Developing an *in vitro* model of haemorrhagic shock to investigate the molecular mechanism of valproic acid in the treatment of massive blood loss

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Declaration of Authorship

I, Alexandra Zuckermann, hereby declare that this thesis and the work presented in it are entirely my own. Where I have consulted the work of others this is always clearly stated.

Signed,

Date:

Abstract

Haemorrhagic shock is a lethal condition caused by massive blood loss. Recent investigations in animal models have found that valproic acid (VPA), a well-established and widely used treatment for bipolar disorder and epilepsy, provides acute protection against haemorrhagic shock-induced mortality. Under these conditions, VPA prevents a decrease in glycogen synthase kinase 3β (GSK3β) Ser9 phosphorylation, an effect suggested to relate to histone deacetylase (HDAC) inhibition.

Here, the molecular signalling events during haemorrhagic shock-like conditions were investigated using a human liver cell line (Huh7). Cells exposed to hypoxia, hypercapnia, and hypothermia mimic the signalling changes seen in haemorrhagic shock, showing reduced GSK3β Ser9 phosphorylation. This *in vitro* reduction is blocked by VPA at physiologically relevant concentrations as it is *in vivo*. VPA treatment also prevents apoptotic signalling, improving cell survival in this model.

VPA-related compounds which are known activators of the peroxisome proliferator-activated receptor γ (PPARγ) were found to reproduce its protective effect independently of their HDAC inhibitory activity. PPARγ inhibition blocks this protection, demonstrating that PPARγ activity is essential for the therapeutic action of VPA in this model. This work therefore establishes an *in vitro* model for haemorrhagic shock signalling, identifies PPARγ regulation as a new molecular target in haemorrhagic shock, and provides novel candidate compounds for the development of life-saving treatment.

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Abbreviations

Akt/PKB	protein kinase B
Bcl-2	B-cell leukemia/lymphoma 2
CBP	CREB-binding protein
coA	coenzyme A
CREB	cAMP response element-binding protein
DA	decanoic acid
ERK1/2	extracellular regulated kinase 1/2 (MAPK 44/42)
FWB	fresh whole blood
GSK3β	glycogen synthase kinase 3β
HAT	histone acetyl transferase
Hc	hypercapnia
HDAC	histone deacetylase
Hp	hypothermia
Hx	hypoxia
H3K9	histone 3 lysine residue 9
MAPK	mitogen-activated protein kinase
Nx	normoxia
OA	octanoic acid
PCAF	p300/CBP-associated factor
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol 3,4,5-trisphosphate
PI3K	phosphoinositide-3-kinase
PPAR	peroxisome proliferator-activated receptor
PPRE	PPAR response element
PTEN	phosphatase and tensin homolog
RXR	retinoid X receptor
SA	sebacic acid
SAHA	suberoylanilide hydroxamic acid
SO	saline-only
TSA	trichostatin A
VPA	valproic acid
VPD	valpromide
2eVPA	2-ene-VPA
2POA	2-propyloctanoic acid (arundic acid)

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1

Introduction

1.1 Blood loss and haemorrhagic shock

Haemorrhage is the significant loss of intravascular blood volume leading to issues with tissue perfusion and cellular hypoxia, while shock describes a condition in which tissue perfusion is not sufficient to sustain aerobic metabolism (Gutierrez *et al.*, 2004; Rossaint *et al.*, 2006). During haemorrhage, loss of blood triggers local vascular signalling and the neuroendocrine system, which induces a systemic reaction (Runciman *et al.*, 1984). As intravascular volume falls, filling pressure in the heart decreases, in turn reducing cardiac output (Dutton, 2007). Changes in sympathetic and parasympathetic flow increase heart rate and contractility. In response to low blood pressure, selective vasoconstriction preserves blood flow to organs most dependent upon oxygen supply, i.e. brain and heart, and reduces bleeding at the site of injury. If hypoperfusion persists, lack of blood and nutrient flow causes necrotic cell death in affected cells, while other cells respond to insufficient resources with the activation of cellular apoptosis pathways. Massive cell death causes organ damage, multiple organ failure, and death (Alam et al., 2011; Angele et al., 2008; Ulukaya et al., 2011).

During shock, cardiac output is insufficient to provide tissue perfusion adequate for sustaining aerobic metabolism (Gutierrez *et al.*, 2004). Though compensatory mechanisms exist, these reach their limits during severe blood loss, necessitating a switch from aerobic to anaerobic metabolism in cells (Shoemaker, 1996). In skin and muscle cells, anaerobic metabolism produces lactic acid, the accumulation of

which leads to metabolic acidosis, one member of the lethal triad haemorrhagic shock. Another member, coagulopathy, is caused by liver damage during shock, which may continue to progress even after macrocirculation has been re-established, for instance due to oedema preventing microcirculatory recovery (Dutton, 2007). The third, hypothermia, is caused by a slowing of ATP metabolism alongside the macroeffects of blood loss (Rossaint *et al.*, 2006). This lethal triad is followed by refractory cardiogenic shock, circulatory system failure, and patient death (Dutton, 2007). Early resuscitation is needed to prevent this outcome.

Resuscitation currently relies on fluid transfusion and blood component reconstitution, *e.g.* red blood cells, platelets, and spray-dried plasma (Alam *et al.*, 2009; Gutierrez *et al.*, 2004; Midwinter, 2009). The fundamental difficulty with these approaches is that these substances need to be stored appropriately, have a very limited shelf-life and access is complicated by corresponding transport issues. Additionally, blood and blood components are costly, carry a risk of disease transmission, and most need to be cross-matched to the patient (Holcomb, 2007; Hodgetts *et al.*, 2011; Kauvar *et al.*, 2005). These issues are particularly problematic in austere environments, in remote areas, when the patient is trapped, or during military operations on foreign territory, emphasising the need for novel methods of treating terminal blood loss. One recently suggested means of preventing death by terminal blood loss is through the regulation of cellular apoptotic pathways via pharmacologic agents, *e.g.* valproic acid (VPA; Butt *et al.*,

2009). Pharmacologic resuscitation does not purport to reverse the inevitable in preventing organism death, but rather attempts to extend the period during which sophisticated medical intervention can prevent irreversible shock and cardiovascular failure, thereby increasing the chances of survival. This pre-lethal state is investigated in this project.

1.1.1 Animal models of haemorrhagic shock

The work that formed the theoretical starting point for this project was carried out by Alam *et al.* (2009) in a swine poly-trauma model of massive blood loss. Yorkshire swine were subjected to a femur fracture and 60% haemorrhage, followed by 30 min of shock and infusion of saline (at three times the volume of blood lost). To simulate internal bleeding as a delayed reaction to organ injury, swine were given a grade V liver injury (bilobar maceration) followed by liver packing. Animals were then randomised into three treatment groups: saline-only resuscitation (SO), fresh whole blood-based resuscitation (FWB), and VPA-based resuscitation (VPA). The SO group received only saline, the FWB group received a blood transfusion equal to the amount of blood lost, and the VPA group received saline containing 400mg/kg VPA. Animals were then monitored for four hours. As expected, survival was very low (25%) in the SO group and complete (100%) in the FWB group. Surprisingly, VPA treatment significantly increased the likelihood of survival (86%). In fact, the only death in the VPA group occurred during VPA infusion, before the animal in question had received the complete treatment dose. This demonstrated the marked pro-survival effect of VPA treatment in haemorrhagic shock with four hours, the putative maximum time between injury and arrival at a medical facility.

To investigate the molecular mechanisms involved in this process, the researchers removed liver tissue after the end of the experiment and analysed protein extract by Western blot for changes in levels and phosphorylation of key signal relay enzymes. Under control conditions

treatment did not affect protein status. In contrast, VPA increased the activation of protein kinase B (Akt) and the inhibition of glycogen synthase kinase-3β (GSK3β) both indicated by increased phosphorylation at Ser473 and Ser9 respectively. Additionally, abundance of downstream proteins β-catenin and B-cell leukemia/lymphoma 2 (bcl-2) protein were increased in response to VPA. These results are in line with previous and later work by the group which found increased levels of β-catenin in cells and also showed an increase of *Bcl-2* transcription within one hour (Li *et al.*, 2008; Zacharias *et al.*, 2011). The researchers concluded that VPA improved early survival in their model and that this was due not to improvement in resuscitation but to better tolerance of shock by the cells, via the activation of the PKB/Akt survival pathway.

In addition to PI3K-signalling, animal models of haemorrhagic shock have also implicated extra-cellular regulated kinase 1/2 (ERK1/2) signalling in this condition. Studies have shown that ERK1/2 Thr202/Tyr204 phosphorylation is increased in the lung of Wistar-Kyoto rats after haemorrhagic shock, and that VPA attenuates this increase (Fukudome *et al.*, 2011; Patel *et al.*, 2011). VPA also increased ERK1/2 phosphorylation in a rat model of intracerebral haemorrhage (Sinn *et al.*, 2007). This is consistent with experiments performed in Sprague-Dawley rats, where administration of a specific ERK1/2 inhibitor (PD 98059) stimulated the inflammatory response and reduced ERK1/2 phosphorylation in the liver (Jarrar *et al.*, 2004). These data

suggest that ERK1/2 may also be involved in cellular signalling during haemorrhagic shock.

Research into the nuclear changes during haemorrhagic shock has suggested a role for histone deacetylase (HDAC) inhibition in mediating pathological effects as well as therapeutic action (Shults *et al.*, 2008). In this study, Wistar-Kyoto rats were subjected to 60% blood loss for one hour, treated, and then monitored for 24 hours. Animals received one of two HDAC inhibitors: VPA or suberoyanilide hydroxamic acid. Survival was at 75% and 83% respectively, compared to 25% in the control group that received no resuscitation. Acetylation of histone H3K9 was up-regulated in the VPA group at one and six hours, but had returned to below normal control levels at 24 hours. These researchers suggested that the histone deacetylase inhibitory effect of VPA causes the short-term survival advantage displayed by treated animals. Other research into rat models by the same group also confirmed that haemorrhagic shock and inflammation cause an imbalance in protein acetylation status (Gonzales *et al.*, 2006; Lin *et al.*, 2006) and that histone deacetylase inhibitors rectify this imbalance leading to a pro-survival phenotype (Lin *et al.*, 2007; Sailhamer *et al.*, 2008) and hepatoprotection (Gonzales *et al.*, 2008). *In vivo* work by Dash *et al.* (2010) meanwhile demonstrated that VPA has neuroprotective effects following traumatic brain injury in rats as well, though in this study a selective HDAC inhibitor (SAHA) did not replicate VPA-induced protection.

1.1.2 Modelling haemorrhagic shock *in vitro*

When trying to reproduce the events of a complex, multi-organ event such as haemorrhagic shock, *in vitro* systems face severe challenges compared to animal models. However, these challenges must be overcome if cost-effective high-throughput drug screening methods are to be set up, as these require an *in vitro* model. It is widely agreed that the predominant cellular conditions during haemorrhagic shock are dominated by hypoxia (lack of adequate O_2 supply) and acidosis (unfavourably low pH), which is a consequence of hypercapnia (unfavourably high CO_2 levels; Butt *et al.*, 2009; Gutierrez *et al.*, 2004; Hess *et al.*, 2008; Hierholzer *et al.*, 2001; Li *et al.*, 2008; Midwinter, 2009; Rossaint *et al.*, 2006). These conditions are caused by the lack of gas transport vehicle, *i.e.* blood. After massive blood loss (>60%) O_2 cannot be effectively transported to target tissues, leading to a drop in O_2 levels as cells continue to function, while CO_2 cannot be adequately removed, leading to CO_2 build-up. Though many *in vitro* models of haemorrhagic shock (see above references) consider only hypoxia, the importance of concurrent hypercapnia has also been demonstrated (Hotter *et al.*, 2004). Beyond these facts, however, literature regarding *in vitro* haemorrhagic shock modelling is scarce. It is clear that any model will need thorough testing and that results will need to be compared and verified across numerous systems.

1.2 Valproic acid

Valproic acid (2-propylpentanoic acid; VPA), a branched short-chain fatty acid, was first produced in 1882 (Burton, 1882 as cited in Lagace *et al.*, 2004) and discovered to be pharmacologically active in 1963, when it was used as a solvent for anticonvulsants and found to have intrinsic anti-convulsant properties. It is of therapeutic use as an anticonvulsant in absence, tonic-clonic, and complex partial seizures as well as a mood-stabiliser (Bialer *et al.*, 2007). It has also been shown to be of therapeutic value in depression, migraine and schizophrenia (Isoherranen *et al.*, 2003; Rosenberg, 2007). VPA has been of clinical interest in spinal muscular atrophy (Lunke *et al.*, 2009), Duchenne Muscular Dystrophy (Gurpur *et al.*, 2009), Parkinson's disease (Chen *et al.*, 2007; Pan *et al.*, 2005), and Huntigton's disease (Zadori *et al.*, 2009). Novel research indicates it is widely neuroprotective (Biermann *et al.*, 2011; Monti *et al.*, 2009), has potential applications in HIV and cancer treatment (Blaheta *et al.*, 2002; Terbach and Williams, 2009) and in haemorrhagic shock during massive blood loss (Bielecka *et al.*, 2008) suggested to occur via its function as an inhibitor of HDAC activity (Göttlicher *et al.*, 2001). Though VPA has been linked to several medical conditions besides haemorrhagic shock, knowledge of its mechanism of action in these conditions is incomplete (*Fig. 1*).

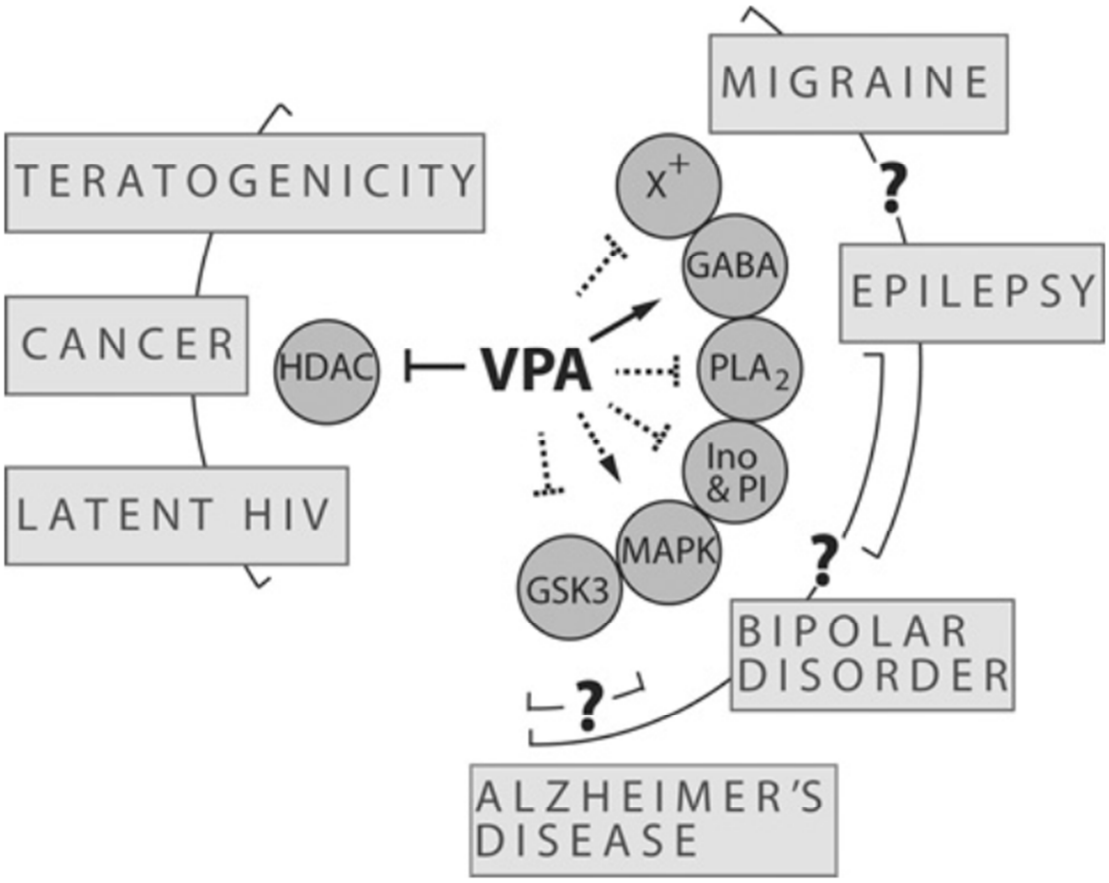

Figure 1.1 – Putative VPA targets and their potential roles in medical conditions. VPA up-regulates (arrows) or down-regulates (⊥) cellular signalling in a known (solid lines) or unknown (broken lines) manner. Limited data exist to correlate each VPA-affected pathway with the medical conditions shown. Figure adapted from Terbach and Williams (2009).

1.2.1 Mechanisms of action

1.2.1.1 Histone deacetylase inhibition

On a molecular level, VPA has been shown to cause a gamut of cellular changes. One undisputed primary mechanisms of action identified has been the direct action of VPA acts as an HDAC inhibitor, a function which has been linked with its teratogenicity and potency as a cancer treatment (Duenas-Gonzales *et al.*, 2008; Gotfryd *et al.*, 2010; Lagace *et al.*, 2004; Phiel *et al.*, 2001). HDAC inhibitors are hypothesised to reverse gene silencing, which occurs via epigenetic regulation and is known to lead to cancerous changes; additionally, HDAC inhibitors are thought to act through transcriptional activation of tumour suppressor genes and induction of cell death (Marks *et al.*, 2000). HDAC activity is of key importance in the nucleus, where it indirectly regulates gene transcription.

In the nucleus, DNA is associated with histone proteins, forming chromatin (Baker, 2011; Lin *et al.*, 2007). The nucleosome, one unit of the repeating pattern which makes up chromatin, contains 146 base pairs of DNA wound around a histone octamere (two each of H2A, H2B, H3, and H4) stabilised by H1. DNA is inaccessible to transcription machinery when tightly wound in its condensed, heterochromatin form and accessible when decondensed into euchromatin (*Fig. 3*). Gene expression can therefore be regulated by structural changes in chromatin via histone modification (Li *et al.*, 2011). All four core histones have lysine-rich terminal tails, which contain sites for methylation, phosphorylation, ubiquination, poly-ADP-ribosylation and

acetylation (Berger, 2002). Methylation of these lysine residues may promote or silence gene expression, while acetylation generally indicates transcriptionally active regions (Barski *et al.*, 2007; Clayton *et al.*, 2006). Histone lysine acetylation is mediated by histone acetyltransferases (HATs), which transfer an acetyl-group from acetyl-coenzyme A to the ε-amino group of specific lysine residues enhancing accessibility for transcription factors (Yang *et al.*, 2008). HDACs achieve deacetylation and structural change by catalysing the hydrolytic removal of this group (Butt *et al.*, 2009; Sinn *et al.*, 2007). Transcription factors may also be acetylated to enhance gene transcription (Li *et al.*, 2011). The balance of HAT and HDAC activity has been termed acetylation homeostasis, highlighting the importance of acetylation in overall cell homeostasis (Saha *et al.*, 2006).

In humans, 18 HDACs have been described (Carey *et al.*, 2006). They are split into four classes according to their homology to yeast HDACs. Class I encompasses HDACs 1-3 and 8, which are related to yeast Rpd3 and ubiquitously present in the nucleus (Chuang *et al.*, 2009; De Ruijter *et al.*, 2003). Class II HDACs are homologous to yeast HDA1 and are further subdivided into class IIa (HDACs 4, 5, 7, & 9) and class IIb (HDACs 6, & 10) based on structural similarities. They are found in both nucleus and cytosol, display tissue-specific expression, and have been strongly implicated in neuronal function. Class III contains the sirtuins (SIRT1-7), NAD^+-dependent metabolic sensors which are named after their relation to Sir2 (Gertz *et al.*, 2010). VPA is

known to inhibit class I and class IIa HDACs, thereby regulating gene transcription (Göttlicher *et al.*, 2001; Gurvich *et al.*, 2004).

Stress, such as in haemorrhagic shock, up-regulates HDAC activity, leading to hypoacetylation, down-regulation of gene transcription, and apoptosis (Lin *et al.*, 2006). These changes are attenuated by HDAC inhibitor action, preventing cell death (Biermann *et al.*, 2011; Gonzales *et al.*, 2006). HDAC inhibitors have now been spotlighted as potential agents for short-term mortality prevention during massive blood loss, though their exact mechanism of action remains unclear. The focus must therefore lie on the interaction of HDACs and their inhibitors, of which VPA is one.

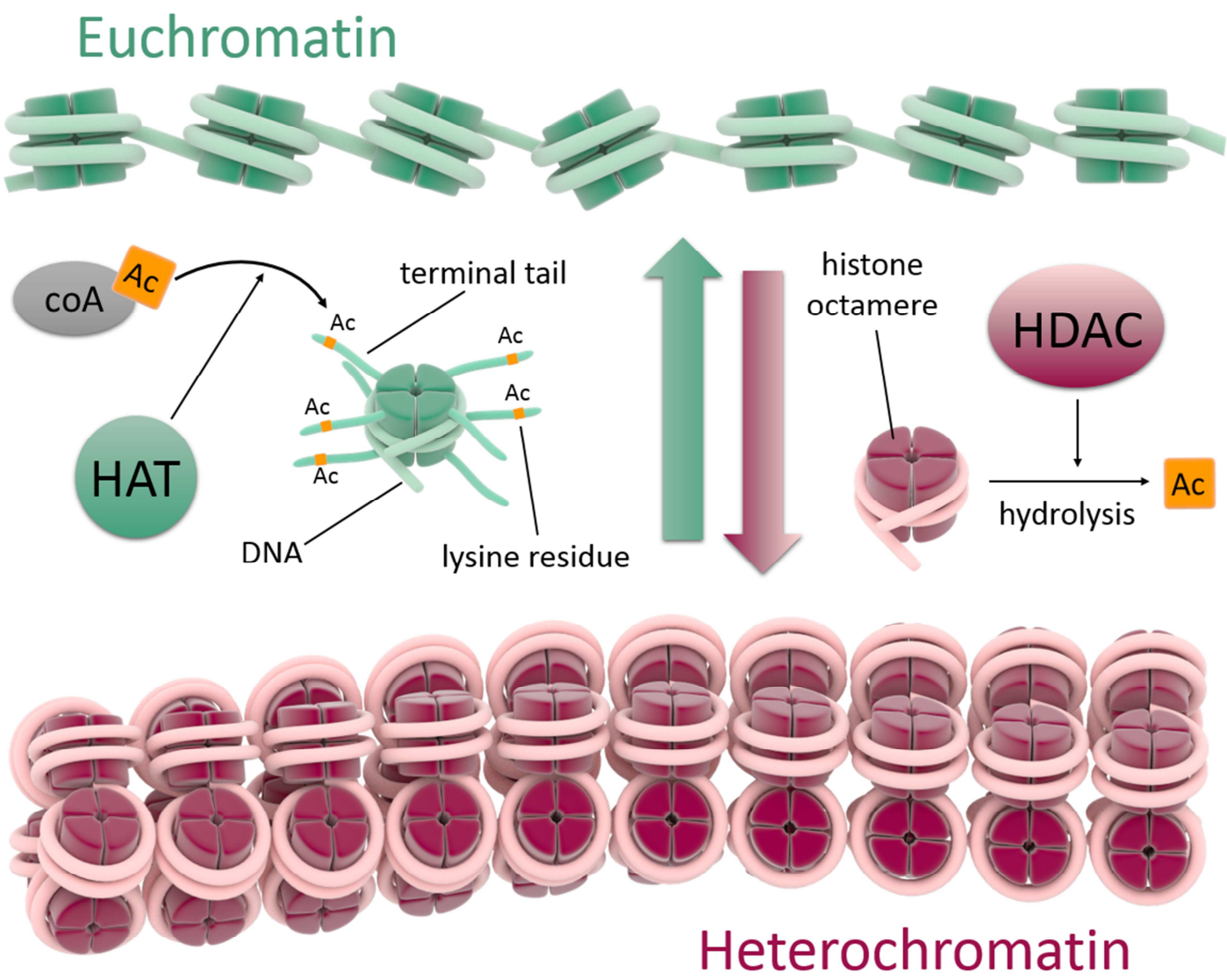

Figure 1.2 – Histone acetylation and deacetylation regulates chromatin state. Chromatin is made up of DNA wrapped around histone proteins. One histone octamere and its associated 146bp of DNA form a nucleosome. DNA is inaccessible to transcription machinery when in its heterochromatin form and accessible when unravelled into euchromatin. Histones have terminal tails containing lysine residues, at which they are acetylated by HATs (histone acetyltransferases). These transfer an acetyl-group from acetyl-coA (coenzyme A) to an available lysine residue, thereby changing histone tail conformation, which in turn increases the distance from one nucleosome to the next and provides transcription machinery with DNA access. HDACs (histone deacetylases) remove these acetyl groups by hydrolysis, thereby causing chromatin to recondense.

In the nucleus, HDAC inhibitors like VPA are able to alter expression of genes involved in cell cycle control, differentiation, and apoptosis (Chen *et al.*, 2009; Rosato *et al.*, 2003; Savickiene *et al.*, 2006). VPA, SAHA, and TSA (trichostatin A) have been shown to up-regulate transcription of pro-survival genes *in vivo* following massive blood loss (Alam *et al.*, 2009; Li *et al.*, 2011; Lin *et al.*, 2006) and suppress inflammation via down-regulation of cytokine gene expression, thereby improving survival (Cao *et al.*, 2008; Li *et al.*, 2009; Zhang *et al.*, 2009). However, HDAC action is not restricted to histones – indeed HDACs are thought to have evolved in the absence of histone proteins (Minucci *et al.*, 2006). HDACs found in the cytoplasm are not part of histone synthesis and acetylation-dependent assembly, and those found in mitochondria also lack obvious substrates as there are no histones present. HDAC inhibitors are therefore likely affect a wide variety of cytosolic pathways as well, and so there might be a cytosolic target for the HDAC inhibitory action of VPA.

1.2.1.2 PPARγ

The peroxisome proliferator-activated receptors (PPARs) are another group of confirmed interaction partners of VPA in the nucleus. These nuclear receptors were identified in the 1990s and have since then primarily been of interest in lipid metabolism research and associated disorders such as diabetes and obesity (Kota *et al.*, 2005). PPARs regulate gene expression by forming a heterodimer with the retinoid X receptor (RXR) and binding to PPAR response elements (PPREs) in gene promoters (*Fig. 3,* Abdelrahman *et al.*, 2005). Three

types of PPAR have been discovered so far, encoded by separate genes: PPARα, PPARβ/δ and PPARγ. VPA activates PPARβ/δ and PPARγ *in vitro*, although it does not appear to be a direct ligand of PPARβ/δ (Lampen *et al.*, 1999; Lampen *et al.*, 2001), and PPARs are known to be part of the VPA-sensitive signalling network (Werling *et al.*, 2001).

PPAR activity is mainly modulated by ligand-binding, but several indirect regulatory mechanisms have also been discovered (Malapaka *et al.*, 2012; Yau *et al.*, 2013). The γ isoform of PPAR has been shown to directly interact with HDACs 3 and 4 via its zinc finger DNA binding domain (Franco *et al.*, 2003), while PPAR expression has been demonstrated to be regulated by Wnt signalling (Bielecka and Obuchowicz, 2008). Additionally, PPAR action on gene expression depends on other chromatin modifications: PPARγ induces histone acetylation, but only in the absence of repressive methylation, indicating that PPAR activity functions as a response to the pre-existing gene activation/repression balance in the nucleus (Lefterova *et al.*, 2010). VPA may therefore also modulate PPARs indirectly, by inhibiting HDACs 3 and 4 or by modifying the gene expression homeostasis by increasing histone acetylation. Histone 3 lysine 9 (H3K9) acetylation is increased at PPARγ binding regions (Lefterova *et al.*, 2010) and this increase has been observed in response to both acute VPA treatment in haemorrhagic shock (Alam *et al.*, 2009) and in response to chronic treatment in bipolar disorder (Machado-Vieira *et al.*, 2011). Conversely, PPARs may act on VPA, as they localise to genes involved in lipid metabolism (Schupp *et al.*, 2009).

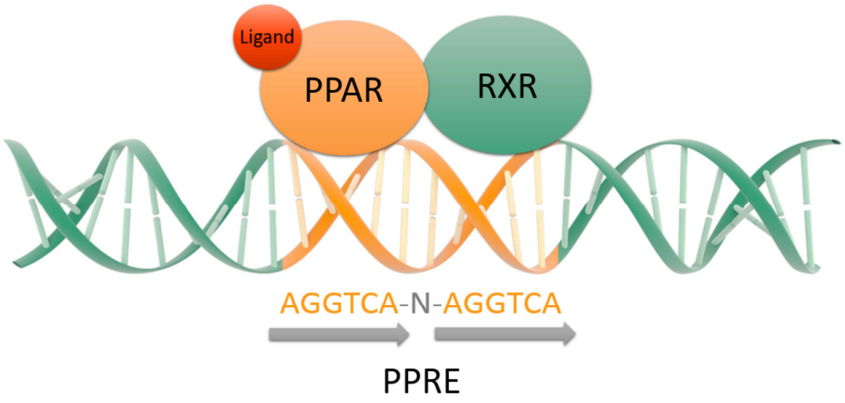

Figure 1.3 – Peroxisome proliferator-activated receptors (PPARs) regulate gene transcription. When bound by a ligand, PPARs form a heterodimer with the retinoid X receptor (RXR) and bind to PPAR response elements (PPREs), made up two AGGTCA repeats separated by a different residue, in gene promoters, thereby inducing gene transcription.

1.2.1.3 Cytosolic effects

In the cytosol, VPA has been shown to regulate cAMP response element binding protein, brain-derived neurotrophic factor, antiapoptotic protein bcl-2 and extracellular signal regulated protein/mitogen-activated protein kinases in a pro-survival manner in mammalian systems (Bielecka *et al.*, 2008). VPA can also act by inducing heat shock protein 70 (HSP70) activity via the phosphoinositide 3-kinase (PI3K) pathway, leading to neuroprotective effects (Marinova *et al.*, 2009). A key protein of the PI3K pathway, GSK3β, is also regulated by VPA (Chen *et al.*, 1999; Werstuck *et al.*, 2004).

1.2.1.3.1 Akt/PKB and GSK3β

The majority of research into the effect of VPA on GSK3β has been carried out in long-term exposure studies in the brain, as both VPA and

GSK3β have been of particular interest in bipolar disorder. However, this research has yielded conflicting evidence of the effect of VPA on neuronal cells, illustrating model-specific differences in VPA action. Chen *et al.* (1999) reported that VPA directly inhibits GSK3β, both in an *in vitro* assay and in SH-SY5Y cells within 24h. However, Ryves *et al.* (2005) showed *in vitro* that while the mood stabiliser lithium inhibits GSK3β in neocortical cells, VPA does not. Dash *et al.* (2010) meanwhile demonstrated that VPA reduces GSK3β activity following traumatic brain injury in a rodent model. Further evidence indicates that VPA does not inhibit GSK3β directly (Hall *et al.*, 2002), but this effect on GSK3β is far from universal (Williams *et al.*, 2002).

GSK3β is one of the two mammalian isoforms of the GSK3 serine/threonine kinase (the other being GSK3α); a highly conserved 433 amino acid protein with a central protein kinase catalytic domain (Frame *et al.*, 2001; Grimes *et al.*, 2001). It was originally discovered to phosphorylate and inactivate glycogen synthase, but is now implicated in a wide range of other signalling processes from neuronal plasticity over general gene expression to cell survival (Bielecka *et al.*, 2008; Grimes *et al.*, 2001). GSK3β is also involved in the regulation of several transcription factors and is a key player in apoptosis and programmed cell death (Grimes *et al.*, 2001; Kaga *et al.*, 2006).

GSK3β activity is partially regulated by the phosphotidylinositol-3-kinase (PI3K) cascade. Stimulation of the insulin receptor leads to activation of PI3K, which phosphorylates the 3'-OH position of the inositol ring of inositol phospholipids, producing phosphatidylinositol-

(3)-phosphate (PIP), phosphatidylinositol-(3,4)-diphosphate (PIP_2), and phosphatidylinositol-(3,4,5)-triphosphate (PIP_3; Cantrell, 2001). This process is reversed by PTEN, which dephosphorylates PIP_3 to PIP_2 on the 3' position (Tamguney *et al.*, 2007). PIP_3 enables phosphorylation of Akt/PKB at Ser-473 and Thr-308, thereby activating it. Akt/PKB then directly interacts with GSK3β, phosphorylating and inactivating it (Alessi *et al.*, 1996; Van Weeren *et al.*, 1998). Akt also promotes cell survival via other pathways, *e.g.* by directly phosphorylating and inhibiting BAD, a pro-apoptotic protein which binds to and inactivates bcl-2 (Manning *et al.*, 2007). Additionally, the PI3K-Akt pathway up-regulates NFκB (nuclear factor κB) survival signalling and inhibits JNK/p38 apoptotic signalling (Dugo *et al.*, 2007; Kim *et al.*, 2001).

GSK3β, when active, targets and phosphorylates β-catenin, leading to its degradation (Manning *et al.*, 2007). This signalling pathway is activated in heat shock and oxidative stress (Shaw *et al.*, 1998). Inflammation is amplified by GSK3β activity via its up-regulation of NFκB activity, which promotes inflammatory processes (Dugo *et al.*, 2007). GSK3β inhibition accordingly protects various model systems from damage via inflammatory processes (Jope *et al.*, 2007). GSK3β can be inactivated by several kinases (*e.g.*, Akt/protein kinase B, some protein kinase C isoforms, and protein kinase A) via phosphorylation of serine-9 (Stambolic *et al.*, 1994; Sutherland *et al.*, 1993). Additionally, threonine-43 may be phosphorylated by extracellular signal-regulated kinases (Ding *et al.*, 2005), while p38 mitogen-activated protein kinase phosphorylates Ser-389 and Thr-390 (Thornton *et al.*, 2008), which is

postulated to increase the likelihood of Ser-9 phosphorylation rather than directly inhibiting GSK3β action (Medina *et al.*, 2011). On the other hand, Tyr-216 phosphorylation leads to an up-regulation of kinase activity (Kim *et al.*, 1999; Kim *et al.*, 2002; Sayas *et al.*, 1999). At high Akt/PKB activity and corresponding low levels of GSK3β, β-catenin accumulates, leading to transcription of the b-cell lymphoma 2 (*bcl-2*) gene. Bcl-2 is an apoptosis regulator protein and, when activated, steers the cell towards a pro-survival phenotype (Dahia, 2000).

