhibitors was investigated next. BV2 microglia were pre-treated for 24 hours with apicidin or the benzamide HDAC inhibitors MI192 or MS-275, followed by activation with the inflammatory stimulant LPS for a further 6 hours (figure 4.2E). Pre-treatment of 500 nM apicidin significantly reduced LPS-induced IL-6 mRNA expression by 98.8 ± 0.313% (P=0.002016) and TNF-α mRNA expression by 72.9 ± 2.039% ($P=8.25 \times 10^{-5}$). Following pre-treatment of 1 μM MI192, LPS-induced expression of IL-6 and TNF-α was significantly reduced by 82.08 ± 1.46% (P=0.01087) and 24.1 ± 1.093% (P=0.0448) respectively. Similarly, pre-treatment of BV2 microglia with 5 μM MS-275 (a HDAC1, 2 & 3 selective inhibitor, table 1.4) before stimulating with LPS also significantly reduced the LPS-induced expression of IL-6 (by 96.8 ± 0.646%, P=0.00213) and TNF-α (by 87.8 ± 0.695%, $P=1.79 \times 10^{-5}$) mRNA.

To summarise, the data presented thus far suggested that combined inhibition of class I HDACs 1, 2 & 3 or HDAC2 & 3 but not HDAC6 & 8, was sufficient to suppress the expression of pro-inflammatory mediators IL-6 and TNF-α in BV2 microglia stimulated with LPS.

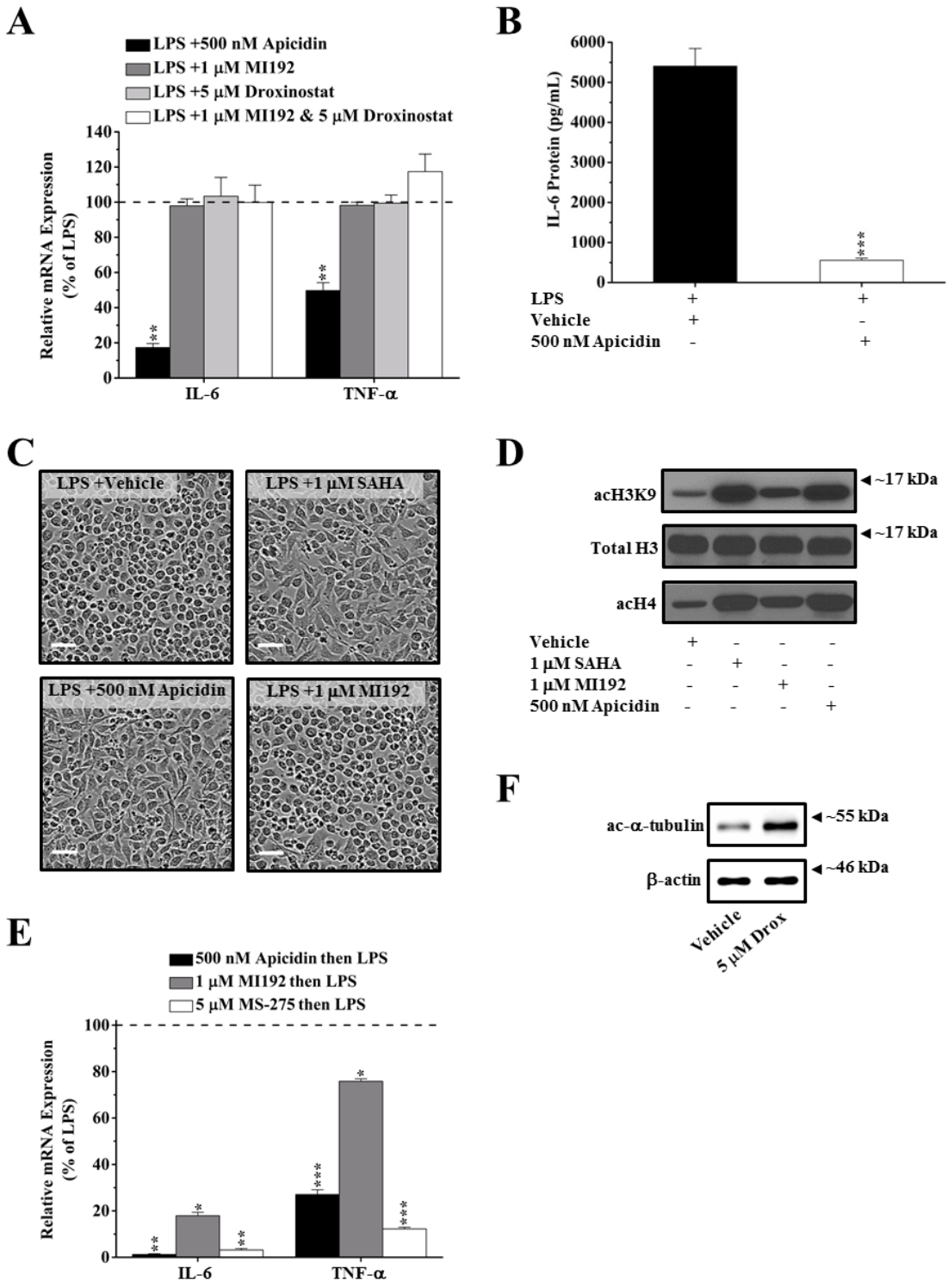


Figure 4.2. Selective inhibition of HDAC1, 2 & 3 or HDAC2 & 3, but not HDAC6 & 8 suppresses pro-inflammatory cytokine expression in microglia.

Figure 4.2 ***continued…*** **(A)** BV2 microglia were treated for 6 hours with 500 ng/mL lipopolysaccharide (LPS) with either vehicle (0.1% v/v DMSO), 500 nM apicidin (a cyclic tetrapeptide HDAC1, 2, 3 & 8 selective inhibitor), 1 μM MI192 (a benzamide HDAC2 & 3 selective inhibitor), 5 μM Droxinostat (a hydroxamate HDAC6 & 8 selective inhibitor) or combined treatment of MI192 and Droxinostat. The mRNA expression of the cytokines interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) was then measured by quantitative PCR. Transcript levels for each treatment were normalised to U6 and data shown are mean mRNA expression levels expressed as a percentage of the expression in LPS +vehicle ± SEM. **(B)** BV2 microglia were treated with 500 ng/mL LPS and either vehicle or 500 nM apicidin for 24 hours followed by measurement of IL-6 protein secreted into the cell culture supernatant using an ELISA. Data shown is mean IL-6 protein (pg/mL) ± SEM. **(C)** Representative phase-contrast images of the effects of HDAC inhibitors on BV2 microglia morphology after 6 hours of co-treatment with LPS. Scale bar 50 μm. **(D)** Representative Western blots of histone H3 lysine 9 acetylation (acH3K9) and histone H4 acetylation (acH4) in BV2 microglia after treatment with 1 μM SAHA, 1 μM MI192 or 500 nM apicidin for 6 hours. Total histone H3 was used as the loading control. **(E)** BV2 microglia were pre-treated for 24 hours with either vehicle, 500 nM apicidin, 1 μM MI192 or 5 μM MS-275 (a benzamide HDAC1, 2 & 3 selective inhibitor), then stimulated with LPS for a further 6 hours. The expression of IL-6 and TNF-α mRNA was then measured and expressed as in (A). **(F)** Representative Western blots of acetylated α-tubulin (ac-α-tubulin) in BV2 microglia after treatment with 5 μM Droxinostat (Drox) for 6 hours. β-actin was used as the loading control. In all experiments, n=3, *P<0.05, **P<0.01 and ***P<0.001 vs. LPS +vehicle.

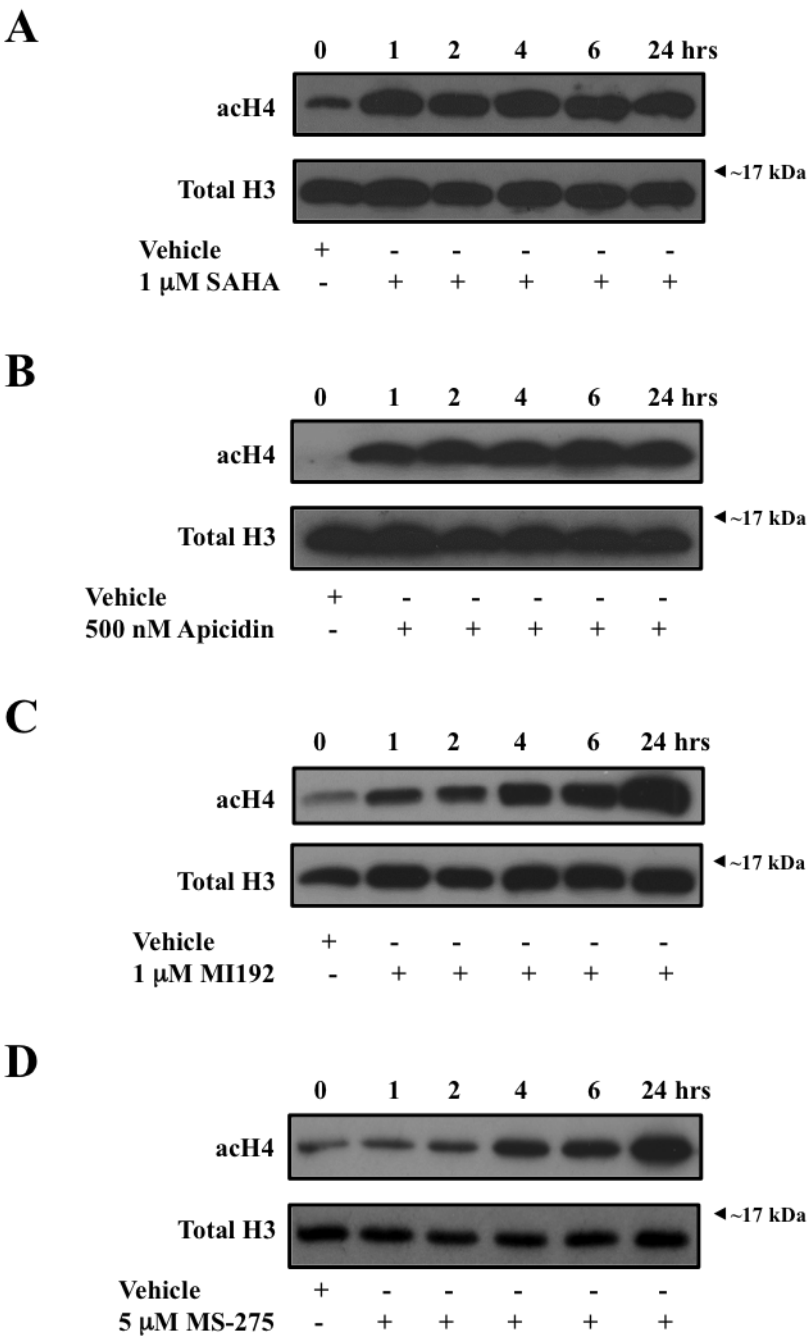


Figure 4.3. Time-course of histone H4 acetylation (acH4) following treatment with HDAC inhibitors of different pharmacophore groups. BV2 microglia were treated to vehicle (0.1% v/v DMSO), or a **(A)** hydroxamate (1 μM SAHA), **(B)** a cyclic tetrapeptide (500 nM apicidin), or benzamide HDAC inhibitors **(C)** 1 μM MI192, **(D)** or 5 μM MS-275 over a 24 hour period. Total histone H3 was used as the loading control.

4.4. HDAC2 knockdown suppresses pro-inflammatory cytokine expression in microglia

To determine if the inhibition of HDAC2 on its own is enough to suppress the expression of pro-inflammatory mediators in BV2 microglia, HDAC2 was knocked down (HDAC2KD) and the effect of this on the inflammatory response was examined. To knockdown HDAC2, BV2 microglia were transfected with siRNA targeted against HDAC2 mRNA. Forty-eight hours post-transfection, the expression of HDAC1, 2 and 3 proteins was determined by Western blot (figure 4.4A & 4.4B). HDAC2 was found to be significantly reduced by 68.7 ± 3.44% (P=0.0114) compared to the expression in the scrambled siRNA control (Scr siRNA). There was no effect of HDAC2KD on the expression of HDAC1 (109.4 ± 1.57% of Scr siRNA) or HDAC3 (99.5 ± 11.8% of Scr siRNA). To examine if HDAC2KD had any effect on the inflammatory response of BV2 microglia, following knockdown cells were stimulated with 500 ng/mL LPS for 6 hours. Then, the expression of LPS-induced pro-inflammatory cytokines IL-6 and TNF-α was assessed by qPCR (figure 4.4C). It was found that knocking down HDAC2 significantly suppressed the expression of IL-6 mRNA by 48.2 ± 1.25% (P=0.00668) and TNF-α mRNA by 22 ± 3.57% (P=0.0134) compared to Scr siRNA +LPS.