Upstream of GSK3β, Akt/PKB has been shown to be the major player in insulin-triggered GSK3β regulation in the PI3K cascade (Cross *et al.*, 1995; Shaw *et al.*, 1997) and is also affected by VPA in haemorrhagic shock (Alam *et al.*, 2009) which regulates the PI3K pathway in this condition (Hwabejire *et al.*, 2014). Several studies have implicated the PI3K/Akt pathway in seizure generation and epilepsy (Buckmaster and Lew, 2011; Shinoda *et al.*, 2004; Zeng *et al.*, 2009; Zhang and Wong, 2012). PIP_3, the active second messenger produced by PI3K activity, induces Akt phosphorylation and is therefore a key component of the PI3K pathway (Delcommenne *et al.*, 1998; Toker and Cantley, 1997). VPA has been shown to modulate PIP_3 turnover in the simple biomedical model *Dictyostelium discoideum* (Chang *et al.*, 2012; Xu *et al.*, 2007) which is replicated in *in vitro* seizure control efficacy in mammalian seizure models (Chang *et al.*, 2012; Chang *et al.*, 2013). Recent research indicates that seizure activity correlates with PIP_3 depletion, a change which is reversed by VPA, therefore suggesting VPA may act by this novel mechanism to attenuate epileptic activity (Chang

et al., 2014). PI3K activity is also reduced in haemorrhagic shock and rescued by VPA treatment (Hwabejire *et al.*, 2014). Although no data has been published at present to indicate this effect is modulated by PIP_3 levels, knowledge gained from research into the mechanisms of action of VPA in other disorders may provide a crucial insight into regulation of pathways of interest in haemorrhagic shock.

1.2.1.3.2 ERK 1/2

ERK 1/2 is a mitogen-activated protein kinase (MAPK) associated with nearly all major cell signalling processes (Martin *et al.*, 2009). It is accepted to be the major effector of the Ras oncoprotein (Mendoza *et al.*, 2011). Raf, the Ras GTPase-regulated kinase, phosphorylates the intermediate kinase (MAPKK) MEK, thereby activating it. MEK, in turn, phosphorylates and activates ERK, the effector kinase of the pathway. The pathway is activated by growth factors, polypeptide hormones, neurotransmitters, chemokines, and G protein-coupled receptors, or by direct activation of protein kinase C (PKC). Downstream cytosolic signalling targets include p90 ribosomal S6 kinase (RSK) and transcription factors. In the nucleus, ERK targets transcription factors that mediate gene transcription for cell survival, division, and motility (Dhillon *et al.*, 2007). In haemorrhagic shock, ERK is activated, a process which is significantly attenuated by VPA (Fukudome *et al.*, 2010). Activation of ERK is also associated with a decrease in Akt activity (Sinha *et al.*, 2004). However, the effect of VPA on ERK has been found to be independent of the HDAC inhibition effect of the drug (Boeckeler *et al.*, 2006; Gotfryd *et al.*, 2010; Ludtmann *et al.*, 2011). The

regulation of ERK by VPA has also been implicated in neuroprotection (Creson *et al.*, 2009; Pan *et al.*, 2005 Yuan *et al.*, 2001). Finally, the PI3K and ERK pathways show significant cross-talk (Mendoza *et al.*, 2011), both inhibitory and stimulatory, so research on one of them must take care to not discount the other.

1.2.1.4 VPA action on haemorrhagic shock pathways

In swine *in vivo* poly-trauma models VPA has been shown to induce a pro-survival phenotype via up-regulation of some of the elements of the PI3K pathway (Alam *et al.*, 2009, 2011; Dash *et al.*, 2010), though VPA has been demonstrated to induce its effects independent of PI3K activity, inositol recycling, and inositol synthesis (Chang *et al.*, 2012). The exact mechanism of action of VPA on the PI3K pathway remains unproven, though one hypothesis implicates regulation of PIP_3 by PCAF and PTEN. VPA treatment leads to increased levels of p-Akt, p-GSK3β, β-catenin and bcl-2 (Alam *et al.*, 2009). Its target is therefore likely to be up-stream of Akt signalling. One possible route for Akt modulation is via the tumor suppressor protein phosphatase and tensin homolog (PTEN), a dual protein/lipid phosphatase involved in cell migration, growth and apoptosis (Dahia, 2000; Tamguney *et al.*, 2007). Its main substrate is PIP_3, which it inactivates by dephosphorylating it to PIP_2. PTEN is acetylated and inactivated by p300/CBP-associated factor (PCAF), a histone acetyltransferase (Okumura *et al.*, 2006). PCAF is inhibited by HDAC3, which is inhibited by VPA (Goettlicher *et al.*, 2001). Thus, a potential

mode of action for VPA in lethal blood loss could be cytosolic HDAC inhibition and PIP_3 regulation.

1.2.2 Side-effects

As VPA has a broad spectrum of action and shows tissue-specificity, VPA treatment can result in numerous side-effects in humans. These include weight gain, changes in cholesterol and fast glucose levels, dermatologic issues like stomatitis, cutaneous leukoclastic vasculitis, and psoriasiform eruption, and neurological effects like encephalopathy, parkinsonism and, rarely, exacerbation of existing epileptic symptoms (Chateauvieux *et al.*, 2010). Children are particularly susceptive to liver-dependent coagulopathies, with symptoms like thrombocytopenia, platelet dysfunction, Von Willebrand disease, Factor XIII deficiency, hypofibrinogenemia, and vitamin K-dependent factor deficiency (Gerstner *et al.*, 2006). In addition, degradation products of VPA have been shown to be hepatotoxic (Kassahun *et al.*, 1991; Bryant *et al.*, 1996). Hepatotoxicity (microvesicular steatosis or abnormal cellular lipid retention) is thought to be caused by VPA metabolites with a terminal double-bond (4-ene-VPA and 2,4-diene-VPA), which are transformed to chemically reactive intermediates and consequently affect fatty acid metabolism via an acyl-coenzyme A (CoA) thioester formation, leading to CoA depletion and the observed toxicity (Bialer *et al.*, 2007; Grillo *et al.*, 2001; Neuman *et al.*, 2001). This effect has been reproduced in several model systems, including Huh7 cells and *Dictyostelium discoideum* (Elphick *et al.*, 2012).

Though several off-target effects of VPA are explored in the literature, teratogenicity is the most noted side-effect. Major birth defects such as spina bifida are seen three times as often in the child if the mother is undergoing VPA treatment, producing a heightened risk of 6.2-7.6% compared to the 2.9-3.6% increase in risk for other anti-epileptic treatments (Genton *et al.*, 2006; Harden *et al.*, 2008; Ornoy, 2009). Children born to mothers undergoing VPA treatment also show an increased likelihood of developmental problems, including autism spectrum disorder and lowered IQ (Ornoy, 2009). Several effects of VPA have been implicated as the reason for its teratogenicity, including regulation of the folic acid cycle (Alonso-Alperte *et al.*, 1999; Ubeda-Martin *et al.*, 1998), increased oxidative stress (Ornoy, 2009), regulation of peroxisome proliferator-activated receptor δ (PPARs; Werling *et al.*, 2001), and the presence of a tertiary carbon bound to a carboxylic group, a hydrogen atom, and two alkyl chains (Nau *et al.*, 1991; Nau *et al.*, 1992). However, research particularly supports histone deacetylase inhibition as the key teratogenic process (Bialer *et al.*, 2007; Eikel *et al.*, 2006; Elphick *et al.*, 2012). Treatment of mice with the potent HDAC inhibitor trichostatin A (TSA) mimicked birth defects seen in response to VPA (Menegola *et al.*, 2005). A Structure-function study of 20 VPA derivatives showed that only teratogenic molecules induced histone hypoacetylation and that HDAC inhibitory activity quantitatively correlated to teratogenic potential (Eikel *et al.*, 2006). If VPA does indeed exert its therapeutic effects in haemorrhagic shock via histone deacetylase inhibition, analogues with more pronounced inhibitory activity may be more beneficial in treating severe blood loss.

1.2.3 Congeners

VPA is unusual in that over a hundred congeners, analogues, and derivatives have been investigated as therapeutic treatments for a variety of conditions, in part in an effort to discover more potent therapeutic treatments and in part to determine and potentially eliminate the structural basis for some of the many side-effects of VPA (Bialer and Yagen, 2007; Galit *et al.*, 2007; Liu *et al.*, 1992; Perrino *et al.*, 2008; Redecker *et al.*, 2000; Rekatas *et al.*, 1996; Terbach and Williams, 2009; Winkler *et al.*, 2005). Less than 3% of VPA is excreted unchanged in urine, indicating that VPA undergoes extensive biotransformation, which mainly takes place in the liver (Silva *et al.*, 2008). The two major metabolic pathways of VPA are glucuronidation and β-oxidation in the mitochondria, accounting for 50% and 40% of dose respectively, while a third pathway, cytochrome P450 (CYP-mediated oxidation, accounts for approximately 10% (Argikar and Remmel, 2009; Ghodke-Puranik *et al.*, 2013; Tan *et al.*, 2010). VPA biotransformation gives rise to more than 50 known metabolites (Abbott and Anari, 1999), some of which exert therapeutic or toxic effects distinct from those of VPA itself (Silva *et al.*, 2008).

VPA derivatives with a terminal double bond (4-ene-VPA, 2,4-diene-VPA), for instance, are more hepatotoxic than the parent drug (Nanau and Neuman, 2013). This led to the development of tetramethylcyclopropanecarboxylic acid (TMCA), a cyclopropyl analogue of VPA, which cannot be biotransformed to terminal double bond metabolites (Bialer and Yagen, 2007). Although TMCA does not show

anti-convulsant activity, its amide derivatives do, leading to a new class of potential anti-epileptic compounds, some of which show increased therapeutic potency compared to VPA (Isoherranen *et al.*, 2003; Okada *et al.*, 2006; Rogawski, 2006; Sobol *et al.*, 2004).

The mechanism of action of VPA is not comprehensively understood, but investigations of the effects of congeners and derivatives have succeeded in clarifying some aspects of the structure-function relationships at play. For instance, early studies showed that VPA as a branched acid with eight carbons had the optimal structure to produce a balance of anti-epileptic potency and sedative effects compared to a wide variety of branched monocarboxylic acids (Isoherranen *et al.*, 2003; Keane *et al.*, 1983; Morre *et al.*, 1984). Both the anti-cancer activity and the teratogenicity of VPA have been found to depend on its action as a histone deacetylase inhibitor (Blaheta and Cinatl, 2002). It has been proposed that branching at the α-position with a side chain longer than a methyl group is required for teratogenicity (Narotsky *et al.*, 1994; Okada *et al.*, 2004), while length of the side chain correlates with teratogenic potential (Bojic *et al.*, 1998). As there are well-established animal models for teratogenicity, existing research on this topic may inform future research into anti-cancer congeners of VPA and facilitate novel discoveries.

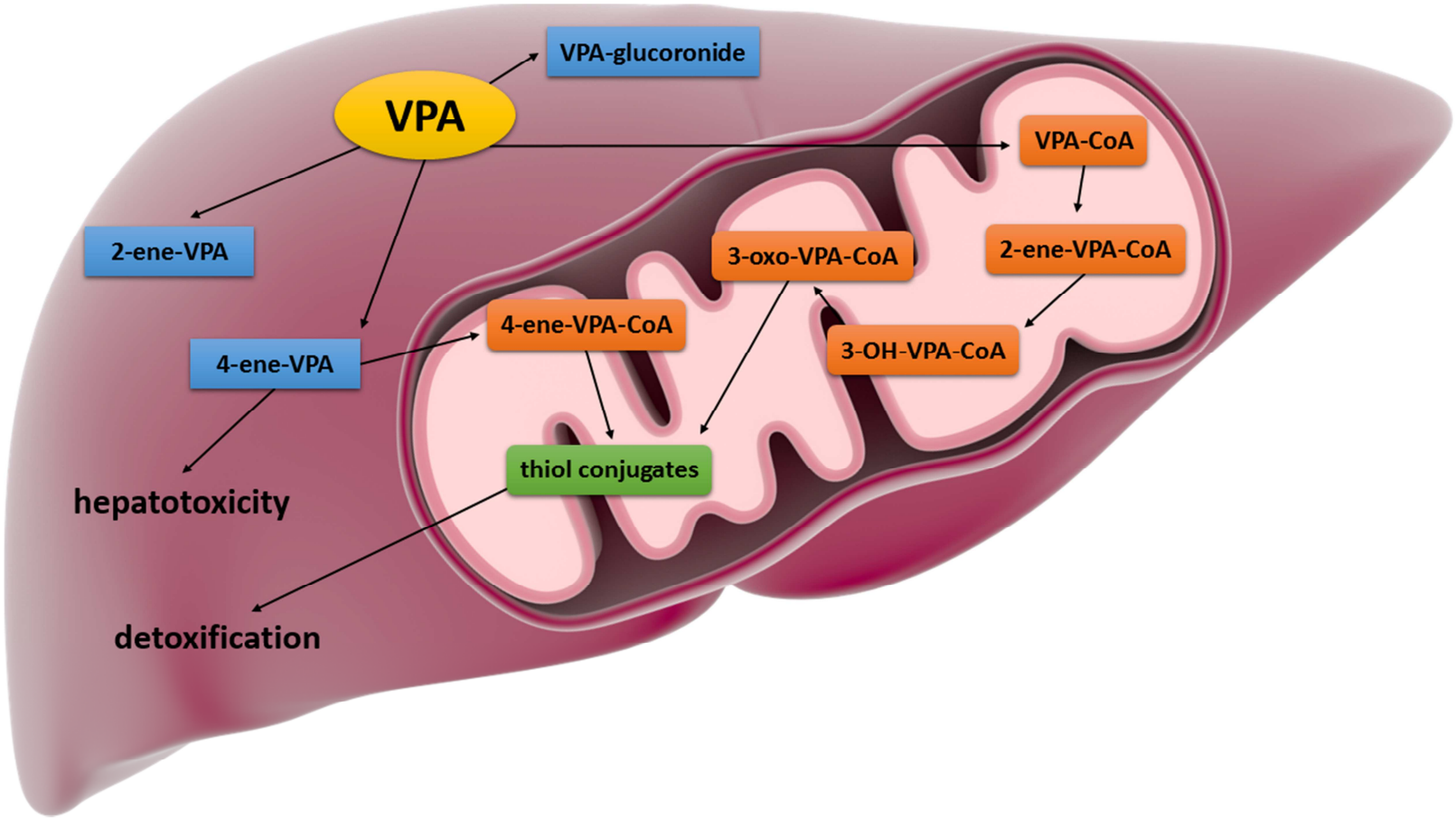

Figure 1.4 – VPA is metabolised in the liver. A small selection of the metabolic pathway of VPA is shown. VPA is metabolised by three pathways: glucuronidation, which produces VPA-glucoronide, cytochrome P450-mediated oxidation, which produces 4-ene-VPA (a cause of hepatotoxicity), and mitochondrial β-oxidation. VPA crosses the mitochondrial membrane, where the formation of VPA-CoA (VPA-co-enzme A) is catalysed. One possible oxidation pathway leads to the production of thiol conjugates of VPA, which act to detoxify the liver. 4-ene-VPA may also be metabolised by some stages of this pathway. Figure adapted from Ghodke-Puranik *et al.* (2013).

1.3 Model systems

1.3.1 Hepatocellular

1.3.1.1 Huh7

The human hepatoma cell line Huh7, an immortal cell line of epithelial-like tumourigenic cells, was established by Nakabayashi *et al.* (1982) from well-differentiated carcinoma cells from a liver tumour in a 57-year-old Japanese male. The cell line retains hepatocellular differentiated functions and gene expression which is thought to be stable in culture. Huh7 cells have been found to be closer to *in vivo* human liver cells than other cell lines, *e.g.* HepG2 cells (Meex *et al.*, 2011). Valproic acid has been found to inhibit human hepatocellular cancer cell growth in Huh7 and *in vivo*, down-regulating anti-tumour Notch-1 signalling (Machado-Vieira *et al.*, 2011). The PI3K pathway and GSK3β have also been studied in Huh7 cells, though not in context relevant to this project (Chen *et al.*, 2012; Johnston *et al.*, 2011; Muraoka *et al.*, 2012). Due to the similarity of Huh7 to human liver tissue, this cell line was selected as the primary model system for this study.

1.3.1.2 HepG2

The cells of the HepG2 cell line, which were derived from the well-differentiated liver carcinoma of a 15-year-old Caucasian male from Argentina, show an epithelial morphology compatible with that of parenchymal hepatocytes and are widely used for cancer research (Aden *et al.*, 1979; Constantini *et al.*, 2013; Meex *et al.*, 2013). Though HepG2

cells have not been used to study ischaemic conditions, some research exists on the effects of VPA in this cell line. VPA decreases HepG2 cell viability and induces a hepatotoxic response (Ji *et al.*, 2010). It also causes the dysfunction of mitochondrial respiration and increases the abundance of radical oxygen species (Komulainen *et al.*, 2015). HepG2 cells were chosen as a secondary hepatic cell line to provide a cellular environment comparable to Huh7, as this would allow comparison of molecular signalling responses and further inform any discoveries made.

1.3.2 Neuronal

1.3.2.1 SH-SY5Y

SH-SY5Y, a human neuroblastoma cell line, is a third sequential sub-clone derived in 1978 from the cell line SK-N-SH, which was first established in 1970 from a large neuroblastoma found in the chest of a 4-year-old girl (Biedler *et al.*, 1973; Biedler *et al.*, 1978). SH-SY5Y has previously been used to investigate molecular signalling in ischaemic conditions and in order to explore neuroprotective effects of anti-ischaemic agents (Seetapun *et al.*, 2013). VPA has been shown to prevent cell death in SH-SY5Y (Daniel *et al.*, 2005) and mediate anti-apoptotic action via HSP70 induction (Pan *et al.*, 2005). In addition, VPA has been reported to inhibit GSK3β in this cell line (Chen *et al.*, 1999), though this result is controversial (Ryves *et al.*, 2005). This cell line was selected to investigate haemorrhagic shock signalling in a neuron-like environment.

1.3.2.2 Neuro-2a

The mouse neuroblastoma cell line Neuro-2a (N2a) was established by Klebe & Ruddle (1969; as cited at bioinformatics.istge.it/cldb/cl3684.html) from a spontaneous tumour of a strain A albino mouse. Work on the PI3K pathway, as well as some on HDAC inhibition, has been done in this model system (Castino *et al.*, 2008; Graham *et al.*, 2006; Politis *et al.*, 2008). As several animal models of haemorrhagic shock have used rodents, N2a was selected for this investigation to provide a signalling environment similar to that found in *in vivo* models.

1.4 Project Aims

This project aims to establish an *in vitro* model system for the testing of drugs for combating negative effects of haemorrhagic shock, modelled by both hypoxia and hypercapnia, in the short term. The starting point for this research project is the drug that has shown most promising efficacy *in vivo*: valproic acid.

> **Hypothesis**: Molecular events during haemorrhagic can be modelled in a human cell line in a manner sufficient for signalling research and as a first step to high-throughput drug discovery.

Four **project aims** are selected in an effort to disprove this hypothesis:

I. Develop a cell line model of haemorrhagic shock which reproduces the regulation of GSK3β Ser9 phosphorylation seen *in vivo*.
II. Verify whether VPA regulates *in vitro* signalling up- and downstream of GSK3β in the same manner as observed *in vivo*.
III. Determine the efficacy of VPA congeners in this model to inform further signalling investigations and provide candidates for drug discovery.
IV. Elucidate the mechanism through which VPA acts to regulate GSK3β in the *in vitro* model system.

Reaching these aims is likely to advance the research effort in relation to both haemorrhagic shock and VPA.

2

Materials and Methods

2.1 Cell line culture

Huh7 were supplied by Japanese Collection of Research Bioresources Cell Bank (#JCRB0403, Japan), HepG2 and SH-SY5Y were supplied by ATCC (#HB-8065 and #CRL-2266 respectively, United Kingdom), HEK293 and Neuro-2a were kindly supplied by Dr Murdoch and Dr Foster respectively (both Centre for Biomedical Sciences, Royal Holloway University of London).

2.1.1 Long-term Storage

Cells were washed with PBS and rapidly detached with 0.05% w/v Trypsin (Invitrogen Life Technologies #15400-054). A haemocytometer was used to ascertain cell number. Cells were re-suspended in freezing medium (Sigma #C6164) at 1-2x10^5 cells/ml and allowed to freeze gradually over 24h at -80°C prior to transferral to liquid nitrogen (gaseous phase).

2.1.2 Culture

Frozen cells were rapidly thawed in a water bath at 37°C, re-suspended in 10ml culture medium (for Huh7, HepG2, and HEK293: DMEM high glucose from Sigma #D5796; for SH-SY5Y and N2a: DMEM F-12 Ham from Sigma #D6421) and pelleted (3min, 265xg). The supernatant was removed and the pellet re-suspended in 10ml complete cell culture medium: DMEM high glucose or DMEM F-12 Ham containing 10% foetal bovine serum (FBS; Invitrogen Life Technologies Ltd. #10082-147), 1X Penicillin / Streptomycin (Sigma #P4333) and non-essential amino acids (Sigma #M7145). Cells were incubated in

vented flasks (37°C, 5% CO_2) and passaged at 70-80% confluency. Cells were detached for passaging using a solution of 0.05% Trypsin in PBS (Severn Biotech #20-7460-01) and brief incubation at 37°C (1-2min Huh7, HepG2, N2a, HEK293; 30sec SH-SY5Y) followed by re-suspension in serum-containing DMEM for passaging. Cells of all types were used experimentally up to passage 15.

2.1.3 Treatment

Cells were seeded into 6-well plates at $5x10^4$ (HEK293), $1x10^5$ (SH-SY5Y, N2a), or $2x10^5$ (Huh7, HepG2) cells/well and allowed to recover for 48h in normoxic conditions (5% CO_2, 37°C). Serum starvation was carried out for 12h prior to treatment with non-complemented serum-free DMEM. Treatment compounds were added directly into culture medium. Cells were treated for 4h either under standard (37°C, 5% CO_2) conditions or in stress conditions (2% O_2, 10% CO_2, 32°C) with a vehicle control (DMSO unless otherwise indicated) or compound of interest: 2-ene-VPA (MolPort), 2POA (Sigma), Ciglitazone (Tocris), DA (Sigma), GSK3787 (Tocris), GW6471 (Tocris), OA (Sigma), SA (Sigma), T0070907 (Tocris), VPA (Sigma, vehicle dH2O), VPD (Katwijk Chemie, The Netherlands). Final concentration of DMSO did not exceed 0.01%. After treatment, cells were rinsed once with ice-cold PBS and scraped directly in RIPA buffer (Sigma) containing proteinase and phosphatase inhibitors (Roche #04693159001 and #04906845001). Protein extract was immediately stored at -20°C.

2.2 Primary cell culture

2.2.1 Rat cortical neuron culture

Primary cortical rat neurons were prepared by Dr Ursu (Centre for Biomedical Sciences, Royal Holloway University of London). All animal experiments were performed according to Home Office regulations in appliance with the Animals Scientific Act 1986. Briefly, E18 embryos from Sprague-Dawley rats were dissected and brains were removed. Cortical neurons were dissociated and suspended in DMEM (as before) supplemented with 1X penicillin/streptomycin (as before), 1X Glutamax (Sigma), and 5% FBS (as before) at a density of $2.5x10^5$ cells/ml. Cells were then seeded into poly-d-lysine-treated (0.1mg/ml; Sigma in 0.1 M Borate buffer) 6-well plates at a density of $5x10^5$ cells/well (2ml/well). Cells were allowed to recover for 24h (37°C, 5% CO_2) and DMEM was replaced with Neurobasal medium (Gibco #21103-049) containing 1X penicillin/streptomycin, 1X B27 supplement (Gibco #17504044) and 1X GlutaMax (Life Technologies #35050-061) and cells were further maintained at 37°C and 5% CO_2. Cultures were supplemented with 0.5ml Neurobasal medium (as before) on a weekly basis from the 7^{th} day of *in vitro* culture, until maturation at 18^{th} - 21^{st} day *in vitro*. On day 21, cells were treated with compound of interest or vehicle control added directly into medium for 4h in standard (37°C, 5% CO_2) or stress conditions (2% O_2, 10% CO_2, 32°C). After treatment, cells were rinsed once with ice-cold PBS and scraped directly in RIPA buffer containing proteinase and phosphatase inhibitors (all as before). Protein extract was stored at -20°C.

2.2.2 Human hepatocellular culture

Cryopreserved pooled human hepatocytes were supplied by Life Technologies (HMCS10, Lot HUE117). Cells were thawed and transferred to culture medium according to supplier's instructions. All reagents used were provided by Life Technologies. Cells were rapidly thawed in a water bath at 37°C, re-suspended in Thawing Medium (#CM7500) supplemented with hepatocyte maintenance supplement pack (#CM4000), and pelleted (200xg, 10min). Supernatant was discarded, cell viability was determined by trypan blue exclusion (Hay, 2002), and cells were suspended in incubation medium (#A1217601) at a rate of $1x10^6$ viable cells/ml. After a brief recovery (incubation at 37°C, 5% CO_2 for 30min), cells were treated for 4h either under standard conditions or in stress conditions (2% O_2, 10% CO_2, 32°C) with vehicle control or compound of interest. After treatment, cells were pelleted, washed once in ice-cold PBS, and lysed in RIPA buffer (Sigma) containing proteinase and phosphatase inhibitors (Roche #04693159001 and #04906845001). Protein extract was immediately stored at -20°C.

2.3 Primary tissue slices

2.3.1 Rat whole brain slices

Rat whole brain slices were provided by Dr Chang (Centre for Biomedical Sciences, Royal Holloway University of London). Briefly, male Sprague-Dawley rats were kept under controlled environmental conditions (24–25°C; 50–60% humidity; 12h light/dark cycle) with free access to food and tap water. Rats were killed using pentobarbital; the brain was removed and placed in ice-cold sucrose solution (NaCl 87mM, KCl 2.5mM, $MgCl_2$ 7mM, $CaCl_2$ 0.5mM, NaH_2PO_4 1.25mM, sucrose 75mM, glucose 25mM, equilibrated with 95% O_2 / 5% CO_2). Transverse slices (350µm) were prepared with an Epilepsia Leica Vibratome (Leica VT1200S) and were then stored in an interface chamber containing artificial cerebrospinal fluid solution (ACSF; NaCl 119mM, KCl 2.5mM, $MgSO_4$ 4mM, $CaCl_2$ 4mM, $NaHCO_3$ 26.2mM, NaH_2PO_4 1mM, glucose 11mM) gassed continuously with a 95% O_2 / 5% CO_2 gas mixture and kept at 37°C in a water bath. Treatment was added directly to ACSF (*Fig. 1*) and allowed to diffuse for five minutes prior to brain slice transfer. After 4h slices were homogenised manually in PBS. The cell suspension was pelleted (3min at 1,500xg), the pellet re-suspended in RIPA buffer containing proteinase and phosphatase inhibitors (750µl/slice) and centrifuged a second time (3min at 10,000xg) to pellet cell debris. The supernatant was removed and stored at -20°C.

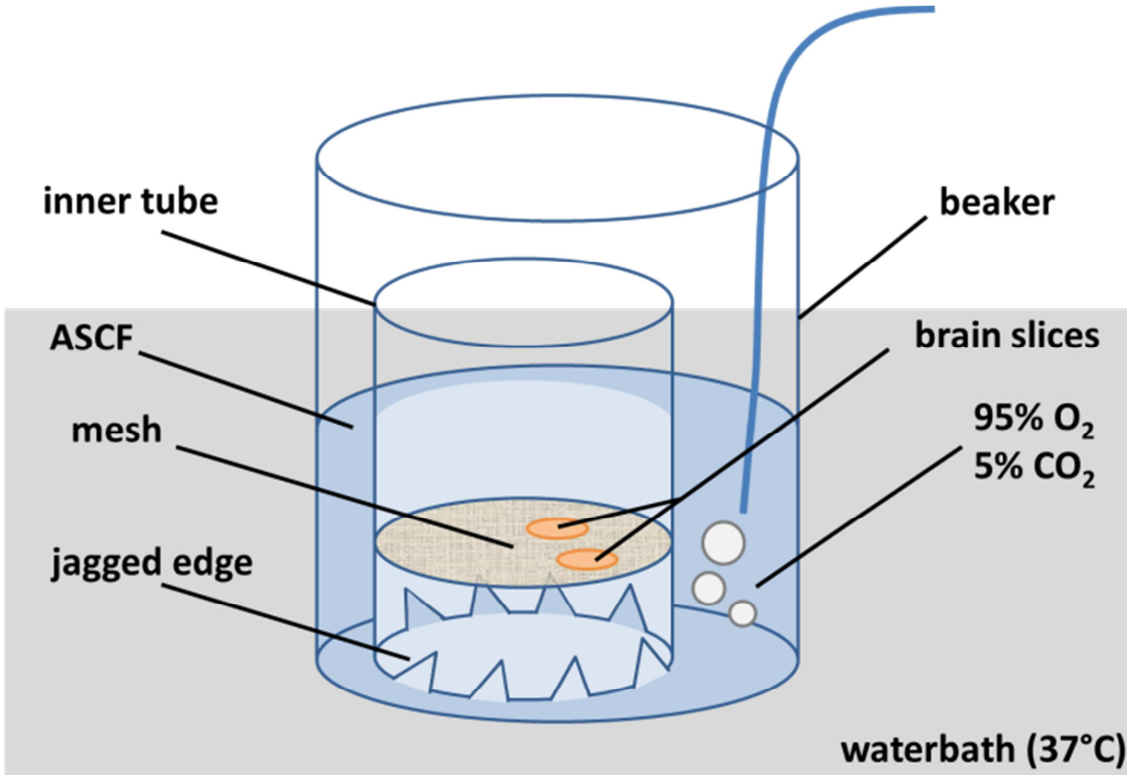

Figure 2.1 – Brain slice treatment set-up. The brain slices rest on a mesh embedded in a tube, open at both ends, which is placed inside a beaker containing oxygenated artificial cerebrospinal fluid (ASCF). The carrier tube is either suspended from the beaker at a slightly elevated point or rests on a jagged edge, allowing ASCF to perfuse freely around the tissue. The ASCF is continuously gassed with 95% O_2 / 5% CO_2 throughout the experiment.

2.3.2 Mouse liver slices

For the pilot liver slice experiment, mice were sacrificed by cervical dislocation, the liver immediately excised and the gallbladder removed. The liver was rapidly moved to ice-cold Earle's balanced salt solution (EBSS; Sigma #E7510) containing D-glucose (25mM; Sigma) which had been gassed for 1h with a 95% O_2 / 5% CO_2 gas mixture. Cylindrical tissue cores (8mm diameter) were prepared using a hand-held coring tool. Cores were placed into the core mount of a Krumdieck tissue slicer (Alabama Research and Development Corporation) and 250µm slices prepared according to equipment instructions. Slices were transferred to pre-incubated (30min at 37°C, 5% CO_2) 12-well plates containing RPMI 1640 culture medium (1.5ml/well; Invitrogen #31870082) supplemented with 5% FBS (as before) as well as the following (all supplied by Sigma): 0.5mM L-methionine (#M5308), 1µM insulin (#I9278), 0.1mM hydrocortisone-21-hemisuccinate (#H2270) and gentamicin 50µg/ml (#G1397). Slice-containing plates were placed on a gyratory shaker (90rpm) to insure adequate media circulation and incubated inside a humidified incubator (37°C, 5% CO_2). After a brief pre-incubation (30min), slices were moved to new, pre-incubated plates containing 1.5ml/well RPMI with treatment compounds or vehicle controls. After treatment (4h), plates were removed and immediately stored on ice. Slices were briefly rinsed in ice-cold PBS before being transferred to ice-cold 500µl RIPA+PP buffer and homogenised manually. Tissue suspensions were centrifuged briefly at high speed and the supernatant stored at -20°C.

2.4 Western Blotting

Extracted protein was boiled (10min at 95°C) in 1X loading buffer (5X: 160mM Tris pH 6.8, 240mM Glycerol, 50mM SDS, 1.8M β-mercaptoethanol, Bromophenol blue) and loaded into a 12.5% acrylamide/bisacrylamide (Sigma #A3699) gel. Protein was separated by SDS-PAGE (150V, 1h) alongside a pre-stained size marker (Fermentas #26619) and transferred to PVDF membrane (Merck Millipore #IPFL00010) via Western Blot (200mA, 1h). All following incubation/washing steps were performed on a bench rocker. Membranes were blocked in blocking buffer containing 5% BSA V (Sigma #A2153) in TBST (Severn Biotech) for 1h. Antibody was added directly to blocking buffer (1:1000) and membranes were incubated at 4°C overnight. All primary antibodies provided by Cell Signaling Technology: GSK3β (#12456), Ser9 pGSK3β (#5558), Akt (#9272), Ser473 pAkt (#4060), PPARγ (#2443), PTEN (#9188), Ser380/Thr382/383 pPTEN (#9549), β-catenin (#8480), acetylated lysine (#9441), β-actin (#4970), β-tubulin (#2128). Membranes were washed in TBST (3x5min) and incubated with secondary antibody (1:15,000; Li-Cor #926-32211 Goat anti-Rabbit) in Odyssey Blocking Buffer (Li-Cor #927-50000) for 1h at room temperature, followed by another washing step in TBST (3x5min) and rinse with TBS. Membranes were visualised and quantified using the Odyssey Sa system (Li-Cor). The prestained protein size marker was used to verify target protein size.

2.5 Commercial assays

2.5.1 Histone deacetylase (HDAC) assay

HDAC inhibition assays were performed using a fluorimetric *in vitro* histone deacetylase assay (Merck Millipore #17-372) and according to instructions using HeLa cell enzyme extract (Enzo #BML-KI140-0100) at a 1:10 dilution. During assay incubation, the enzymatic activity from HeLa cell extract deacetylates a substrate unless inhibited by the compound of interest. Deacetylation of substrate allows a fluorescent compound (the activator) to bind, leading to an amplified signal with increasing deacetylation. Inhibitor-containing reactions are compared to a vehicle control sample.

2.5.2 Bicinchoninic acid (BCA) Assay

Protein abundance was measured using the Pierce BCA assay, which was supplied by Life Technologies (#23225) and carried out according to manufacturer's instructions.

2.5.3 Lactate dehydrogenase (LDH) activity assay

LDH release was measured using LDH Cytotoxicity Assay (Pierce #88953) and according to the manufacturer's instructions.

2.5.4 Viability, cytotoxicity, and apoptosis assay

Huh7 cells were analysed for cell viability, cytotoxicity, and apoptosis using ApoTox Glo (Promega #G6320) according to manufacturer instructions.

2.6 siRNA knock-down

Specific PPARγ siRNAs and negative control siRNA (Qiagen #GS5468 and #SI03650325 respectively) were used in conjunction with Lipofectamine RNAiMAX (Inivtrogen) according to manufacturer protocols. Cells were seeded into 6-well plates and cultured to 70% confluence (48h, 37°C, 5% CO_2). Cells were transfected in 250µl unsupplemented culture medium (DMEM high glucose, as before) with all four PPARγ siRNAs or the negative control siRNA for 6h, after which 750µl DMEM containing 10% FBS was added to each well. Cells were rested overnight (16h), at which point medium was replaced with fresh DMEM (containing 10% FBS) and cultured for a further 24h prior to experiments.

2.7 Reverse transcription quantitative RT-PCR

Huh7 cells were trypsinised after treatment and re-suspended in 200µl PBS / well for RNA extraction. RNA was extracted using High Pure RNA Isolation kit (Roche #11828665001) and according to the manufacturer's instructions. RNA was then purified using DNA free (Life Technologies #AM1906) and coding DNA (cDNA) was synthesised using First Strand cDNA Synthesis (Life Technologies #K1612). Real-time amplification with SYBR Green (BioRad #172-5120) was performed using a Rotor-Gene 6000 (Qiagen). Levels of transcription were quantified using the method proposed by Livak and Schmittgen (2001; $2^{-\Delta\Delta C_T}$).