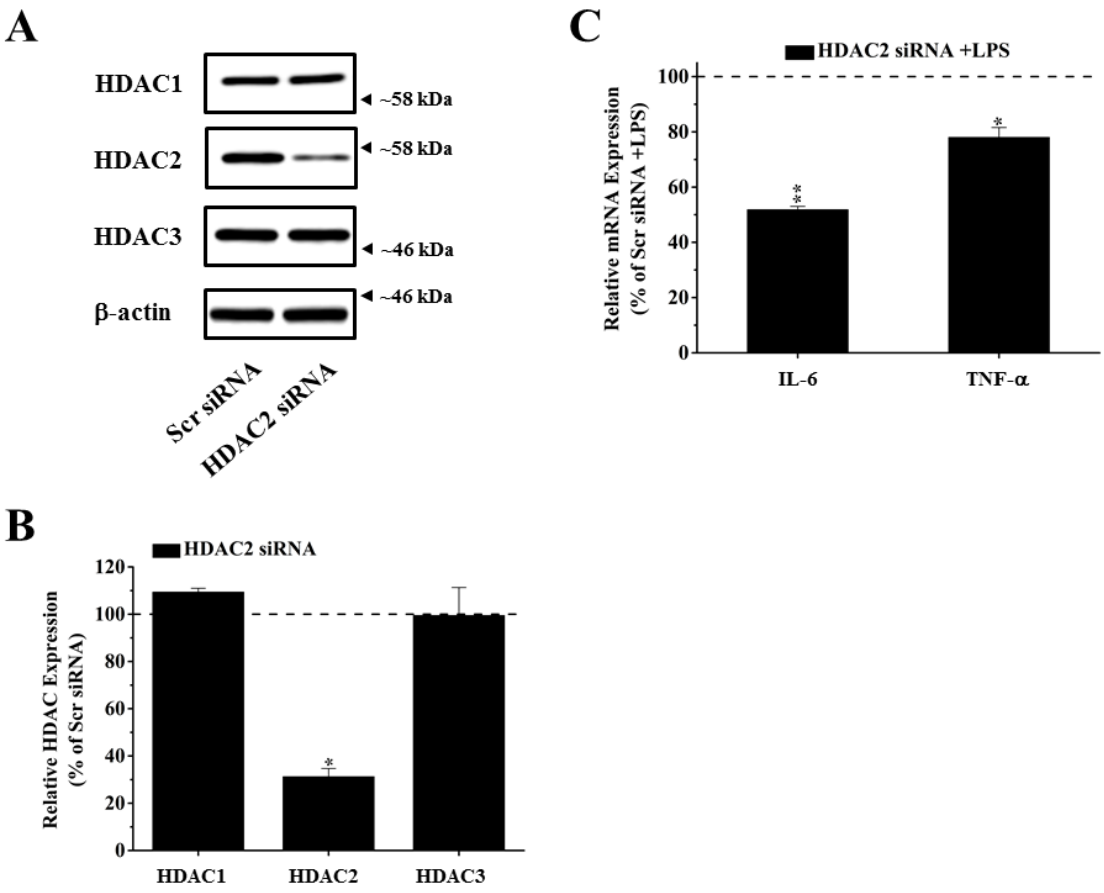


Figure 4.4. HDAC2 knockdown suppresses pro-inflammatory cytokine expression in BV2 microglia. **(A)** Representative Western blots of HDAC1, 2 and 3 proteins expressed in BV2 microglia following transfection with scrambled (Scr) or HDAC2 siRNA for 48 hours. β-actin was used as the loading control. **(B)** Quantification of (A), band intensities were normalised to β-actin and data shown are relative mean HDAC expression levels expressed as a percentage of the expression in the Scr siRNA control ± SEM, *n*=3, *P<0.05 vs. Scr siRNA. **(C)** BV2 microglia transfected with Scr or HDAC2 siRNA for 48 hours were stimulated with 500 ng/mL lipopolysaccharide (LPS) for 6 hours. Then, the mRNA expression of the cytokines interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) was measured by quantitative PCR. Transcript levels for each treatment were normalised to U6 and data shown are relative mean mRNA expression levels expressed as percentage of the expression in Scr siRNA +LPS ± SEM, *n*=3, *P<0.05 and **P<0.01 vs. Scr siRNA +LPS.

4.5. HDAC1 knockdown suppresses pro-inflammatory cytokine expression in microglia

The non-selective HDAC inhibitors SAHA and VPA and the class I selective HDAC inhibitors apicidin and MS-275 all suppress the expression of pro-inflammatory mediators in BV2 microglia stimulated with LPS. They may do this by inhibiting HDAC1 as well as HDAC2. To determine if the inhibition of HDAC1 on its own is enough to suppress the expression of pro-inflammatory mediators in BV2 microglia, HDAC1 was knocked down (HDAC1KD) and the effect of this on the inflammatory response was examined. To knockdown HDAC1, BV2 microglia were transfected with siRNA targeted against HDAC1 mRNA. Forty-eight hours post-transfection, the expression of HDAC1, 2 and 3 proteins was determined by Western blot (figure 4.5A & 4.5B). HDAC1 protein was found to be significantly reduced by 62.56 ± 4.52% (P=0.000134) compared to the expression in the scrambled siRNA control (Scr siRNA). Following HDAC1KD, HDAC2 protein was found to be significantly upregulated (194 ± 7.49% of Scr siRNA, P=0.000773) but there was no change in the expression of HDAC3 (101.4 ± 4.79% of Scr siRNA). To examine if HDAC1KD with the accompanying increase in HDAC2 levels had any effect on the inflammatory response, forty-eight hours post-transfection, BV2 microglia were stimulated with 500 ng/mL LPS for 6 hours and the mRNA expression of pro-inflammatory cytokines IL-6 and TNF-α was assessed by qPCR (figure 4.5C). HDAC1KD and the accompanying upregulation of HDAC2 did not affect the LPS-induced mRNA expression of IL-6 and TNF-α, which were 116 ± 7.73% and 112 ± 6.84% of the expression in Scr siRNA +LPS respectively.

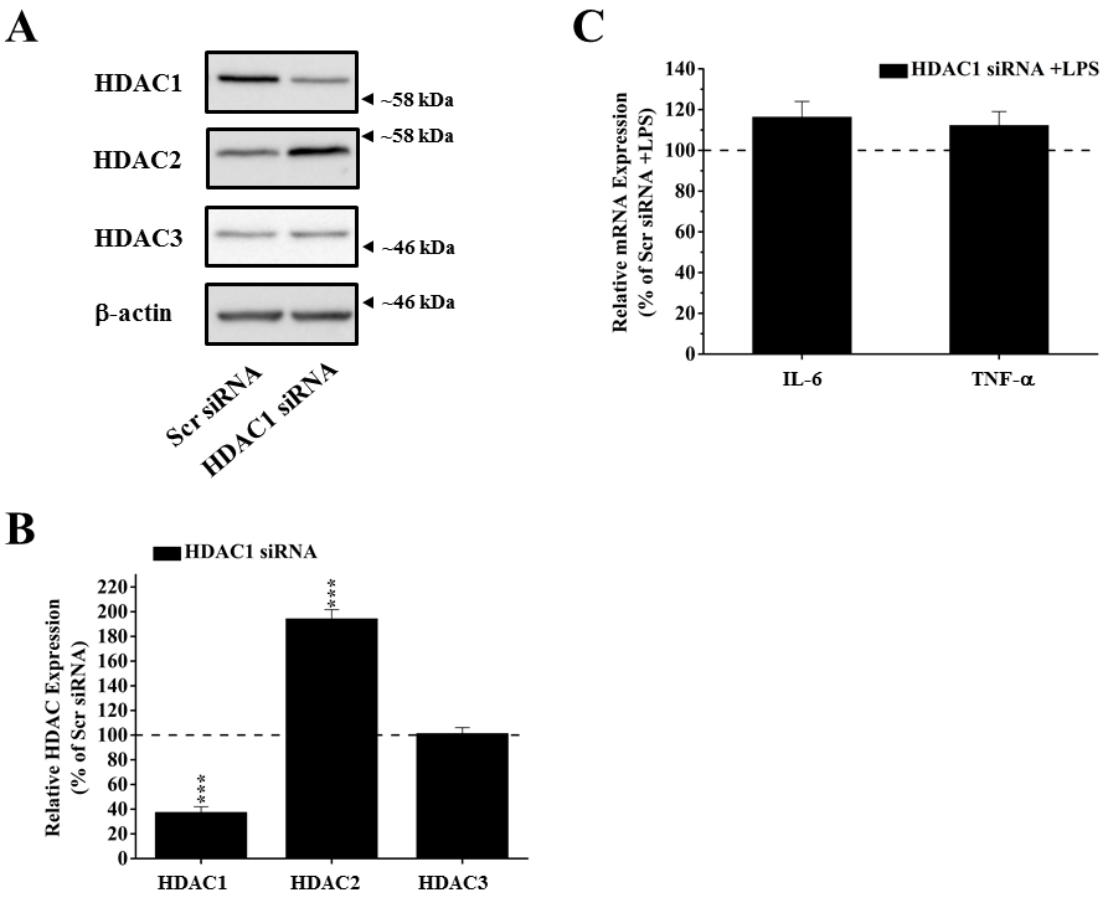


Figure 4.5. HDAC1 knockdown causes a compensatory upregulation of HDAC2 and has no effect on pro-inflammatory cytokine expression in BV2 microglia. **(A)** Representative Western blots of HDAC1, 2 and 3 proteins expressed in BV2 microglia following transfection with scrambled (Scr) or HDAC1 siRNA for 48 hours. β-actin was used as the loading control **(B)** Quantification of (A), band intensities were normalised to β-actin and data shown are relative mean HDAC expression levels expressed as a percentage of the expression in the Scr siRNA control ± SEM, *n*=3, ***P<0.01 vs. Scr siRNA. **(C)** BV2 microglia transfected with Scr or HDAC1 siRNA for 48 hours were stimulated with 500 ng/mL lipopolysaccharide (LPS) for 6 hours. Then, the mRNA expression of the cytokines interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) was measured by quantitative PCR. Transcript levels for each treatment were normalised to U6 and data shown are relative mean mRNA expression levels expressed as percentage of the expression in Scr siRNA +LPS ± SEM, *n*=3, ***P<0.001 vs. Scr siRNA +LPS.

It was hypothesised that the upregulation of HDAC2 following HDAC1KD counteracted an anti-inflammatory effect mediated by a reduction in HDAC1 expression and activity. To investigate this, the inflammatory response of BV2 microglia was examined after knocking down HDAC1 whilst simultaneously preventing the upregulation of HDAC2. To do this, cells were transfected with both HDAC1 and HDAC2 siRNA. Forty-eight hours post-transfection, the expression of HDAC1, 2 and 3 proteins was determined by Western blot (figure 4.6A & 4.6B). HDAC1 protein was significantly reduced by 63.5 ± 2.36%, (P=0.004026 compared to Scr siRNA) and the upregulation of HDAC2 was prevented (89.7 ± 6.23% which was not significantly different from Scr siRNA). The expression of HDAC3 was not significantly altered (116 ± 4.77% of Scr siRNA). BV2 microglia were then stimulated with LPS for 6 hours. Now, a reduction in HDAC1 expression without the upregulation of HDAC2, significantly reduced LPS-induced expression of IL-6 mRNA by 34.8 ± 2.95% (P=0.0153) and TNF-α mRNA by 35.7 ± 4.8% (P=0.0169) compared to the expression in Scr siRNA +LPS (figure 4.6C).