2.8 Cell fixing and staining

Huh7 cells were seeded at $8x10^4$ cells/well into 6-well plates containing poly-d-lysine-coated (0.1mg/ml; Sigma in 0.1 M Borate buffer) coverslips. Cells were allowed to recover for 24h prior to the experiment, after which culture medium was aspirated, coverslips were rinsed with PBS, and cells were fixed in 4% paraformaldehyde (20min). Cells were then permeabilised with 0.2% Triton in PBS (3min) and blocked in 10% FBS in PBS (15min). Fixed cells were incubated with PPARγ antibody (1:100) at 4°C overnight, followed by incubation with fluorescent secondary antibody (1:1000, 30min) in the dark. Finally, cells were stained with diamidino-2-phenylindole (DAPI; Life Technologies #62248). Coverslips were mounted onto microscope slides using Fluoromount (Sigma #F4680) and allowed to dry overnight prior to analysis.

2.9 Software

Statistical analysis and graphing were performed using Prism 5 (GraphPad Software). Microscopic images were captured using Image Pro Plus 6.3 (Media Cybernetics). Cell fluorescence was analysed using Image J (Rasband, 1997).

2.10 Statistical analysis

Data were analysed using one-way ANOVA, two-way ANOVA, or Student's t-test as appropriate after confirmation of normal distribution with Kolmogorov-Smirnov test. Results were further analysed using post-hoc Tukey test where appropriate. Probability (p) values larger than 0.05 were considered insignificant (n.s.), 0.01–0.05 significant (*), 0.001–0.01 very significant (**), and <0.001 highly significant (***).

3

Investigating the effect of VPA on molecular signalling in standard culture conditions

3.1 Introduction

VPA is known to modulate glycogen synthase kinase-3β (GSK3β) and extracellular regulated kinase 1/2 (ERK1/2) signalling *in vivo*. VPA increases GSK3β phosphoylation during haemorrhagic shock in liver tissue (Alam *et al.*, 2009; Alam *et al.*, 2011; Hwabijre *et al.*, 2014) and is neuroprotective in the brain by regulating ERK1/2 signalling (Fukudome *et al.*, 2010; Kochanek *et al.*, 2012; Pramod *et al.*, 2010). These effects are tissue-specific (Gotfryd *et al.*, 2010) and occur within four hours (Alam *et al.*, 2009). A broad approach encompassing these factors in experimental design was therefore necessary to establish an *in vitro* model.

Four cell lines, two primary cell types, and two types of primary tissue were selected to investigate their response to VPA treatment. Two human hepatocarcinoma cell lines (Huh7 and HepG2) were chosen to model changes in GSK3β phosphorylation and two neuroblastoma cell lines, Neuro2a (mouse) and SH-SY5Y (human) to model those in ERK1/2 phosphorylation. The use of two independent cell lines per tissue was necessary to control for line-dependent signalling differences for which VPA is known (Gotfryd *et al.*, 2010). Primary cells and tissue were utilised as they more accurately reflect *in vivo* conditions compared to immortalised cell lines. As ERK1/2 and GSK3β are the protein of interest in neuronal and hepatic tissues respectively, the study of VPA effects on ERK1/2 in liver- and GSK3β in brain-derived cells and tissue served as a control for pathway-specific activation. The

extent of cell and tissue types used provided a substantial range of environments for new discovery.

In cell culture, serum-starvation is used as a standard methodology to prepare cells for experimental procedure. However, the removal of serum from cell culture medium has recently been highlighted as a stressor in itself (Pirkmajer and Chibalin, 2011). Therefore, to ensure the use of appropriate cell culture techniques, all cell lines were treated in both serum-starved and serum-supplemented conditions in order to discover whether serum state affects the response of ERK1/2 and GSK3β phosphorylation to VPA. Experimental results were interpreted with the aim of establishing the correct model environment.

The experiments described in this chapter were performed to discover the effect of VPA at a range of concentrations on the phosphorylation state of two proteins (GSK3β and ERK1/2) in four cell lines (Huh7, HepG2, Neuro2a, and SH-SY5Y) and primary cells and tissue within four hours. This approach studied the effect of VPA on two known protein targets while taking into account some complexities of the tissue-dependent VPA mechanism of action and providing a suitable framework for result interpretation.

3.2 Results

3.2.1 Neuronal tissue

3.2.1.1 Effect of VPA on neuroblastoma-derived cell lines

3.2.1.1.1 GSK3β phosphorylation

In a pilot experiment, Neuro2a cells were serum-starved and treated with VPA at two physiologically relevant (0.5 and 1mM) and one clinically excessive (3mM) concentration (Levy & Shen, 1995) for four hours. GSK3β phosphorylation levels were calculated as phosphorylated protein to total protein ratio and compared to an untreated control *(Fig. 1)*. No change in GSK3β phosphorylation was observed in response to VPA treatment at any concentration in Neuro2a cells ($p>0.05$), nor did treatment affect the level of total GSK3β.

To examine the effect of VPA on a second neuroblastoma-derived cell line, SH-SY5Y cells were treated with VPA. In order to discover the effect of serum starvation on protein phosphorylation in response to VPA, cells received fresh media with or without serum 16h before treatment. GSK3β phosphorylation was quantified as a phosphorylated protein to total protein ratio and compared to an untreated control *(Fig. 2)*. VPA did not affect pGSK3β levels in SH-SY5Y cells, a result unaffected by the presence or absence of serum in culture media ($p>0.05$). Total levels of GSK3β were also not affected in cells regardless of serum state. The analysis indicates that VPA does not modulate GSK3β phosphorylation in SH-SY5Y cells in standard cell culture conditions over four hours of treatment.

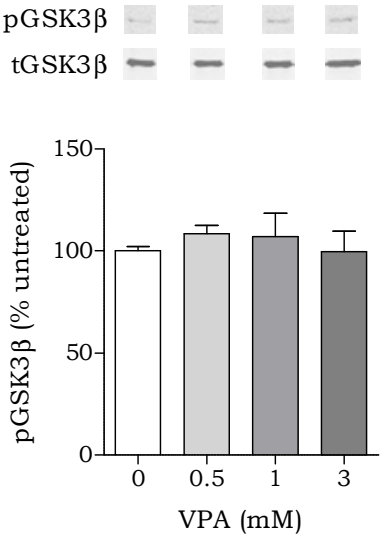

Figure 3.1 – Effect of VPA treatment on GSK3β phosphorylation in Neuro2a. Neuro2a cells were cultured under standard conditions (37°C, 5% CO2) receiving fresh serum-free medium 16h prior to the start of treatment. Cells were treated with VPA at indicated concentrations or vehicle control (dH_2O) for four hours. Protein extract was probed with phosphorylated GSK3β (pGSK3β; Ser9) and total GSK3β (tGSK3β) antibodies and visualised with fluorescent secondary antibody. Levels of pGSK3β were calculated as ratio to tGSK3β and are shown as mean ± SEM. Data (n=4, technical duplicates) were statistically analysed (ANOVA and Tukey test) and * indicate significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

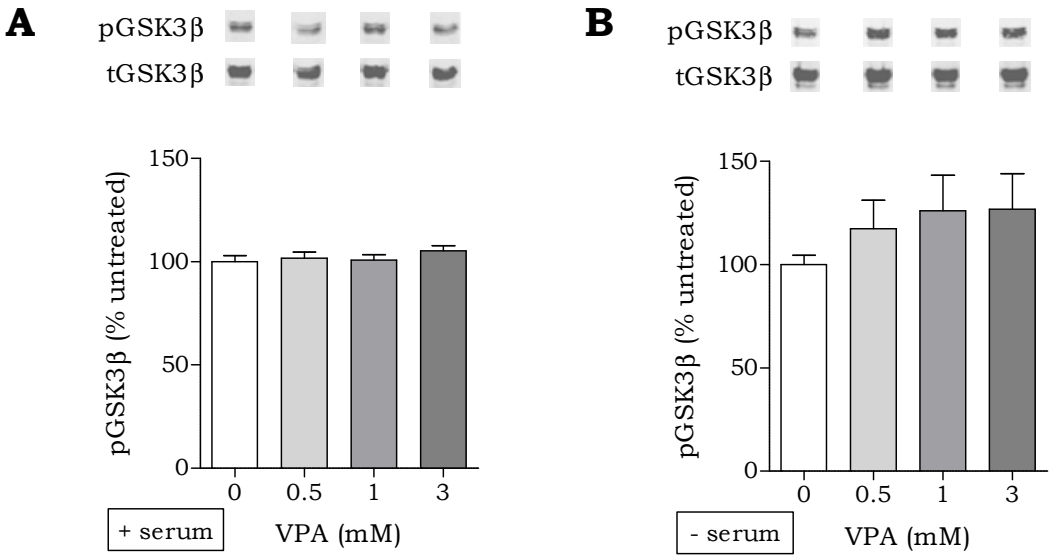

Figure 3.2 – Effect of VPA treatment on GSK3β phosphorylation in SH-SY5Y. SH-SY5Y cells were cultured under standard conditions (37°C, 5% CO2) receiving fresh media with or without serum 16h prior to the start of treatment. Cells were treated with VPA at indicated concentrations or vehicle control (dH2O) for four hours in (A) serum-supplemented or (B) serum-free medium. Protein extract was probed with phosphorylated GSK3β (pGSK3β; Ser9) and total GSK3β (tGSK3β) antibodies and visualised with fluorescent secondary antibody. Levels of pGSK3β were calculated as ratio to tGSK3β and are shown as mean ± SEM. Data (n=3, technical duplicates) were statistically analysed (ANOVA and Tukey test) and * indicate significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.2.1.1.2 ERK1/2 phosphorylation

SH-SY5Y cells were treated with VPA to discover any effect on ERK1/2 phosphorylation. After four hours, protein was extracted and pERK1/2 (Thr202/Tyr204) levels quantified (*Fig. 3*) as a ratio of phosphorylated protein to total protein and compared to an untreated control. VPA did not have a significant effect on ERK1/2 phosphorylation in SH-SY5Y cells regardless of serum state ($p>0.05$), nor did the amount of total ERK1/2 change. The data therefore suggest that VPA does not affect ERK1/2 phosphorylation in SH-SY5Y in standard cell culture conditions during short-term treatment.

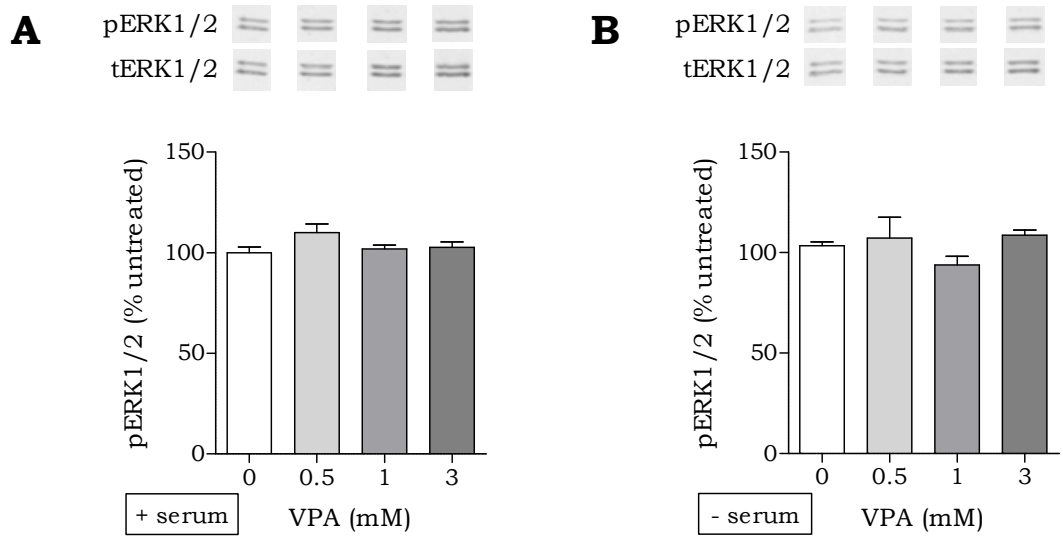

Figure 3.3 – Effect of VPA treatment on ERK1/2 phosphorylation in SH-SY5Y. SH-SY5Y cells were cultured under standard conditions (37°C, 5% CO_2) receiving fresh media with or without serum 16h prior to the start of treatment. Cells were treated with VPA at indicated concentrations or vehicle control (dH2O) for four hours in (A) serum-supplemented or (B) serum-free medium. Protein extract was probed with phosphorylated ERK1/2 (pERK1/2; Thr202/Tyr204) and total ERK1/2 (tERK1/2) antibodies and visualised with fluorescent secondary antibody. Levels of pGSK3β were calculated as ratio to tGSK3β and are shown as mean ± SEM. Data (n=3, technical duplicates) were statistically analysed (ANOVA and Tukey test) and * indicate significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.2.1.2 Effect of VPA on primary neuronal cells and tissue

Primary tissue more accurately represents *in vivo* tissue compared to cell lines. To elucidate potential differences in GSK3β and ERK1/2 phosphorylation between cell lines and primary tissue, pilot studies were performed on isolated rat neuronal tissue. Rat cortical cells and whole brain tissue slices were exposed to VPA for four hours and protein phosphorylation quantified. VPA effects on protein phosphorylation in primary tissue served as a comparison to that seen in cell lines.

Rat cortical neurons were extracted, cultured for six days, and then treated with VPA. Due to small extract volume, only phosphorylated and total GSK3β levels were visualised and quantified (*Fig. 4*). VPA did not significantly change Ser9 phosphorylation of GSK3β ($p>0.05$) and did not affect total GSK3β levels. These data suggest that VPA does not affect GSK3β signalling via phosphorylation in primary rat cortical neurons.

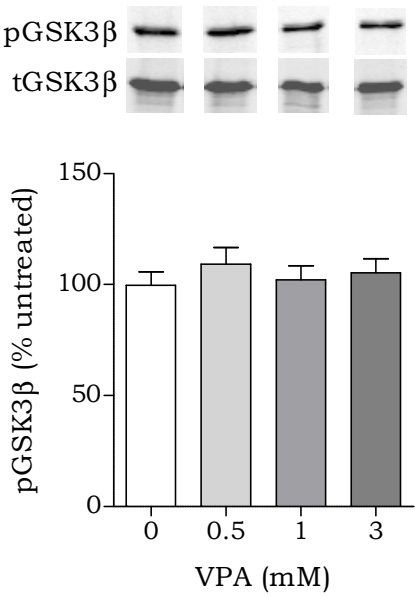

Figure 3.4 – Effect of VPA treatment on GSK3β phosphorylation in primary murine cortical neurons. Cortical neurons extracted from E18 murine embryos were provided in monolayer culture six days post-extraction. Cells were treated in standard conditions (37°C, 5% CO2) with VPA at indicated concentrations or vehicle control (dH_2O) for four hours. Protein extract was probed with phosphorylated GSK3β (pGSK3β; Ser9) and total GSK3β (tGSK3β) antibodies and visualised with fluorescent secondary antibody. Levels of pGSK3β were calculated as ratio to tGSK3β and are shown as mean ± SEM. Data (n=6, technical duplicates) were statistically analysed (ANOVA and Tukey test) and * indicate significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Whole rat brain slices (350µm thick) were prepared as described previously (Chang *et al.*, 2010) and stored for treatment in an interface chamber containing artificial cerebrospinal fluid (aCSF) solution. VPA was added directly to the aCSF and slices were treated for four hours and then homogenised manually. Protein was extracted for visualisation and analysis of GSK3β (Ser9) and ERK1/2 (Thr202/Tyr204) phosphorylation levels (*Fig. 5*). VPA does not significantly affect GSK3β (*Fig. 5A*) or ERK1/2 (*Fig. 5B*) phosphorylation in rat whole brain slices ($p>0.05$). VPA treatment also had no effect on total protein levels. The data therefore indicate that VPA does not affect GSK3β and ERK1/2 signalling through phosphorylation in *ex vivo* brain slice tissue.

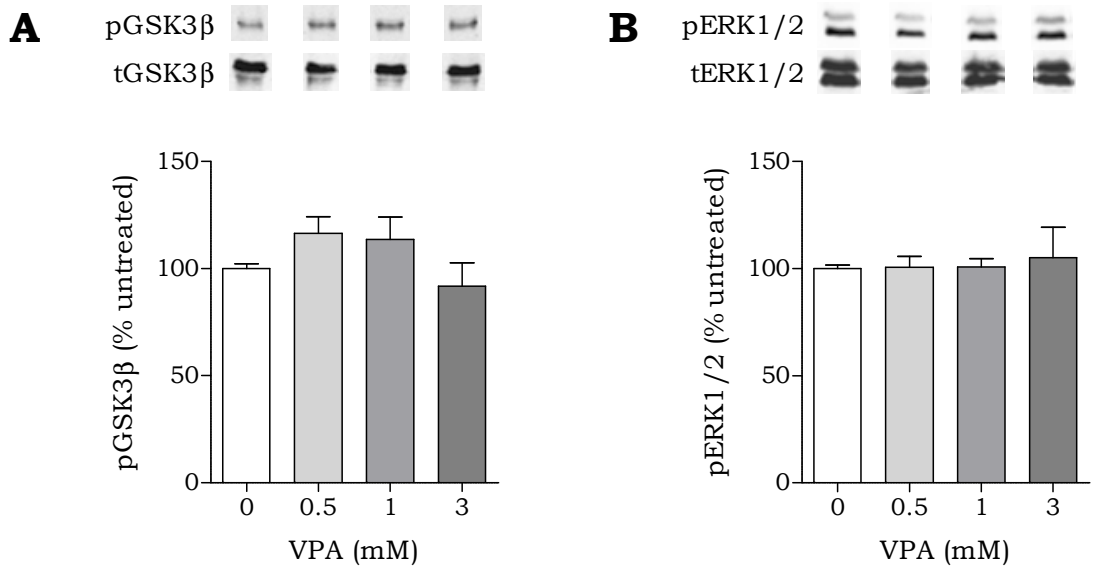

Figure 3.5 – Effect of VPA treatment on GSK3β and ERK1/2 phosphorylation in rat brain slices. Rat brains were removed and sliced using a Leica Vibratome slicer. Slices treated with VPA at indicated concentrations in an interface chamber containing artificial cerebral spinal fluid for four hours. Protein extract was probed with (A) phosphorylated GSK3β (pGSK3β; Ser9) or total GSK3β (tGSK3β) antibody, or (B) phosphorylated ERK1/2 (pERK1/2; Thr202/Tyr204) or total ERK1/2 (tERK1/2) antibody and visualised with fluorescent secondary antibody. Levels of pGSK3β and pERK1/2 were calculated as ratio to tGSK3β and are shown as mean ± SEM. Data (n=6 for GSK3β; n=4 for ERK1/2; technical duplicates) were analysed (ANOVA and Tukey test) and are shown as mean ± SEM and * indicate significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.2.2 Hepatic tissue

3.2.2.1 Effect of VPA on hepatocarcinoma-derived cell lines

3.2.2.1.1 GSK3β phosphorylation

VPA affects GSK3β phosphorylation in liver during haemorrhagic shock (Alam *et al.*, 2009). Therefore, liver-derived cell lines were utilised to discover the effect of VPA on GSK3β phosphorylation *in vitro*. Two human cell lines, Huh7 and HepG2, were selected to enable comparison of any signalling changes observed in response to VPA between two cell lines from hepatic source tissue. Cells were treated with VPA for four hours in serum-free and serum-supplemented media to analyse the

effect of serum state on GSK3β (Ser9) phosphorylation in response to VPA. Effects on Ser9 phosphorylation were quantified as a phosphorylated protein to total protein ratio and compared to an untreated control.

In Huh7 cells treated in serum-supplemented media, VPA did not significantly affect pGSK3β levels (*Fig. 6A*). In serum-starved Huh7, VPA increased GSK3β phosphorylation (to 121±8% compared to untreated cells) at 3mM (*Fig. 6B*), but this change was not significant ($p>0.05$). In HepG2, VPA did not significantly affect GSK3β phosphorylation in either serum-starved or serum-supplemented cells even at high concentrations *(Fig. 7)*. VPA did not modulate total protein level in either cell line. The results therefore indicate that VPA does not affect GSK3β phosphorylation in Huh7 or HepG2 in standard cell culture conditions.

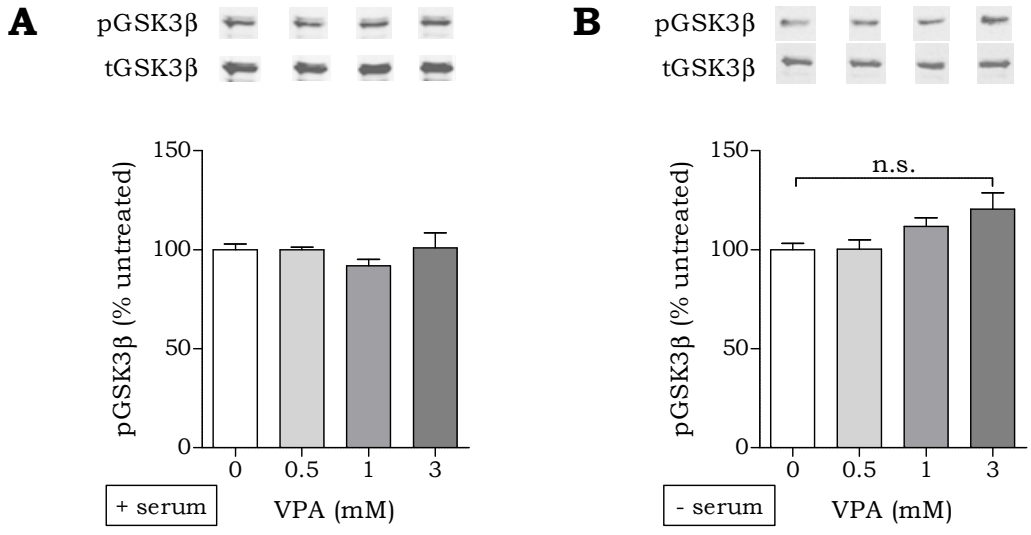

Figure 3.6 – Effect of VPA treatment on GSK3β phosphorylation in Huh7. Huh7 cells were cultured under standard conditions (37°C, 5% CO_2) receiving fresh media 16h prior to the start of treatment. Cells were treated with VPA at indicated concentrations or vehicle control (dH_2O) for four hours in (A) serum-supplemented or (B) serum-free medium. Protein extract was probed with phosphorylated GSK3β (pGSK3β; Ser9) and total GSK3β (tGSK3β) antibodies and visualised with fluorescent secondary antibody. Levels of pGSK3β were calculated as ratio to tGSK3β and are shown as mean ± SEM. Data (n=6, technical triplicates) were statistically analysed (ANOVA and Tukey test) and * indicate significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

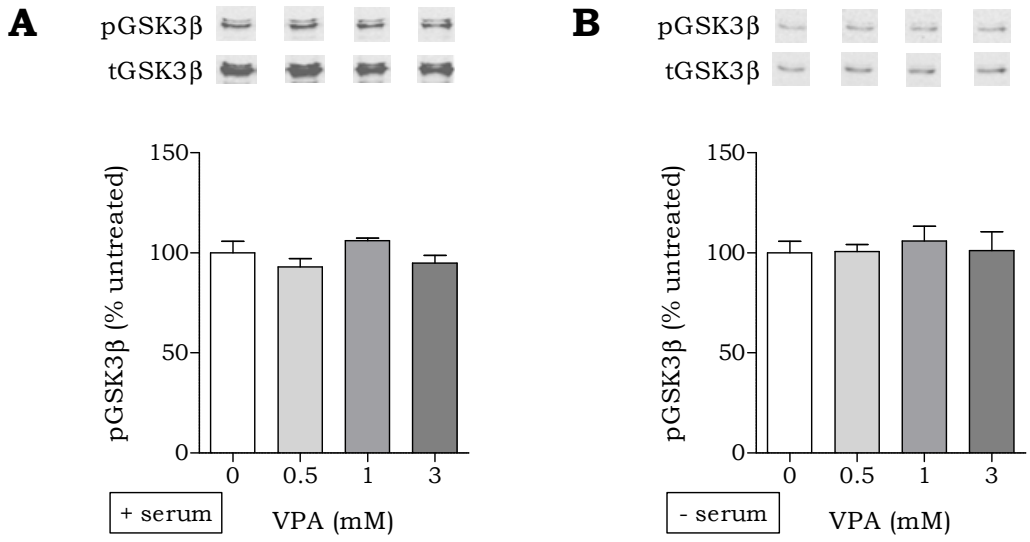

Figure 3.7 – Effect of VPA treatment on GSK3β phosphorylation in HepG2. HepG2 cells were cultured under standard conditions (37°C, 5% CO_2) receiving fresh media 16h prior to the start of treatment. Cells were treated with VPA or vehicle control (dH_2O) for four hours in (A) serum-supplemented or (B) serum-free medium. Protein extract was probed with phosphorylated GSK3β (pGSK3β; Ser9) and total GSK3β (tGSK3β) antibodies and visualised with fluorescent secondary antibody. Levels of pGSK3β were calculated as ratio to tGSK3β and are shown as mean ± SEM. Data (n=6, technical triplicates) were statistically analysed (ANOVA and Tukey test) and * indicate significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.2.2.1.2 ERK1/2 phosphorylation

Though ERK1/2 phosphorylation has been shown to be modulated *in vivo* during haemorrhagic shock, this regulation has not been observed in liver (Fukudome *et al.*, 2010). To discover potential differences in pathway activation between GSK3β and ERK1/2, the same two hepatocarcinoma-derived cell lines were exposed to VPA and analysed for ERK1/2 phosphorylation. Cells were treated in serum-supplemented or serum-free media to discover the effect of serum state on ERK1/2 phosphorylation in response to VPA. Effects on protein phosphorylation were then compared to those seen on GSK3β.

Huh7 cells were treated with VPA and pERK1/2 (Thr202/Tyr204) levels were quantified as a phosphorylated protein to total protein ratio and compared to an untreated control (*Fig. 8*). ERK1/2 phosphorylation in Huh7 cells did not differ significantly between treated and untreated cells, regardless of serum state ($p>0.05$). To examine the effect of VPA in a distinct liver-derived cell line, HepG2 cells were treated with VPA and ERK1/2 phosphorylation (Thr202/Tyr204) was quantified (*Fig. 9*). VPA treatment does not have a significant effect on ERK1/2 phosphorylation in HepG2 cells at any concentration used, neither in serum-supplemented nor in serum-free media ($p>0.05$). The data suggest that VPA does not affect ERK1/2 phosphorylation in Huh7 or HepG2 cells and that these results are not modulated by the presence or absence of serum.

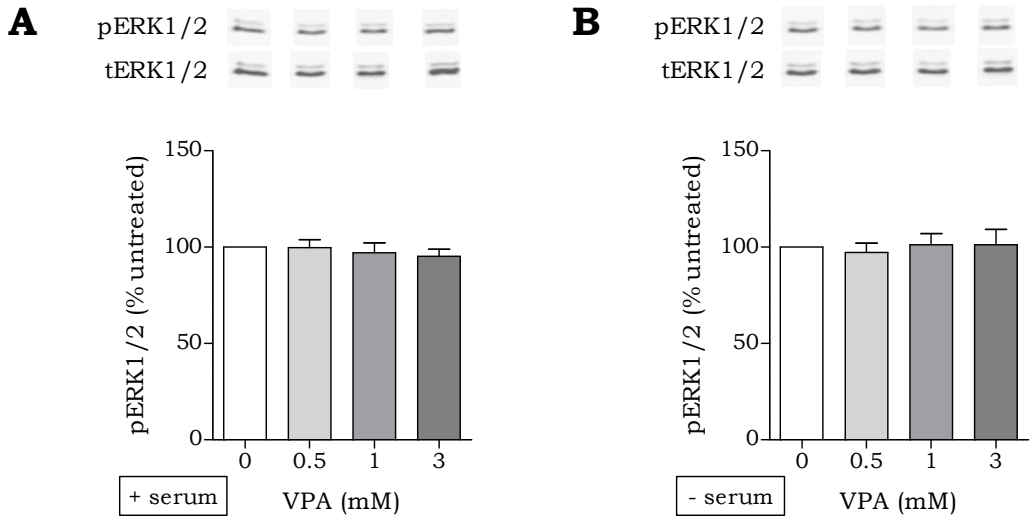

Figure 3.8 – Effect of VPA treatment on ERK1/2 phosphorylation in Huh7. Huh7 cells were cultured under standard conditions (37°C, 5% CO_2) receiving fresh media 16h prior to the start of treatment. Cells were treated with VPA or vehicle control (dH_2O) for four hours in (A) serum-supplemented or (B) serum-free medium. Protein extract was probed with phosphorylated ERK1/2 (pERK1/2; Thr202/Tyr204) and total ERK1/2 (tERK1/2) antibodies and visualised with fluorescent secondary antibody. Levels of pERK1/2 were calculated as ratio to tERK1/2 and are shown as mean ± SEM. Data (n=6, technical triplicates) were statistically analysed (ANOVA and Tukey test) and * indicate significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

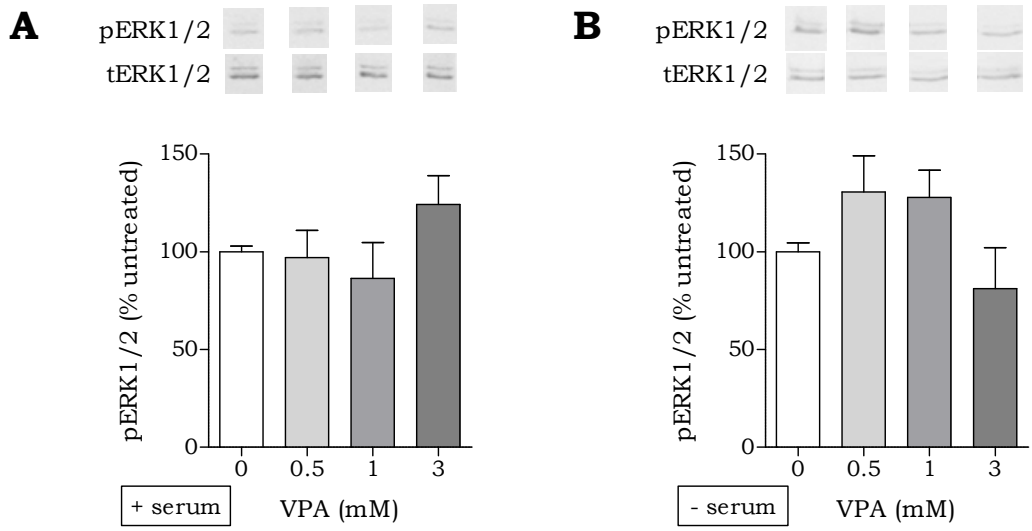

Figure 3.9 – Effect of VPA treatment on ERK1/2 phosphorylation in HepG2. HepG2 cells were cultured under standard conditions (37°C, 5% CO_2) receiving fresh media 16h prior to the start of treatment. Cells were treated with VPA or vehicle control (dH_2O) for four hours in (A) serum-supplemented or (B) serum-free medium. Protein extract was probed with phosphorylated ERK1/2 (pERK1/2; Thr202/Tyr204) and total ERK1/2 (tERK1/2) antibodies and visualised with fluorescent secondary antibody. Levels of pERK1/2 were calculated as ratio to tERK1/2 and are shown as mean ± SEM. Data (n=6, technical triplicates) were statistically analysed (ANOVA and Tukey test) and * indicate significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.2.2.2 Effect of VPA on primary hepatic cells and tissue

Cell lines reflect conditions in healthy *in vivo* tissue less accurately than primary cells used *ex vivo*. Therefore, pilot studies on primary hepatic cells tissue were performed to investigate potential effects of VPA on GSK3β and ERK1/2 phosphorylation. Results were compared to those from cell lines treated *in vitro* to discover any differences in protein phosphorylation.

Cryopreserved rodent hepatocytes were treated with VPA in an approach similar to that used with cell lines. GSK3β phosphorylation (Ser9) was visualised, quantified, and compared to an untreated control (*Fig. 10A*). Due to a limited amount of available cells, the number of experimental repeats were not sufficient to determine whether VPA had

a significant ($p<0.05$) effect on GSK3β phosphorylation in primary hepatocytes, but preliminary data did not reproduce those found *in vivo* (Alam *et al.*, 2009).

A pilot experiment on hepatic tissue was performed using heterogeneous murine liver tissue core slices. GSK3β phosphorylation levels in murine liver slice protein extract were visualised and quantified (*Fig. 10B*). VPA treatment significantly ($p<0.01$) decreased pGSK3β levels by 58±10% at 3mM concentration compared to untreated control but did not modulate GSK3β levels at lower concentrations ($p>0.05$). These results, obtained in in non-cancerous tissue, indicate that the lack of signalling response to VPA seen in Huh7 and HepG2 is likely not specific to carcinoma-derived cells and therefore due to treatment conditions rather than carcinoma-specific effects of VPA.

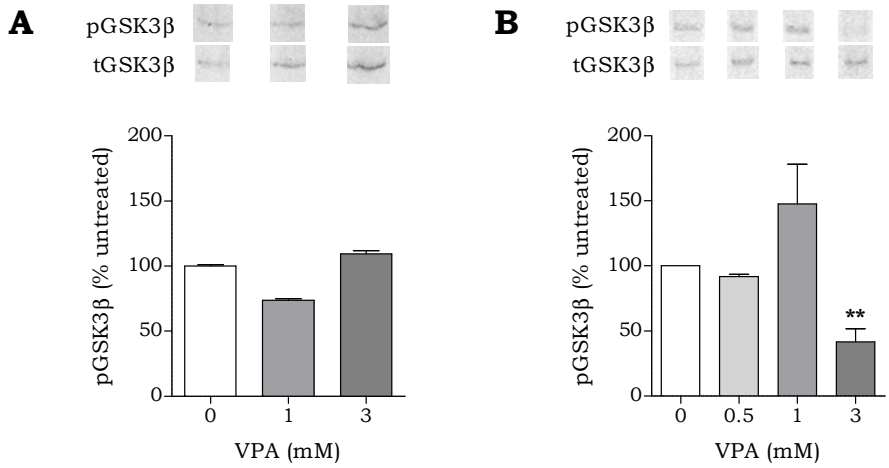

Figure 3.10 – Effect of VPA treatment on GSK3β phosphorylation in primary hepatic tissue (pilot). After VPA treatment (4h) protein was extracted and probed with phosphorylated GSK3β antibody (Ser9) or total GSK3β antibodies, visualised, and quantified. Data are shown as mean (bar) ± SEM (error bar). (A) Rat primary hepatocytes were thawed, allowed to form a monolayer and to rest overnight. After 16h, cells were treated with VPA or vehicle control (dH_2O) (n=2 technical). Due to the limited number of repeats, statistical analysis of the data was not possible. (B) Murine livers were excised and liver cores produced using a hand-held coring tool. A Krumdieck tissue slicer produced 250µm thick slices which were allowed to rest for 30min prior to treatment with VPA or vehicle control (dH_2O). Data (n=3 technical) were analysed using ANOVA and Tukey test. * indicate significance compared to 0mM VPA: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.3 Discussion

3.3.1 Effect of VPA on protein phosphorylation in neuron cells

VPA treatment has been shown to be neuroprotective in brain injury and during haemorrhagic shock (Dash *et al.*, 2010; Sinn *et al.*, 2007; Williams *et al.*, 2006). Acute protective cellular changes after traumatic brain injury and intracerebral haemorrhage in response to VPA have been shown to be effected by ERK1/2 signalling (Dash *et al.*, 2010; Sinn *et al.*, 2007). VPA also affects GSK3β signalling in the brain, but studies have focused on the effects of chronic treatment (Hall *et al.*, 2002; Leng *et al.*, 2008). Neuronal cells were therefore selected to investigate the acute effect of VPA on ERK1/2 and GSK3β phosphorylation.