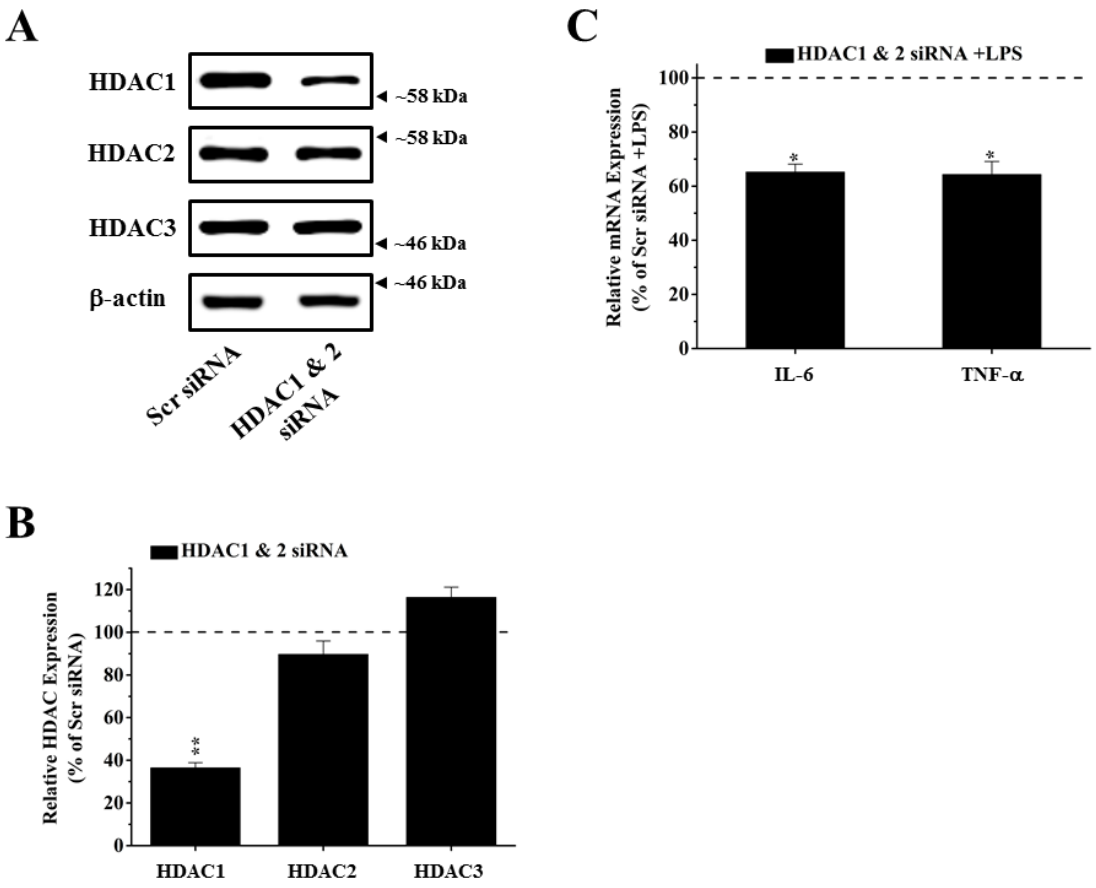


Figure 4.6. Knocking down HDAC1 whilst preventing the accompanying upregulation of HDAC2, suppresses pro-inflammatory cytokine expression in BV2 microglia. **(A)** Representative Western blots of HDAC1, 2 and 3 proteins expressed in BV2 microglia following transfection with scrambled (Scr) or combined HDAC1 & HDAC2 siRNA for 48 hours. β-actin was used as the loading control. **(B)** Quantification of (A), band intensities were normalised to β-actin and data shown are relative mean HDAC expression levels expressed as a percentage of the expression in the Scr siRNA control ± SEM, *n*=3, **P<0.01 vs. Scr siRNA. **(C)** BV2 microglia transfected with Scr or HDAC1 & HDAC2 siRNA for 48 hours were stimulated with 500 ng/mL lipopolysaccharide (LPS) for 6 hours. Then, the mRNA expression of the cytokines interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) was measured by quantitative PCR. Transcript levels for each treatment were normalised to U6 and data shown are relative mean mRNA expression levels expressed as percentage of the expression in Scr siRNA +LPS ± SEM, *n*=3, *P<0.05 vs. Scr siRNA +LPS.

4.6. Exploration of the mechanisms underlying the anti-inflammatory effects of HDAC inhibition in microglia

Through the use of selective HDAC inhibitors and knocking down specific HDAC isoforms it was established that inhibiting the activity of HDAC1 and/or 2 suppresses the expression of pro-inflammatory mediators in activated BV2 microglia. The focus of the study turned to exploring potential mechanisms by which the inhibition of these two isoforms produced this anti-inflammatory phenotype.

One mechanism could be that HDAC inhibitors prevent an increase in HDAC activity associated with an LPS-induced upregulation of HDAC1 and 2. To investigate this hypothesis, BV2 microglia were stimulated with 500 ng/mL LPS for 6 hours and the expression of HDAC1, 2 and 3 proteins was determined by Western blot. This analysis showed that LPS treatment did not alter HDAC expression (figure 4.7A & 4.7B) and suggested that HDAC upregulation is not involved in the inflammatory response of BV2 microglia during the 6 hours of LPS stimulation.

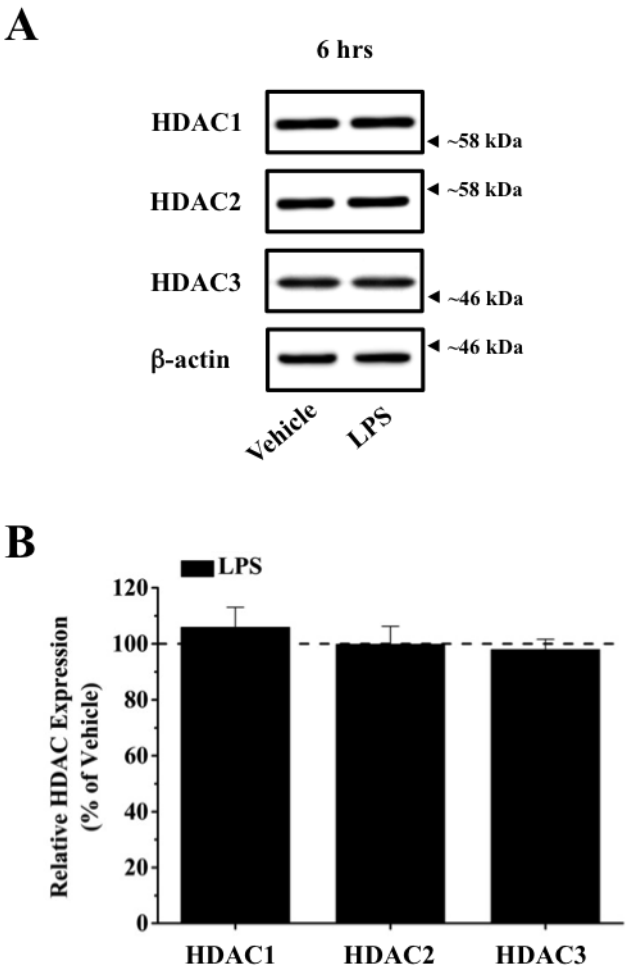


Figure 4.7. Lipopolysaccharide (LPS) treatment does not affect the expression of HDAC1, 2 or 3 in BV2 microglia. **(A)** BV2 microglia were treated with 500 ng/mL LPS for 6 hours followed by Western blot for HDAC1, 2 & 3. β-actin was used as the loading control. **(B)** Quantification of (A), band intensities were normalised to β-actin and data shown are relative mean HDAC expression levels expressed as percentage of the expression in the vehicle control ± SEM, *n*=3.

Another way inhibition of HDAC1 and/or 2 may be anti-inflammatory could be through increasing histone acetylation and the expression of genes encoding anti-inflammatory proteins. To investigate if the synthesis of new proteins is required for the anti-inflammatory effects of HDAC inhibitors, BV2 microglia were treated for 1 or 3 hours with LPS with or without the protein synthesis inhibitor cycloheximide (1 μg/mL, CHX) and in the presence of either vehicle, 1 μM SAHA or 500 nM apicidin. A protein synthesis determination assay was performed and cells that were able to synthesise proteins incorporated O-propargyl-puromycin into newly synthesised peptides, which could be subsequently labelled with a green fluorescent dye and visualised by fluorescence microscopy. At both time points, CHX treatment robustly reduced protein synthesis in all treatments as indicated by a reduction in the intensity of green fluorescence in cells (figure 4.8A). After treating BV2 microglia for 3 hours, the LPS-induced expression of IL-6 mRNA was analysed by qPCR (figure 4.8B). Both SAHA and apicidin treatment after 3 hours, significantly reduced the expression of LPS-induced IL-6 by 81.7 ± 2.59% ($P=7.32 \times 10^{-5}$) and 84.7 ± 1.084% ($P=6.22 \times 10^{-5}$) respectively. Protein synthesis inhibition by CHX did not prevent the suppression of IL-6 mRNA expression by SAHA (100.68 ± 9.80% of LPS +SAHA) or apicidin (107.3 ± 9.20% of LPS +Apicidin). This data rules out a mechanism whereby HDAC inhibitors mediate anti-inflammatory effects through increasing protein expression. Conversely, HDAC inhibitors may be anti-inflammatory by suppressing the synthesis of proteins important for inducing the expression of pro-inflammatory mediators. However, global protein synthesis inhibition in BV2 microglia did not prevent the production of IL-6 mRNA in response to LPS stimulation (107.6 ± 9.69% of the expression in LPS with vehicle), therefore did not recapitulate the effects of HDAC inhibitors on the expression of pro-inflammatory mediators. This rules out a mechanism whereby HDAC inhibitors are anti-inflammatory by inhibiting protein synthesis.

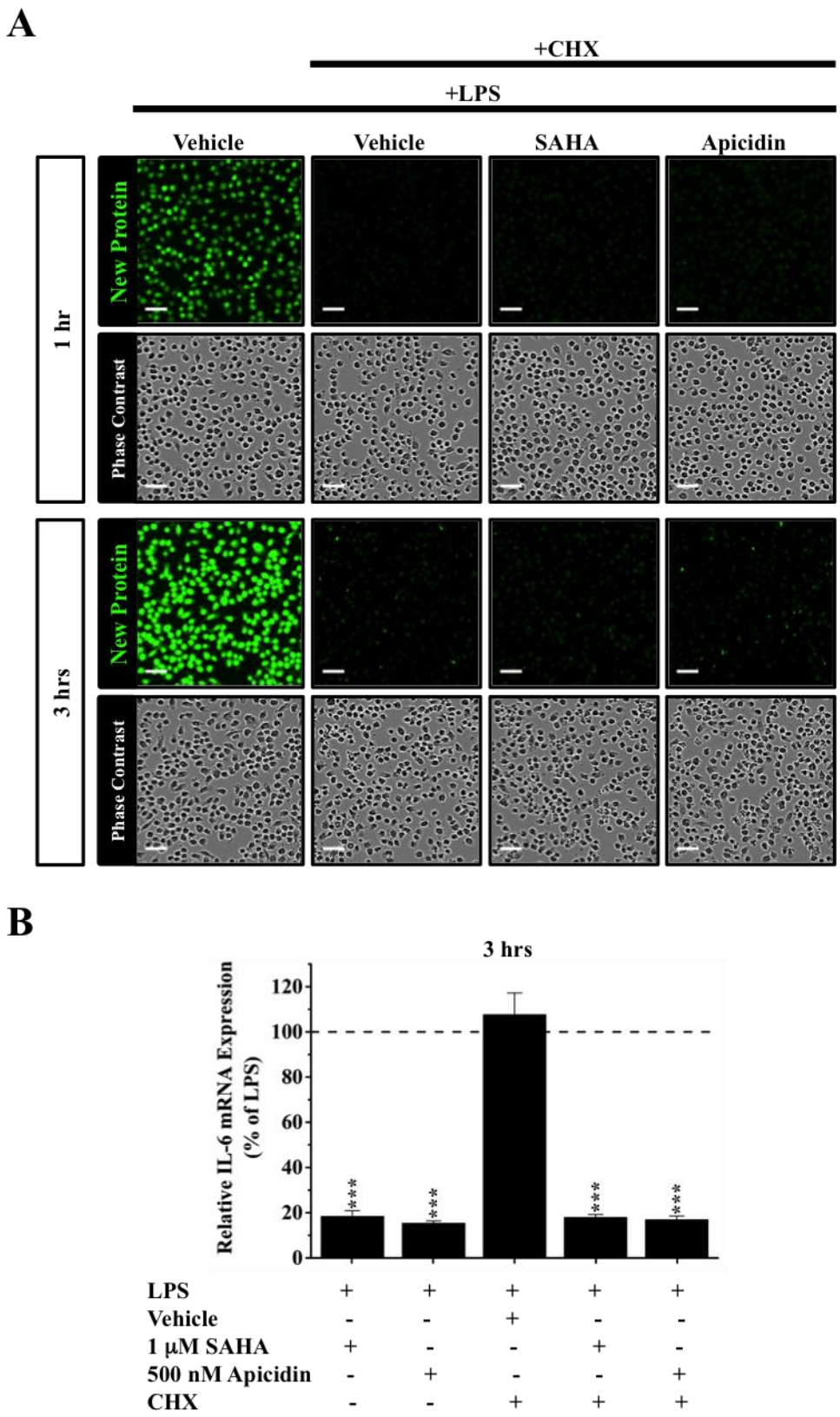


Figure 4.8. Histone deacetylase inhibitors do not induce novel protein synthesis to suppress pro-inflammatory cytokine expression in microglia.