Two neuroblastoma cell lines, Neuro2a (mouse) and SH-SY5Y (human), both of which have been shown to respond to VPA treatment, were used in these experiments. In SH-SY5Y, VPA has an anti-apoptotic effect via p53 and bcl-2 (Song *et al.*, 2012) and decreases the vulnerability of cells to oxidative stress (Monti *et al.*, 2009). VPA also induces bcl-2 regulation via the MAPK and PI3K pathways in SH-SY5Y and shows activity similar to that in cultured cortical cells (Creson *et al.*, 2009). In Neuro2a however, VPA induces differentiation (Chen *et al.*, 2011) but does not modulate GSK3β activity (Phiel *et al.*, 2001). Neuro2a was selected for experiments as *in vivo* studies of the effect of VPA in haemorrhagic shock have utilised rodent models (Kochanek *et*

al., 2012; Li *et al.*, 2010) while SH-SY5Y was chosen due to its increased similarity to the human cellular environment.

Neuro2a and SH-SY5Y were both exposed to VPA at a series of concentrations, both clinically relevant (0.5, 1mM) and excessive (3mM; Levy & Shen, 1995). A pilot experiment on Neuro2a showed no effect of VPA on GSK3β phosphorylation. Treatment of SH-SY5Y with VPA replicated the lack of response seen in Neuro2a, both in serum-free and serum-supplemented media. Neither GSK3β phosphorylation nor ERK1/2 phosphorylation were acutely modulated by VPA treatment in SH-SY5Y, regardless of drug concentration. These data demonstrate the need to modify the treatment or culturing conditions of SH-SY5Y cells to gain the opportunity of creating a functional model system.

Primary neuronal tissue was utilised to investigate the effect of VPA on protein phosphorylation in a cellular environment more similar to *in vivo* conditions than can be found in cell lines. Isolated rat cortical cells were chosen for VPA exposure to allow for use of a treatment protocol almost identical (save for changes in incubation medium) to that used with cell lines. VPA did not affect GSK3β phosphorylation and low protein yield precluded the analysis of ERK1/2 phosphorylation status in cortical neurons. These results indicated that the lack of significant effect on protein phosphorylation seen in SH-SY5Y was not specific to the cell line.

To investigate whether the acute effect of VPA in the brain relies on the action of non-neuronal supporting cells, heterogenous brain tissue was exposed to VPA. Rat whole brain slices were dissected and

treated in an interface chamber at identical drug concentrations to those used *in vitro*. This exposure of primary brain tissue to VPA did not produce significant changes to protein phosphorylation status, neither of GSK3β nor in ERK1/2. These data suggest that the absence of a significant effect of VPA on protein phosphorylation in SH-SY5Y was not due to the homogenous cellular environment of the cell line cells.

3.3.2 Effect of VPA on protein phosphorylation in hepatic cells

As the main focus of haemorrhagic shock research has been on GSK3β signalling in the liver (Hwabejire *et al.*, 2014) *ex vivo* hepatic tissue and hepatocarcinoma-derived cell lines were chosen as the basis for the primary line of experimental enquiry into modelling haemorrhagic shock. *In vivo* studies have often relied upon GSK3β phosphorylation at Serine 9 as a modulation marker (Alam *et al.*, 2009; Li *et al.*, 2008; Li *et al.*, 2011) which was therefore of interest as a primary target. ERK1/2 phosphorylation status was investigated as a control to demonstrate the selective impact of VPA on GSK3β phosphorylation.

Two hepatocarcinoma-derived cell lines, Huh7 and HepG2 (both modified from human tumours), were selected for their proven response to VPA treatment and prevalence of data regarding the effect of VPA on liver tissue during haemorrhagic shock. HepG2 has been used more extensively than Huh7, but the latter is beneficial for experiments due to evidence for its increased similarity to human tissue (Krelle *et al.*, 2013; Meex *et al.*, 2011). In Huh7, VPA inhibits cell proliferation

(Machado *et al.* 2011) and in HepG2, VPA decreases cell viability (Neuman *et al.*, 2013). VPA primes both Huh7 and HepG2 for TRAIL-dependent apoptosis (Pathil *et al.*, 2006). However, some apoptotic action of VPA has been shown to be balanced by VPA itself: The histone deacetylase (HDAC) inhibitory activity of VPA induces *clusterin* over-expression which renders cancer cells resistant to HDAC inhibition-induced apoptosis (Liu *et al.*, 2009). In primary tissue, evidence is equally conflicting: VPA has been shown to sensitise primary hepatocytes to the death receptor agonist CD95L (Weiller *et al.*, 2011), but Armeanu *et al.* (2005) found that primary hepatocytes tolerate VPA treatment well. These data further reinforce the need for cross-system comparisons.

Huh7 and HepG2 were acutely treated with VPA to investigate potential effects on protein phosphorylation. VPA treatment had no significant effect on GSK3β Ser9 phosphorylation in Huh7 or HepG2, irrespective of the presence or absence of serum in culture media. ERK1/2 phosphorylation (Thr202/Tyr204) was also unaffected in Huh7 and HepG2, regardless of treatment concentration and serum state. The lack of response indicates that the cellular conditions used in this chapter do not model those found in haemorrhagic shock.

3.3.3 Effect of serum on VPA action

The experiments on cell lines discussed in this chapter were performed on cells incubated in culture media supplemented with foetal bovine serum (FBS) and in media not supplemented with FBS. Cell lines are traditionally cultured in serum-supplemented media but are often

serum-starved prior to experiments to ensure homogenous cell states (e.g. Di Daniel *et al.*, 2005; Gurvich *et al.*, 2004; Jo *et al.*, 2011; Li *et al.*, 2009). However, cells show reduced basal cellular activity in serum-free media (Codeluppi *et al.*, 2011) and individual cell lines react differently to serum starvation (Levin *et al.*, 2010). Removal of serum from culture media has even been shown to induce apoptosis (Wang *et al.*, 2013). Pirkmajer and Chibalin (2011) warn that serum starvation, though widely used as a standard method in cell culture experiments, modulates signalling response to yield results divergent from or opposite to those produced in cells treated in serum-supplemented media, accordingly producing conflicting results and hindering data interpretation. Experiments were therefore performed in both serum-free and serum-supplemented media to investigate the effect of serum state on VPA-dependendent changes to protein phosphorylation.

Cell serum state did not significantly affect protein phosphorylation in response to VPA treatment. In neuronal cells, the presence or absence of serum did not modulate ERK1/2 or GSK3β phosphorylation levels during exposure to VPA. In hepatic cells, no effect of VPA on pERK1/2 or pGSK3β was observed to be dependent on serum supplementation. As serum starvation lacks a parallel in the clinical context as well as in whole organism models of haemorrhagic shock, future experiments on cell lines were performed in serum-supplemented media.

3.3.4 Implications and conclusion

This chapter investigated the acute effect of VPA treatment on GSK3β and ERK1/2 phosphorylation in neuronal and hepatic cell lines and primary tissue with the aim of establishing an *in vitro* model of haemorrhagic shock. However, VPA did not affect protein phosphorylation in the majority of model systems studied. A review of the literature on the short-term protective effects of VPA observed *in vivo* highlights a potential reason for these results: Though several studies have shown the beneficial therapeutic effects of VPA in disease, the relevant feature of these data is that the humans, animals, or *in vitro* models under discussion underwent pathological conditions prior to or during treatment with VPA (e.g. Alam *et al.*, 2009; Hwabejire *et al.*, 2014; Kochanek *et al.*, 2014; Williams *et al.*, 2006). This is a key difference between these studies and the model systems exposed to VPA in this chapter, which were treated in standard culturing conditions.

Unlike organ tissue during lethal blood loss, cells undergoing VPA treatment in the experiments discussed here were not in the process of responding to adverse circumstances. The absence of significant change to protein phosphorylation in response to VPA may be due to cells being cultured in standard cell culture conditions favourable to cellular activity and growth. Thus, a central consideration for future experiments must be the successful reproduction of the pathological circumstances found during haemorrhagic shock. Any effect of VPA on in these systems will specifically be due to its effect on cellular compensation to the adverse environment provided, as it is *in vivo*. The

response to VPA treatment in these pathological environments will provide a solid foundation on which to establish a successful model of haemorrhagic shock.

3.4 Summary

The results in this chapter demonstrate that VPA does not affect GSK3β or ERK1/2 phosphorylation in cell lines or *ex vivo* cells and tissue at clinically relevant concentrations. The data suggest that this is unlikely to be due to differential effects on immortalised cell lines and primary tissue or the homogeneous environment of cell lines and isolated primary cells. Serum-starvation, a common methodology in cell culture, has been demonstrated to not modulate the effect of VPA on GSK3β and ERK1/2 phosphorylation. Due to these data and the lack of a clinical parallel for this methodological step, its inclusion in future experiments is unwarranted. The models studied in this chapter do not accurately reflect the signalling changes observed in response to VPA treatment *in vivo*, thus further approaches need to be investigated with a focus on inducing pathological environments mimicking those found in terminal blood loss.

4

Investigating the effect of VPA on molecular signalling in stress conditions

4.1 Introduction

Haemorrhagic shock, caused by significant loss of blood, affects the entire organism (Kauvar and Wade, 2005). Loss of intravascular volume leads to decreased tissue perfusion, which affects cellular respiration: a lack of a gas transport vehicle (blood) leads to a decrease in essential components required for respiration (oxygen, nutrients) and an accumulation of waste product such as carbon dioxide, thereby increasing cellular stress (Gutierrez *et al.*, 2004). As the effects of regional hypoperfusion spread, the core temperature of the organism falls (Kheirbek *et al.*, 2009). Together with coagulopathies, these effects combine and exacerbate each other to make massive blood loss and haemorrhagic shock a potentially lethal condition (Findlay *et al.*, 2007).

Animal models of haemorrhagic shock aim to recreate the conditions found in human patients as closely as possible. This includes loss of at least 40% of total blood as in the most severe cases of lethal blood loss (Gutierrez *et al.*, 2004), artificial control of core temperature to ensure precise reduction, and bone and internal soft tissue injury (Alam *et al.*, 2009; Butt *et al.*, 2009; Kochanek *et al.*, 2012). While it is necessary to accurately mimic events seen during lethal blood loss *in vivo* in animal treatment protocols in order to investigate model organism survival, these experiments are cost- and time-intensive which may prevent in-depth investigations of molecular signalling.

To effectively model the impact of lethal blood loss on cellular signalling *in vitro* the individual components of this whole organism

condition must be distinguished and simulated on a cellular scale. During haemorrhagic shock, loss of blood leads to a decrease in available oxygen (hypoxia) and an increase in carbon dioxide (hypercapnia), which in turn causes a reduction of pH (acidosis; Hotter *et al.*, 2004). A decrease in local or global temperature (hypothermia) also affects cellular gene expression and molecular signalling (Jia *et al.*, 2014; Kelly *et al.*, 2005; Shore *et al.*, 2013), for instance decreasing GSK3β phosphorylation in swine liver (Alam *et al.*, 2009) and increasing ERK1/2 phosphorylation in rat lung (Fukudome *et al.*, 2010). The presence of the components of this pathological state is therefore likely to be of key importance in producing a haemorrhagic shock-like environment *in vitro*.

In the experiments described in this chapter, human cell lines were exposed to pathological conditions mimicking those present in haemorrhagic shock (hypoxia, hypercapnia, and hypothermia) and treated with VPA to determine the effect on cellular signalling compared to that seen in standard cell culture conditions (normoxia). The presence of conditions in isolation or in combination and with or without VPA treatment was characterised with regards to the effects on GSK3β and ERK1/2 phosphorylation. These changes in levels of phosphorylated protein were compared to those found *in vivo* to determine which, if any, stress conditions replicated the phosphorylation changes observed in animal models, thereby yielding a suitable *in vitro* model of haemorrhagic shock signalling.

4.2 Results

4.2.1 Stress conditions in Huh7

To determine the effect of pathological gas and temperature states on GSK3β phosphorylation in Huh7, which is reduced after haemorrhagic shock *in vivo* (Alam *et al.*, 2009 Hwabejire *et al.*, 2014), cells were exposed to hypoxia (2% O_2), hypercapnia (10% CO_2), and hypothermia (32°C). Huh7 cells were incubated for four hours in stress or standard (atmospheric O_2, 5% CO_2, 37°C) cell culture conditions with or without VPA at clinically relevant concentrations (0.1-1mM; Levy & Shen, 1995). Cell protein extract was analysed for GSK3β (Ser9) or ERK1/2 (Thr202/Tyr204) phosphorylation compared to total GSK3β and ERK1/2 levels while β-tubulin levels were established to provide a reference for total protein present.

Huh7 cells were first treated with hypoxia and hypercapnia at a standard cell culture temperature (37°C) to determine whether these conditions sufficed to modulate protein phosphorylation (*Fig. 1*). Exposure to low oxygen and high carbon dioxide for four hours did not significantly affect phosphorylation of GSK3β and ERK1/2 compared to cells cultured in standard conditions (normoxia; $p>0.05$). Neither GSK3β nor ERK1/2 phosphorylation levels were modulated by VPA during these conditions at any concentration tested. Levels of total ERK1/2 and GSK3β were also unaffected by gas stress without concurrent VPA treatment ($p>0.05$).

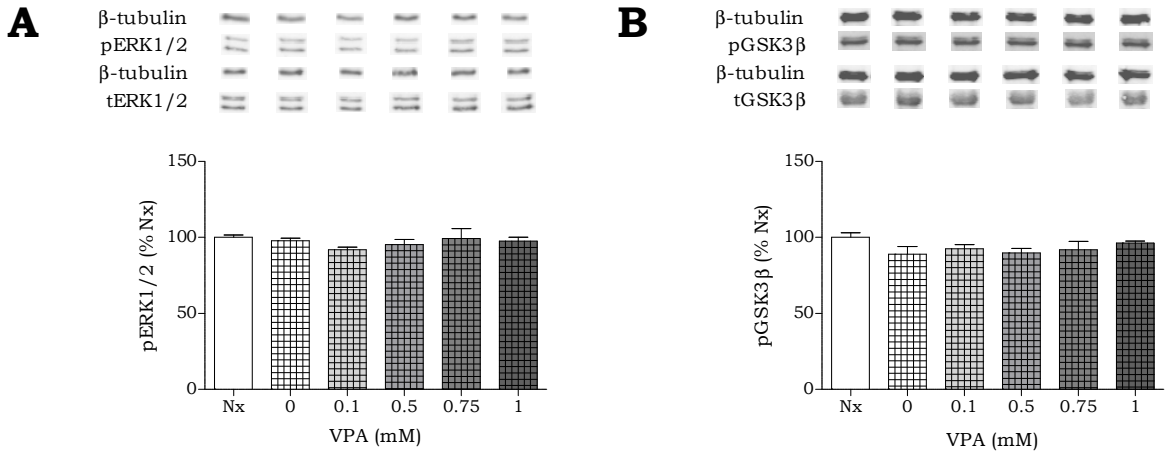

Figure 4.1 - Effect of VPA treatment on ERK1/2 and GSK3β phosphorylation in Huh7 undergoing hypoxia and hypercapnia. Huh7 cells were cultured under standard conditions (37°C, 5% CO2) receiving fresh medium 16h prior to the start of treatment. After VPA was added to media at indicated concentration, cells were transferred to adverse gas conditions (2% O_2, 10% CO_2) for four hours. Control cells were treated with vehicle control (dH_2O) and concurrently incubated in standard conditions (normoxia; Nx). Protein extract was probed with (A) phosphorylated ERK1/2 (pERK1/2; Thr202/Tyr204) and total ERK1/2 (tERK1/2) antibodies or (B) phosphorylated GSK3β (pGSK3β; Ser9) and total GSK3β (tGSK3β) antibodies, as well as β-tubulin antibody as loading control, and visualised with fluorescent secondary antibody. ERK1/2 and GSK3β fluorescence were corrected for loading differences with β-tubulin and phosphorylated protein levels were calculated as ratio to total protein and are shown as mean ± SEM. Data (n=9, technical triplicates) were statistically analysed (ANOVA and Tukey test) and * indicate significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

As adverse gas conditions did not significantly affect protein phosphorylation, Huh7 cells were exposed to hypoxia and hypercapnia in combination with hypothermia (32°C) to establish any effect on ERK1/2 or GSK3β phosphorylation (*Fig. 2*). The three combined stressors (HxHcHp) did not significantly affect ERK1/2 phosphorylation (*Fig. 2A*), nor total ERK1/2 levels compared to those found in cells cultured in standard conditions (Nx). The adverse conditions also did not significantly modulate total GSK3β levels, however, they significantly ($p<0.001$) reduced GSK3β Ser9 phosphorylation to 50±6% of untreated standard culture control (Nx; *Fig. 2B*). Treatment with VPA significantly ($p<0.05$ at 0.5mM and $p<0.001$ at 0.75mM) reversed the

reduction of pGSK3β in a dose-dependent manner, increasing phosphorylation levels to 91±10% of control at 0.5mM and to 128±16% at 0.75mM. VPA did not have this effect on GSK3β phosphorylation during standard cell culture conditions where treatment at 0.75mM did not significantly affect pGSK3β levels. These results suggest that the increase of GSK3β phosphorylation observed in response to VPA treatment during hypoxia, hypercapnia, and hypothermia is specific to cells treated in stress conditions similar to those observed in severe blood loss.

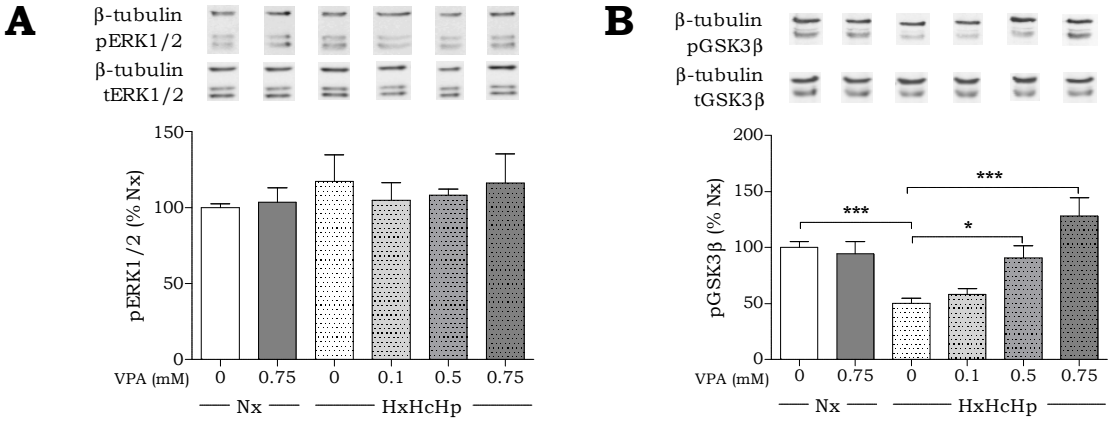

Figure 4.2 - Effect of VPA treatment on ERK1/2 and GSK3β phosphorylation in Huh7 undergoing hypoxia, hypercapnia, and hypothermia. Huh7 cells were cultured under standard conditions (37°C, 5% CO2) receiving fresh medium 16h prior to the start of treatment. After VPA was added to media at indicated concentration, cells were transferred to adverse gas and temperature conditions (2% O_2, 10% CO_2, 32°C; HxHcHp) for four hours. Control cells were treated with vehicle control (dH_2O) or VPA as indicated and concurrently incubated in standard conditions (Normoxia; Nx). Protein extract was probed with (A) phosphorylated ERK1/2 (pERK1/2; Thr202/Tyr204) and total ERK1/2 (tERK1/2) antibodies or (B) phosphorylated GSK3β (pGSK3β; Ser9) and total GSK3β (tGSK3β) antibodies, as well as β-tubulin antibody as loading control, and visualised with fluorescent secondary antibody. ERK1/2 and GSK3β fluorescence were corrected for loading differences with β-tubulin and phosphorylated protein levels were calculated as ratio to total protein and are shown as mean ± SEM. Data (n=9, technical triplicates) were statistically analysed (ANOVA and Tukey test) and * indicate significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

To determine whether the effect on GSK3β phosphorylation observed during combined hypoxia, hypercapnia, and hypothermia was dependent upon the presence of all three conditions, Huh7 cells were exposed to each condition separately and to all possible pairs of adverse conditions (*Fig. 3*). Cells exposed to hypoxia, hypercapnia, or hypothermia individually did not show a decrease in pGSK3β levels, nor did any two conditions combined lead to a reduction of phosphorylation. Therefore, these results suggest that the presence of all three conditions is necessary to cause the reduction in GSK3β Ser9 phosphorylation in Huh7 cells which mimics that seen during haemorrhagic shock *in vivo* (Alam *et al.*, 2009).

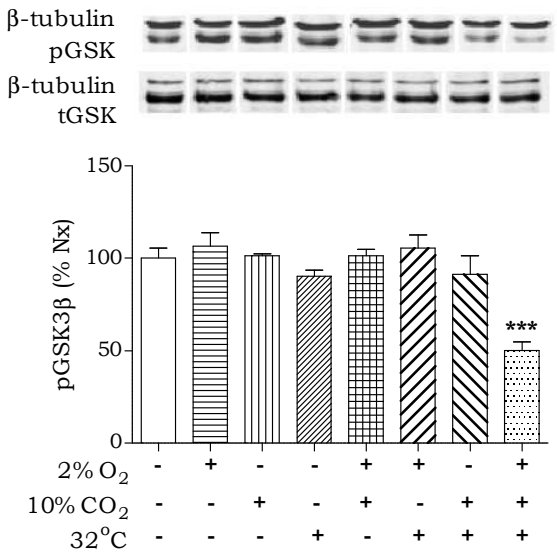

Figure 4.3 – Effect of hypoxia, hypercapnia, and hypothermia on GSK3β phosphorylation in Huh7. Huh7 cells were cultured under standard conditions (37°C, 5% CO2) receiving fresh medium 16h prior to the start of treatment. Cells were then exposed for four hours to standard cell culture conditions or to single or combined stress conditions (2% O_2, 10% CO_2, 32°C) as indicated. Protein extract was probed with phosphorylated GSK3β (pGSK3β; Ser9) and total GSK3β (tGSK3β) antibodies, as well as β-tubulin antibody as loading control, and visualised with fluorescent secondary antibody. GSK3β fluorescence was corrected for loading differences with β-tubulin. Phosphorylated protein levels were calculated as ratio to total protein and are shown as mean ± SEM. Data (n=9, technical triplicates) were statistically analysed (ANOVA and Tukey test) and * indicate significance compared to Nx 0: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4.2.2 Stress conditions in HepG2 and HEK293

To establish whether other cell lines may provide cellular environments suitable for modelling haemorrhagic shock signalling two human cell lines, HepG2 and HEK293, were selected for further experiments. Signalling changes in the HepG2 line, a hepatocarcinoma-derived cell line, would serve as a comparison to the changes observed in Huh7. HEK293 cells, derived from human embryonic kidney, were of interest as the GSK3β pathway has been shown to be activated during massive blood loss in kidney in a similar manner to that seen in liver (Zacharias *et al.*, 2011). Both cell lines were exposed to hypoxia (2% O_2), hypercapnia (10% CO_2), and hypothermia (32°C) with or without concurrent VPA treatment for four hours. GSK3β phosphorylation (Ser9) was quantified to show any changes in phosphorylation state and highlight potential pathway activation.

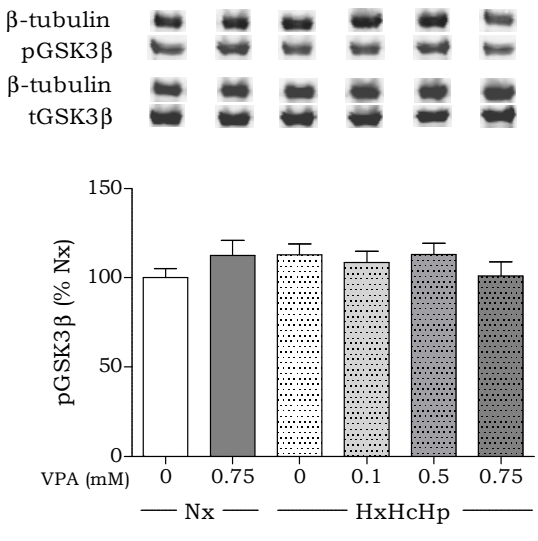

Figure 4.4 – Effect of VPA treatment on GSK3β phosphorylation in HepG2 during hypoxia, hypercapnia, and hypothermia. HepG2 cells were cultured under standard conditions (37°C, 5% CO2) receiving fresh medium 16h prior to the start of treatment. After VPA was added to media at indicated concentration, cells were transferred to suboptimal gas and temperature conditions (2% O_2, 10% CO_2, 32°C; HxHcHp) for four hours. Control cells were treated with vehicle control (dH_2O) or VPA as indicated and incubated in standard conditions (Normoxia; Nx) concurrently. Protein extract was probed with phosphorylated GSK3β (pGSK3β; Ser9) and total GSK3β (tGSK3β) antibodies. Levels of β-tubulin served as loading control. GSK3β fluorescence was corrected for loading differences with β-tubulin and phosphorylated protein levels were calculated as ratio to total protein and are shown as mean ± SEM. Data (n=3, technical triplicates) were statistically analysed (ANOVA and Tukey test) and * indicate significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

In HepG2 cells, the adverse gas and temperature conditions did not have a significant effect on GSK3β phosphorylation (*Fig. 4)*. As in standard cell culture conditions, VPA did not affect pGSK3β levels in HepG2 exposed to the simulated haemorrhagic shock environment. Despite sharing source tissue with Huh7 (human hepatocarcinoma), HepG2 did not replicate the signalling changes seen in the first cell line which mimic those *in vivo* and was therefore excluded from further experiments.

In HEK293 Hypoxia, hypercapnia, and hypothermia did not significantly affect pGSK3β levels (*Fig. 5*). However, VPA treatment significantly increased GSK3β Ser9 phosphorylation during stress conditions even at low concentrations; to 363±37% at 0.1mM ($p<0.01$), 372±54% at 0.5mM ($p<0.001$) and 381±38% at 0.75 ($p<0.001$). As the adverse culture conditions used did not affect pGSK3β, VPA treatment was not corrective as seen *in vivo* during haemorrhagic shock. Therefore HEK293 was excluded from further experiments in favour of Huh7, which models *in vivo* signalling changes more accurately.

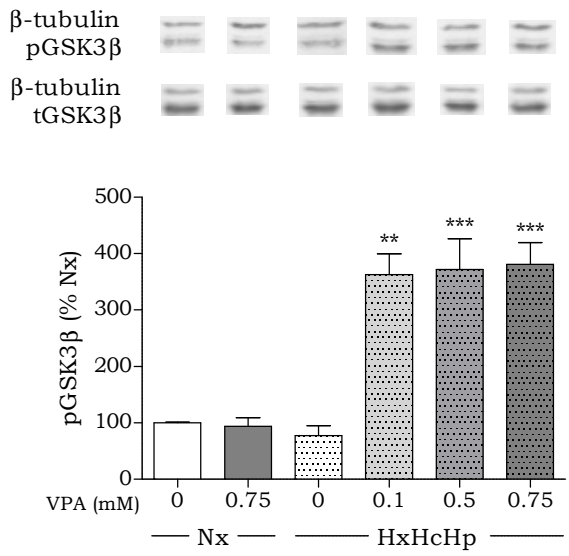

Figure 4.5 – Effect of VPA on GSK3β phosphorylation in HEK293 undergoing hypoxia, hypercapnia, and hypothermia. HEK293 cells were cultured under standard conditions (37°C, 5% CO2) receiving fresh medium 16h prior to the start of treatment. After VPA was added to media at indicated concentration, cells were transferred to suboptimal gas and temperature conditions (2% O_2, 10% CO_2, 32°C; HxHcHp) for four hours. Control cells were treated with vehicle control (dH_2O) or VPA as indicated and incubated in standard conditions (Normoxia; Nx) concurrently. Protein extract was probed with phosphorylated GSK3β (pGSK3β; Ser9) and total GSK3β (tGSK3β) antibodies.
Levels of β-tubulin were used as loading control, and visualised with fluorescent secondary antibody. GSK3β fluorescence was corrected for loading differences with β-tubulin and phosphorylated protein levels were calculated as ratio to total protein and are shown as mean ± SEM. Data (n=3, technical triplicates) were statistically analysed (ANOVA and Tukey test) and * indicate significance compared to Nx 0: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4.2.3 Stress conditions in SH-SY5Y

To determine whether hypoxia, hypercapnia, and hypothermia and concurrent VPA treatment affect protein phosphorylation in neuronal tissue the neuroblastoma-derived cell line SH-SY5Y was exposed to combined stress conditions with or without VPA and analysed for GSK3β Ser9 and ERK1/2 Thr202/Tyr204 phosphorylation. Haemorrhagic shock-like conditions did not significantly decrease levels of pGSK3β and VPA did not affect GSK3β phosphorylation at any concentration (*Fig. 6A*). Adverse gas conditions also did not affect ERK1/2 phosphorylation, regardless of the presence or absence of VPA (*Fig. 6B, C).* However, while adverse incubation conditions did not significantly affect total ERK1/2 levels, concurrent treatment with VPA did ($p<0.05$), increasing total ERK1/2 by 75±23% at 1mM (*Fig. 6D*).

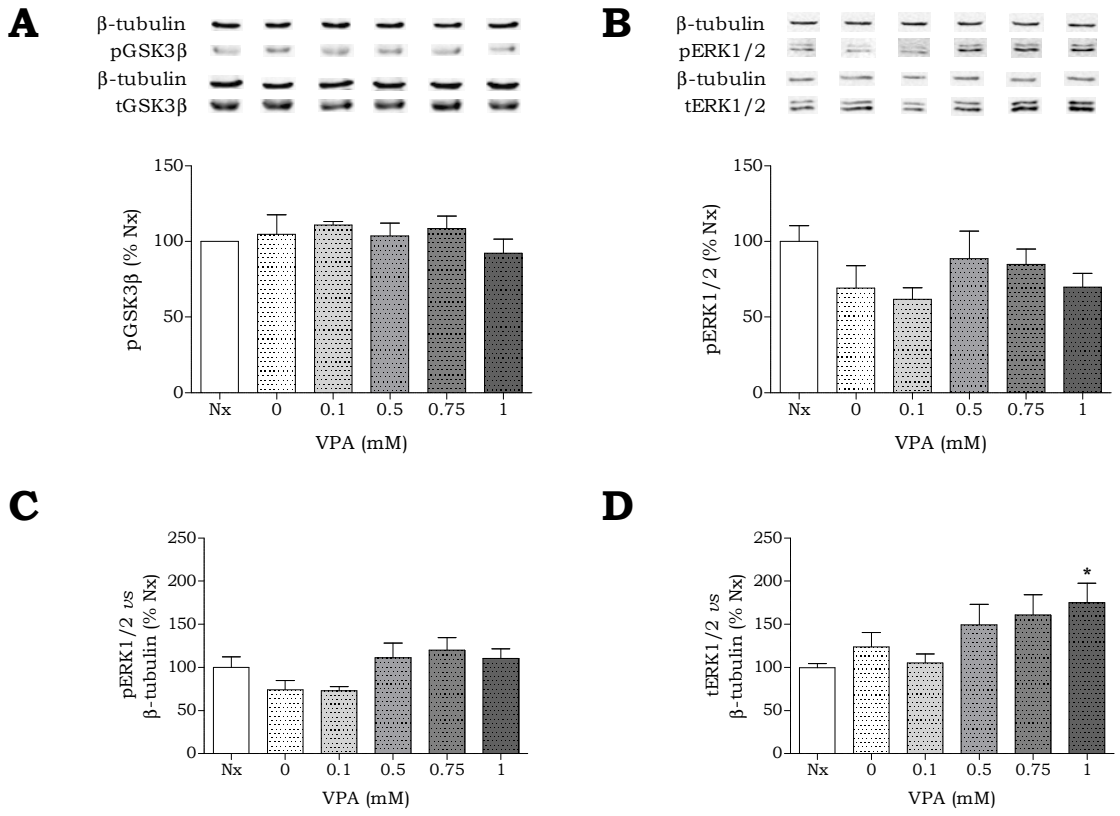

Figure 4.6 - Effect of hypoxia, hypercapnia, and hypothermia and VPA treatment on GSK3β and ERK1/2 in SH-SY5Y. SH-SY5Y cells were cultured under standard conditions (37°C, 5% CO2) receiving fresh medium 16h prior to the start of treatment. After VPA was added to media at indicated concentration, cells were transferred to suboptimal gas and temperature conditions (2% O_2, 10% CO_2, 32°C) for four hours. Control cells were treated with vehicle control (dH_2O) and concurrently incubated in standard conditions (Normoxia; Nx). Protein extract was probed with (A) phosphorylated GSK3β (pGSK3β; Ser9) and total GSK3β (tGSK3β) antibodies or (B) phosphorylated ERK1/2 (pERK1/2; Thr202/Tyr204) and total ERK1/2 (tERK1/2) antibodies, as well as β-tubulin antibody as loading control. Levels of ERK1/2 and GSK3β were corrected for loading differences with β-tubulin and phosphorylated protein levels were calculated as (A, B) ratio to total protein or (C, D) ratio to β-tubulin and are shown as mean ± SEM. Data (n=9, technical triplicates) were statistically analysed (ANOVA and Tukey test) and * indicate significance compared to Nx 0: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

To further investigate a potential change in total ERK1/2 levels in response to VPA treatment during pathological conditions, a reverse transcription quantitative real-time PCR assay was performed to quantify *erk1/2* expression, using GAPDH levels as a control. The expression of *erk1/2* increased significantly in cells undergoing gas and temperature stress, by 2.5±0.3 fold in untreated cells and by 2.8±0.4

fold in cells treated with 1mM VPA (*Fig. 7*). However, *erk1/2* expression did not vary significantly depending on the presence of absence of VPA regardless of incubation conditions. The data therefore suggest that cells respond to stress conditions by increased *erk1/2* expression but that VPA treatment does not have a significant effect on gene expression in stress or standard conditions.