Figure 4.8 ***continued…*** BV2 microglia were treated for 1 or 3 hours with 500 ng/mL lipopolysaccharide (LPS) with or without 1 μg/mL cycloheximide (a protein synthesis inhibitor, CHX) and in the presence of either vehicle (0.1% v/v DMSO), 1 μM SAHA or 500 nM apicidin. **(A)** Representative green fluorescent and phase contrast images of each treatment. Cells that are positive for green fluorescence have synthesised new proteins during the treatment period. Scale bar 50 μm. **(B)** After 3 hours of treatment, the mRNA expression of the cytokine interleukin-6 (IL-6) was measured by quantitative PCR. Transcript levels for each treatment were normalised to U6 and data shown are mean mRNA expression levels expressed as a percentage of the expression in LPS +vehicle ± SEM, *n*=3, ***P<0.001 vs. LPS +vehicle.

In addition to increasing the acetylation of histone proteins, HDAC inhibitors have the potential to increase the acetylation of all proteins that make-up the acetylome. The identity and function of proteins that make-up the acetylome in microglia is unknown but it is likely that some of these are involved in or can regulate the inflammatory response. One way of visualising the changes in the acetylome after treating cells with HDAC inhibitors is to perform a Western blot to identify all lysine-acetylated proteins in the cell. Comparing the acetylome of BV2 microglia treated with HDAC inhibitors that suppress the inflammatory response within a given time frame with the acetylome of BV2 microglia treated with a HDAC inhibitor that does not, may reveal the acetylation of specific proteins responsible for suppressing the inflammatory response. As shown earlier, during 6 hours of treatment, SAHA and apicidin both suppress the LPS-induced expression of pro-inflammatory mediators in BV2 microglia. However, during 6 hours of treatment MI192 is unable to suppress the inflammatory response of BV2 microglia. This suggests that during the first 6 hours of treatment, HDAC inhibitors that are anti-inflammatory increase the acetylation of specific proteins that suppress the inflammatory response whereas MI192 does not. In support of this, it has already been shown that SAHA and apicidin increase the acetylation of histone proteins to a greater extent compared to MI192 (figure 4.2D), so a similar comparison was performed to look for differences in the acetylation state of non-histone proteins in microglia treated with these HDAC inhibitors (figure 4.9).

Following treatment of LPS and either vehicle, 1 μM SAHA, 500 nM apicidin or 1 μM MI192 for 6 hours, it was observed that all three HDAC inhibitors increased the acetylation of proteins in BV2 microglia compared to LPS +vehicle. SAHA treatment caused an increase in acetylation of a protein with an approximate molecular weight of 55 kDa and this protein was not hyperacetylated following treatment with apicidin or MI192. This band is likely to be α-tubulin, which has the same molecular weight and is uniquely deacetylated by HDAC6 (Hubbert et al., 2002), a target of the non-selective HDAC inhibitor SAHA but not apicidin or MI192 (see section 1.8). Two proteins were observed to be hyperacetylated following treatment of SAHA or apicidin but not in MI192. One protein was observed between molecular weight markers 55 and 72 kDa (indicated by the red box in figure 4.9) and the other between markers 26 and 34 kDa (indicated by the green box in figure 4.9). The acetylation of these two proteins may be important for the anti-inflammatory effects of SAHA and apicidin during 6 hours of treatment. The possible identity and function of these proteins will be discussed later.

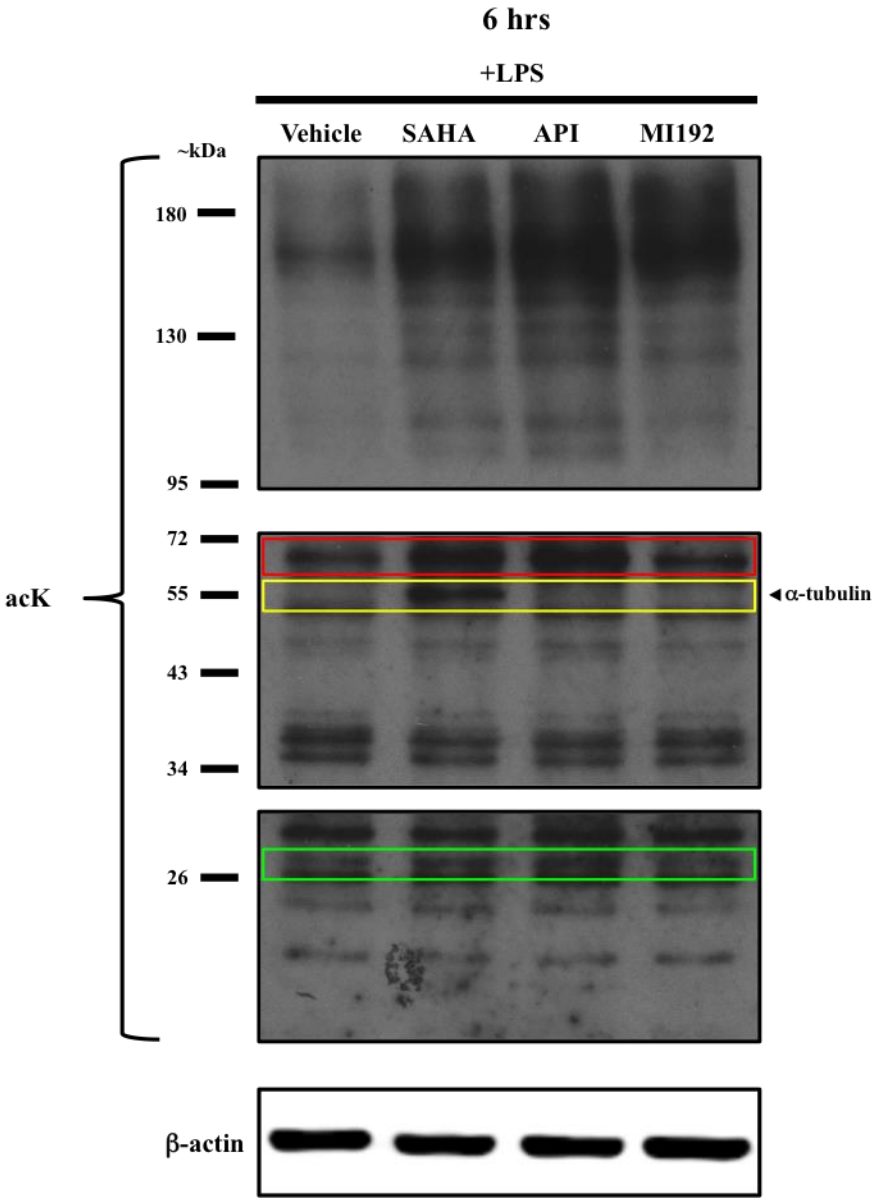


Figure 4.9. Changes in the acetylome in BV2 microglia following treatment with histone deacetylase inhibitors. BV2 microglia were treated for 6 hours with 500 ng/mL lipopolysaccharide (LPS) and vehicle (0.1% v/v DMSO), or 1 μM SAHA or 500 nM apicidin (API) which are anti-inflammatory during this timeframe, or 1 μM MI192 which is not anti-inflammatory during this timeframe. Whole-cell lysates were subjected to Western blotting and probed with an antibody recognising acetylated lysine residues (acK), β-actin was used as a loading control. Coloured boxes indicate differences in acetylated proteins between SAHA and both apicidin & MI192 (yellow) and between MI192 and both SAHA & apicidin (red and green).

4.7. Discussion

4.7.1. Inhibition of either HDAC1 or HDAC2 suppresses the inflammatory response of microglia

In this chapter, the effects of various HDAC inhibitors on the inflammatory response of activated BV2 microglia were investigated. It was found that selective inhibition of class I HDACs 1, 2 and 3 suppresses the inflammatory response of these cells as indicated by a reduction in the expression of pro-inflammatory mediators IL-6 and TNF-α. This is consistent with the anti-inflammatory effects of inhibiting these HDAC isoforms in other innate immune cells such as mononuclear cells and macrophages (see section 4.1 and table 4.1). Taken together, this suggests the role of these three HDACs in the inflammatory response is conserved and the inhibition of these HDACs may lead to the acetylation of the same proteins in all innate immune cells, which when acetylated inhibits the inflammatory response. Having identified HDAC1, 2 & 3 as targets to suppress pro-inflammatory mediator expression in microglia, the effect of knocking down these HDACs was investigated with the aim to identify the isoform important for this response. Knocking down HDAC2 significantly suppressed the expression of pro-inflammatory mediators in activated BV2 microglia whereas HDAC1KD had no effect. As a consequence of knocking down HDAC1 there was a significant upregulation of HDAC2 but no change in HDAC3. This upregulation also occurs in immortalised murine macrophages following HDAC1KD (Jeong et al., 2014) and in peripheral nerves (Jacob et al., 2011) and embryonic stem cells (Dovey et al., 2010b) from HDAC1KO mice. Knocking down HDAC2 in BV2 microglia had no consequential effect on the expression of either HDAC1 or HDAC3 and this is consistent with the effects of knocking out HDAC2 in mouse embryonic stem cells (Dovey et al., 2010b). However, other studies have shown that like HDAC2 when HDAC1 is silenced or knocked out, HDAC1 is upregulated following HDAC2KD in immortalised murine macrophages (Jeong et al., 2014), as well as in peripheral nerves (Jacob et al., 2011) and in the brains of HDAC2KO mice (Guan et al., 2009). The mechanisms underlying the upregulation of HDAC1 or 2 in response to a loss in the other are not fully understood. It has been suggested that HDAC1 can negatively regulate the

expression of HDAC2 (Jeong et al., 2014). This is based on the observation that histones in the promoter of the HDAC2 gene become hyperacetylated in HDAC1-deficient cells (Lagger et al., 2002). However, Dovey et al. (2010b) report that despite measuring an increase in HDAC2 protein levels in HDAC1KO cells, there was no change in the expression of HDAC2 at the mRNA level and propose that the increase in HDAC2 protein in the absence of HDAC1, is through enhancement of mRNA translation, or an increase in HDAC2 protein stability. The reason why HDAC1 is not upregulated following HDAC2 knockdown in BV2 microglia, or HDAC2 knockout in embryonic stem cells, but is upregulated in other cells and tissues is currently not known. It is possible that the compensatory upregulation of a HDAC isoform following the loss of another, depends on the expression of this other HDAC falling below a certain threshold, or the levels being reduced for a certain length of time (as demonstrated by Jacob et al. (2011)). Moreover, the residual activity of the remaining HDAC and multi-protein complexes, the ability of one HDAC to regulate the expression of another, may also contribute to whether a HDAC is upregulated or not and all these mechanisms may be dependent on the particular cell type and circumstance.

Because knocking down HDAC2 suppressed pro-inflammatory mediator expression, one might hypothesise that an upregulation of HDAC2 is pro-inflammatory. However, when HDAC2 was upregulated following knockdown of HDAC1 there was no effect on the expression of the pro-inflammatory mediators measured. One possible explanation is that a pro-inflammatory effect of HDAC2 upregulation was being counteracted by an anti-inflammatory effect of HDAC1KD. An alternative explanation is that the induction of HDAC2 expression following HDAC1KD is not pro-inflammatory, but the upregulation of HDAC2 and increase in activity may compensate for the reduction in HDAC1 expression and loss of activity, and therefore prevent an anti-inflammatory effect mediated by a loss of HDAC1. This latter hypothesis was based on the observations reported in other studies which show in some circumstances HDAC1 and HDAC2 are functionally redundant. This means these two isoforms can perform the same function in certain cellular processes and when one is reduced, the upregulation of the other is able to compensate for this loss of activity and prevent phenotypic changes. This functional redundancy of HDAC1

and 2 has been observed during brain development in mice where individual knockout of either HDAC1 or 2 has no effect on the development of the brain, but when both HDAC isoforms are simultaneously knocked out there is a significant reduction in neural cell viability and impairment in neural cell differentiation and brain histoarchitecture (Montgomery et al., 2009). Furthermore, in peripheral nerves from HDAC1KO and HDAC2KO mice, HDAC2 and HDAC1 are upregulated in these two knockout mice respectively and when this upregulation is prevented there are changes in the expression of genes which were previously unobserved (Jacob et al., 2011). The authors of this study go on to propose that when a HDAC becomes upregulated, this HDAC compensates for a loss in activity of the other and prevents changes in the expression of specific genes (Jacob et al., 2011). Similarly, in BV2 microglia, when the upregulation of HDAC2 upon HDAC1KD was prevented, there was an anti-inflammatory effect when HDAC1 was knocked down as indicated by the suppression of pro-inflammatory mediator expression in activated cells. Together the data presented indicates that both HDAC1 and HDAC2 can regulate the inflammatory response of microglia and inhibition of either, when there is no change in the expression of the other, is anti-inflammatory in these cells.