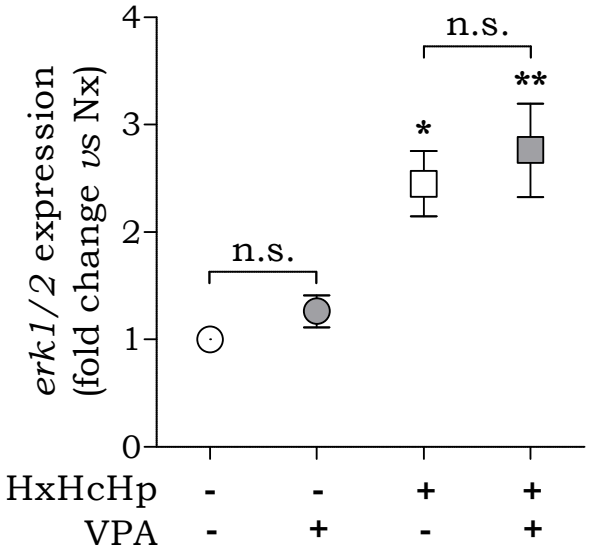

Figure 4.7 – Effect of VPA on ERK1/2 transcription in SH-SY5Y in hypoxia, hypercapnia, and hypothermia. SH-SY5Y cells were cultured under standard conditions (37°C, 5% CO2) receiving fresh medium 16h prior to the start of treatment. After VPA (1mM) or vehicle control (dH_2O) was added to media, cells were transferred to stress conditions (2% O_2, 10% CO_2, 32°C; HxHcHp) for four hours. Control cells were treated with vehicle control (dH_2O) or VPA as indicated and incubated concurrently in standard conditions (Normoxia; Nx). SH-SY5Y mRNA was extracted and cDNA produced which served as the template for quantitative real-time PCR to quantify erk1/2 expression (GAPDH expression was quantified as control). Data (n=3, technical duplicates) is presented as fold-change compared to untreated Nx samples. * indicate significance compared to Nx 0 unless otherwise indicated: * $p<0.05$, ** $p<0.01$, *** $p<0.001$; n.s. not significant.

4.3 Discussion

In this chapter, four cell lines were exposed to haemorrhagic shock-like culture conditions of hypoxia (2% O_2), hypercapnia (10% CO_2), and hypothermia (32°C) in order to investigate whether any changes in GSK3β and ERK1/2 phosphorylation showed similarity to those observed *in vivo* during severe blood loss. Hepatic, nephric, and neuronal cell lines were selected in order to determine the similarity in signalling response to each host tissue respectively. Cells were treated with VPA to establish whether phosphorylation changed in the protective manner seen in animal models. The experiments discussed provide evidence for the suitability of selected cell lines as model systems for signalling in haemorrhagic shock.

4.3.1 Stress conditions in Huh7

Huh7 cells were exposed to hypoxia and hypercapnia to investigate any acute effects on GSK3β Ser9 and ERK1/2 Thr202/Tyr204 phosphorylation. These gas conditions were selected as potential *in vitro* model components because one of the first effects of massive blood loss is tissue hypoperfusion (Peitzman *et al.*, 1995) which causes cellular hypoxia (Rossaint *et al.*, 2006) and hypercapnia (Johnson and Weil, 1991). Huh7 cells were cultured in severe hypoxia of 2% oxygen (Miyamoto *et al.*, 2015), which is approximately equal to 2kPa, a value below the clinical threshold of 8kPa for significant [sic] hypoxia (Findlay *et al.*, 2007). Carbon dioxide levels were increased during cell treatment to 10%, approximately equal to 10kPa, which is

above the *in vivo* clinical threshold for hypercapnia (6kPa; Findlay *et al.*, 2007). Under these adverse conditions total or phosphorylated GSK3β or ERK1/2 levels were unaffected. These results are dissimilar from those observed during haemorrhagic shock *in vivo*, where GSK3β phosphorylation is decreased (Alam *et al.*, 2009; Hwabejire *et al.*, 2014) and ERK1/2 phosphorylation is increased (Fukudome *et al.*, 2010). Concurrent VPA treatment did not affect protein phosphorylation in Huh7, a result distinct from those observed in published work, where VPA has been shown to modulate both pGSK3β and pERK1/2 by increasing and reducing phosphorylation levels respectively. The data therefore suggest that Huh7 cells exposed to hypoxia and hypercapnia do not appropriately reflect the cellular conditions during haemorrhagic shock.

As acute exposure of Huh7 to hypoxia and hypercapnia was not sufficient to produce the changes in protein phosphorylation observed *in vivo*, cells were exposed to adverse gas conditions in conjunction with suboptimal temperature (hypothermia). The presence of hypothermia, which often occurs spontaneously during or after massive blood loss, is associated with increased lethality in clinical practice (Shafi *et al.*, 2005). Cells were exposed to a temperature of 32°C which is classified as moderate hypothermia (Kheirbek *et al.*, 2009) and beyond the clinical threshold for coagulopathy risk (Cosgriff *et al.*, 1997) alongside the previously used adverse gas concentrations. Combined hypoxia, hypercapnia, and hypothermia significantly reduced GSK3β Ser9 phosphorylation to 60% of normoxic levels. This reduction was reversed

in a dose-dependent manner during VPA treatment, to 91% of untreated normoxic control at a treatment concentration of 0.5mM and to 128% at 0.75mM. These results mimic those found *in vivo*, where Hwabejire and colleagues (2014) showed that VPA dose-dependently reverses the decrease of GSK3β phosphorylation caused by blood loss within an acute time period (1-6h) in a rodent model of haemorrhagic shock. In this study, GSK3β phosphorylation was decreased by approximately 65% by haemorrhagic shock, and rescued by VPA to approximately 150% of healthy animal values. Recovery was dose-dependent, with higher doses of VPA resulting in increased protein phosphorylation. The changes in GSK3β phosphorylation observed in Huh7 therefore replicate those seen *in vivo* to a remarkable extent which suggests that culturing Huh7 in simultaneous hypoxia, hypercapnia, and hypothermia may simulate signalling changes seen in severe blood loss *in vivo*.

Haemorrhagic shock-like conditions did not significantly affect ERK1/2 phosphorylation or total ERK1/2 levels in Huh7, nor did concurrent VPA treatment modulate these levels within four hours of treatment. These data contrast with a previously referenced study, in which Kochanek and colleagues (2012) showed that haemorrhagic shock increased ERK1/2 phosphorylation 2.5-fold after four hours in a Wistar-Kyoto rat model of shock, while VPA treatment induced a decrease of pERK1/2 to healthy levels. However, these researchers observed ERK1/2 regulation in lung and as the effects of haemorrhagic shock and VPA have been shown to be tissue-specific, data may vary

according to tissue types studied. This conclusion is supported by data presented by Patel *et al.* (2011), which demonstrates that pERK1/2 increases more than 2-fold in Wistar-Kyoto rat liver after haemorrhagic shock and is attenuated by therapeutic intervention with VPA. However, data on pERK1/2 in Huh7 do not replicate those seen in rodent liver after haemorrhagic shock and was therefore not investigated further.

To establish whether low oxygen, high carbon dioxide, and low temperature were sufficient, either individual or in paired treatment, to cause the reduction in pGSK3β observed during concurrent exposure, Huh7 cells were incubated in all possible adverse condition combinations for four hours. Only simultaneous exposure to all three treatments significantly reduced GSK3β Ser9 phosphorylation, while all other combinations had no significant effect. During lethal blood loss *in vivo* these three pathological changes cause and exacerbate each other *in vivo* (Gutierrez *et al.*, 2004; Hotter *et al.*, 2004; Kheirbek *et al.*, 2009) and are therefore unlikely to occur individually during severe haemorrhage. These data suggest that this model system can be used to analyse cell signalling events in blood loss.

4.3.2 Stress conditions in HepG2 and HEK293

To investigate whether the effect of adverse gas and temperature conditions on GSK3β phosphorylation in Huh7 could be replicated in another liver-derived cell line, HepG2 cells were exposed to these conditions for four hours with and without VPA treatment. In HepG2, neither adverse conditions nor VPA treatment significantly affected GSK3β phosphorylation. This difference in response to hypoxia,

hypercapnia, and hypothermia between Huh7 and HepG2 may be explained by differences in cell metabolism between cell lines. Dettmer and colleagues (2013) investigated metabolic regulation across a number of cell lines, including HepG2 and Huh7. Their analysis of relative amount of metabolites showed that these two cell lines differ in terms of kynurenine levels, which were higher in HepG2 than in Huh7. Kynurenine, a metabolite of tryptophan, has been of therapeutic interest as a GSK3β inhibitor in diabetes, cancer, affective disorders, and neurodegenerative diseases (Pele-Shulman *et al.*, 2008). In the liver, kynurenine is produced by tryptophan-2,3-dioxygenase while indoleamine-2,3-dioxygenase performs this role in most other tissues (Stone and Darlington, 2013). It is therefore possible that the tryptophan-kynurenine pathway is targeted by distinctive regulatory mechanisms in the liver compared to other tissues, which could provide an explanation for the tissue-specific effect of VPA on GSK3β phosphorylation. A recent study indicated that the up-regulation of the kynurenine pathway may be a component in VPA action, as demonstrated by an increase in kynurenine levels in response to acute VPA treatment (Maciejak *et al.*, 2013). This evidence combined highlights the possibility that the differences observed in pGSK3β between Huh7 and HepG2 in response to VPA treatment are due to different baseline activity of the kynurenine pathway. The difference in regulation may also play a part in the lack of change in pGSK3β observed in HepG2 in response to haemorrhagic shock-like conditions. Supporting evidence shows that both kynurenine and quinolinic acid (a kynurenine pathway metabolite) were increased in patients after

haemorrhage and therapeutic intervention (Heyes *et al.*, 1995). This suggests that a high basal level of kynurenine in HepG2 may prevent a replication of the pathway response seen *in vivo*, which may affect regulation of GSK3β phosphorylation or VPA action.

Differences in regulation of the kynurenine pathway are merely one potential cause for the distinct signalling responses observed in Huh7 and HepG2, as they also differ with regards to the presence of drug-metabolising enzymes as shown by Guo *et al.* (2010). This study analysed 251 enzymes in terms of their relative abundance in HepG2 and Huh7, of which several are present in significantly different levels in the two cell lines under discussion. Any one of these enzymes may be a key component in the cellular reaction to haemorrhagic shock and/or VPA treatment and therefore be responsible for the signalling differences observed. Thorough investigation of these differences may therefore yield further insight into the effect of simulated blood loss on cellular signalling. However, as Huh7 closely replicates changes in pGSK3β observed *in vivo*, this cell line was selected for in-depth experimental investigation and, as the effect of adverse culture conditions and VPA treatment did not regulate GSK3β in HepG2 this manner, HepG2 cells were excluded from further modelling experiments.

In order to explore the possibility of modelling haemorrhagic shock signalling in a cell line derived from independent source tissue, the kidney-derived cell line HEK293 was exposed to haemorrhagic shock-like conditions and treated with VPA. Stress conditions did not

significantly affect pGSK3β levels, but simultaneous VPA treatment caused a significant increase of GSK3β phosphorylation up to 381±38% compared to untreated normoxic controls. Patel *et al.* (2012) observed a similar 3-fold increase *in vivo* in kidney in response to therapeutic intervention after acute injury, where pGSK3β is not affected in response to haemorrhagic shock. Results gained from a rat kidney, however, show that GSK3β is significantly reduced (by approximately 40%) after haemorrhagic shock compared to healthy animals (Nandra *et al.,* 2013). The decrease in pGSK3β in response to these conditions is not replicated in HEK293 cells. However, the animals used in this study were treated with erythropoietin for three days prior to haemorrhage, which may have affected signalling in a manner not seen in patients with severe blood loss. As GSK3β inhibitors have been shown to attenuate renal injury (Dugo *et al.*, 2006) and GSK3β is highly phosphorylated at Ser9 and therefore inhibited in HEK293, further investigation into this cell line may determine whether it is suitable to model renal signalling during haemorrhagic shock.

4.3.3 Stress conditions in SH-SY5Y

In SH-SY5Y cells exposed to hypoxia, hypercapnia, and hypothermia levels of pGSK3β did not change significantly. Concurrent treatment with VPA had no effect, regardless of concentration. Total GSK3β protein levels were unaffected both by adverse conditions and VPA treatment. *In vivo* studies on the effect of VPA on GSK3β pathway protein phosphorylation during haemorrhage and brain injury have focused on Akt (Sinn *et al.*, 2007; Wang *et al.*, 2013), therefore the

results described in this chapter cannot be compared directly. However, Akt phosphorylation is reduced in shock and increased in response to VPA, which may cause a concurrent increase and reduction of GSK3β phosphorylation respectively. No such change was observed in SH-SY5Y undergoing haemorrhagic shock-like conditions. This is in contrast with results from a study into VPA action in SH-SY5Y, which found that GSK3β was dose-dependently inhibited by VPA, as indicated by increased Ser9 phosphorylation and reduced kinase activity (Chen *et al.*, 1999). Though this study was performed in standard cell culture conditions those data still diverge from the results discussed in this and the previous chapter, where VPA did not affect GSK3β regardless of culture environment. The differences seen between published data and those obtained in the experiments performed for this work show that the SH-SY5Y cell line did not replicate either *in vivo* or *in vitro* results. As the stress conditions used induce a change in GSK3β phosphorylation in Huh7 the lack of effect on pGSK3β in SH-SY5Y indicates that pathway regulation in response to adverse conditions is tissue specific. GSK3β Ser9 phosphorylation was therefore not further investigated in SH-SY5Y.

SH-SY5Y cells were then analysed for ERK1/2 phosphorylation and total protein level post-exposure to adverse conditions and VPA. Hypoxia, hypercapnia, and hypothermia did not significantly affect pERK1/2 (Thr202/Tyr204) compared to total ERK1/2 levels, but total ERK1/2 was significantly increased compared to β-tubulin loading control during VPA treatment (1mM) in haemorrhagic shock-like

conditions. In a related study, ERK1/2 phosphorylation was reduced in SH-SY5Y during simulated ischaemia and reperfusion (Zuo *et al.*, 2006). However, these experiments were performed under combined glucose and oxygen deprivation to simulate ischaemic conditions. The presence of an additional stressor such as glucose deprivation may explain why these results differ from those discussed in this chapter. *In vivo*, VPA increases ERK1/2 phosphorylation in rat hippocampal neurons after traumatic brain injury (Dash *et al.*, 2010), a result replicated in rat after intracerebral haemorrhage (Sinn *et al.*, 2007). It is likely that cellular signalling in SH-SY5Y is modulated differently than that in mature whole brains and that this dissimilar regulation is the cause for the disparity in results. When Noël and colleagues (2015) exposed SH-SY5Y cells to hypothermia (30°C), they found that phosphorylated ERK1/2 levels were reduced in response. It is possible that the combination of hypothermia with additional stressors prevented this change in pERK1/2 from occurring here. However, in all of the studies referenced above, total ERK1/2 levels were unaffected by stress conditions or therapeutic intervention. It is therefore unlikely that the data on ERK1/2 phosphorylation in SH-SY5Y discussed in this chapter replicate those seen *in vivo*.

To further investigate the effect of VPA treatment on total ERK1/2 levels during haemorrhagic shock-like conditions, *erk1/2* transcription levels were analysed utilising RT-PCR. Compared to untreated cells in standard culture, adverse conditions significantly increased *erk1/2* expression 2.5±0.3-fold. VPA treatment, however, did not significantly

modulate gene expression in standard or stress conditions compared to standard and stressed untreated cells respectively. This result contrasts with the previously discussed protein data, where VPA treatment does significantly increase ERK1/2 levels while stress conditions do not. This potential incongruity suggests that ERK1/2 may be post-transcriptionally regulated in this model. The RNA-binding protein Hu antigen R (HuR), which regulates transport and stability of mRNAs containing AU-rich domains, is known to regulate both MEK-1 and ERK1/2 (Brennan and Steitz, 2001; Wang *et al.*, 2010). HuR is involved in anti-apoptotic signalling and has been shown to mediate the cellular response to oxidative stress via MAPK phosphatase 1 (Abdelmohsen *et al.*, 2007; Kuwano and Gorospe, 2008). This RNA-binding protein has also been studied in SH-SY5Y, where it regulates HSP70 in oxidative stress (Amadio *et al.*, 2008). Therefore, Therefore, HuR may be involved in ERK1/2 signalling in haemorrhagic shock. Additionally, dysregulation of HuR has been implicated in a number of cancers (Blaxall *et al.*, 2000; Danilin *et al.*, 2010; Heinonen *et al.*, 2005). Distinct modes of regulation of HuR in SH-SY5Y compared to the non-immortalised cells found *in vivo* could potentially explain the differences in ERK1/2 regulation discussed in this chapter. Further investigation into HuR may therefore yield insight into ERK1/2 signalling in haemorrhagic shock-like conditions in SH-SY5Y. However, as the cell line did not accurately mimic the cellular response to severe blood loss in animal models, it was excluded from further experiments.

4.4 Summary

In this chapter, exposure of cell lines to haemorrhagic shock-like conditions of hypoxia, hypercapnia, and hypothermia produced cell line-dependent responses in total protein level and protein phosphorylation. These three stress conditions combined induced a significant reduction of GSK3β phosphorylation in Huh7 which was dose-dependently reversed by VPA treatment. These data replicate those seen in animal models *in vivo* and accordingly suggest that the *in vitro* system discussed in this chapter accurately models the regulation of pGSK3β observed in haemorrhagic shock. Therefore, further investigations into associated signalling changes are necessary to establish the suitability of Huh7 as a model system for severe blood loss.

Haemorrhagic shock-like conditions did not affect pGSK3β levels in HepG2, HEK293, or SH-SY5Y cells. However, concurrent VPA treatment increased pGSK3β levels 3.8-fold in HEK293 and increased total ERK1/2 levels in SH-SY5Y. The pGSK3β results from HEK293 partially reproduced those seen *in vivo*, while further investigation of *erk1/2* expression in SH-SY5Y suggested that post-transcriptional regulation may play a role in the effects observed. Signalling changes in these cell lines did not mimic those seen in the respective source tissues during haemorrhagic shock in animal models, and though either cell line may be used to gain insight into modulation of cell signalling during stress, both were excluded in favour of Huh7 which more accurately models *in vivo* signalling.

5

Characterising the effect of haemorrhagic shock-like conditions and the VPA mechanism of action in Huh7

5.1 Introduction

Haemorrhagic shock has a multitude of effects on an organism on a cellular level, as detrimental changes in the heart and vascular system leads to reduced tissue perfusion and oxygen transport which widely affects cellular processes (Angele *et al.*, 2008). Lack of oxygen triggers anaerobic metabolism and carbon dioxide accumulates, leading to acidosis (Rossaint *et al.*, 2006). This fall in pH alongside hypoxia, hypercapnia, and hypothermia causes cell stress, causing apoptosis and necrosis (Ulukaya *et al.*, 2011). VPA prevents cell death via pro-survival pathways in the liver after massive blood loss (Gonzales *et al.*, 2008; Hwabejire *et al.*, 2014) but does not modulate acidosis (Alam et al., 2009). It was therefore of interest to establish how VPA affected these parameters in the *in vitro* model system under investigation.

Haemorrhagic shock and VPA treatment affect cellular activity by inducing cytosolic signalling changes in affected cells *in vivo*. In liver cells after massive blood loss, GSK3β Ser9 phosphorylation is reduced (Alam *et al.*, 2009), which correlates with increased activity. GSK3β, when active, contributes to the degradation of β-catenin (Manning et al., 2007), the accumulation of which is associated with a pro-survival phenotype via induction of bcl-2 (Dahia, 2000). Akt, which inhibits GSK3β by phosphorylation at Ser9 (Frame et al., 2001; Buttrick and Wakefield, 2008), is dephosphorylated at Ser473 and thereby inactivated during blood loss conditions (Alessi *et al.*, 1996; Hwabejire *et al.*, 2014). Akt phosphorylation at Ser473 is mediated by PI3K, which in turn is inhibited by PTEN (Maehama and Dixon, 1998) and itself

inhibited by phosphorylation (Ross and Gericke, 2009). PTEN activity therefore decreases Akt activity (Stambolic *et al.*, 1998) leading to increased GSK3β action which, by the degradation of β-catenin, leads to increased apoptosis. This process is reversed by VPA treatment *in vivo* (Hwabejire *et al.*, 2014; Li *et al.*, 2008; Zacharias *et al.*, 2011). PTEN, Akt, and β-catenin are therefore prime targets for investigation of the suitability of Huh7 in adverse culture conditions as a model for haemorrhagic shock signalling.

In addition to cytosolic changes VPA treatment also modulates nuclear histone acetylation during massive blood loss (Li *et al.*, 2011) by inhibiting histone deacetylases (HDACs; Gurvich *et al.*, 2004; Marinova *et al.*, 2009; Phiel *et al.*, 2001; Sinn *et al.*, 2007). HDACs remove acetyl groups from histone amino acid residues (Thiagalingam *et al.*, 2006), thereby increasing the abundance of tightly packed heterochromatin (De Rujiter *et al.*, 2003) which is inaccessible to transcription machinery. In haemorrhagic shock, VPA treatment induces histone 3 lysine 9 acetylation which increases the amount of loosely packed euchromatin available to gene transcription machinery (Butt *et al.*, 2009; Gonzales *et al.*, 2008). Although there is evidence that HDAC inhibitors structurally unlike VPA have pro-survival effects in haemorrhagic shock (Lin *et al.*, 2007; Shults *et al.*, 2014; Zacharias *et al.*, 2011), there is also evidence to suggest HDAC inhibitory activity is not sufficient to mimic the beneficial effects of VPA (Dash *et al.*, 2010). This suggests that VPA may act through a separate mechanism to attenuate the effects of haemorrhagic shock. Investigation of the effect

of haemorrhagic shock-like conditions and concurrent VPA treatment on histone acetylation in Huh7 was therefore of interest in order to further characterise this cell line as model of massive blood loss.

In this chapter, the mechanism of VPA in providing protection against haemorrhagic shock-like signalling was investigated. Changes in response to stress conditions and concurrent VPA treatment in acidosis, cell viability, necrosis and apoptosis were quantified alongside their effects on cytosolic signalling and their impact on nuclear histone acetylation. These data were obtained to further characterise Huh7 as a potential *in vitro* model for haemorrhagic show and to allow comparison with the changes observed in animal models of severe blood loss to further indicate the suitability of this Huh7-dependent system as a model system.

5.2 Results

5.2.1 Huh7 cell state and viability

VPA treatment after haemorrhagic shock *in vivo* decreases the expression of genes which modulate necrosis (Dekker *et al.*, 2014) and reduces the size of necrotic lesions (Sinn *et al.*, 2007). To investigate the effect of VPA treatment on necrosis an assay was performed to indicate the production of LDH, a necrosis marker enzyme, in Huh7 cells after exposure to stress culture conditions (*Fig. 2*). Surprisingly, these conditions significantly reduced LDH release to 58±5% compared to untreated cells undergoing normoxic conditions. VPA treatment significantly reduced LDH release regardless of culture conditions, to 61±9% in normoxia compared to untreated cells, and to 63±6% of untreated cells undergoing adverse conditions in hypoxia, hypercapnia, and hypothermia (37±7% of untreated normoxia control). These data suggest that VPA protects Huh7 cells from necrosis regardless of culture conditions.

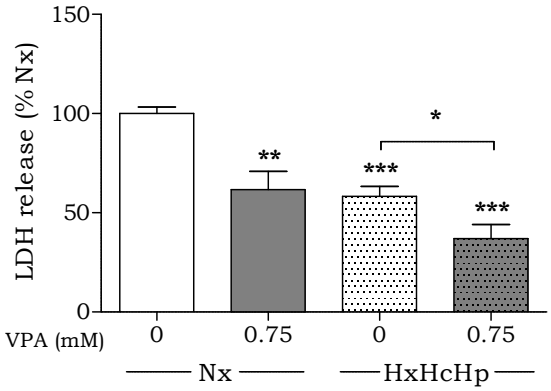

Figure 5.1 – Effect of hypoxia, hypercapnia, and hypothermia with concurrent VPA treatment on LDH release in Huh7. Huh7 cells were grown under standard conditions (37°C, 5% CO2) receiving fresh medium 16h prior to the start of treatment. Cells were treated with VPA (0.75mM) or vehicle control (dH_2O) and exposed for four hours to standard cell culture conditions (Nx) or to haemorrhagic shock-like conditions (HxHcHp; 2% O_2, 10% CO_2, 37°C) as indicated. At treatment end point, cell culture medium analysed. LDH presence was investigated by measuring formazan formation, where increase in LDH resulted in increased formazan levels and therefore increased light absorbance at 490nm. Data (n=5, technical triplicates) were analysed using ANOVA and Tukey test. * indicate significance compared to Nx 0 unless otherwise indicated: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. HxHcHp significantly ($p<0.001$) reduced LDH release compared to untreated Nx control.

Haemorrhagic shock induces apoptosis and reduces cell viability, changes which are partially blocked by VPA treatment in rat liver, porcine brain, and rat kidney (Butt *et al.*, 2009; Dekker *et al.*, 2014; Zacharias *et al.*, 2011). To study how haemorrhagic shock-like conditions affect Huh7 with regards to three enzymatic measures for cell state, cells were exposed to these conditions and analysed for viability, cytoxicity, and apoptosis. Viability was measured via cleavage of a specific substrate that is only performed by live cells, Cytotoxicity was measured by amount of substrate cleaved outside of cells by protease leaked from dead cells, and apoptosis was quantified via measurement of caspase-3/7 activity (*Fig. 3*). In normoxic conditions, VPA treatment did not affect cell viability, cytotoxicity, or apoptosis. Hypoxia, hypercapnia, and hypothermia did not significantly affect viability or cytotoxicity either, regardless of VPA treatment. However, these conditions significantly ($p<0.01$) increased apoptosis by 15±4%,

which concurrent VPA treatment reduced by 28±5% to a level of activity significantly ($p<0.001$) lower than that seen in untreated cells both in stress and in standard conditions. These results suggest that while the haemorrhagic shock-like model environment investigated does not affect cell membrane integrity within four hours, it does increase apoptotic signalling; an effect which is reversed if cells are simultaneously exposed to VPA. The data therefore provide evidence for an anti-apoptotic effect of VPA in Huh7 cells undergoing hypoxia, hypercapnia, and hypothermia.

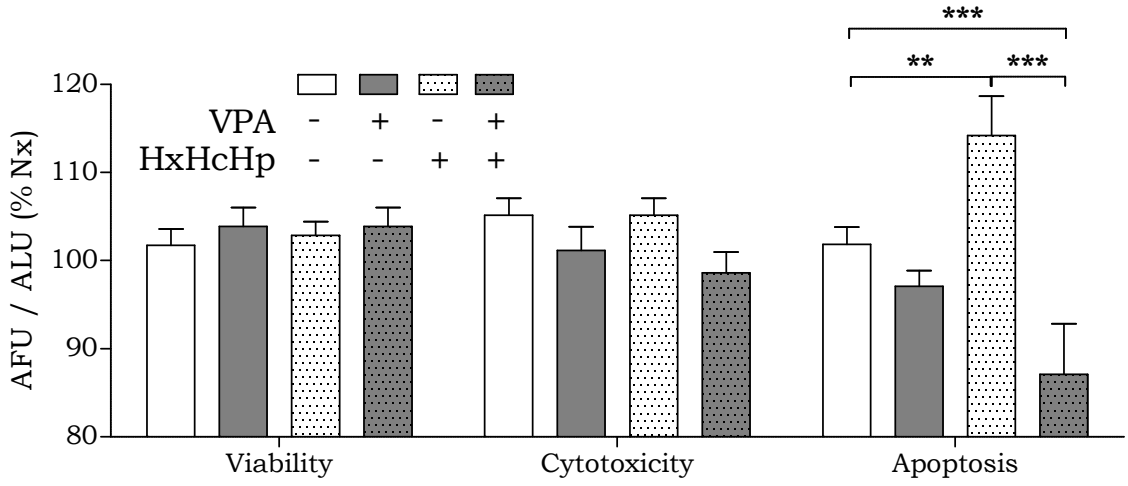

Figure 5.2 – Effect of hypoxia, hypercapnia, and hypothermia with concurrent VPA treatment on Huh7 cell viability, cytotoxicity, and apoptosis. Huh7 cells were grown under standard conditions (37°C, 5% CO2) receiving fresh medium 16h prior to the start of treatment. Cells were then treated with VPA (0.75mM) or vehicle control (dH_2O) and exposed for four hours to standard cell culture conditions (Nx) or to haemorrhagic shock-like conditions (HxHcHp; 2% O_2, 10% CO_2, 37°C) as indicated. Indicators of cell state (Viability, Cytotoxicity, and Apoptosis) were measured as in-well enzyme activity using a commercial assay (Promega). Briefly, substrate for the viability measure crosses cell membranes and is cleaved by a live-cell protease to yield fluorescence while substrate for the cytotoxicity measure cannot cross cell membranes and is cleaved to yield fluorescence by dead-cell protease leaked from dead cells which have lost membrane integrity. Apoptosis is investigated by addition of substrate for caspase-3/7 activity which luminesces when cleaved. Signal from cell state reactions was measured in arbitrary fluorescent units (AFU) for viability and cytotoxicity and arbitrary luminescence units (ALU) for apoptosis. Data (n=5, technical triplicates) were statistically analysed using ANOVA and Bonferroni test and compared to values from untreated Nx cells. * indicate significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Haemorrhagic shock *in vivo* causes acidosis – a reduction of pH below 7.1 (Angele *et al.*, 2008) – but this effect is not reversed by VPA treatment (Alam *et al.*, 2009). Huh7 cells were therefore exposed to haemorrhagic shock-like conditions of hypoxia, hypercapnia, and hypothermia to investigate the effect of these conditions and concurrent VPA treatment on culture medium pH (*Fig. 3*). The haemorrhagic shock-like conditions of hypoxia (2% O_2), hypercapnia (10% CO_2), and hypothermia (32°C) significantly reduced pH in Huh7 culture medium to 6.80±0.05 from 7.73±0.03 measured in standard (normoxic) conditions. These results suggest that VPA treatment does not significantly affect environmental (media) pH in standard or adverse culture conditions.

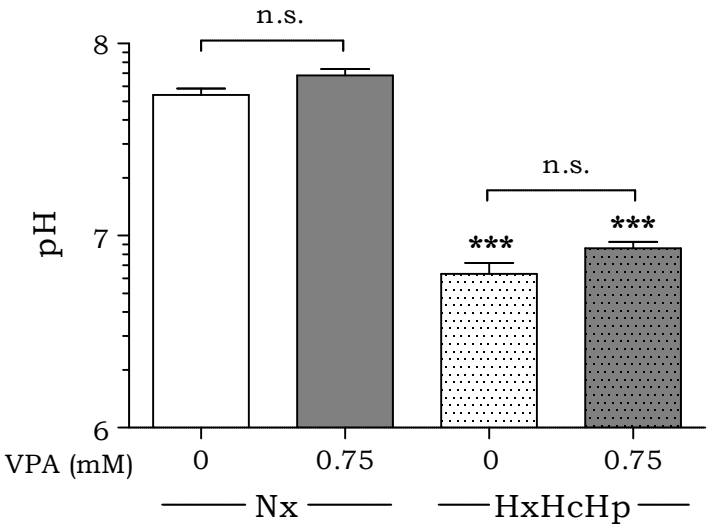

Figure 5.3 – Effect of hypoxia, hypercapnia, and hypothermia with concurrent VPA treatment on Huh7 culture media pH. Huh7 cells were grown under standard conditions (37°C, 5% CO_2) receiving fresh medium 16h prior to the start of treatment. Cells were treated with VPA (0.75mM) or vehicle control (dH_2O) and exposed for four hours to standard cell culture conditions (Nx) or to haemorrhagic shock-like conditions (HxHcHp; 2% O_2, 10% CO_2, 37°C) as indicated, after which pH was measured in culture medium. Data (n=5, technical triplicates) are presented as mean ± SEM and were statistically analysed (ANOVA and Tukey test). * indicate significance compared to Nx 0 unless otherwirse indicated: * $p<0.05$, ** $p<0.01$, *** $p<0.001$; n.s. not significant.

5.2.2 Cytosolic signalling in haemorrhagic shock-like conditions

Since earlier experiments described in this work indicated an inhibiting effect of VPA on GSK3β Ser9 phosphorylation, a range of experiments were employed to investigate the signalling mechanism leading to this result. First, the effect of haemorrhagic shock-like conditions and concurrent VPA treatment on PTEN regulation was examined. PTEN indirectly regulates GSK3β, as it drives dephosphorylation of PIP_3 to PIP_2, opposing PI3K action, which induces Akt activity, which in turn phosphorylates GSK3β at Ser9, thereby inhibiting this enzyme (Frame *et al.*, 2001; Buttrick and Wakefield, 2008; Maehama and Dixon, 1998; Stambolic *et al.*, 1998). As PTEN may be inhibited by phosphorylation itself (Rahdar *et al.*, 2009; Ross and Gericke, 2009; Vazquez *et al.*, 2001), total and phosphorylated PTEN abundance in protein extract from Huh7 cells was measured (*Fig. 4*). Hypoxia, hypercapnia, and hypothermia significantly increased PTEN levels to 140±12% compared to normoxic control, but VPA treatment did not affect total PTEN regardless of culture environment. However, a significant decrease in PTEN phosphorylation (to 69±9% of normoxia) caused by stress conditions was prevented by VPA (at 89±4% of normoxia). These results suggest that VPA blocks a drop in PTEN phosphorylation caused by simulated severe blood loss conditions, which increase total protein level, thereby potentially inducing heightened protein activity.

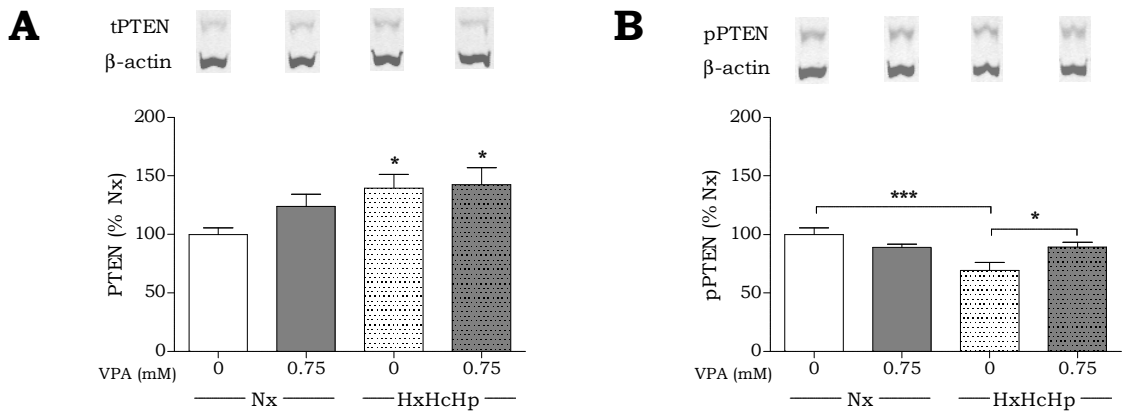

Figure 5.4 – Effect of hypoxia, hypercapnia, and hypothermia with concurrent VPA treatment on PTEN levels and phosphorylation in Huh7. Huh7 cells were grown under standard conditions (37°C, 5% CO2) receiving fresh medium 16h prior to the start of treatment. Cells were then treated with VPA or vehicle control (dH_2O) and exposed for four hours to standard cell culture conditions (Nx) or to haemorrhagic shock-like conditions (HxHcHp; 2% O_2, 10% CO_2, 37°C) as indicated. Protein extract was probed with phosphorylated PTEN (pPTEN; Ser380/Thr382/Thr383) and total PTEN (tPTEN) antibodies. Levels of β-actin served as loading control. GSK3β fluorescence was corrected for loading differences with β-actin. Total protein levels were calculated as ratio to β-actin and phosphorylated protein levels were calculated as ratio to total protein compared to levels in untreated Nx. Data (n=5, technical triplicates) are shown as mean ± SEM and were statistically analysed using ANOVA and Tukey test. * indicate significance compared to Nx 0 unless otherwise indicated: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

PTEN provides a critical role in regulating downstream Akt, the activity of which is inhibited by haemorrhagic shock via decreased Ser473 phosphorylation, an effect prevented by VPA treatment (Hwabejire *et al.*, 2014). To determine whether Akt responded similarly to haemorrhagic shock-like conditions *in vitro*, total and phosphorylated Akt levels from Huh7 cells exposed to hypoxia, hypercapnia, and hypothermia with concurrent VPA treatment were quantified (*Fig. 5*). Exposure of Huh7 to these conditions significantly increased total levels of Akt to 162±19% of normoxia control with no effect of VPA treatment regardless of culture environment. Hypoxia, hypercapnia, and hypothermia significantly reduced Akt Ser473 phosphorylation to 60±6% of normoxia control. This effect was reversed by concurrent VPA

treatment, which increased phosphorylation levels to 87±5% of untreated normoxia control (145±5% of untreated hypoxia, hypercapnia, and hypothermia control). These data suggest that adverse gas and temperature culture conditions increase Akt levels while decreasing phosphorylation, suggesting reduced kinase activity, and that this effect is reversed by simultaneous VPA treatment.