I propose that in BV2 microglia the activity of the upregulated HDAC2 compensates for a loss in HDAC1 expression and activity. In support of this, both HDAC1 and HDAC2 are members of the same multi-protein complexes (see section 1.4.4) and when either is reduced, the other isoform if upregulated, is incorporated into the CoREST, NuRD and Sin3 multi-protein complexes in its place (Dovey et al., 2010b; Guan et al., 2009). It is possible that the functional redundancy and compensatory action of these two HDAC isoforms is mediated by the interchangeable incorporation of these HDACs into the same specific functional multi-protein complex. In BV2 microglia, when HDAC1 is knocked down, the upregulated HDAC2 may be incorporated into a specific multi-protein complex in place of the lost HDAC1 and because of this, the number of functional complexes, which would otherwise be reduced if there was no upregulation, is maintained. These complexes, of which a greater number now contain HDAC2 instead of HDAC1, are still functional and can appropriately and sufficiently deacetylate proteins involved in, or that regulate the inflammatory response. Because there is not a reduction in the activity of these

specific complexes there is no shift towards the acetylation of specific proteins, which upon acetylation no longer promote the inflammatory response or when acetylated actively suppress it. This hypothesis could explain why an anti-inflammatory phenotype is not produced when HDAC1 is knocked down and HDAC2 is upregulated and implies that in regulating the inflammatory response of BV2 microglia, HDAC1 & 2 through a complex they share perform the same function. Therefore, it doesn't matter which of the two isoforms is inhibited, an anti-inflammatory effect is dependent on a reduction in the number and activity of specific functional multi-protein complexes which HDAC1 and 2 are interchangeable members of and which are recruited to and deacetylate specific protein substrates. This implies that inhibition of either one of these isoforms in isolation would be sufficient to suppress the inflammatory response of innate immune cells. In support of this, MI192 which is selective for HDAC2 over HDAC1 (see section 1.8) suppresses the inflammatory response in BV2 microglia (figure 4.2E) and in peripheral monocytes (Gillespie et al., 2011). In these instances, one would have to assume that selectively inhibiting HDAC2 with MI192 does not cause an upregulation of HDAC1 and an associated compensatory effect because of this.

In immortalised murine macrophages, the compensatory effect associated with an upregulated class I HDAC following knockdown of others also extends to HDAC3. Here, knockdown of HDAC1 leads to an upregulation of HDAC2 and vice versa. When the authors knocked down both HDAC1 & 2 to levels below those found endogenously (rather than knocking down one HDAC and preventing the upregulation of the other as performed in this thesis), this caused an increase in the levels of HDAC3. In this scenario, it was only when all three isoforms were knocked down, that an anti-inflammatory phenotype was observed (Jeong et al., 2014). It is surprising that this upregulated HDAC3 had a compensatory effect because HDAC3 is a member of a different multi-protein complex to HDAC1 and 2. Yet HDAC3 upregulation and an increase in the activity of HDAC3-containing complexes must be sufficient to maintain the acetylation state of a specific protein or proteins, which if hyperacetylated would lead to an inhibition of the inflammatory response. This implies that this substrate(s) can be deacetylated by all three of these HDACs and the specific multi-protein complexes they are members of. Therefore, it is possible that

inhibiting HDAC3 on its own may also reduce the inflammatory response of BV2 microglia. In support of this, other studies have shown that knocking down or knocking out HDAC3 in macrophages (Chen et al., 2012a), in human embryonic kidney (HEK)-293 cells and murine embryonic fibroblasts (Ziesche et al., 2013) before activating the inflammatory response with appropriate stimuli, significantly suppresses the expression of pro-inflammatory genes in those cells. At present, there is no direct evidence to suggest HDAC3 is involved in the inflammatory response of microglia or that the selective HDAC3 inhibition using a pharmacological agent or HDAC3KD can suppress it. However, the non-selective or class I selective HDAC inhibitors which suppressed the expression of pro-inflammatory mediators in BV2 microglia, all inhibit HDAC3 and the inhibition of this isoform as well as inhibition of HDAC1 and/or 2, could contribute to the anti-inflammatory phenotype observed.

As proposed earlier, in BV2 microglia and other innate immune cells, HDAC1 and HDAC2 may deacetylate the same protein substrate(s) and in doing so regulate the inflammatory response. It is important to note that in other cellular processes outside of those discussed; the upregulation of one of these two isoforms following the loss of the other is not always compensatory. A notable example of this in the field of neuroscience is how HDAC1 upregulation in HDAC2KO mice does not compensate for a loss in HDAC2 activity because these mice exhibit a phenotype of improved learning and memory (Guan et al., 2009). Also, in mouse embryonic stem cells, despite an upregulation of HDAC2 after knocking out HDAC1 the specific acetylation of histone H3 at lysine 56 is increased (Dovey et al., 2010b). These two studies as well as others suggest that in some circumstances one HDAC isoform dominates the other when deacetylating a specific protein substrate, or that not all proteins that are regulated by acetylation, are targeted by both HDAC1 and 2, and these two isoforms have a degree of substrate specificity. How this substrate specificity and lack of it in different cellular processes is brought about is an interesting question for further study. One obvious way HDACs 1 and 2 could deacetylate the same substrates and therefore both regulate the same cellular process is because they are both able to form part of the same multi-protein complex either together or individually and it is this specific complex, rather than the particular HDAC isoform, which is targeted to a substrate. So in BV2 microglia, one particular

complex regardless of which or the combination of HDACs 1 & 2 are present is recruited to and deacetylates a particular protein substrate, which in turn regulates the inflammatory response. On the other-hand, substrate specificity of HDAC1 and 2 could be mediated through one HDAC being present in one complex, which the other HDAC is not and these complexes target specific protein substrates. An example of this has been seen in embryonic mouse cells where HDAC1 is found in the Sin3 complex to a greater extent than HDAC2 (Dovey et al., 2010b). This Sin3 complex containing only HDAC1 may be how this isoform regulates a specific function in embryonic stem cells. Together this poses an interesting question for further research with respect to identifying the HDAC1 and/or 2 containing multi-protein complex that when inhibited is responsible for the suppression of pro-inflammatory mediator expression in BV2 microglia. Bantscheff et al. (2011) have reported that VPA and MS-275, two HDAC inhibitors which are anti-inflammatory in microglia (figure 4.1B and 4.2E respectively), do not inhibit HDAC1 or HDAC2 in the Sin3 multi-protein complex, but inhibit these HDAC isoforms in the CoREST and NuRD complexes. Furthermore, Guan et al. (2009) show that following HDAC2KO in the mouse brain, HDAC1 is upregulated and this isoform then becomes incorporated into the Sin3 and NuRD complexes but not the CoREST complex. Based on these data, one could hypothesise a role of the NuRD complex or a yet to be identified complex in regulating the inflammatory response in microglia.

4.7.2. How is histone deacetylase inhibition anti-inflammatory?

This chapter has presented evidence that shows the inhibition of class I HDACs and more specifically HDAC1 and/or HDAC2, has anti-inflammatory effects in microglia. The underlying mechanisms of this response remain to be fully elucidated, but this thesis has begun to answer this question by investigating if the following mechanisms are involved:

1) An increase in the acetylation of histone proteins which leads to an increase in the expression of anti-inflammatory proteins,
2) An increase in the acetylation of non-histone proteins which regulate the inflammatory response.

Firstly, inhibiting HDACs increases histone acetylation in BV2 microglia and this may lead to an increase in the expression of genes, particularly those encoding proteins that suppress the inflammatory response. However, SAHA and apicidin suppressed LPS-induced mRNA expression of the pro-inflammatory cytokine IL-6 in BV2 microglia when protein synthesis was simultaneously inhibited. This phenomenon has also been observed in human HeLa cells where non-selective HDAC inhibition suppressed TNF-α induced IL-8 mRNA expression when protein synthesis was inhibited with cycloheximide (Furumai et al., 2011). Also, inhibiting protein synthesis in BV2 microglia did not prevent the LPS-induced expression of IL-6 mRNA. Together these data rule out a mechanism whereby HDAC inhibitors are anti-inflammatory through increasing the expression of existing or new proteins or by inhibiting protein synthesis in general. A more likely mechanism is that HDAC inhibition increases the acetylation of other pre-existing proteins, which in-turn regulates the inflammatory response.

There is experimental evidence in the literature that shows HDAC inhibitors inhibit the inflammatory response by reducing the activity of the transcription factor NF-κB. Furumai et al. (2011) reported that in HeLa cells stimulated with the inflammatory stimulus TNF-α, non-selectively inhibiting HDACs with TSA, significantly suppressed the transcriptional activity of NF-κB, reduced its recruitment to and

binding at the gene promoter of the pro-inflammatory cytokine IL-8, reduced the recruitment of RNA polymerase II to the IL-8 gene promoter and ultimately reduced the transcription and expression of this cytokine. Although not examined by Furumai et al. (2011), it is possible that NF-κB recruitment is also reduced at other NF-κB dependent pro-inflammatory genes that are suppressed by HDAC inhibitors, such as IL-6 and TNF-α. A key mechanism by which NF-κB activity is regulated is through acetylation and deacetylation of specific lysine residues in the subunits that come together to form this transcription factor. Therefore inhibiting HDACs will lead to the increase in the acetylation of these lysine residues and affect the activity of NF-κB. Specifically, class I HDACs are responsible for deacetylating NF-κB, HDAC3 deacetylates the RelA[p65] subunit of NF-κB at lysine residues 122, 123, 314 and 315 and as discussed in section 1.2.3, the acetylation of these lysine residues reduces the transcriptional activity of NF-κB (Kiernan et al., 2003; Ziesche et al., 2013). Therefore, HDAC3 by removing these acetyl groups from these lysine residues may facilitate NF-κB dependent transcription of pro-inflammatory genes in response to inflammatory stimuli. Consistent with this idea, acetylation mimicking RelA[p65] mutants at the lysine residues mentioned (Ziesche et al., 2013) or selective knockdown or knockout of HDAC3 (discussed in sections 4.1 and 4.7.1), reduce the expression of pro-inflammatory mediators in activated cells. Furthermore, HDAC3KD reduces the recruitment of RelA[p65] to the gene promoter of the pro-inflammatory cytokine IL-8 (Ziesche et al., 2013). In addition to HDAC3, HDAC1 & 2 are also known to interact with RelA[p65] (Ashburner et al., 2001) and combined knockout of HDAC1 & 2 increases the acetylation of RelA[p65] (Chen et al., 2011). In the latter study, knocking out HDAC1 was accompanied with an upregulation of HDAC2 and vice versa, and when this happened the acetylation of RelA[p65] was not increased (Chen et al., 2011). This observation is consistent with the idea that the upregulated HDAC isoform compensates for a loss in the other, preserves the number of a specific functional HDAC complex, so there is no change in the acetylation state of RelA[p65]. Based on these data, it is possible that in BV2 microglia following non-selective HDAC inhibition, selective inhibition of class I HDACs, or following HDAC2KD, or HDAC1KD when the upregulation of HDAC2 is prevented, the acetylation and inhibition of RelA[p65]/NF-κB is involved in suppressing the inflammatory response. In support of this, studying the acetylome of

activated BV2 microglia treated to the anti-inflammatory non-selective HDAC inhibitor SAHA and the anti-inflammatory class I selective HDAC inhibitor apicidin for 6 hours, one observed an increase in the acetylation of a protein with an approximate molecular weight of 65 kDa. The identity of this protein and the acetylated lysine residues need to be confirmed but based on its molecular weight, this protein correlates well with acetylated RelA[p65]. The acetylation of this 65 kDa protein was not as abundant after 6 hours of MI192 treatment. This is consistent with the observed lack of an effect on the inflammatory response with this inhibitor during this time frame. Benzamide HDAC inhibitors like MI192 and MS-275 have slow binding kinetics so an increase in protein acetylation following HDAC inhibition takes longer to occur. If the acetylation of this 65 kDa protein is important in reducing the inflammatory response following class I HDAC inhibition, then one would predict the acetylation of this protein to be greater after 24 hours of MI192 treatment, when after this length of time MI192 suppresses the expression of pro-inflammatory mediators in activated BV2 microglia. Studying the acetylome following HDAC1KD, HDAC2KD and HDAC1KD with the upregulation of HDAC2 prevented, will also help determine if the acetylation of this 65 kDa protein is key to suppressing the inflammatory response. If the acetylation of this protein is important for an anti-inflammatory effect then I would predict that following HDAC2KD or HDAC1KD when the compensatory increase in HDAC2 is prevented, this protein would also become hyperacetylated like that induced by SAHA and apicidin. I would also predict that when knocking down HDAC1, the accompanying upregulation and increase in activity of HDAC2 would compensate for a loss of HDAC1 activity so there would be no change in the acetylation state of this 65 kDa protein compared to the control.

Another signalling pathway that is activated upon inflammatory stimuli and induces the expression of pro-inflammatory mediators involves mitogen-activated protein kinases (MAPKs). When innate immune cells are stimulated with inflammatory stimuli, MAPKs are first phosphorylated by MAPK kinases and then enter the nucleus and activate transcription factors leading to the expression of pro-inflammatory cytokines (reviewed by Dong et al. (2002)). MAPKs are inactivated when dephosphorylated by phosphatases located in the nucleus (reviewed by Jeffrey

et al. (2007)). One such phosphatase, MKP-1 (mitogen-activated protein kinase phosphatase 1), has been shown to be positively regulated by acetylation (Cao et al., 2008). In macrophages stimulated with LPS, inhibiting HDACs 1, 2 & 3 with MS-275, or silencing their expression leads to an increase in the acetylation of MKP-1, which dephosphorylates/inactivates p38 MAPK and ultimately reduces the LPS-induced expression of pro-inflammatory mediators (Jeong et al., 2014). MKP-1 is a negative regulator of the inflammatory response not just in macrophages but also in microglia (Eljaschewitsch et al., 2006). However, an increase in the acetylation of a protein with the molecular weight of MKP-1 (39 kDa) was not detected in BV2 microglia following class I HDAC inhibition.

In addition to the acetylation of RelA[p65] and MKP-1, HDAC inhibition may be anti-inflammatory by increasing the acetylation state of other pre-existing proteins involved in the inflammatory cascade. As well as the 65 kDa acetylated protein, another protein with a molecular weight between 27 and 33 kDa was identified to be hyperacetylated by anti-inflammatory HDAC inhibitors in BV2 microglia (figure 4.9). In an attempt to learn the identity of this protein a bioinformatics analysis was performed. All the proteins expressed in humans with a molecular weight between 27 and 33 kDa (TagIdent tool, ExPASy, Swiss Institute of Bioinformatics, Artimo et al. (2012); Gasteiger et al. (2003); Gasteiger et al. (2005)) were compared with the proteins found to be hyperacetylated in the MV4-11 human monocyte/macrophage leukaemia cell-line when treated with SAHA or MS-275 (Choudhary et al., 2009). This analysis revealed seven proteins that matched these criteria (shown in table 4.2).

Table 4.2. Acetylated proteins with a molecular weight between 27 and 33 kDa in MV4-11 human monocytes/macrophages following treatment with HDAC inhibitors.

UniProt ID	Protein name	~MW (kDa)	Function
Q16629	Serine/arginine-rich splicing factor 7	27	mRNA processing
Q9UKM9	RNA-binding protein Raly	32	mRNA processing
O75586	Mediator of RNA polymerase II transcription subunit 6	28	Transcription
P18669	Phosphoglycerate mutase 1	29	Glycolysis
Q8N954	G patch domain-containing protein 11	30	NA binding
P00338-2	L-lactate dehydrogenase A chain	30	Anaerobic respiration
P00491	Purine nucleoside phosphorylase	32	Purine metabolism

Abbreviations, MW: Molecular Weight, NA: Nucleic acid. Protein name, molecular weight and function were sourced from UniProt (2015).

Because these proteins are involved in important cellular processes such as gene expression, DNA synthesis and respiration, it is likely that most will be ubiquitously expressed in all cell types including microglia. However, these proteins are unlikely to be involved in specifically activating or inhibiting the inflammatory response in innate immune cells. Next, a more comprehensive analysis was performed to look at the entire acetylome in both humans and mice (Liu et al., 2014) to determine if any acetylated proteins with a molecular weight between 27 and 33 kDa are involved in the inflammatory response of any of the innate immune cells in the whole organism, including microglia. Comparing all the proteins within this molecular weight range in each species with the respective organism's acetylome (Liu et al., 2014), short-listed 199 proteins in humans and 186 proteins in mice. Cross-referencing these acetylated proteins with those classified as being involved in the inflammatory response for each species (176 and 156 proteins for humans and mice respectively, UniProt (2015)) did not lead to the identification of any proteins of the desired molecular weight known to be involved in inflammation. If the protein with a molecular weight between 27 and 33 kDa observed to be acetylated following HDAC inhibition in BV2 microglia is indeed one of those identified by the bioinformatics analysis (table 4.2), then it is unlikely that this protein is responsible

for the anti-inflammatory effects seen with HDAC inhibitors. To confirm the identity of this acetylated protein, the protein could be isolated from the rest of the proteins in the acetylome and then analysed using mass spectrometry.

Though not investigated in this thesis, another way HDAC inhibitors could suppress pro-inflammatory gene expression in microglia without requiring protein synthesis, is by increasing the acetylation of histone proteins or transcription factors, which leads to an increase in the expression of microRNAs (miRNAs). Mature miRNAs reduce protein expression by binding to target sites within mRNA encoding a particular protein. This interaction prevents mRNA translation or initiates mRNA degradation (reviewed by Bartel (2004)) and could account for an observed reduction in both mRNA expression and protein secretion of pro-inflammatory mediators in BV2 microglia and other innate immune cells. Grabiec et al. (2012) have observed a significant acceleration of IL-6 mRNA degradation in human fibroblast-like synoviocytes (inflammatory cells involved in the pathogenesis of arthritis) when stimulated with LPS in the presence of TSA, compared to LPS alone. This mRNA degradation could possibly be brought about by HDAC inhibition mediated expression of miRNAs. Indeed, in human fibroblast-like synoviocytes, SAHA treatment significantly increases the expression of miR-146a, which in turn suppresses IL-1β induced RelA[p65] phosphorylation/activation, inhibits the expression of proteins involved in the TNF-α and IL-1 receptor-mediated signalling cascades and significantly reduces IL-6 protein secretion. The anti-inflammatory effects of SAHA in these cells could be prevented with a miR-146a inhibitor or by overexpressing either HDAC1, 4 or 6 (Wang et al., 2013a). Another miRNA, miR-124, is also upregulated following SAHA or apicidin treatment (Wang et al., 2014) and has been shown to negatively regulate the inflammatory response. This miR-124 is expressed in microglia of the brain and spinal cord in mice and is down-regulated in activated microglia in mice following experimental autoimmune encephalomyelitis and in isolated microglia stimulated with LPS & IFNγ (Ponomarev et al., 2011). Furthermore, when murine macrophages are transfected with mimics of miR-124, this leads to a reduction in the expression of the pro-inflammatory mediators iNOS (Ponomarev et al., 2011), IL-6 (Sun et al., 2013) and TNF-α (Ponomarev et al., 2011).

In addition to suppressing the expression of pro-inflammatory mediators like cytokines by the possible mechanisms discussed, HDAC inhibitors may also be neuroprotective in brain insults, injury and neurodegenerative disease where the inflammatory response is involved by suppressing the signalling pathways activated by them. Upon the expression of pro-inflammatory cytokines such as IL-1β, IL-6, IFNγ and TNF-α, these proteins are secreted by innate immune cells into the extracellular space and are free to bind to the their respective receptors in the cell membrane of other cells. In turn, this activates intracellular signalling cascades that lead to further cytokine expression and physiological responses such as the apoptosis of neurones. Components of these cytokine receptor mediated signalling cascades may also be regulated by acetylation. An example of this is STAT1 (signal transducer and activator of transcription 1) a protein activated by the IL-6 and IFNγ receptor-signalling pathways (Ihle, 2001), which is positively regulated by acetylation. Class I HDACs, HDAC1 & 3 but not HDAC2 or HDAC8 can deacetylate STAT1 (Kramer et al., 2006). The acetylation of this protein is increased in human derived cell-lines when HDACs are inhibited with VPA or TSA. Upon acetylation, STAT1 interacts with RelA[p65] reducing its affinity for DNA and triggering its export out of the nucleus (Kramer et al., 2006).

4.8. Conclusion

BV2 microglia when stimulated with LPS is an appropriate model to study the inflammatory response of primary microglia and neuroinflammation *in vivo*. Using this model, it has been demonstrated that inhibiting class I HDACs, 1, 2 & 3 inhibits the inflammatory response of microglia as indicated by a suppression of the expression of pro-inflammatory mediators. Knocking down specific HDAC isoforms revealed that HDAC1 and HDAC2 are important for this response. Knockdown of HDAC2, or knockdown of HDAC1 when the accompanying upregulation and compensatory activity of HDAC2 was prevented, suppressed the expression of pro-inflammatory cytokines. This suggests HDAC1 and HDAC2 are functionally redundant in regulating the inflammatory response of microglia. Mechanistically, HDAC1 & 2 may be interchangeable members of a specific multi-protein complex, which deacetylates specific proteins that regulate the inflammatory response. Reducing the activity of either or both of these isoforms and therefore a specific multi-protein complex, may lead to the acetylation of these proteins, which upon acetylation either no longer function to promote the inflammatory response or actively suppress it. Investigating some of the mechanisms of how HDAC inhibition could be anti-inflammatory suggests that HDAC inhibitors do not require novel protein synthesis to have their effect, rather they increase the acetylation of pre-existing proteins which can regulate the inflammatory response directly. One promising candidate identified in this thesis is a protein with a molecular weight of ~65 kDa. This protein correlates well with the RelA/p65 subunit of NF-κB, a transcription factor that induces pro-inflammatory gene expression and has been shown by others to be negatively regulated when acetylated at specific lysine residues.

Chapter 5

General Discussion

5.1. Is selective inhibition of HDAC1 & 2 a therapeutically viable option to treat brain insults, injury and neurodegenerative disease?

Over the last decade many studies have shown that HDAC inhibition has neuroprotective and neuro-restorative effects in *in vitro, ex vivo* and *in vivo* models relevant to brain insults such as cerebral ischaemia, injuries such as traumatic brain injury and neurodegenerative diseases such as Alzheimer's, Parkinson's, motor neurone disease and multiple sclerosis. Chronic neuroinflammation is thought to exacerbate neuronal injury and death in these conditions (reviewed by Block and Hong (2005); Block et al. (2007); Glass et al. (2010); Smith et al. (2012)) and one-way HDAC inhibitors may be neuroprotective is by suppressing the inflammatory response of resident microglia and infiltrating peripheral innate immune cells. Despite substantial evidence suggesting HDAC inhibitors may be therapeutically useful in preventing neural cell death in brain conditions, these drugs have not been extensively tested in clinical trials for this purpose. Only two trials have studied the neuroprotective efficacy of HDAC inhibitors in patients (U.S. National Institutes of Health, 2015) one of which investigated the effects of sodium butyrate in individuals with motor neurone disease. However, inhibiting HDACs had no therapeutic effect as determined by measuring muscle function (Cudkowicz et al., 2009). Currently a clinical trial is in progress to investigate if the clinically available HDAC inhibitor VPA protects the brain and improves recovery of brain function in patients after traumatic brain injury (Zhou, 2013). HDAC inhibitors are also currently being tested in clinical trials for other conditions and at present there are 493 registered clinical trials worldwide that have tested or are currently testing HDAC inhibitors to treat injury and disease (U.S. National Institutes of Health, 2015). SAHA is used in the clinic to treat cutaneous T-cell lymphoma and the majority of the completed or ongoing clinical trials have or are investigating the use of HDAC inhibitors as a form of chemotherapy to treat a variety of other cancers. HDAC inhibitors have also been tested in the inflammatory condition of juvenile-onset arthritis. Here the non-selective HDAC inhibitor Givonstat (ITF2357) significantly reduces the number of swollen/inflamed and arthritic joints and improves the range of motion of joints in patients with this condition (Vojinovic et al., 2011).

A problem identified when performing clinical trials and using non-selective HDAC inhibitors in the clinic is that these drugs can cause significant side effects. This is presumably from an inappropriate inhibition of particular HDACs when using non-selective HDAC inhibitors and in the case of VPA, interacting with other molecular targets (see section 1.8). Some of the adverse side effects associated with sodium butyrate, SAHA and VPA treatment include liver damage, dizziness, drowsiness & tiredness, headaches, mood changes, confusion, loss of motor coordination, nausea, vomiting, diarrhoea, loss of appetite, unusual weight gain or loss, peripheral oedema, anaemia, bleeding & bruising and respiratory difficulties (Cudkowicz et al., 2009; MedlinePlus, 2009; MedlinePlus, 2014). Furthermore, children exposed to HDAC inhibitors in the womb are at a high risk of serious developmental disorders (MedlinePlus, 2009; MedlinePlus, 2014). One-way these side effects could be minimised would be to use selective HDAC inhibitors that target only the HDAC isoforms important to produce a therapeutic response. In this thesis, progress has been made in identifying the specific HDACs to inhibit in order to reduce neuroinflammation in brain insults, injuries and neurodegenerative disease. Here, it has been shown that HDAC inhibitors, which are selective for isoforms HDAC1, 2 & 3 or more specifically HDAC2 & 3, suppress the inflammatory response of microglia. In support of these findings, other studies have shown that the inhibition of these three HDAC isoforms reduces neuroinflammation and is neuroprotective in *in vivo* models of cerebral ischaemia (Lanzillotta et al., 2013; Murphy et al., 2014), experimental autoimmune neuritis (Zhang et al., 2010) and an amyloidosis model of Alzheimer's disease (Zhang and Schluesener, 2013). HDAC inhibitors selective for these isoforms have also been shown to reduce the inflammatory response in rodent models of arthritis (Cantley et al., 2015; Lin et al., 2007). At present it remains to be determined if selectively inhibiting HDAC1, 2 & 3 is therapeutically beneficial in a clinical trial to treat brain insults, injuries and neurodegenerative disease, and if selectively inhibiting these HDAC isoforms has fewer side effects in patients compared to those caused by non-selective HDAC inhibitors. To understand these side effects, it is important to understand more about the substrates of HDACs and the function of these proteins in cells and physiological systems.