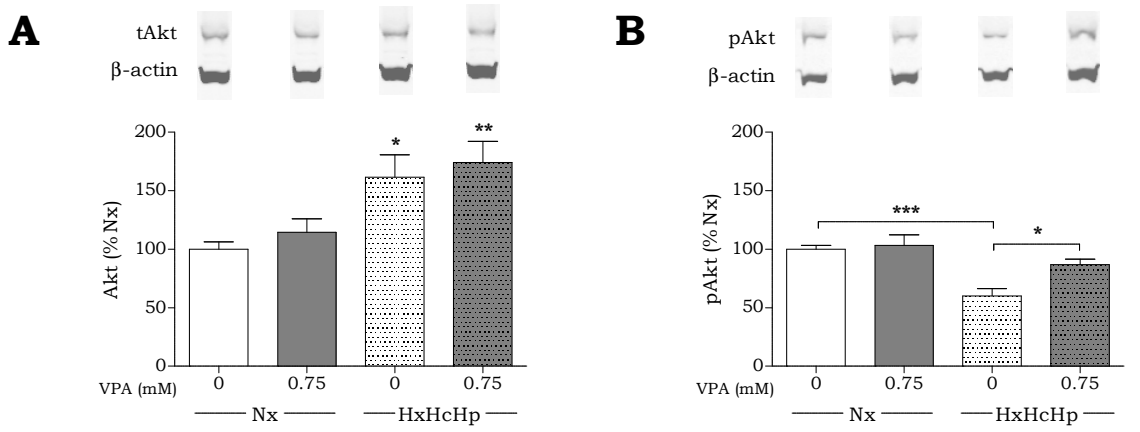

Figure 5.5 – Effect of hypoxia, hypercapnia, and hypothermia with concurrent VPA treatment on Akt levels and phosphorylation in Huh7. Huh7 cells were grown under standard conditions (37°C, 5% CO2) receiving fresh medium 16h prior to the start of treatment. Cells were then treated with VPA or vehicle control (dH_2O) and exposed for four hours to standard cell culture conditions (Nx) or to haemorrhagic shock-like conditions (HxHcHp; 2% O_2, 10% CO_2, 37°C) as indicated. Protein extract was probed with phosphorylated Akt (pAkt; Ser473) and total Akt (tAkt) antibodies. Levels of β-actin served as loading control. GSK3β fluorescence was corrected for loading differences with β-actin. Total protein levels were calculated as ratio to β-actin and phosphorylated protein levels were calculated as ratio to total protein compared to levels in untreated Nx. Data (n=5, technical triplicates) are shown as mean ± SEM and were statistically analysed using ANOVA and Tukey test. * indicate significance compared to Nx 0 unless otherwise indicated: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

The inhibition of GSK3β by VPA leads to an increase of β-catenin abundance, as active GSK3β primes β-catenin for ubiquitination and degradation (Liu *et al.*, 1999). This accumulation of β-catenin is associated with a pro-survival phenotype after severe blood loss (Alam *et al.*, 2009; Beurel and Jope, 2006). To determine whether haemorrhagic shock-like conditions affect β-catenin *in vitro*, total β-catenin levels were investigated in Huh7 treated with VPA during stress conditions (*Fig. 6*). Hypoxia, hypercapnia, and hypothermia significantly reduced β-catenin levels in Huh7 to 59±7% compared to Huh7 cultured in standard conditions. Concurrent VPA treatment blocked this trend and increased total β-catenin levels to 91±5% of control. These results provide evidence that VPA prevents the decrease of β-catenin levels in Huh7 caused by a haemorrhagic shock-like culture environment.

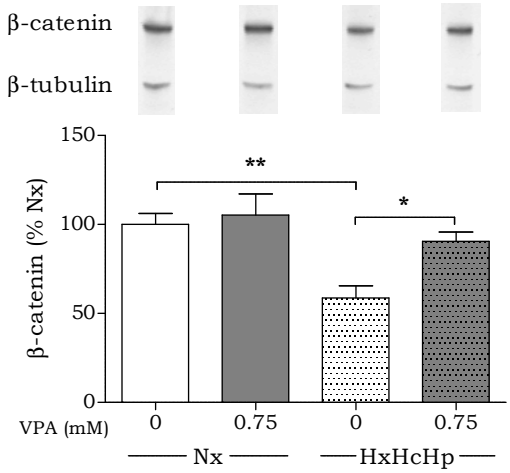

Figure 5.6 – Effect of hypoxia, hypercapnia, and hypothermia with concurrent VPA treatment on β-catenin levels in Huh7. Huh7 cells were grown under standard conditions (37°C, 5% CO2) receiving fresh medium 16h prior to the start of treatment. Cells were then treated with VPA or vehicle control (dH_2O) and exposed for four hours to standard cell culture conditions (Nx) or to haemorrhagic shock-like conditions (HxHcHp; 2% O_2, 10% CO_2, 37°C) as indicated. Protein extract was probed with β-catenin and β-tubulin antibodies, the latter serving as loading control. Total protein levels were calculated as ratio to β-tubulin and compared to β-catenin levels in untreated cells in Nx. Data (n=5, technical triplicates) are shown as mean ± SEM and were statistically analysed using ANOVA and Tukey test. * indicate significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

5.2.3 VPA regulates histone acetylation

The protective pro-survival effects of VPA treatment in animal models of haemorrhagic shock have been attributed to histone deacetylase inhibition, as VPA increases H3K9 acetylation *in vivo* (Butt *et al.*, 2009; Gonzales *et al.*, 2008; Sinn *et al.*, 2007; Zacharias *et al.*, 2011). To examine the effect of VPA on histone regulation in Huh7 undergoing haemorrhagic shock-like conditions, cell extract was quantified for histone acetylation levels in the presence and absence of VPA (*Fig. 7*). In normoxia, VPA treatment significantly ($p<0.01$) increased H4 lysine acetylation in normoxic culture conditions (to 406±39% compared to untreated control), but not H2/3 acetylation. In hypoxia, hypercapnia, and hypothermia, histone acetylation was

unchanged. VPA treatment in these stress conditions significantly ($p<0.01$) increased the level of H4 acetylation compared to untreated cells, to an extent not significantly different to that observed during VPA treatment in normoxia (395±116%). However, exposure to VPA significantly ($p<0.001$) increased H2/3 acetylation in stress conditions to 681±124%, a value significantly higher that seen in normoxia, both in the presence and absence of VPA. These data demonstrate that regulation of H4 acetylation by VPA independent of culture environment. This is in contrast to the effect of VPA on H2/3 acetylation, which is not affected in normoxia but observed to be significantly increased in cells exposed to hypoxia, hypercapnia, and hypothermia.

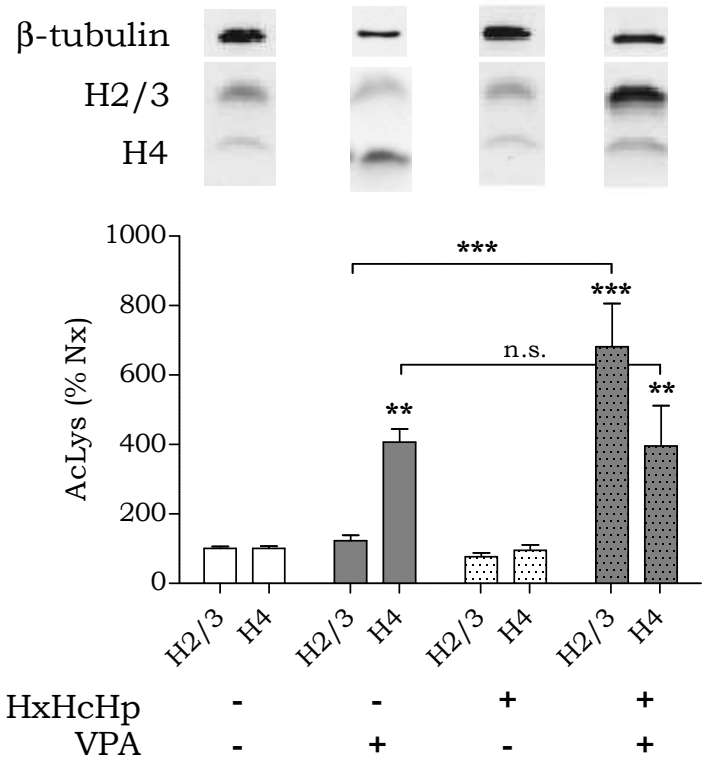

Figure 5.7 – Effect of hypoxia, hypercapnia, and hypothermia with concurrent VPA treatment on histone acetylation in Huh7. Huh7 cells were grown under standard conditions (37°C, 5% CO2) receiving fresh medium 16h prior to the start of treatment. Cells were then treated with VPA or vehicle control (dH_2O) and exposed for four hours to standard cell culture conditions (Nx) or to haemorrhagic shock-like conditions (HxHcHp; 2% O_2, 10% CO_2, 37°C) as indicated. Protein extract was probed with acetylated lysine and β-tubulin antibodies, the latter serving as loading control. Total acetylation levels were calculated as ratio to β-tubulin and compared to acetylation levels in untreated cells in Nx. Data (n=5, technical triplicates) are shown as mean ± SEM and were statistically analysed using ANOVA and Bonferroni test. * indicate significance compared to Nx 0 unless otherwise indicated: * $p<0.05$, ** $p<0.01$, *** $p<0.001$; n.s. not significant.

5.3 Discussion

5.3.1 Huh7 cell state and viability

In order to investigate the translational accuracy of Huh7 cells undergoing hypoxia, hypercapnia, and hypothermia as a model of haemorrhagic shock signalling, the effect of these stress conditions on cells was investigated in the presence or absence of VPA. In whole animal models, haemorrhage and shock have several negative effects on a cellular level. Firstly, inadequate tissue perfusion and a drop in oxygen levels induces anaerobic metabolism and therefore the accumulation of carbon dioxide, which leads to acidosis, a pathology so consequential it is billed as part of the lethal triad of haemorrhagic shock (Rossaint *et al.*, 2006). Secondly, adverse tissue state increases necrosis, and thirdly, cellular stress induces apoptosis, which may continue after animal resuscitation and therefore have a delayed effect (Ulukaya *et al.*, 2011). These parameters of cell state and viability were analysed in Huh7 in order to further characterise the proposed model system.

Severe blood loss causes acidosis (Alam *et al.*, 2009; Angele *et al.*, 2008; Hess and Holcomb, 2008), which negatively affects cell viability (Gores *et al.*, 1989). VPA treatment does not affect acidosis in animal models (Alam *et al.*, 2009) and therefore likely promotes cell survival by an independent mechanism. The pH of culture medium of Huh7 undergoing hypoxia, hypercapnia, and hypothermia fell to below the clinical acidosis threshold to 6.80±0.05, mimicking conditions *in vivo*.

This decrease was not reversed by VPA treatment, which was comparable to the lack of impact on pH by VPA observed in whole animal models. This result suggests that any beneficial effects observed in Huh7 in response to VPA were not mediated by a change in pH, analogous to *in vivo* results.

Cellular stress caused by hypoxia during haemorrhagic shock has been observed to cause necrosis in human liver (Helling, 2005; Ulukaya *et al.*, 2011). VPA has been shown to protect against necrotic processes in a swine model of haemorrhage as indicated by a decrease in necrotic gene expression (Dekker *et al.*, 2014). VPA also attenuated necrosis in a rat model of septic shock (Shang *et al.*, 2010) and protected against perihematomal cell death in intracerebral haemorrhage (Sinn *et al.*, 2007). However, there are some conflicting data from an *ex vivo* study on rat hepatocytes, where VPA treatment increased lactate dehydrogenase (LDH) release, a marker for necrosis (Kiang *et al.,* 2010). This disparity may be explained by a difference in culture environment, as the primary hepatocytes in this study were not exposed to stress conditions. Necrosis can be analysed by measuring LDH activity in culture medium, which is leaked from dead cells (Ulukaya *et al.*, 2011). Therefore, LDH release into Huh7 culture medium was investigated during stress conditions. VPA reduced LDH activity in cells undergoing both standard and adverse culture conditions, suggesting a decrease in necrosis similar to that observed *in vivo*. However, LDH activity was also reduced in cells exposed to stress conditions compared to those incubated in normoxia. These data may be explained by the complexity

of the pathological model environment used. Though ischaemia and concurrent hypoxia cause necrosis, hypothermia and acidosis have been shown to protect against and delay the hypoxia-induced necrotic process within an acute time frame respectively (Gores *et al.*, 1989; Hale *et al.*, 1997). To understand how these processes may attenuate each other, the underlying mechanism of ATP metabolism must be discussed.

One contributing cause of cellular effects during haemorrhagic shock is the process of ATP depletion caused by a change in cellular metabolism in response to blood loss. During haemorrhage, reduced tissue perfusion leads to a decrease in oxygen delivery while cellular oxygen uptake remains constant (Kheirbek *et al.*, 2009). Though compensatory mechanisms exist, these reach their limits during severe blood loss, necessitating a switch from aerobic to anaerobic metabolism in cells (Shoemaker, 1996). As cells are in a pathological (hypoxic, hypovolemic) state, ATP use exceeds ATP production, leading to ATP depletion and cell death (Keller *et al.*, 2002; Kheirbek *et al.*, 2009). Though this process continues unless prevented by resuscitation, the speed of progression may be affected by intra- and extra-cellular conditions such as acidosis and hypothermia. Both of these conditions were present in Huh7 cells undergoing haemorrhagic shock-like conditions and may therefore have affected cell death and LDH release.

Hypothermia may be beneficial or harmful *in vivo*, depending on timing and severity. Controlled hypothermia, as used in elective surgery, has long been known to be improve patient outcome and also

protects against a decrease in ATP levels (Kheirbek *et al.*, 2009). Spontaneous hypothermia caused by a slowing of ATP metabolism during haemorrhagic shock, however, is associated with an increase in patient death (Rossaint *et al.*, 2006). These two cases of hypothermia differ primarily in terms of time of onset: surgical hypothermia is induced prior to any potential ischaemia, while spontaneous hypothermia occurs after and because of haemorrhage (Kheirbek *et al.*, 2009; Midwinter, 2009). In the model system discussed, Huh7 were exposed to hypothermia alongside hypoxia and hypercapnia, which may have increased the protective effect of hypothermia, compared to a delayed onset of temperature reduction and therefore may explain the reduction in LDH release. Huh7 cells were also exposed to mild to moderate hypothermia, which is less likely to be harmful to patients *in vivo* (Kheirbek *et al.*, 2009) and is commonly used as a protective technique (Hale *et al.*, 1997; Jin *et al.*, 2014; Miyauchi *et al.*, 2014), supporting the hypothesis that hypothermia may protect Huh7 cells against death in this model. Therefore, both timing and severity of hypothermia in the Huh7 model system may provide protective cellular effects. However, as VPA action reduces LDH release, which indicates anti-necrotic action, in Huh7 and this result mimics those observed *in vivo*, the timing and severity of hypothermia induction was preserved.

Acidosis may also protect Huh7 cells against cell death and therefore lead to a decrease in LDH release and activity. Intracellular acidosis has been shown to delay cell death after ATP depletion (Gores *et al.*, 1989), but this protective effect of acidosis is time-limited and

does not ultimately prevent cell death. As cells are exposed to acidosis in the Huh7 model of haemorrhagic shock-signalling, the low pH may protect against necrosis in this system. As VPA does not affect culture medium pH, cells may benefit from this protective mechanism regardless of concurrent treatment, suggesting that LDH release in VPA-treated cells may be further reduced due to this combination of protective environmental condition and VPA treatment. However, though the preventative delaying effect of acidosis on LDH release and therefore necrosis is relevant within the acute time period under investigation, the selected conditions may ultimately overcome this protection via apoptotic mechanisms.

To further analyse the effect of hypoxia, hypercapnia, and hypothermia on cell state, marker enzyme activity in Huh7 was measured to investigate cell viability, cytotoxicity, and apoptosis. Cell viability and cytotoxicity measures depended on conserved and lost cell membrane integrity respectively. The substrate used in the viability measure may cross the cell membrane to be cleaved by a live cell protease, while the substrate for the cytotoxicity measure cannot and is therefore only cleaved by cell protease leaked from dead cells. Neither measure was affected by hypoxia, hypercapnia, and hypothermia with or without concurrent VPA treatment. Apoptosis, indicated by the activity of caspase-3/7, was increased during adverse culture conditions and decreased by simultaneous VPA treatment. Apoptosis may be caused by oxidative stress (Ulukaya *et al.*, 2011), which occurs during haemorrhage (Angele *et al.*, 2008), and against which VPA has

been demonstrated to be protective (Lee *et al.*, 2014). The increase of apoptosis during haemorrhagic shock-like conditions and the modulating effect of concurrent VPA treatment therefore suggest that VPA protects against apoptosis in the Huh7 model of severe blood loss in a manner analogous to the pro-survival action observed *in vivo*.

5.3.2 Cytosolic signalling in haemorrhagic shock-like conditions

GSK3β is regulated and modulated by a number of signalling pathways (Jope *et al.*, 2007; Katoh and Katoh, 2006; Phukan *et al.*, 2010; Wu and Pan, 2010) and several interacting enzymes have been implicated in the beneficial effects of VPA treatment in lethal blood loss. During haemorrhagic shock *in vivo*, GSK3β phosphorylation and Akt phosphorylation as well as total levels of β-catenin are reduced (Alam *et al.*, 2009; Hwabejire *et al.*, 2014; Li *et al.*, 2008). PTEN may modulate these changes via activity antagonistic to PI3K, which induces Akt phosphorylation by PIP_3 production, thereby affecting GSK3β and β-catenin activity (Hwabejire *et al.*, 2014; Wang *et al.*, 2011). GSK3β phosphorylation in Huh7 undergoing haemorrhagic shock-like conditions and VPA treatment mimics the *in vivo* response, the experiments in this chapter investigated changes to PTEN, Akt, and β-catenin to yield further insight into effects on cytosolic signalling.

In this chapter, the effect of hypoxia, hypercapnia, and hypothermia with concurrent VPA treatment on PTEN levels and phosphorylation in Huh7 was investigated. PTEN, like GSK3β, is inhibited by phosphorylation, at Ser380/Thr382/Thr383 (Rahdar *et al.*,

2009; Torres and Polido, 2001; Vazquez *et al.*, 2001). In a mouse model of traumatic brain injury, PTEN phosphorylation was observed to be decreased after ischaemia but increased after therapeutic intervention with Scriptaid, a HDAC inhibitor (Wag *et al.*, 2013). In a rat model of haemorrhagic shock, phosphorylation of Akt and GSK3β, which are downstream of PTEN in this signalling pathway and indirectly regulated by its activity, is reduced after severe blood loss and this effect is attenuated by VPA treatment (Hwabejire *et al.*, 2014). In Huh7, haemorrhagic shock-like conditions increased total PTEN abundance, but decreased PTEN phosphorylation. VPA did not significantly affect total PTEN abundance but did prevent the fall in pPTEN levels. Therefore, the combined increase of total protein level and decrease in protein phosphorylation suggests PTEN activity increases in Huh7 during haemorrhagic shock-like conditions. As observed *in vivo*, VPA treatment inhibits both GSK3β and PTEN activity in Huh7 undergoing these conditions by increasing protein phosphorylation. These data provide evidence that the regulation of PTEN signalling in Huh7 in haemorrhagic shock-like conditions functions analogously to that observed during haemorrhagic shock *in vivo*.

The literature on potential direct or indirect interaction between VPA and PTEN is scarce. VPA has been shown to increase PTEN expression in human prostate and ovarian cancer (Gravina *et al.*, 2009; Lin *et al.*, 2008) and in the macrophage-like RAW 264.7 cell line, thereby inhibiting components of the lipopolysaccharide-induced inflammatory response (Jambalganiin *et al.*, 2014). There is as yet no

published data on the effect of VPA on PTEN phosphorylation. However, VPA has widely been investigated in connection with PI3K and PIP_3 signalling, which may indirectly inform further work involving PTEN regulation. PI3K phosphorylates PIP_2 to the PIP_3, a second messenger for Akt (Delcommenne et al., 1998; Toker and Cantley, 1997). This process is balanced by PTEN activity, which dephosphorylates PIP_3 to PIP_2 (Maehama and Dixon, 1998). VPA has been shown to prevent a reduction in PIP_3 signalling during seizures (Chang et al., 2014; Xu et al., 2007), and to act in the regulation of inositol phosphates in bipolar disorder (Williams et al., 2002; Harwood and Agam, 2003). While this evidence may implicate PTEN action in pathologies as diverse as haemorrhagic shock, epilepsy, and bipolar disorder, the published evidence does not provide an explanation for the mechanism through which VPA acts on PTEN. Therefore, further investigation is needed to determine how PTEN may be regulated by VPA in the Huh7 model of haemorrhagic shock.

As discussed, PTEN inhibits Akt indirectly by dephosphorylateion of PIP_3 to PIP_2 (Stambolic *et al.*, 1998) which leads to a reduction in GSK3β Ser9 phosphorylation. An increase in PTEN activity in stress conditions would therefore be expected to decrease Akt activity by inhibiting phosphorylation at Ser473 and concurrent activation (Alessi *et al.*, 1996). To investigate whether Akt is regulated in a manner similar to PTEN and GSK3β during hypoxia, hypercapnia, and hypothermia, total and phosphorylated protein levels in Huh7 were analysed. These stress conditions increased total Akt levels and

decreased Akt phosphorylation. VPA treatment did not affect total Akt levels in these conditions, but it did significantly increase Akt phosphorylation at Ser473. The decrease in pAkt induced by hypoxia, hypercapnia, and hypothermia suggests a reduction of Akt activity, which correlates with an increase in PTEN and GSK3β activity and mimics results seen in haemorrhagic shock *in vivo* (Hwabejire *et al.*, 2014). These results suggest that haemorrhagic shock-like conditions and VPA treatment regulate GSK3β phosphorylation via Akt modulation.

Levels of β-catenin are reduced in animal models during severe blood loss and rescued by concurrent VPA treatment (Alam *et al.*, 2009; Li *et al.*, 2008). As β-catenin is primed for degradation by GSK3β (Hart *et al.*, 1998; Kitagawa *et al.*, 1999; Liu *et al.*, 1999), levels of total β-catenin were measured in Huh7 undergoing severe blood loss-like conditions with simultaneous VPA treatment. Exposure of cells to hypoxia, hypercapnia, and hypothermia induced a significant decrease in β-catenin levels, while concurrent VPA treatment protected against this change. These results mimic those observed *in vivo* and therefore suggest that β-catenin regulation in Huh7 during haemorrhagic shock-like conditions appropriately models that seen in animal models.

In Huh7 undergoing haemorrhagic shock-like conditions enzyme activation is regulated in a manner distinct from that observed Huh7 cells cultured in standard conditions and this regulation is further modulated by concurrent VPA treatment (*Fig. 8*). PTEN and Akt phosphorylation is decreased, which may contribute to the concurrent

decrease in GSK3β phosphorylation discussed in the previous chapter. These changes in signalling may contribute to the degradation of β-catenin also observed in adverse culture conditions. VPA treatment reverses the balance on PTEN, Akt, and (as previously discussed) GSK3β phosphorylation, as well as increasing total levels of β-catenin. Inhibition and activation of this pathway is analogous to the pathway regulation observed during haemorrhagic shock and concurrent VPA treatment *in vivo*, which provides evidence for the suitability of Huh7 undergoing hypoxia, hypercapnia, and hypothermia as a model for cytosolic signalling during haemorrhagic shock.

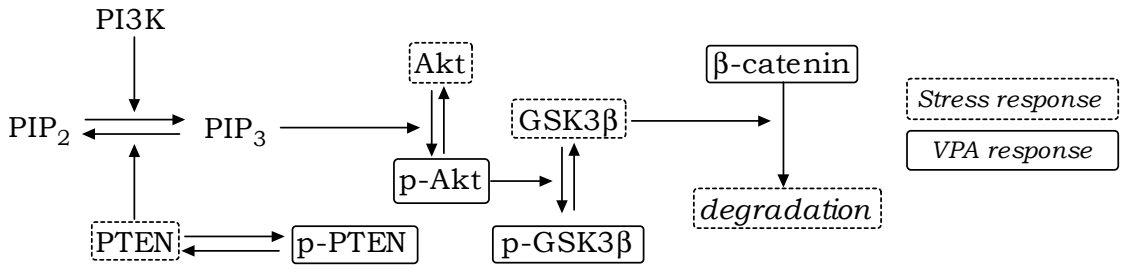

Figure 5.8 – Summary of enzyme regulation in Huh7 undergoing haemorrhagic shock-like conditions with or without VPA treatment. The experiments discussed in this chapter show that adverse culture conditions lead to a reduction of PTEN, Akt, and GSK3β phosphorylation as well as total β-catenin levels in Huh7. Simultaneous VPA treatment reverses the balance of enzyme activity by inducing PTEN, Akt, and GSK3β phosphorylation and β-catenin accumulation. Regulation of this pathway mimics results observed during haemorrhagic shock *in vivo*.

5.3.3 VPA regulates histone acetylation

VPA treatment increases H3K9 acetylation during haemorrhagic shock *in vivo* and much of the beneficial pro-survival effect of VPA has been attributed to its action as a histone deacetylase (HDAC) inhibitor (Butt *et al.*, 2009; Shults *et al.*, 2011; Zacharias *et al.*, 2011). VPA inhibits both predominantly nuclear and cytosolic HDACs directly, which may explain the broad effect treatment has on cellular signalling which has allowed VPA to be therapeutically utilised in a diverse range of pathological conditions (de Rujiter *et al.*, 2003; Göttlicher *et al.*, 2001; Hoffmann *et al.*, 2008; Phiel *et al.*, 2001; Thiagalingam *et al.*, 2003). In addition to indirect modulation of cellular signalling via an effect on gene expression, cytosolic proteins may be regulated directly by acetylation and de-acetylation (Abe *et al.*, 2000; Kouzarides, 2000; Li *et al.*, 2006; Wangsen *et al.*, 2008). As it has been shown that oxidative stress increases class I and II HDAC activity (Niu et al., 2015), it is possible that VPA may attenuate this increase in activity and exert some pro-survival effects in haemorrhagic shock via HDAC inhibition.

Investigation of the effect of VPA treatment on histone acetylation in Huh7 demonstrated that VPA affected histone acetylation in both standard and haemorrhagic shock-like conditions. Histone 4 acetylation was increased regardless of cell state, indicating that regulation of H4-acK was not specific to adverse culture conditions. However, H2/3-acK was regulated in a manner dependent on cellular gas and temperature environment: acetylation was up-regulated by VPA only in hypoxia, hypercapnia, and hypothermia and not affected in normoxia conditions.

This result suggests that the effect of VPA on H2/3 is dependent on the modified cell state induced by haemorrhagic shock-like conditions. As VPA increased H3 acetylation in Huh7 in a manner similar to that observed during severe blood loss *in vivo*, the evidence does not indicate that Huh7 undergoing simulated blood loss conditions are unsuitable as a model for changes in cellular signalling.

5.4 Summary

The experiments discussed in this chapter were performed to investigate the suitability of Huh7 cells exposed to hypoxia, hypercapnia, and hypothermia for the modelling of haemorrhagic shock signalling by comparing and contrasting changes in Huh7 cell state, protein modification, and total protein abundance to those observed in animal models. VPA treatment reduced necrosis and apoptosis in adverse cell culture conditions, suggesting a pro-survival effect. The decreases in PTEN and Akt phosphorylation as well as in total β-catenin levels induced by adverse gas and temperature conditions were rescued by concurrent VPA treatment, data which mimic those observed *in vivo*. VPA regulated H3 acetylation in a manner dependent on cell state which is analogous to changes observed in animal models. Therefore, the evidence discussed suggests that Huh7 undergoing hypoxia, hypercapnia, and hypothermia may be an appropriate *in vitro* model system for cellular signalling in haemorrhagic shock *in vivo*.

6

Investigating the therapeutic mechanism of action of VPA congeners and derivatives

6.1 Introduction

A key benefit to *in vitro* models of human pathologies is the potential use for high-throughput drug discovery. Often, structure-function studies based on existing medical drugs discover related structures with improved therapeutic potency (e.g. Chang *et al.*, 2015; Sobol *et al.*, 2006). Novel treatment options may also inform signalling research by virtue of comparing enzymatic or ligand activity of varying chemical structure to pre-existing knowledge of cellular signalling mechanisms (e.g. Maar *et al.*, 1997; Redecker *et al.*, 2000). The previously established Huh7 model for haemorrhagic shock signalling was therefore utilised to investigate compounds structurally related to VPA for enhanced potency in this model.

For this investigation, six branched and straight chain fatty acids and their derivatives were selected: 2-ene-valproic acid (2eVPA), 2-propyloctanoic acid (arundic acid; 2POA), valpromide (VPD), decanoic acid (DA), sebacic acid (SA), and octanoic acid (OA). These congeners of VPA were selected based upon potential for HDAC inhibition, as the therapeutic effect of VPA in haemorrhagic shock has been attributed to this mechanism (Butt *et al.*, 2009; Gonzales *et al.*, 2008; Li *et al.*, 2011). However, as HDAC inhibition has also been shown to not fully replicate a pro-survival effect in a model of cerebral ischaemia (Dash *et al.*, 2010), the divergent efficacies of the chosen molecules in other clinical contexts increased the likelihood of discovering an alternative mechanism of action. Finally, investigation of molecules structurally

similar to VPA was pursued in aid of discovering a novel, more potent therapeutic treatment for haemorrhagic shock.

VPA may affect nuclear processes not only by inhibiting HDACs but also through its action on transcription machinery, for instance by targeting peroxisome proliferator-activated receptors, part of the nuclear receptor family, which are known to be a part of the VPA-sensitive signalling network (Blaheta and Cinatl, 2002). Having bound a ligand, PPARs form a heterodimer with the retinoid X receptor and activate gene transcription by binding to PPAR response elements in gene promoters (Abdelrahman *et al.*, 2005). The subfamily of PPARs includes PPARα, β/δ, and γ, each with a range of ligands, target genes and biological roles (Burns *et al.*, 2007; Kota *et al.*, 2005). As PPAR activity has been implicated as an attenuating influence in ischaemic conditions (Collino *et al.*, 2006; Collino *et al.*, 2008; Culman *et al.*, 2007) and VPA is known to act as a ligand to all three receptor isoforms (Lampen *et al.*, 1999; Lampen *et al.*, 2001), PPAR involvement in Huh7 during haemorrhagic shock-like conditions and VPA treatment was investigated.

6.2 Results

6.2.1 VPA congeners regulate GSK3β phosphorylation

Six derivatives, analogues, and congeners of VPA were selected for investigation in the Huh7 model of haemorrhagic shock signalling (*Fig. 1*). As the pro-survival effect of VPA in haemorrhagic shock has been attributed to HDAC inhibition, all compounds were investigated for HDAC inhibitory activity using a commercial *in vitro* assay utilising nuclear HeLa extract as the source of HDAC activity (*Fig. 2*;). Of all compounds tested, 2POA was determined to be the most potent HDAC inhibitor analysed with an IC_{50} of 0.4mM, 6-fold lower than the IC_{50} of VPA at 2.3mM. The IC_{50} values for SA, DA, OA, and 2eVPA activity were found to be within the same order of magnitude as VPA, while VPD did not inhibit half of HDAC activity up to 10mM.

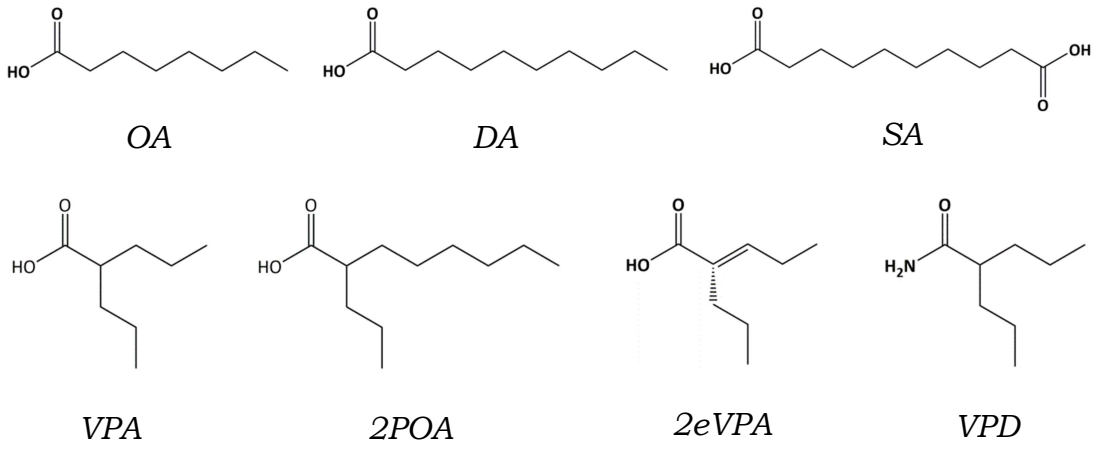

Figure 6.1 – Structural overview of VPA analogues, congeners, and derivatives. Three branched and three straight chain congeners and derivatives of VPA were selected for investigation in the Huh7 model of haemorrhagic shock signalling: decanoic acid (DA), sebacic acid (SA), octanoic acid (OA), 2-ene-VPA (2eVPA), 2-propyloctanoic acid (2POA), and valpromide (VPD).