Histones are not the only proteins that are regulated by lysine acetylation. There are now 4817 proteins in humans that are known to be modified in this way (Liu et al., 2014). Because of this, one has to consider that even with the currently available inhibitors selective for HDAC1, 2 & 3, it is likely that these too will effect the normal acetylation-deacetylation of a significant proportion of proteins in the acetylome and doing so may lead to unwanted changes in many physiological processes. Work by Choudhary et al. (2009) comparing the acetylome in MV4-11 human monocyte/macrophage leukaemia cells following treatment with the non-selective HDAC inhibitor SAHA and the HDAC1, 2 & 3 selective HDAC inhibitor MS-275 has provided some insight into this problem. Analysis of the available raw data reveals that of the 1058 acetylated sites examined in these cells, the acetylation of 8.22% of these is increased at least 1.5 fold by both SAHA and MS-275. SAHA also increases the acetylation of an additional 12.67% of the sites, which MS-275 does not and MS-275 increases the acetylation of an additional 8.98% of the sites, which SAHA does not. Although there are differences in which sites become acetylated, the overall increase in acetylation following selective HDAC1, 2 & 3 inhibition is similar to that induced by SAHA which inhibits HDACs 5, 6 & 8 in addition to HDACs 1, 2 & 3 (table 1.4). This analysis was performed on one cell-type and so if this extrapolated to the effects of HDAC inhibition in the whole organism; it is likely that selective inhibition of HDAC1, 2 & 3 will still lead to changes in many cellular processes and physiological systems. With this in mind, it might be possible to minimise these side effects by inhibiting just one of these three HDAC isoforms. Identifying the most appropriate isoform to inhibit will require a clear understanding of the roles of each in physiological processes.

In this thesis it has been demonstrated that specific knockdown of either HDAC1 or HDAC2 suppresses the inflammatory response of microglia. This suggests HDAC1 and HDAC2 have functional redundancy in the inflammatory response of microglia and because of this; it may be possible to inhibit only one of these isoforms is to suppress neuroinflammation in brain insults, injuries and neurodegenerative disease. However, this would depend on HDAC inhibitors selective for HDAC1 over HDAC2 and vice versa to not cause an increase in the expression of the other isoform, which the HDAC inhibitor would not inhibit. If one could inhibit just one of

these isoforms without causing an up-regulatory increase in the other, is there any evidence to favour inhibiting one over the other? Some experimental evidence reported in the literature supports the idea that inhibiting HDAC1 is neuroprotective, yet another set of studies oppose this and show specific inhibition of HDAC1 in neurones causes neurotoxicity (see section 3.1.1). The reasons underlying these contradictions are not known, so inhibiting HDAC1 in the brain comes with the potential risk of causing damage not neuroprotection. If inhibiting HDAC1 is to be avoided, will selective inhibition of HDAC2 be sufficient to have neuroprotective and anti-inflammatory effect in brain insults, injuries and neurodegenerative disease? In support of this, silencing HDAC2 reduces the inflammatory response in microglia (figure 4.4), HDAC2KD prevents CGNs from undergoing apoptosis due to oxidative stress (Peng et al., 2015) and HDAC2 is found upregulated in the cortex of individuals with Alzheimer's disease and in the cortex and hippocampus of the p25/Cdk5 mouse model of this condition (Graff et al., 2012). In this mouse model, mice display Alzheimer's disease related pathologies such as neuronal loss (Cruz et al., 2003), β-amyloid accumulation (Cruz et al., 2006), Tau hyperphosphorylation (Cruz et al., 2003), neurofibrilliary tangles (Cruz et al., 2003), reactive astrogliosis (Muyllaert et al., 2008), microgliosis (Muyllaert et al., 2008), neuroinflammation (Muyllaert et al., 2008) and reduced synaptic density (Fischer et al., 2005) in both the hippocampus and the cerebral cortex of mouse brains. The mechanism that causes HDAC2 upregulation in Alzheimer's disease and in the p25/Cdk5 mouse model has not been explained. However the same laboratory has previously shown that p25 inhibits HDAC1 in this mouse model (Kim et al., 2008). HDAC1 expression is unchanged in the post-mortem brain of patients with Alzheimer's disease and p25/Cdk5 mice (Graff et al., 2012) but a loss of HDAC1 activity in this mouse model and inhibition of HDAC1 in isolated neurones causes neuronal death (Kim et al., 2008). Although not examined in these studies, it is possible that a reduction in HDAC1 activity may cause a compensatory upregulation and increase in activity of HDAC2. This could explain why HDAC2 is found upregulated when HDAC1 activity is reduced in the p25/Cdk5 mouse model of Alzheimer's disease. Despite this upregulation of HDAC2, HDAC2 would not adopt the independent and specific functions of HDAC1. This is presented as neurotoxicity because HDAC1 is important for DNA repair processes in neurones (Kim et al., 2008; Wang et al.,

2013b). Also, following the upregulation of HDAC2, the independent function of this isoform as a negative regulator of learning and memory would be enhanced (Graff et al., 2012; Guan et al., 2009) and in part cause the cognitive decline associated with Alzheimer's disease (Graff et al., 2012). Is the upregulation of HDAC2 just a consequence of a loss of HDAC1 activity and it is this loss that causes neurotoxicity? Or does an increase in HDAC2 activity following upregulation also cause neurotoxicity? Knocking down HDAC2 in the hippocampus of p25/Cdk5 mice reduces the expression of HDAC2 back to normal levels and in doing so restores neuronal function and improves cognition but does not halt neurodegeneration in these mice. The lack of a neuroprotective effect would suggest HDAC2 upregulation does not cause neurotoxicity but contributes to cognitive decline by reducing functionality of neurones. However, knocking down HDAC2 below normal levels suppresses CGN apoptotic cell death (Peng et al., 2015) and the data presented in this thesis shows this knockdown also suppresses the inflammatory response of microglia. So why isn't HDAC2KD neuroprotective in the *in vivo* model of Alzheimer's disease by suppressing apoptosis or the neuroinflammatory component? By only knocking down HDAC2 in p25/Cdk5 mice to levels of expression found normally, HDAC2 will still be able to regulate the inflammatory response and/or other mechanisms which cause neurotoxicity and drive neurodegeneration. A reduction in HDAC2 expression below normal levels, like shown in BV2 microglia (figure 4.4) will be required to prevent the inflammatory response and associated neurotoxicity, because the expression level of HDAC2 is now reduced below that, which ordinarily would permit it. An alternative reason why neuroprotection is not seen in the hippocampus of p25/Cdk5 mice when the level of HDAC2 expression is returned to normal levels is due to the methodology used to reduce HDAC2 expression. Graff et al. (2012) infected cells of the hippocampus with an AAV vector containing HDAC2 short hair-pin RNA (shRNA) and show HDAC2 levels are reduced in neurones. However, no mention is made of any changes in HDAC2 levels in microglia in the brains of the p25/Cdk5 mice compared to wild-type controls and following AAV-mediated HDAC2KD. Previous work by Dr Ian C Wood's laboratory (School of Biomedical Sciences, University of Leeds, UK, unpublished data) has shown that the infection rate of AAV in BV2 microglia cultures is very low. Therefore, it is likely that in the hippocampus of p25/Cdk5 mice, only neurones

are infected with the virus and not microglia. This means that HDAC2 is not knocked down in microglia. Therefore, the inflammatory response initiated in the mouse model of Alzheimer's disease is unaffected and continues to drive neurotoxicity and neurodegeneration in this model. A future area of study would be to determine if specific knockdown or selective inhibition of HDAC2 in microglia is neuroprotective *in vivo* by reducing neuroinflammation in models of brain insults, injuries and neurodegenerative disease. Nevertheless, experimental evidence reported in the literature shows reducing HDAC2 but not HDAC1 in mice improves cognitive functions such as learning and memory (Graff et al., 2012; Guan et al., 2009) and this in itself may be of therapeutic benefit in conditions where a restoration in cognition is required.

Although selective inhibition of HDAC1 and/or HDAC2 will avoid the side effects associated with inhibiting other HDACs, inhibiting these two isoforms together or individually will still produce side effects associated with inhibiting cellular processes they both regulate, or inhibiting their individual functions. For example, HDAC1 and HDAC2 are important in regulating intestinal function and the inhibition of this function could be the cause of the digestive problems reported by patients taking HDAC inhibitors. In support of this, specific knock out of both these isoforms in intestinal cells in mice causes digestive system defects. These mice weigh less and produce looser stools (Turgeon et al., 2013). There is also an increase in the proliferation and migration of epithelial cells in the intestine, but a decrease in cell lineage commitment. Together this leads to cellular disorganisation, a thickening of the intestinal epithelium, an increase in the overall length of the intestine and disruption of normal intestinal function (Turgeon et al., 2013). Knockout of these two isoforms also increases the infiltration of immune cells into the intestine, which secrete inflammatory mediators (Turgeon et al., 2013). However, this pro-inflammatory effect is not a consequence of the loss of HDAC1 & 2 in immune cells, because HDAC1 and 2 expression levels were only manipulated in intestinal cells. Therefore, immune cell infiltration and inflammation is an indirect effect, which may be stimulated by disrupted intestinal cells, digestion and behaviour of commensal bacterial in the intestine.

A loss of HDAC2 expression is however correlated with chronic inflammation in the lungs of patients with chronic obstructive pulmonary disorder (reviewed by Barnes (2009)). Therefore the inhibition of HDAC2 may be the cause of breathing difficulties reported by some patients taking HDAC inhibitors. Why and how class I HDAC inhibition is anti-inflammatory in *in vivo* brain conditions (Lanzillotta et al., 2013; Murphy et al., 2014; Zhang and Schluesener, 2013; Zhang et al., 2010), in patients with and in *in vivo* models of inflammatory arthritis (Cantley et al., 2015; Lin et al., 2007; Vojinovic et al., 2011), in isolated microglia (figure 4.2A & E and 4.4), monocytes (Gillespie et al., 2011), macrophages (Jeong et al., 2014; Zhang and Schluesener, 2013) and fibroblasts (Choo et al., 2010), but pro-inflammatory in the pulmonary system, is not understood.

Taking side effects into consideration, is selective inhibition of HDAC1 and/or 2 a viable therapeutic strategy to treat brain insults, injuries and neurodegeneration? With every pharmacological intervention it is expected that there will be side effects. Currently available non-selective HDAC inhibitors used in the clinic have a numerous side effects, yet these drugs have still been approved and are in use because the gains of using them to treat cancer, epilepsy and bipolar disorder, which are severely debilitating conditions, outweigh the negative side effects these drugs may cause. Selectively inhibiting HDAC1 and/or 2 using the currently available selective HDAC inhibitors such as MS-275 or MI192, or newly developed compounds may not remove all the side effects associated with HDAC inhibition, but will prevent any side effects associated with inhibiting class II HDAC isoforms. The work presented in this thesis suggests there may be an alternative way of reducing the activity of HDAC1 and HDAC2, which generates fewer side effects compared to using selective HDAC inhibitors. Here it has been suggested that the anti-inflammatory effects seen when using HDAC inhibitors is brought about by the inhibition of a specific multi-protein complex which HDAC1 and HDAC2 are interchangeable members of. It may be possible to identify then target a specific non-HDAC component, which is unique to this specific complex, such as a component important for targeting the complex to the appropriate protein substrate. Inhibiting such a component will reduce the side effects associated with inhibiting all of the functional complexes which HDAC1 and/or HDAC2 are members of.