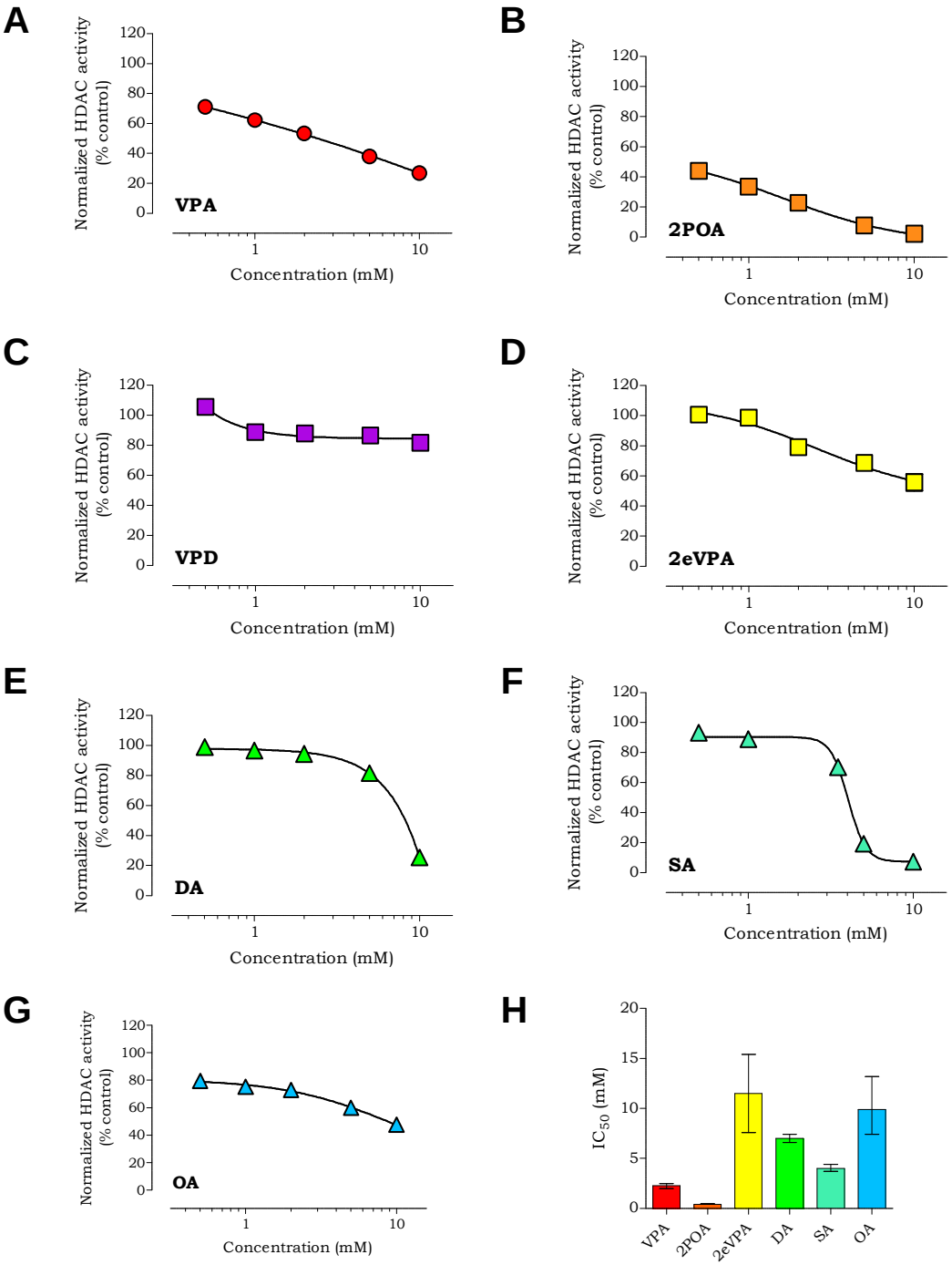

Figure 6.2 – Histone deacetylase inhibition activity of VPA and six related compounds. (A-G) HDAC inhibition activity of VPA, congeners, and derivatives was quantified employing HeLa nuclear enzyme extract as the source of HDAC activity and shown as fitted dose-response curves. Data (n=3, technical duplicates) are shown as mean ± SEM, however, error bars are not visible due to small SEM. (H) Comparison of HDAC inhibition IC_{50} values of VPA, congeners, and derivatives for three independent measurements at five concentrations (0.5-10mM). No IC_{50} for VPD was calculated as this compound did not inhibit half of activity at the highest concentration tested (10mM). Data are shown as mean ± 95% CI.

Since the effect of VPA in increasing GSK3β Ser9 phosphorylation in haemorrhagic shock has been linked to HDAC inhibition (Butt *et al.*, 2009; Shults *et al.*, 2008), compounds investigated for HDAC inhibition were then assessed in Huh7 to determine their efficacy in up-regulating GSK3β Ser9 phosphorylation. Huh7 cells were exposed to haemorrhagic shock like conditions and treated with these six molecules at 0.25 and 0.75mM (*Fig. 3*). Compounds found to have an effect at either of these treatment levels were then investigated at additional concentrations. In the absence of treatment, pGSK3β levels were reduced to 50±6% of normoxic levels. At 0.25mM, DA significantly increased pGSK3β levels to 110±22% and 2eVPA increased phosphorylation to 125±18% ($p<0.01$), compared to the increase seen after 0.75mM VPA treatment, which up-regulated phosphorylation to 128±16%. At 0.1mM, DA and 2eVPA increased phosphorylation still further, to 138±16% and 158±14% ($p<0.01$ and $p<0.001$) respectively. These two molecules did not significantly affect GSK3β phosphorylation at lower (0.05mM) or higher (0.75mM) concentrations. SA significantly increased pGSK3β at 0.75mM to 123±15%, comparable to the increase observed in response to VPA, and lower concentrations (0.25, 0.1mM) did not affect Ser9 phosphorylation. OA, 2POA, and VPD did not affect GSK3β phosphorylation at either 0.25mM or 0.75mM. The data therefore suggest that DA, and 2eVPA may be more potent than VPA under these conditions in increasing pGSK3β levels, while SA may replicate the dose-dependent increase in Ser9 phosphorylation seen after VPA treatment. Though DA, 2eVPA, and SA all inhibit HDAC activity as previously discussed, the increases in pGSK3β levels observed do not

correlate with higher IC_{50} values regarding HDAC inhibition. Surprisingly, the results therefore also suggest that the efficacy of congeners in increasing pGSK3β levels in the Huh7 model of haemorrhagic shock is independent of HDAC inhibitory activity.

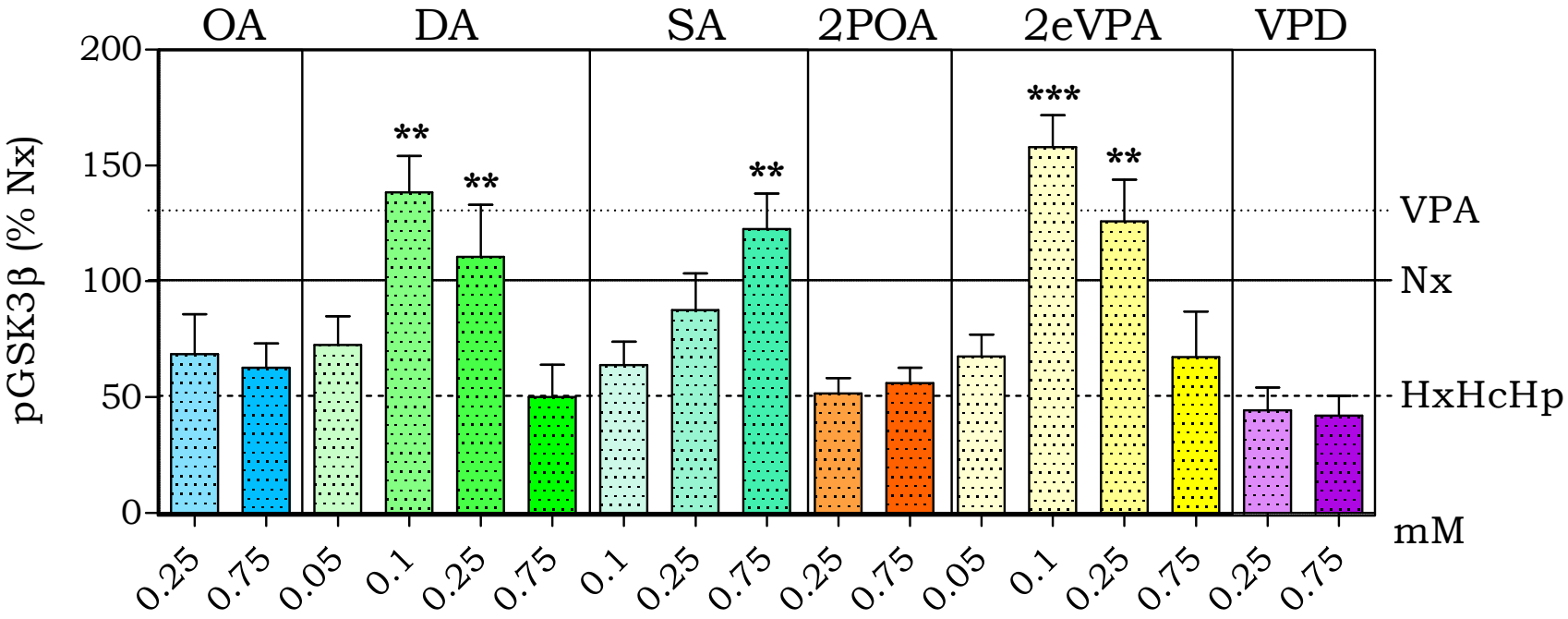

Figure 6.3 – VPA derived and congenic PPARγ ligands increase GSK3β phosphorylation in Huh7 during haemorrhagic shock-like conditions independent of HDAC inhibitory activity. Huh7 cells were cultured under standard conditions (37°C, 5% CO2) receiving fresh medium 16h prior to the start of treatment. Cells were then transferred to stress conditions (2% O_2, 10% CO_2, 32°C) and treated with VPA congeners and derivatives at two concentrations for four hours. Levels of pGSK3β (Ser9) and tGSK3β were corrected for loading with β-tubulin, quantified as phosphorylated to total GSK3β ratio, and presented as percentage of levels in untreated cells incubated in standard conditions (Nx). Data are shown as mean ± SEM. Previously obtained average pGSK3β levels in Nx, HxHcHp, and HxHcHp + VPA (0.75mM) are indicated by horizontal lines for ease of interpretation. Data (n=3, technical triplicates) were statistically analysed (ANOVA and Tukey test) to discover any significant difference to HxHcHp. * indicate significance compared to HxHcHp: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

6.2.2 VPA acts via PPARγ

VPA and the two analogues most efficacious in increasing GSK3β phosphorylation, 2eVPA and DA, are ligands of the transcription regulating peroxisome proliferator-activated receptors (PPARs; Lampen *et al.*, 2001; Malapaka *et al.*, 2012). To examine this mechanism, Huh7 cells were simultaneously treated with selective PPARα, β/δ, and γ inhibitors and VPA in haemorrhagic shock-like conditions. Inhibitors of PPARα (GW6471; 50μM; Abu Aboud *et al.*, 2013) and PPARβ/δ (GSK3787; 10μM; Palkar *et al.*, 2010) did not attenuate the effect of VPA on pGSK3β. However, the PPARγ inhibitor T0070907 (50μM; An *et al.*, 2014), prevented the VPA-dependent increase in GSK3β phosphorylation; this did not change significantly compared to that observed in untreated cells exposed to stress conditions (*Fig. 5A*). To verify whether PPARγ activation was sufficient to increase pGSK3β phosphorylation in these conditions, Huh7 cells were treated with Ciglitazone (Lehmann *et al.*, 1995), a specific PPARγ agonist structurally unrelated to VPA (*Fig. 5B*). Ciglitazone, like VPA, dose-dependently protected cell signalling against the reduction in pGSK3β levels under haemorrhagic shock-like conditions (122±16% at 20μM, 127±20% at 40μM, and 164±22% at 80μM). These results support a role for activation of PPARγ as a therapeutic treatment for haemorrhagic shock.

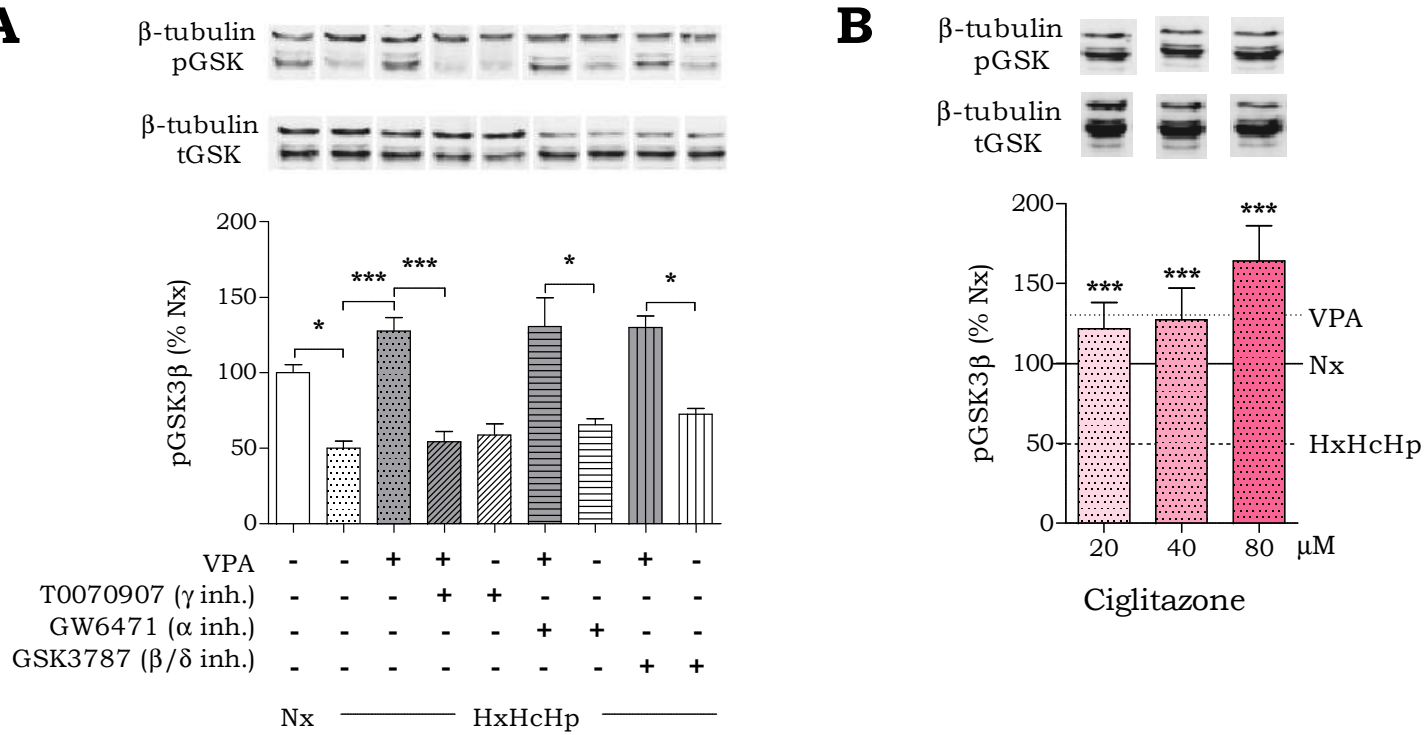

Figure 6.4 –PPARγ inhibition prevents VPA-induced GSK3β phosphorylation which is mimicked by PPARγ agonist Ciglitazone. Huh7 cells were cultured under standard conditions (Nx; 37°C, 5% CO2) receiving fresh medium 16h prior to the start of treatment. Cells were then transferred to stress conditions (HxHcHp; 2% O_2, 10% CO_2, 32°C) for four hours and treated with (A) VPA (0.75mM) in combination with the PPARα (GW6471, 50μM), β/δ (GSK3787, 10μM), and γ inhibitors (T0070907, 50μM), and with the inhibitors alone, or (B) the specific PPARγ agonist Ciglizatone. Levels of pGSK3β (Ser9) and tGSK3β were corrected for loading with β-tubulin, quantified as phosphorylated to total protein ratio, and are presented as percentage of levels in untreated cells incubated in standard conditions (Nx). Data are shown as mean ± SEM. Data (n=3, technical triplicates) were statistically analysed (ANOVA and Tukey test) to discover any significant difference to HxHcHp. * indicate significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

To investigate whether VPA and congener mechanism of action regulates gene transcription via PPARγ or a separate mechanism, Huh7 cells were exposed to stress conditions and concurrently treated with VPA and the transcription inhibitor Actinomycin at 1µg/ml (*Fig. 4*). GSK3β phosphorylation was significantly reduced by 31±5% in the presence of Actinomycin compared to cells which were treated only with VPA. This result suggested that the mechanism of action of VPA in regulating GSK3β Ser9 phosphorylation relies to some extent on gene transcription.

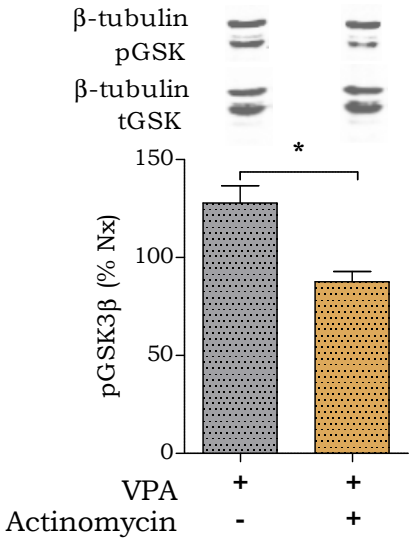

Figure 6.5 – Effect of Actinomycin on VPA-induced GSK3β Ser9 phosphorylation in Huh7 during hypoxia, hypercapnia, and hypothermia. Huh7 cells were cultured under standard conditions (37°C, 5% CO2) receiving fresh medium 16h prior to the start of treatment. Cells were then exposed for four hours to stress conditions (2% O_2, 10% CO_2, 32°C) and simultaneously treated with VPA (0.75mM) and Actinomycin (1µg/ml) as indicated. Protein extract was probed with phosphorylated GSK3β (pGSK3β; Ser9) and total GSK3β (tGSK3β) antibodies, as well as β-tubulin antibody as loading control, and visualised with fluorescent secondary antibody. GSK3β fluorescence was corrected for loading differences with β-tubulin. Phosphorylated protein levels were calculated as ratio to total protein and are shown as mean ± SEM compared to normoxia (100%). Data (n=6, technical triplicates) were statistically analysed (Student's t-test). * indicate significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

As pharmacological inhibitors may modulate signalling through off-target effects, a genetic approach to deplete PPARγ levels in VPA-treated cells was also employed to corroborate the evidence for involvement of this receptor. Treatment of Huh7 cells with an equal combination of four individual PPARγ siRNAs (Echeverri and Perriman, 2006) significantly reduced PPARγ protein abundance by 78±5% compared to untreated cells (*Fig. 6A*), whereas treatment with scrambled negative control (Ctrl) siRNA did not. Changes in pGSK3β levels in these cells were then assessed in haemorrhagic shock-like conditions, in the presence and absence of VPA (*Fig. 6B*). Cells treated with scrambled siRNA retained the VPA-dependent protection against pGSK3β reduction (93±5% compared to control). However, cell treated with PPARγ-specific siRNAs and showing a concurrent reduction in PPARγ abundance lost the protective effect of VPA on GSK3β Ser9 phosphorylation in these conditions, which resulted in a further reduction of pGSK3β levels (35±7% compared to control). These data further support an essential role for PPARγ activation in protection against haemorrhagic shock-like signalling in Huh7.

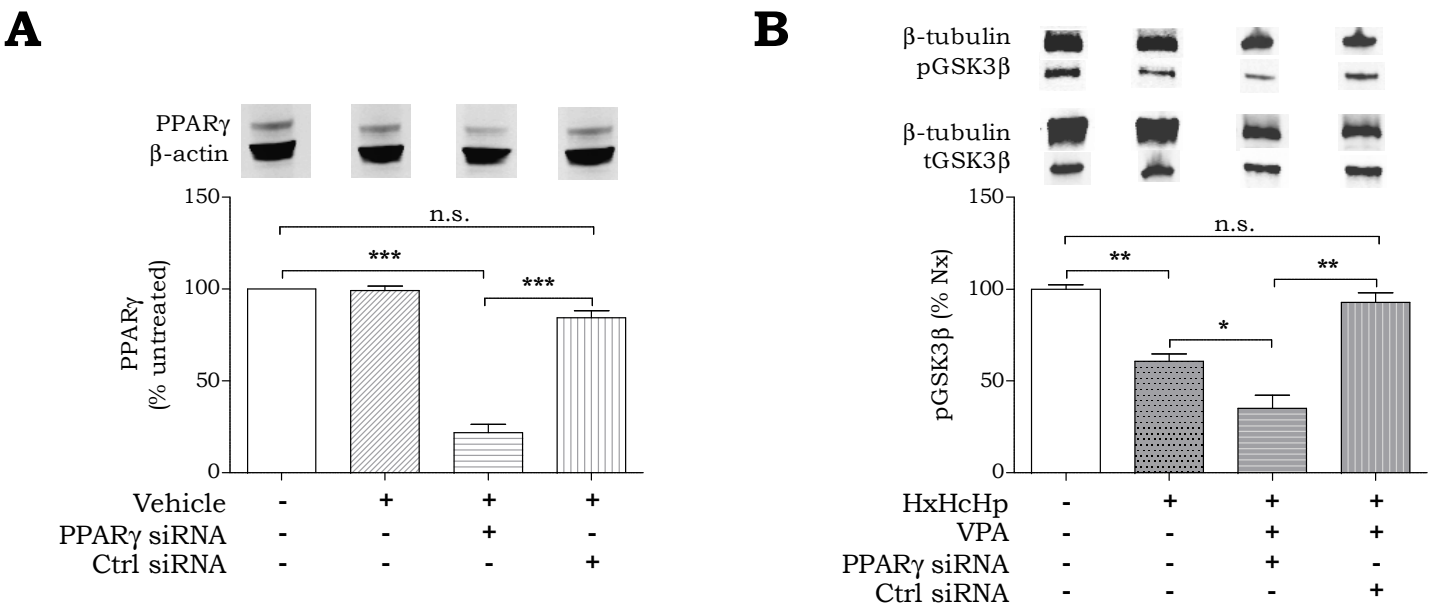

Figure 6.6 – Protection against haemorrhagic shock-Like signalling depends on PPARγ activity. (A) Huh7 cells were transfected with PPARγ siRNA, negative control (scrambled) siRNA, or transfection vehicle (RNAiMAX) for 48h. Protein extract was probed with PPARγ and β-actin antibodies. PPARγ levels were quantified and corrected for loading with β-actin. (B) Huh7 cells were transfected with PPARγ or control siRNA for 48h and then exposed to stress conditions (HxHcHp; 2% O_2, 10% CO_2, 32°C) with VPA treatment (0.75mM) as indicated for four hours. Control cells were treated with vehicle control (dH_2O). Levels of pGSK3β (Ser9) and tGSK3β were corrected for loading with β-tubulin, quantified as phosphorylated to total protein ratio. Protein levels are presented as percentage of levels in untreated cells incubated in standard conditions. (A, B) Data (n=3, technical triplicates) were statistically analysed (ANOVA and Tukey test) and are presented as mean ± SEM. * indicate significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

6.2.3 VPA treatment increases PPARγ transcription

PPARγ may be regulated by VPA through a variety of mechanisms, including via an increase in total PPARγ abundance (Abdelrahman *et al.*, 2004; Victor *et al.*, 2006). To investigate this possibility, mRNA was extracted from Huh7 cells undergoing stress conditions and simultaneous VPA treatment to quantify the expression levels of the *Pparγ* gene using a reverse transcription quantitative real-time PCR assay, with *Actin* mRNA abundance serving as a control (*Fig. 7*). VPA treatment during haemorrhagic shock-like conditions significantly increased *Pparγ* expression 1.5±0.1 fold compared to untreated normoxic cells ($p<0.05$). However, stress conditions alone did not significantly regulate abundance of PPARγ mRNA. These results suggest that PPARγ may be regulated as part of VPA-induced response.

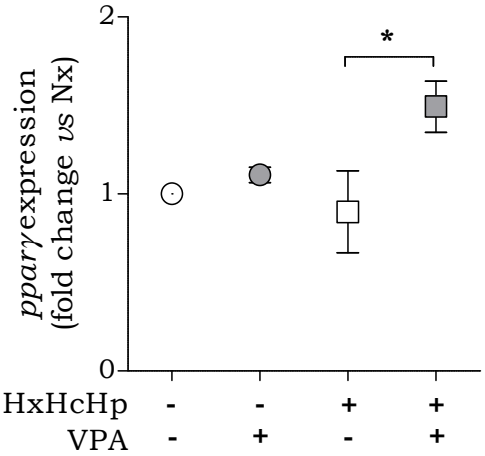

Figure 6.7 – VPA increases PPARγ mRNA in haemorrhagic shock-like conditions. Huh7 cells were cultured under standard conditions (37°C, 5% CO2) receiving fresh medium 16h prior to the start of treatment. Cells were then exposed to stress (2% O_2, 10% CO_2, 32°C) or standard conditions for four hours and treated with VPA (0.75mM) as indicated. Control cells were treated with vehicle control (dH_2O) or VPA as indicated and incubated concurrently in standard conditions. Huh7 mRNA was extracted and cDNA produced which served as the template for quantitative real-time PCR to quantify *Pparγ* expression (*Actin* expression was quantified as control). Data (n=3, technical duplicates) is presented as fold-change compared to untreated Nx samples. * indicate significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

6.2.4 PPARγ may be regulated by intracellular localisation

PPARγ functions in both the nucleus and the cytoplasm (Seger and Burgermeister, 2007) and has been variously observed to reside mainly in the nucleus (Berger *et al.*, 2000) or to be evenly distributed between nucleus and cytosol (Thuillier *et al.*, 1998). To investigate whether PPARγ localisation changed in haemorrhagic shock-like conditions or during VPA treatment, Huh7 cells were fixed, probed with PPARγ antibody, and stained to show nuclei (DAPI DNA stain; *Fig. 8A*). Quantification of fluorescence showed that 48±1% of total PPARγ was localised in the nucleus in normoxic conditions. Stress conditions significantly reduced nuclear PPARγ levels to 45±1%, while concurrent VPA treatment significantly increased cytosolic abundance of PPARγ to 57±1% compared to 55±1% in stress conditions alone (*Fig. 8B*). However, total levels of PPARγ were not affected (*Fig. 8C*). These data suggest that VPA have a small but significant effect on localisation of PPARγ as a component of the therapeutic mechanism of action observed in haemorrhagic shock signalling.

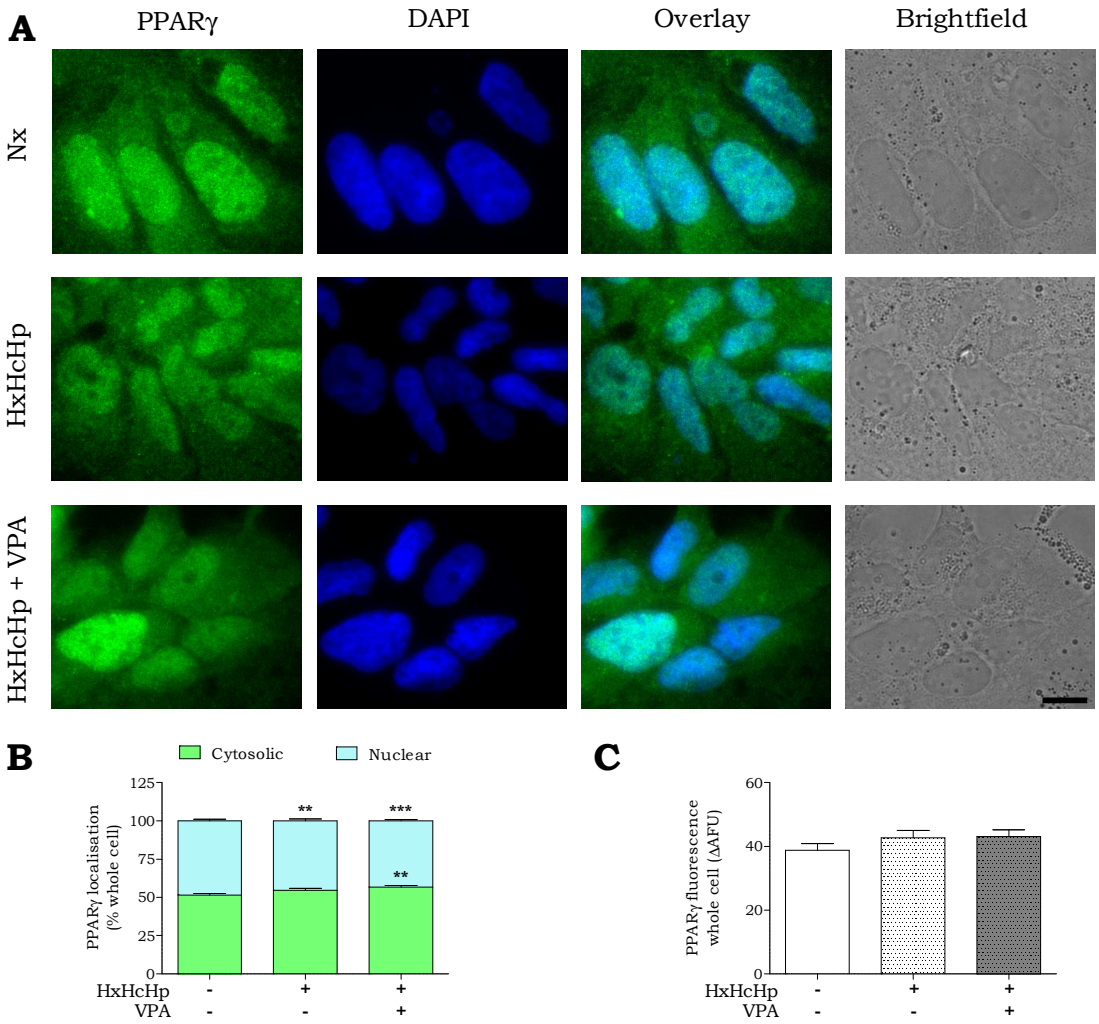

Figure 6.8 – Cellular localisation of PPARγ is affected by stress conditions and VPA treatment. Huh7 cells were cultured under standard conditions (Nx; 37°C, 5% CO2) receiving fresh medium 16h prior to the start of treatment. Two thirds of cells were then transferred to stress conditions (HxHcHp; 2% O_2, 10% CO_2, 32°C) and treated with VPA (0.75mM) as indicated. After treatment, cells were fixed with paraformaldehyde and probed with PPARγ antibody and fluorescent secondary antibody while cell nuclei were stained with DAPI. (A) Representative cells are shown. Size bar represents 10µm. (B) Cells (n=24 cells per treatment group per each of 3 independent experiments) were quantified for PPARγ nucleic and cytosolic fluorescence. The data were statistically analysed (ANOVA and Bonferroni test) and are shown as mean ± SEM stacked to 100% observed cell fluorescence. (C) Cells were quantified for PPARγ total cell fluorescence. The data were statistically analysed (ANOVA and Tukey test) and are shown as mean ± SEM. * indicate significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

6.2.5 PPARγ ligands prevent apoptotic signalling in Huh7

To establish whether PPARγ regulation was essential for the pro-survival effect of VPA, Huh7 cells were cultured in stress conditions with concurrent VPA and specific PPARγ inhibitor (T0070907) treatment and caspase-3/7 activity was measured as an indicator of apoptosis (*Fig. 9A*). T0070907 treatment prevented the anti-apoptotic effect of VPA (0.75mM, 80±4% of control), increasing caspase-3/7 activity by 28% to 108±4% of control and did not significantly affect apoptotic signalling in isolation (120±11% of control). To investigate whether PPARγ activation was sufficient to inhibit apoptotic signalling, Huh7 cells in haemorrhagic shock-like conditions were treated with the specific PPARγ agonist Ciglitazone (60μM) as well as 2eVPA (0.1mM) and DA (0.1mM). All three of these treatments significantly reduced apoptotic signalling to a similar extent as VPA, to 77±7%, 73±6%, and 77±4% respectively. These results suggest that the pro-survival effect of VPA is dependent on PPARγ activity and that specific PPARγ ligands successfully decrease Huh7 cell apoptosis.