5.2. Is activation of specific deacetylase enzymes also neuroprotective?

Although this thesis and much of the literature has focussed on the use of HDAC inhibitors as neuroprotective agents, in some circumstances would it be beneficial to increase the activity of specific deacetylase enzymes to be neuroprotective and suppress neuroinflammation in brain insults, injuries and neurodegenerative disease? If inhibiting HDACs that cause neurotoxicity is neuroprotective, then it is plausible that increasing the activity of HDACs that promote neurone survival or are anti-inflammatory will be neuroprotective also. Although, the role of HDAC1 in neuronal death and survival is debated (see section 3.1.1), one group is patenting the use of HDAC1 activators to treat Alzheimer's disease, Parkinson's disease, motor neurone disease, traumatic brain injury and cerebral ischaemia (Tsai et al., 2015). This application is based on their earlier observations that a loss of HDAC1 activity is toxic to isolated neurones by causing DNA damage (Kim et al., 2008; Wang et al., 2013b) and HDAC1 activity is reduced in the brain of the p25/Cdk5 mouse model of Alzheimer's disease (Kim et al., 2008). Furthermore, over-expressing HDAC1 provides neuroprotection in this model as well as in forebrain ischaemia in rats (Kim et al., 2008). Apparent HDAC1 activators have been identified by this group following a screen of 1760 small molecules including synthetic compounds, natural products such as flavonoids and a subset of United States Food & Drug Administration (FDA) approved drugs (Tsai et al., 2015). These compounds were tested for effects on the activity of recombinant HDACs 1, 2, 6, 8 & 10 in an *in vitro* substrate deacetylation assay. The most promising compound, ChemBridge 5104434, increased HDAC1 activity by 2.2 fold with little effect on the other HDAC isoforms (Tsai et al., 2015). How this compound activates HDAC1 and the effect of this on neurone survival in mouse models of the aforementioned brain conditions has yet to be examined. But, based on the work presented in this thesis, if inhibiting HDAC1 suppresses neuroinflammation then it is possible that activating HDAC1 will augment it. Furthermore, other studies have shown an increase in the activity of HDAC1 causes toxicity to neurones (Bardai et al., 2012; Kim et al., 2010). Because of this, HDAC1 activators identified by Tsai et al. (2015), may actually promote the inflammatory response and exacerbate neurotoxicity in brain insults, injuries and neurodegenerative disease.

Class III HDACs the sirtuins have not been discussed in this thesis however, experimental evidence reported in the literature suggests that increasing the activity of specific sirtuins may be neuroprotective and anti-inflammatory. All seven sirtuins (SIRT1-7) are expressed in the brain (Michishita et al., 2005) and SIRT1 and SIRT2 are two isoforms that have been implicated in neuronal degeneration (reviewed by Harting and Knoll (2010); Raghavan and Shah (2012)). SIRT1 activity has mainly been attributed with neuroprotective effects; increasing SIRT1 activity through overexpression or by using apparent pharmacological activators like resveratrol protects neurones from OGD (Lanzillotta et al., 2013; Wang et al., 2009a). Inhibitors of SIRT1 & 2 such as sirtinol (Grozinger et al., 2001) prevent this neuroprotective effect. Increased activity of SIRT1 also protects neurones from DNA damage (Dobbin et al., 2013; Hasegawa and Yoshikawa, 2008), models of Alzheimer's disease (Chen et al., 2005; Dobbin et al., 2013; Kim et al., 2007a), Parkinson's disease (Okawara et al., 2007) and motor neurone disease (Kim et al., 2007a). Interestingly, increasing SIRT1 or SIRT2 activity with pharmacological agents including resveratrol also suppresses the inflammatory response of microglia (Chen et al., 2005; Pais et al., 2013; Yang et al., 2012; Ye et al., 2013). In neurone and microglia co-cultures, this anti-inflammatory effect prevents neuronal death triggered by the inflammatory response of activated microglia (Chen et al., 2005). Mechanistically it is thought that SIRT1 and SIRT2 suppress the inflammatory response of microglia by specifically deacetylating p65[RelA] at lysine 310 (Pais et al., 2013; Rothgiesser et al., 2010a; Yeung et al., 2004). Consistent with this, increasing SIRT1 activity with resveratrol leads to a decrease in the acetylation state of this lysine residue (Lanzillotta et al., 2013). Acetylation of this specific site has been shown to increase the transcriptional activity of NF-κB (Chen et al., 2002; Ziesche et al., 2013). Therefore promoting the deacetylation of this site by overexpressing SIRT1 & 2, or treating with a pharmacological activator, may reduce the transcriptional activity of this transcription factor. This could explain how increasing SIRT1 & 2 activity suppresses the expression of NF-κB dependent pro-inflammatory mediators in microglia. Combining an increase in the deacetylation at K310 in p65[RelA] by using a SIRT1 or 2 activator, together with an increase in the acetylation of lysine residues 122, 123, 315 and 315 in p65[RelA] by using a class I selective HDAC inhibitor, may negatively regulate NF-κB function to a greater

extent compared to using these drugs on their own. In support of this idea, when MS-275 and resveratrol are co-administered, there is less ischaemic brain damage in mice following MCAO compared to the protection provided when mice are treated to either compound alone (Lanzillotta et al., 2013). This study would suggest that combining these drugs would have a greater neuroprotective effect in clinical conditions compared to administering one drug or the other. However, it is important to consider that increasing SIRT1 activity and consequently decreasing the acetylation of SIRT1-regulated proteins in the acetylome (Chen et al., 2012b), may evoke additional side effects alongside those caused by inhibiting HDACs 1, 2 & 3.

5.3. Further work

5.3.1. Investigating the mechanisms underlying the functional redundancy of HDAC1 & 2 in the inflammatory response

The data presented in this thesis suggests HDAC1 and HDAC2 are functionally redundant in regulating the inflammatory response of microglia. Others have also shown these two isoforms are functionally redundant in the inflammatory response of other innate immune cells (Jeong et al., 2014). The functional redundancy of HDAC1 and HDAC2 means both these enzymes target and deacetylate the same protein substrates. When either enzyme is inhibited, the deacetylation of these specific proteins is reduced and this leads to an increase in the acetylation state and upon this, anti-inflammatory effects. The identity of these proteins has yet to be fully elucidated but a possible candidate protein with a molecular weight of 65 kDa has been identified in this thesis. This protein is suspected to be the p65/RelA subunit of NF-κB which is a key regulator of the inflammatory response. An obvious direction of further work would be to perform Western blots and mass spectrometry to confirm the identity of this protein, determine which lysine residues are acetylated and then perform functional assays to determine if and how this acetylation is responsible for the anti-inflammatory effects seen when using HDAC inhibitors or knocking down HDACs 1 or 2.

Further work is also required to understand the molecular mechanisms underlying the functional redundancy of HDAC1 and 2 in the inflammatory response. One of the potential mechanisms is that HDAC1 and HDAC2 are interchangeable members of a specific multi-protein complex, which is responsible for deacetylating a specific protein that can regulate the inflammatory response. Identifying which multi-protein complex is responsible i.e. the Sin3, NuRD or the CoREST complex and the proteins it is comprised of is one avenue for further study. Using a combination of co-immunoprecipitation experiments and mass spectrometry one could examine the interacting partners of the protein substrate, which when acetylated is responsible for the suppressing the inflammatory response. Then having identified which multi-protein complex interacts with the protein substrate, this can be compared to the complex that no longer interacts with the protein substrate following HDAC inhibition, HDAC2KD or HDAC1KD when the associated upregulation of HDAC2 is prevented. One could also look at the composition of the complex that interacts with the substrate when HDAC1 is knocked down but HDAC2 is upregulated.

5.3.2. Further investigations of the anti-inflammatory effects of inhibiting HDAC1 and/or 2 in microglia

In this thesis, the effects of various HDAC inhibitors as well as silencing of HDACs 1 or 2 on neuroinflammation have been examined. This involved using a LPS-stimulated BV2 murine microglia model system. Although using the BV2 murine microglia cell-line appropriately models the inflammatory response of microglia (see section 4.1), it is important to determine if selective HDAC1 and/or 2 inhibition has any clinical value in reducing neuroinflammation in humans. To do this, one could examine the effects of selective inhibition of HDAC1 and/or 2 in isolated human microglia using the protocols described by Suh et al. (2010).

In this thesis, the inflammatory response of microglia was examined by measuring changes in IL-6, iNOS and TNF-α expression. Even though these are good markers of neuroinflammation in the brain, it would also be good to look at a wider range of pro-inflammatory mediators in order to get a clearer idea of how effective selective inhibition of HDAC1 and/or 2 is in suppressing the inflammatory response of

microglia. To analyse the changes in expression of many neuroinflammatory markers at once, one experimental approach would be to use PCR arrays, which will allow the researcher to profile the expression of up to 384 genes simultaneously.

The primary goal of suppressing the inflammatory response of microglia and reducing neuroinflammation by using HDAC inhibitors selective for HDAC1 and/or 2, is to prevent the neurotoxic effects associated with the production and release of pro-inflammatory mediators. To investigate if HDAC inhibitor-mediated suppression of pro-inflammatory mediator expression is sufficient to significantly reduce the toxicity of the inflammatory response, one could either add the supernatants of stimulated microglia to isolated neurone cultures, or co-culture microglia with neurones (as used by Gresa-Arribas et al. (2012)). This would allow one to examine changes in neurone viability in response to microglia-mediated inflammation in the absence or presence of a HDAC1 and/or 2 selective inhibitor, or after specifically knocking down HDAC1 or HDAC2 in microglia. Another approach would be to stimulate the microglial inflammatory response in organotypic slice cultures of brain tissue such as cortex, hippocampus or cerebellum, or in the brains of rodents and then study the effects of this on neurone integrity, function and survival in the absence and presence of selective inhibitors of HDAC1 and 2, or knockdown of these isoforms in specific brain regions and cell types.

Much has been discussed with respect to suppressing the expression of pro-inflammatory mediators in microglia to have a neuroprotective effect in models relevant to brain insults, injuries and neurodegeneration. It is important to mention that not all consequences of microglial activation in these brain conditions are detrimental and some are important for tissue repair and recovery. One of these is the ability of microglia to phagocytose cell debris and cells damaged beyond repair. If such objects were not removed, then they would accumulate and possibly interfere with normal neurone connectivity and function, as well as act as stimulants for a chronic inflammatory response, which would continue to exacerbate neurone injury and cause neural cell death. Activated microglia are known to exist as two phenotypes termed M1 and M2. The M1-phenotype is associated with the release of toxic pro-inflammatory mediators such as cytokines and free radicals, on the other-

hand M2 microglia suppress neuroinflammation, release neurotrophic factors and promote tissue repair and recovery. Microglia of the M2-phenotype also exhibit a stronger capacity to phagocytose cell debris, damaged cells and dead cells than M1 microglia (reviewed by Cherry et al. (2014); Hu et al. (2015)). Recently, Wang et al. (2015) have shown that non-selective HDAC inhibitors suppress the expression of pro-inflammatory mediators by microglia in part by switching the phenotype from the pro-inflammatory and toxic M1-type to the protective M2-type (as discussed in section 1.10.5). Following on from this work, it would be interesting to examine if selective class I HDAC inhibition and in particular selective inhibition of HDAC1 and/or HDAC2 also switches the phenotype of activated microglia to the M2-phenotype. This switch may also improve the ability of microglia to phagocytose cell debris and cells that cannot be rescued following injury. Taken together, a reduction in pro-inflammatory mediator expression following HDAC inhibition, combined with improved phagocytosis of cell debris and dead cells, would limit the extent of brain damage and help improve recovery in brain insults, injuries and neurodegenerative disease.

5.4. Final conclusion

Experimental evidence reported in the literature shows HDAC inhibitors are neuroprotective, neuro-restorative and improve neurological outcome in *in vivo* models of brain insults such as cerebral ischaemia, injuries such as traumatic brain injury and neurodegenerative diseases such as Alzheimer's, multiple sclerosis, motor neurone disease and Parkinson's disease. There is strong evidence to implicate neuroinflammation in the pathogenesis of these conditions and it has been shown that HDAC inhibitors can suppress the inflammatory response of microglia and other innate immune cells. However, which of the eleven zinc-dependent HDAC isoforms are important for this response and how inhibition of these leads to the anti-inflammatory and neuroprotective effects is not clear. The data presented in this thesis shows for the first time that selective inhibition of HDAC1 and/or HDAC2 has an anti-inflammatory effect in microglia. The data presented here also suggests there is functional redundancy of these isoforms in regulating the inflammatory response. Based on this, selectively inhibiting either HDAC1 or HDAC2 and not the other HDAC isoforms may be a viable strategy to reduce neuroinflammation in brain conditions whilst minimising the side effects associated with inhibiting other HDACs. In support of this, MI192, which is selective for HDAC2 over HDAC1, suppressed the inflammatory response of BV2 microglia. Further work should now be carried out to understand the mechanisms underlying the functional redundancy of HDAC1 & 2 in the inflammatory response and to determine if selective inhibition of either of these isoforms suppresses neuroinflammation and is neuroprotective in more complex experimental models of brain insults, injuries and neurodegenerative disease.

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Publication number: US 2015/0190411 A1. Filed: 12th August 2014. Patent application number: 14/457,981.

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