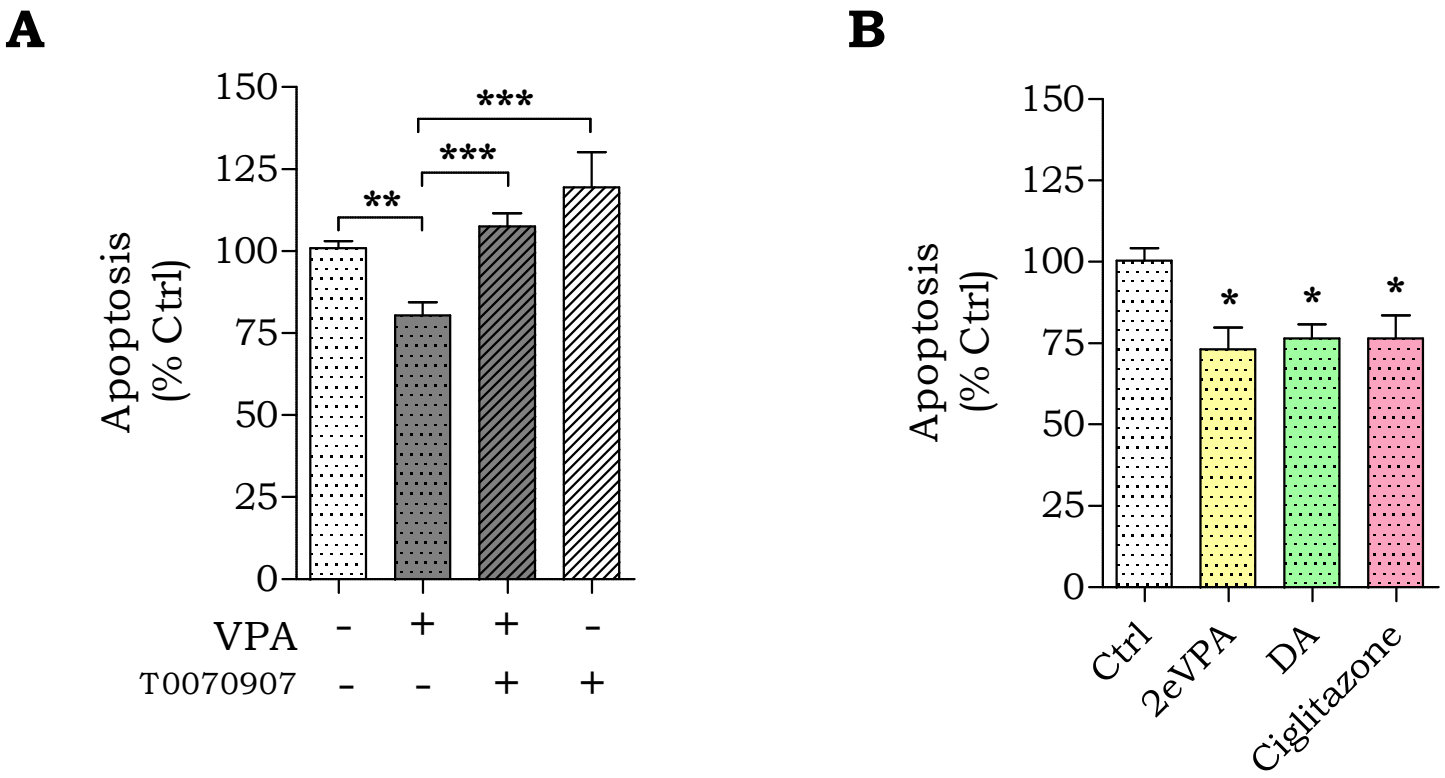

Figure 6.9 – Pro-survival action of VPA depends on PPARγ activity and is replicated by PPARγ ligands. Huh7 cells were grown under standard conditions (37°C, 5% CO2) receiving fresh medium 16h prior to the start of treatment. Cells were then treated with (A) VPA (0.75mM), vehicle control (DMSO), or PPARγ inhibitor T0070907, or (B) vehicle control (DMSO) or PPARγ ligands 2-ene-VPA (2eVPA), decanoic acid (DA), or Ciglitazone. Cells were exposed to haemorrhagic shock-like conditions (2% O_2, 10% CO_2, 37°C) for four hours. Apoptosis was measured as in-well enzyme activity using a commercial assay (Promega). Briefly, apoptosis is investigated by addition of substrate for caspase-3/7 activity which luminesces when cleaved. Signal from cell state reactions was measured in arbitrary luminescence units (ALU). Data (n=3, technical triplicates) were statistically analysed using ANOVA and Tukey test and compared to values from untreated cells. * indicate significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

6.3 Discussion

6.3.1 VPA congeners regulate GSK3β phosphorylation

The Huh7 model of haemorrhagic shock signalling was employed to determine whether a set of six molecules structurally similar to VPA could improve upon its ability to stimulate GSK3β Ser9 phosphorylation. Any such improvement would supply additional clues to the mechanism of action of VPA in this model. The six congeners, analogues, and derivatives of VPA under investigation were selected based on structural or functional relation to VPA and medical efficacy in other pathological conditions. 2eVPA is a major, non-teratogenic metabolite of VPA with improved anti-epileptic potency, but clinical trials for epilepsy were discontinued because 2eVPA is partially bio-transformed back to VPA *in vivo*, which was detectable in patient serum from 2 hours after administration (Bialer and Yagen, 2007; Düsing, 1992; Isoherranen *et al.*, 2003; Palaty and Abbott, 1995). 2POA, a homologue of VPA, has been of interest for the treatment of stroke and neurodegenerative disorders, although clinical trials for stroke were discontinued due to lack of proven efficacy (Bialer and Yagen, 2007; Ishibashi *et al.*, 2007). VPD, the less teratogenic primary amide analogue and prodrug of VPA, is approved as an anti-psychotic and anti-epileptic drug in several European countries as it is three to five times more potent than VPA in these pathologies (Bialer *et al.*, 1996; Isoherranen *et al.*, 2003; Radatz *et al.*, 1998). DA, a ten carbon straight chain fatty acid, accumulates in patients that follow the (anti-epileptic) medium-chain triglyceride ketogenic diet and has been shown to reduce

epileptiform activity more effectively than VPA (Chang *et al.*, 2013; Haidukewych *et al.*, 1982; Sills *et al.*, 1986). SA, the dicarboxylic form of DA, has been shown to improve plasma glucose levels in mouse models of diabetes (Kim *et al.*, 2008; Membrez *et al.*, 2010). OA is rapidly metabolized in rats *in vivo* and does not control epileptiform activity *in vitro*, though it has been shown to regulate ERK1/2 but not Akt in the PC12 cell line (Chang *et al.*, 2015; Kamata *et al.*, 2007; Liu and Pollack, 1994). These six compounds target a wide variety of signalling pathways, which would inform further investigations should a compound more potent than VPA in regulating the Huh7 model of haemorrhagic shock be found.

The beneficial mechanism of action of VPA in haemorrhagic shock has been thought to be via HDAC inhibitory activity (Butt *et al.*, 2009; Gonzales *et al.*, 2008; Li *et al.*, 2011), though Dash *et al.* (2010) found that HDAC inhibition alone was not sufficient to be protective in traumatic brain injury, as the selective HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) failed to produce behavioural improvements in their rodent model. To establish the HDAC inhibitory potency of each compound, an *in vitro* HDAC assay containing HeLa cell nuclear extract was employed. The IC_{50} values of these compounds mainly fell within one order of magnitude of one another (0.5-10mM). However, as VPD did not inhibit half of HDAC activity at the highest concentration studied (10mM), its IC_{50} value could not be calculated. However, when Huh7 cells were treated with these analogues, efficacy in increasing GSK3β Ser9 phosphorylation under these conditions was found not to

correlate with HDAC inhibitory activity. These data suggested, as did the *in vivo* work presented by Dash and colleagues (2010), that HDAC inhibitory activity is not sufficient to produce a change in GSK3β phosphorylation.

6.3.2 VPA acts via PPARγ

2eVPA and DA, two VPA analogues which significantly increased GSK3β phosphorylation in haemorrhagic shock-like conditions, are ligands of the peroxisome proliferator-activated receptors (PPARs; Lampen *et al.*, 2001). To determine whether PPARs are involved in VPA action in the Huh7 model of haemorrhagic shock, cells were treated with VPA and selective PPAR inhibitors in haemorrhagic shock-like conditions. Only the PPARγ inhibitor T0070907 prevented VPA from increasing GSK3β Ser9 phosphorylation, while the PPARα and PPARβ/δ inhibitors GW6471 and GSK3787 had no effect. None of these inhibitors affected Ser9 phosphorylation in the absence of other treatment. These data suggest that VPA acts specifically via PPARγ to modulate pGSK3β levels in the model of haemorrhagic shock developed here. To verify whether PPARγ activation is sufficient to produce an increase in Ser9 phosphorylation, Huh7 cells were treated with the selective PPARγ agonist Ciglitazone, the prototypical compound of the thiazolidinedione (TZD) family, other members of which are licensed for treatment of diabetes mellitus (Consoli and Formoso, 2013; Yau *et al.*, 2013). Ciglitazone, like VPA, increased GSK3β dose-dependently in haemorrhagic shock-like conditions, therefore providing further evidence for the involvement of

PPARγ in the mechanism of action of VPA and analogous molecules. To determine whether the VPA-induced increase of GSK3β phosphorylation was dependent upon gene transcription potentially mediated by PPARγ, Huh7 cells were co-treated with VPA and the transcription inhibitor Actinomycin. Actinomycin significantly inhibited the increase of pGSK3β levels by VPA, indicating the involvement of transcriptional processes in this regulation of GSK3β phosphorylation.

The results discussed here are supported by evidence from published literature. The PPARs are part of the nuclear receptor superfamily and promote target genes when in a heterodimer with the retinoid X receptor (RXR), binding to PPAR response elements in DNA (Abdelrahman *et al.*, 2005; Lefterova *et al.*, 2010). These receptors have been implicated in anti-inflammatory signalling and are known to be part of a VPA-sensitive signalling network (Lampen *et al.*, 1999; Werling *et al.*, 2001). Lampen *et al.* (2001) showed that while VPA ligand activity is approximately equal for all three PPAR receptors, 2eVPA displays double the affinity for PPARγ than it does for α and β/δ. DA is also a selective agonist of PPARγ, while OA, which did not increase GSK3β phosphorylation in Huh7, is not (Malapaka *et al.*, 2012; Nascimento *et al.*, 2012). The DA binding site of PPARγ is different to that occupied by TZDs like ciglitazone, occupying only a small part of the binding pocket and leaving the remainder untouched (Malapaka *et al.*, 2012). This snug fit may explain why shorter straight chain fatty acids (C6-C8) do not bind to PPARγ and why longer chain molecules (C12–C22) fit into a different part of the site, the same that rosiglitazone occupies. There are

no published data indicating SA may act as a ligand to PPARγ. Though the chains in both molecules contain the same number of carbon atoms, the structural dissimilarity between SA and DA (the presence of a second charged group in SA) suggests that if SA were to occupy the DA binding site, it would bind with lower affinity. This mechanism would correlate with the comparatively high concentration required to produce a significant increase of pGSK3β levels. Overall, the evidence discussed does suggest PPARγ may be involved in the cellular response to VPA in haemorrhagic shock-like conditions.

To corroborate the evidence for PPARγ involvement in the signalling mechanism under investigation and to exclude any off-target effects of the pharmacological PPARγ inhibitor T0070709, a genetic approach utilising siRNA to deplete PPARγ levels Huh7 cells was employed. Treatment of with PPARγ siRNAs significantly reduced PPARγ protein abundance and simultaneously inhibited the effect of VPA on GSK3β Ser9 phosphorylation. PPARγ depletion resulted in a larger reduction of pGSK3β levels than that seen in stress conditions alone, suggesting that PPARγ may be involved in the inherent cellular response to adverse conditions. This conclusion is supported by evidence from a study on the involvement of PPARγ in stroke, where T0070709 was shown to increase infarct size in the presence or absence of a PPARγ agonist, suggesting that endogenous PPARγ ligands may mitigate the effects of cerebral ischemia (Victor *et al.*, 2006). The results shown in this chapter in combination with the evidence from the literature

therefore provide strong evidence for a role of PPARγ in haemorrhagic shock.

6.3.3 VPA treatment increases PPARγ transcription

Cerebral ischaemia increases PPARγ abundance while simultaneously inhibiting its activity, a process which is fully reversed by the endogenous PPARγ agonist cyclopentenone prostaglandin 15-deoxy-$^{\Delta 12,14}$-PGJ_2 (15*d*-PGJ_2; Victor *et al.*, 2006). As 15*d*-PGJ_2 also protects against hepatic injury in haemorrhagic shock (Abdelrahman *et al.*, 2004), the possibility that stress conditions and VPA treatment in Huh7 may act via a modulation of PPARγ abundance was investigated. Huh7 *Pparγ* expression was analysed using reverse transcription quantitative real-time PCR and showed that VPA treatment significantly increased *Pparγ* expression in haemorrhagic shock-like conditions, while these conditions alone did not affect PPARγ abundance. This is in contrast with *in vivo* data, where ischaemic conditions increase PPARγ abundance which is unaffected by ligand treatments; ligands instead induces PPARγ activity by increasing DNA binding (Luo *et al.*, 2006; Ou *et al.*, 2006; Victor *et al.*, 2006). Therefore, though VPA may exert beneficial effects in Huh7 by promoting the accumulation of PPARγ, there is no evidence for such a mechanism in the literature. In addition, PPARγ levels do not respond to haemorrhagic shock-like conditions in Huh7, suggesting that the attenuating action of VPA in haemorrhagic shock-like conditions is not likely to depend upon a change in PPARγ abundance alone.

6.3.4 PPARγ may be regulated by intracellular localisation

Since PPARγ is found in both nucleus and cytosol and localisation may play a role in regulating activity, intracellular localisation of PPARγ was analysed in cells after undergoing stress conditions and VPA treatment. In these experiments, cells were fixed and incubated with PPARγ antibody, to show that stress conditions significantly reduced nuclear levels of PPARγ by 3% compared to normoxia, and concurrent VPA treatment significantly increased cytosolic abundance by 2% compared to stress conditions alone, while total abundance of PPARγ was unaffected. The magnitude of these changes may seem minor, but it was not possible to compare these changes to published results to ascertain their likely validity, as the literature often only contains primary imaging data without quantification. However, as PPARγ is often thought to exert its effects through regulation of transcription (Culman *et al.*, 2007; Lefterova *et al.*, 2010) these results may highlight a need for an alternative explanation for a putative increase in PPARγ activity correlating to an accumulation in the cytosol.

Aside from ligand activity and total abundance, PPARγ activity is affected by nuclear export and import (Han *et al.*, 2002; Kawahito *et al.*, 2000; Shibuya *et al.*, 2002). This mechanism has been suggested to be responsible for regulating nuclear activity of PPARγ, as it is known to recruit transcription factor co-activators when in the nucleus, thereby limiting access to these co-activators for other transcription factors (Abdelrahman *et al.*, 2005). The full significance of cytosolic PPARγ is as yet unclear and the mechanisms underlying the nuclear-cytoplasmic

shuttling of PPARs are not well characterised, but existing evidence shows that localisation and therefore intracellular transport are key components of PPARγ regulation (Burgermeister *et al.*, 2007; Umemoto *et al.*, 2012). PPARγ movement between intracellular compartments can be effected by ligand activity, Umemoto and colleagues (2012) have shown. These researchers found that the PPARγ ligand pioglitazone enhances both the nuclear import and export of PPARγ, but does not anchor PPARγ in the nucleus. Additionally, PPARγ is often trafficked in conjunction with binding partners (Umemoto *et al.*, 2012; Victor *et al.*, 2006). PPARγ may affect localisation of a bound protein by co-exporting it from the nucleus. This mechanism has been proposed to explain the regulation of NFκB by PPARγ, which binds p65, the transcriptionally active component of NFκB, and co-transports it from the nucleus into the cytosol, thereby inhibiting NFκB action and suppressing inflammation (Kelly *et al.*, 2004). It is therefore possible that VPA, 2eVPA, DA, SA, and Ciglitazone act to increase PPARγ shuttling in Huh7 and that a secondary mechanism may act during haemorrhagic shock to, for instance, down-regulate NFκB by p65 export with PPARγ, thereby promoting a pro-survival effect in treated cells.

6.3.5 PPARγ ligands prevent apoptotic signalling in Huh7

To determine whether VPA-induced cell survival is dependent on PPARγ action, caspase-3/7 activity was measured in Huh7 cells undergoing haemorrhagic shock-like conditions co-treated with T0070907 and VPA. T0070907 prevented the VPA-induced decrease in apoptotic signalling, suggesting that PPARγ activity is necessary for the

pro-survival effect of VPA. To verify these results, cells were incubated under haemorrhagic shock-like conditions and treated with the PPARγ ligands 2eVPA, DA, and Ciglitazone. All three structurally diverse agonists significantly decreased caspase-3/7 activity, indicating decreased apoptotic signalling. These data correlate with published results, which have shown that PPARγ ligands are beneficial in ischaemia-reperfusion injury, inflammation, and shock, as well as improving recovery after ischaemic stroke (Abdelrahman *et al.*, 2005; Culman *et al.*, 2007). Rosiglitazone, an equipotent PPARγ agonist of ciglitazone, attenuates caspase-3 activity in rabbit heart following ischaemia and reperfusion (Liu *et al.*, 2004; Tao *et al.*, 2003). Pioglitazone, another PPARγ ligand from the TZD family, prevents ischaemia-reperfusion injury in rat intestine in a dose-dependent manner (Abdelrahman *et al.*, 2005). Taken together with the data discussed in this chapter, these facts strongly support a role for PPARγ in the pro-survival effect of VPA while suggesting PPARγ ligands as a new class of therapeutic treatments in haemorrhagic shock.

6.4 Summary

Of the six VPA congeners, derivatives, and analogues tested in this chapter, three were effective in reproducing the increase in GSK3β Ser9 phosphorylation seen after VPA treatment in Huh7 cells undergoing haemorrhagic shock-like conditions. The efficacy of 2-ene-VPA, decanoic acid, and sebacic acid did not correlate with their potency as histone deacetylase inhibitors but was surprisingly found to be dependent on PPAR. Pharmacologic inhibition of all three PPAR isoforms showed that only the inhibition of PPARγ resulted in a block of the effect of VPA on GSK3β Ser9 phosphorylation in haemorrhagic shock-like conditions. Conversely, the specific PPARγ ligand Ciglitazone mimicked the dose-dependent effect of VPA on GSK3β phosphorylation. These data provided solid evidence for the involvement of PPARγ in the VPA mechanism of action in this model.

The PPARγ ligands investigated in this work significantly reduced apoptotic caspase-3/7 signalling in Huh7, suggesting they exert a pro-survival effect on Huh7 undergoing haemorrhagic shock-like conditions. *In vivo*, PPARγ agonists have been demonstrated to be beneficial in pathological conditions related to haemorrhage and shock. These results therefore provide evidence for the involvement of PPARγ in haemorrhagic shock-like signalling and indicate the need to investigate the suitability of PPARγ agonists as life-saving treatments in animal models of haemorrhagic shock.

7

Conclusion and Outlook

7.1 Huh7 cells model haemorrhagic shock signalling

In this work, Huh7 cells undergoing hypoxia, hypercapnia, and hypothermia mimicked haemorrhagic shock signalling changes seen in animal models *in vivo* (Alam *et al.*, 2009; Hwabejire *et al.*, 2014). Additionally, the signalling pathway analysed in this model system responded to VPA in the manner observed *in vivo*. These results suggest that Huh7 cells may be useful to provide an environment for in-depth molecular signalling research and as a system for high-throughput drug studies. Further research should investigate alternative cell lines for the variety of tissues characterised in haemorrhagic shock and future work must verify the results shown in this work in animal models to confirm the translational accuracy of the Huh7 model system.

VPA treatment reduced necrosis and apoptosis in Huh7 under haemorrhagic shock-like conditions, suggesting a pro-survival effect. VPA protects swine and rats from a lethal outcome after haemorrhage and shock (Alam *et al.*, 2009; Gonzales *et al.*, 2006; Hwabejire *et al.*, 2014; Shults *et al.*, 2008). As VPA is protective in both these model systems, Huh7 may be as a first-step investigative tool in the process of developing new therapeutica for *in vivo* use. By providing a first indicator of compound efficacy prior to animal testing, this system may significantly reduce the number of animals necessary for these investigations. The model established in this work may therefore contribute to lowering the cost and time required to develop a life-saving treatment for haemorrhagic shock.

7.2 VPA congeners show improved potency

In a first effort to identify compounds with improved efficacy compared to VPA in attenuating haemorrhagic shock-like signalling, the Huh7 model system was treated with several congeners of VPA, some of which are currently being trialled for medical use (Huhges et al., 2014; Asano *et al.*, 2005, as cited in Rogawski *et al.*, 2006; Wlaź *et al.*, 2015). Three compounds, decanoic acid, sebacic acid (a metabolite of DA; Kim *et al.*, 2008), and 2-ene-VPA, the unsaturated metabolite of VPA (Ghodke-Puranik *et al.* 2013), mimicked the dose-dependent increase of GSK3β phosphorylation induced by VPA in haemorrhagic shock conditions. The two more potent compounds, decanoic acid and 2-ene-VPA, were further found to inhibit apoptosis in the Huh7 model of haemorrhagic shock. Future work should investigate these two compounds in *in vivo* models to determine their potential as medical interventions in haemorrhagic shock.

Decanoic acid is a major component of the medium-chain triglyceride ketogenic diet (Hughes et al., 2014). It has been shown to dose-dependently reduce seizures *in vitro* and in an *in vivo* mouse model of epilepsy (Chang *et al.*, 2014; Wlaź *et al.*, 2015) and is currently supplied as a supplement ("Betaquik", Vitaflo International Ltd) to support MCT enrichment. *In vitro*, decanoic acid directly blocks AMPA-receptor generated currents (Williams, personal communication). Decanoic acid shows improved potency over VPA in reducing apoptosis and blocking a reduction in GSK3β Ser9 phosphorylation in the Huh7 model of haemorrhagic shock. Additionally, the primary decanoic acid

metabolite, sebacic acid, was also shown to reduce the decrease in GSK3β Ser9 phosphorylation. This suggests that, should decanoic acid be used as a treatment for haemorrhagic shock, both the compound itself and its metabolite would exert a pro-survival effect, potentiating what may be a life-saving mechanism.

Like decanoic acid, 2-ene-VPA is more efficacious than VPA in blocking dysregulated signalling and reducing apoptotic activity in Huh7 caused by haemorrhagic shock-like conditions. 2-ene-VPA has previously reached stage I clinical trials as a potential second-generation epilepsy treatment with reduced hepatotoxicity compared to VPA (Düsing, 1992). These trials were discontinued because 2-ene-VPA is partially biotransformed to VPA *in vivo*, minimising its non-toxic advantage over a chronic treatment period (Isoherranen *et al.*, 2003). However, this would be of little consequence in a potentially life-saving, acute intervention during the treatment of haemorrhagic shock. Additionally, a one-dose study in healthy male volunteers found that a very high (800mg) dose of 2-ene-VPA was well-tolerated by all subjects, who did not exhibit changes in vital signs, haematological or clinical chemistry parameters, or sedation beyond that seen in placebo controls (Düsing, 1992). Therefore, should 2-ene-VPA protect against lethal events in animal models in the same manner as VPA, further development would be accelerated by existing results from this stage I trial, encouraging a comparatively rapid progression to a life-saving treatment for terminal blood loss.

7.3 Haemorrhagic shock-like signalling depends on PPARγ

One key novel discovery made in this thesis is that PPARγ activity is required for the VPA-induced effect on GSK3β Ser9 phosphorylation and apoptotic signalling in Huh7 under haemorrhagic shock-like conditions. This conclusion is supported by the published literature, as VPA is known to be a ligand of PPARγ and both decanoic acid and 2-ene-VPA bind PPARγ preferentially compared to PPAR isoforms (Lampen *et al.*, 2001; Nascimento *et al.*, 2012). PPARs and PPARγ in particular have been studied as a potential therapeutic target in ischaemia, inflammation, and shock (Abdelrahman *et al.*, 2005; Collino *et al.*, 2008). These results provide strong evidence to recommend further investigation into the role of PPARγ in the VPA mechanism of action in haemorrhagic shock.

The data shown in this thesis suggest that total PTEN levels are increased in Huh7 undergoing haemorrhagic shock-like conditions, while concurrent VPA treatment increases PTEN phosphorylation, thereby down-regulating PTEN and leading to GSK3β inhibition via Akt. There are several potential mechanisms in which PPARγ activity may be linked to these changes. PPARγ binds the transcription co-factors CBP and p300, transrepressing other co-factor binding partners which no longer have access to these two proteins (Abdelrahman *et al.*, 2005). One of these binding partners is PCAF, which regulates gene transcription when bound to CBP and p300 (Li *et al.*, 2006; Nakatani, 2006). PCAF binds PTEN (Okumura *et al.*, 2006) and acetylates lysine residues within the catalytic cleft, thereby altering this structure

essential for PIP_3 binding beyond functionality (Lee *et al.*, 1999). PIP_3 goes on to activate Akt, a process which is inhibited by PTEN dephosphorylating PIP_3 to PIP_2 (Wang *et al.*, 2011). It may therefore be possible that increased PPARγ activity, caused by VPA binding, indirectly functions to decrease PTEN activity by preventing PCAF from acting through gene transcription regulation. This may increase the abundance of PCAF available for other catalytic activities, such as PTEN acetylation (*Fig. 1*). Analogously to histone regulation, conformational changes caused by PTEN acetylation predispose this protein to further inhibitory post-translational modification, such as the phosphorylation observed in Huh7. No data exist on interactions between PTEN acetylation and phosphorylation, so further work is needed. As PTEN acetylation was not examined in this work, future studies could investigate this potential pathway by determining PTEN acetylation in the Huh7 model of haemorrhagic shock.

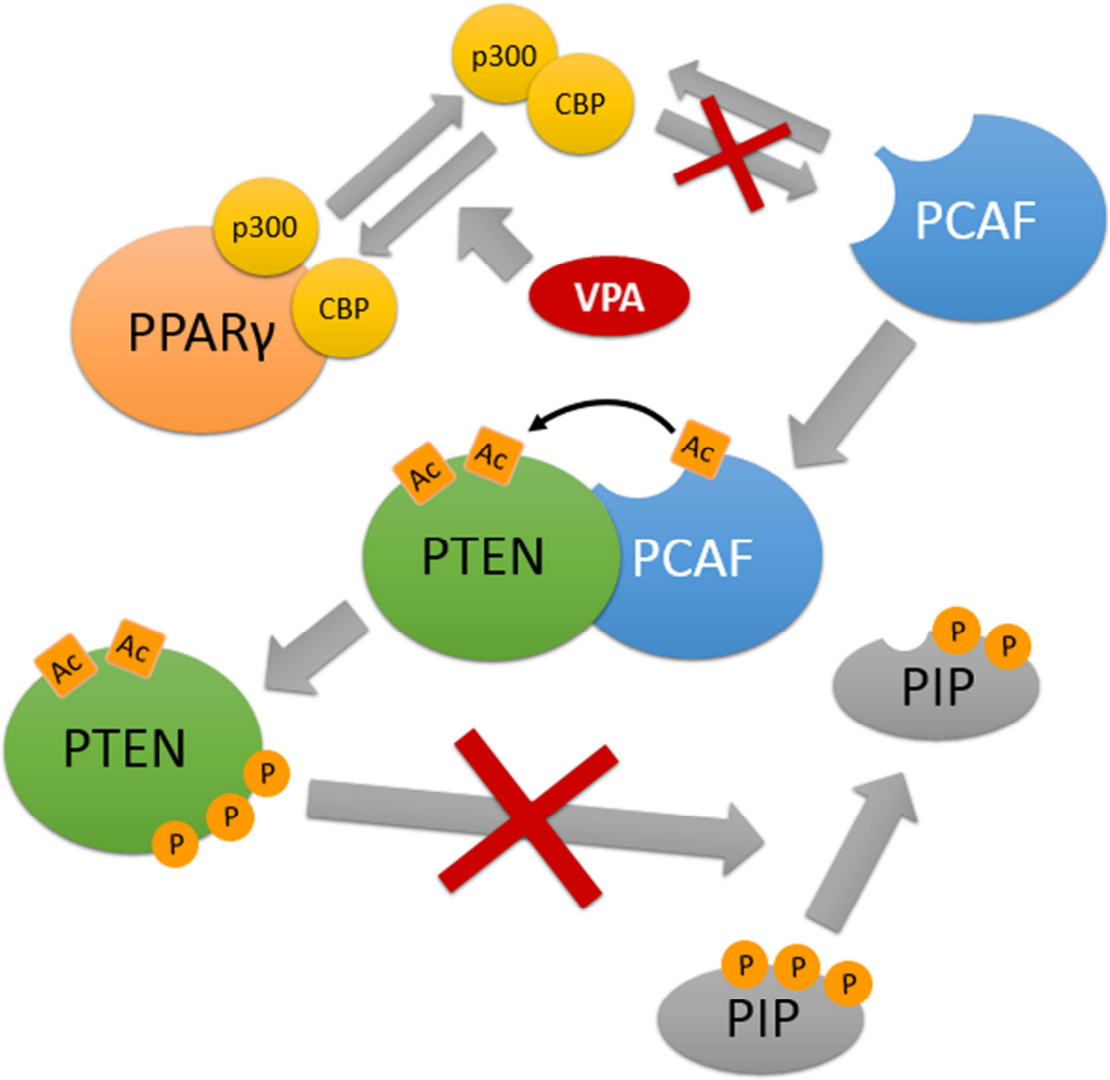

Figure 7.1 – A potential mechanism for VPA-induced PTEN inhibition via PPARγ. VPA interacts with PPARγ, activating it and causing PPARγ to bind the transcription co-factors p300 and CBP, thereby transrepressing other transcription factors, such as PCAF. This may result in high levels of PCAF not bound to DNA, which may then increasingly bind to other interaction partners, such as PTEN. PCAF acetylates (Ac) the PIP_3 binding site of PTEN, preventing the second messenger PIP_3 from being bound and dephosphorylated to PIP_2. This may lead to conformational changes which might predispose PTEN to further inhibition by phosphorylation, thereby increasing PIP_3 levels and downstream activity.

GSK3β, an enzyme of interest in several pathologies, has been linked to PPARγ action before. Akt and GSK3β phosphorylation were induced by the angiotensin II blocker Telmisartan, which thereby protected primary rat cerebellar granule cells against nutrient deprivation-induced apoptosis, an effect inhibited by a PPARγ antagonist (GW9662) and mimicked by the selective PPARγ ligand pioglitazone (Pang *et al.*, 2014). This study therefore describes the same regulatory pathway proposed in this thesis for haemorrhagic shock but in a neuronal context. In the rat brain, VPA protects cells from the detrimental effects of haemorrhagic shock and intracerebral haemorrhage (Li *et al.*, 2008; Sinn *et al.*, 2007). These data suggest that VPA may be able to regulate GSK3β in the brain via PPARγ. This may be of interest for other fields of research, such as that of bipolar disorder, where the action of VPA on GSK3β has long been debated.

The inhibition of GSK3β by VPA has been extensively debated for some time (Harwood and Agam, 2003; Phiel *et al.*, 2001; Ryves *et al.*, 2005; Shimshoni *et al.*, 2007). However, combining existing evidence with the discoveries made in this work may provide a different perspective on the matter. Ryves and colleagues (2005) reported that VPA did not inhibit GSK3β in cultured neocortical cells in standard cell culture conditions. These data do not conflict with the pathway regulation shown here, as the VPA-induced inhibition of GSK3β in Huh7 is dependent on the presence of haemorrhagic shock-like conditions. Instead, these results suggest that VPA may only inhibit GSK3β in certain dysregulating conditions, which may exist in the

brains of bipolar disorder patients. In support of this, Grimes and Jope (2001) found that GSK3β is inhibited by VPA when overexpressed in SH-SY5Y cells, while Werstuck and colleagues (2004) showed that some VPA metabolites act as potent GSK3β inhibitors *in vivo*. The inconsistency of data across the published literature on VPA-based modulation of GSK3β strongly suggests that VPA does not inhibit GSK3β directly and that any inhibitory activity is dependent on the cellular environment studied.

VPA is known to limit the extent of depressive phases in bipolar disorder (Cheng *et al.*, 2005). In an exciting development, a very recent randomized, double-blind, placebo-controlled trial found that co-treatment of bipolar I patients with lithium, the well-established and widely-used bipolar disorder treatment, and the specific PPARγ ligand pioglitazone significantly reduced depressive symptoms (Zeinoddini *et al.*, 2015). An earlier open-label trial found a similar decrease in depression of bipolar disorder patients with pioglitazone treatment in isolation (Kemp *et al.*, 2014). VPA has been reported to also act synergistically with lithium, both in bipolar disorder (Del Grande *et al.*, 2013) and in neuroprotection, where concurrent treatment of both drugs blocked glutamate excitotoxicity (Leng *et al.*, 2008). It is therefore possible that VPA, in acting on GSK3β indirectly via PPARγ, supports lithium action to yield an improved patient outcome.

7.4 Study limitations

As in any scientific work, experiments and the data they produce are interpreted to yield the insight believed to be closest to objective reality, however, both practical and theoretical limitations should be taken into account in this process. While some limitations are universal, tacitly accepted as inevitable, and simultaneously presumed to not be present (e.g. the possibility of consistent human error in experimental technique), some may be rectified in future work. The three most relevant examples of this latter group are discussed here.

As the majority of this work relies on cell culture, the most fundamental caveats must necessarily be concerned with associated techniques. Highly relevant, therefore, is the recent discussion regarding gas concentrations in standard cell culture conditions. Most cell line experiments are performed in atmospheric oxygen conditions (19-21% O_2), while few cells are ever exposed to this level *in vivo* (Tiede *et al.*, 2012). Indeed, physiological oxygen pressure in the liver is generally between 74 and 104mmHg, around half that of atmospheric 160mmHg often used in cell culture (Jungermann and Kietzmann, 2000). As, for instance, mitochondrial radical oxygen species production varies with oxygen level, it is likely that standard culture conditions produce an altered cell state compared to that found *in vivo* (Tiede *et al.*, 2012). Such an impact on cell state would affect any cell line experiments, but are particularly relevant in hypoxic set-ups, as used in this work. Any future work would therefore benefit from a systematic approach to verify the use of physiologically-relevant oxygen levels.

The majority of experimental results discussed in this thesis are quantified from Western blot data on protein abundance. Though the linear fluorescence quantification used is much improved compared to traditional ECL pixel-based quantification, it continues to rely on the presence of "loading control" proteins with a relatively consistent level of abundance, regardless of experimental condition. The two "housekeeping" proteins selected for this thesis, β-tubulin and β-actin, have not been shown to be affected by VPA in short-term experiments, but absence of published data does not indicate a total absence of effect. Another technique used to ensure equal protein loading in this project, preliminary quantification of sample protein concentration, may suffer from a related error; that is, experimental conditions may modulate protein abundance (total or specific) and thereby skew data collection. To circumvent these limitations, future experiments may rely on newer, improved techniques, such an in-cell Western quantification. However, increasing precision in protein quantification is, to a certain extent, dependent on technological advancement, and future progress in data reliability is likely to correlate with the establishing of novel or refined experimental equipment.

Finally, any data-reliant experimental process is reliant on the correct use of statistics; however, aside from misapplication of specific approaches, statistical tests are subject to both inherent and experiment-dependent limitations. In the case of this thesis, some results which seem to show significant differences "by eye" are not statistically significant. Should statistical methods be insufficient in

power or accuracy, they are likely to produce false negatives or positives. The knock-on effects of these erroneous categorisations may drastically change consequent interpretation and therefore affect further experimental decisions. Several options of compensating for these errors exist and careful selection of statistical methods will prevent a large proportion. However, a constant limitation, especially in laboratory-based biological investigations, is small sample size. Ideally, future work would be performed with sufficient time and financial support to allow for larger sample sizes compared to those in this thesis. Extensive biological and technical repetition of experiments relying on so variable a system as mammalian cell lines may go a long way to increasing the statistical accuracy of subsequent data analysis.

7.5 Suggestions for future work

The study hypothesis is confirmed. The aims of this study have largely been met but the discoveries discussed in this thesis may form the base for a far-reaching investigation into haemorrhagic shock signalling. Though there is a near limitless supply of options for future work, the following are suggested as immediately relevant and interesting targets.

I. **Verification** of current results through further investigation of changes in PPARγ protein abundance in the Huh7 model and analysis of the activity of other PPARγ interaction partners, e.g. acetyl-coenzyme A.

II. **Translation** of *in vitro* results discussed through the use of animal models to establish the effect of PPARγ-agonists on organism survival in haemorrhagic shock.

III. **Investigation** of further members of the signalling cascade involved in the Huh7 response to hypoxia, hypercapnia, and hypothermia, e.g. HDAC3/4 activity (VPA targets and PPARγ interactors) and the involvement of the Wnt-pathway in β-catenin accumulation in response to VPA.

IV. **Expansion** of the concepts discussed to cell lines produced from additional organs and signalling pathways affected in haemorrhagic shock, e.g. HEK293 cells, which respond to haemorrhagic shock-like conditions in a manner very similar to *in vivo* kidney.

7.6 Summary

This work has established an *in vitro* model for the dysregulated signalling found in haemorrhagic shock by exposing a human liver cell line, Huh7, to hypoxia, hypercapnia, and hypothermia. This model responds to VPA treatment in the manner seen *in vivo*. Therefore, the model may be of use as a high-throughput system for drug discovery, as compounds with potentially improved efficacy over VPA (2-ene-VPA, a VPA metabolite and decanoic acid, a VPA congener) have already been identified in this thesis. The simplicity of the Huh7 model of haemorrhagic shock also provides an ideal environment for studies of the molecular signalling of haemorrhagic shock. This work has found that the therapeutic action of VPA relies on PPARγ activity, a new revelation which may have far reaching implications for other pathological conditions in which VPA is effective, e.g. bipolar disorder. The availability of the *in vitro* model of haemorrhagic shock signalling established in this thesis is likely to accelerate therapeutic innovation in haemorrhagic shock research and may ultimately save human lives.

8

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