INDUCTION OF MELATONIN RECEPTORS BY VALPROIC ACID

EPIGENETIC MECHANISMS UNDERLYING THE UPREGULATION OF MELATONIN RECEPTOR EXPRESSION BY VALPROIC ACID

BY

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TITLE: Epigenetic mechanisms underlying the upregulation of melatonin receptor expression by valproic acid.

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ABSTRACT

Melatonin is an indoleamine hormone with neuromodulatory and neuroprotective properties. It mediates many of its effects by its two G protein-coupled receptors, MT_1 and MT_2. We have shown that valproic acid (VPA) induces melatonin receptor expression in cultured rat C6 glioma cells, and in the rat hippocampus. VPA is known to affect gene expression through several mechanisms, including the modulation of intracellular kinase pathways and/or transcription factors, as well as the inhibition of histone deacetylase (HDAC) activity.

In this study, we show that HDAC inhibitors of distinct chemical classifications, including suberanilohydroxamic acid (SAHA) and 4-(dimethylamino)-n-[7-(hydroxyamino)-7-oxoheptyl] benzamide (M344), parallel the effects of VPA on MT_1 induction *in vitro*. However valpromide, a VPA analogue that lacks the ability to inhibit HDAC activity, does not. The observed increase in MT_1 expression by VPA is matched by an increase in global histone H3 acetylation. More importantly, an enrichment of histone H3 acetylation occurs along the rat MT_1 promoter following treatment with VPA, indicating that histone acetylation and chromatin remodelling are a primary mechanism underlying this induction.

Independent of VPA, the rat MT_1 gene may be regulated by a number of intracellular kinase pathways and transcription factors, which are also targeted by VPA. KG501-mediated CREB inhibition did not block MT_1 upregulation by VPA.

Blockade experiments targeting the PKC, PI3K/AKT, or GSK3β signaling pathways suggest that VPA induces melatonin receptor expression independent of these intracellular signaling cascades as well. The relevance of melatonin receptor upregulation was assessed using *in vivo* VPA and melatonin combination treatments on neuroprotective gene expression.

The results of this study provide evidence that expression of the melatonin receptor is epigenetically induced by VPA by means of promoter histone acetylation. Melatonin receptor upregulation by VPA, or other HDAC inhibitors, may represent a therapeutic strategy for the management of several nervous system disorders.

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This dissertation would not have been possible without the ongoing guidance, encouragement, and support of my supervisor, Dr. Lennard Niles, whose commitment to helping me learn and develop as a scientist extended well above the call of duty, and I will be forever grateful for his mentorship. I would also like to extend my deepest appreciation to my committee members, Dr. Ram Mishra and Dr. Jane Foster, as well as the members of my examination committee, Dr. Barbara Monti and Dr. Douglas Davidson, for their thoughtful considerations towards my project. I would also like to thank Dr. Jane Foster for all the additional time she spent in assisting me with qPCR data analysis.

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LIST OF ABBREVIATIONS

^{125}I-Mel	2-[125I]Iodomelatonin
18S RNA	18S Ribosomal RNA
5-HT	5-Hydroxytryptamine; Serotonin
5-HTP	5-Hydroxytryptophan
AADC	Aromatic Amino Acid Decarboxylase
AC	Adenylate Cyclase
AcH3	Acetyl Histone H3
AMPA	Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid
AMPK	Adenosine Monophosphate-activated Protein Kinase
AP1	Activator Protein 1
BDNF	Brain-Derived Neurotrophic Factor
BDNF P1	Brain-Derived Neurotrophic Factor Promoter 1
BIM1	Bisindolymaleimide 1
Bmal1	Aryl Hydrocarbon Receptor Nuclear Translocator-Like Protein 1
Ca^{2+}	Calcium
cAMP	Cyclic Adenosine Monophosphate
CBP	CREB Binding Protein
cDNA	Complimentary Deoxyribonucleic Acid
CDNF	Cerebral Dopamine Neurotrophic Factor
cGMP	Cyclic Guanosine Monophosphate
ChIP	Chromatin Immunoprecipitation
Clock	Circadian Locomotor Output Cycles Kaput
CNS	Central Nervous System
CpG	Cytosine immediately followed by Guanine
CREB	Cyclic Adenosine Monophosphate Response Element-Binding

	Protein
Cry 1	Cryptochrome Gene 1
Cry 2	Cryptochrome Gene 2
Ct	Cycle Threshold
DAG	Diacylglycerol
DMEM	Dulbecco'S Modified Eagle'S Medium
DMNT	DNA Methyltransferase
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EGR1	Early Growth Response 1
ERK 1/2	Extracellular-Signal-Regulated Kinase Proteins 1 And 2
FBS	Fetal Bovine Serum
GABA	Gamma Aminobutyric Acid
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GATA2	GATA Transcription Factor 2
GC	Guanylate Cyclase
GDNF	Glial-Derived Neurotrophic Factor
GPCR	G-Protein Coupled Receptors
GSK3β	Glycogen Synthase Kinase 3 Beta
H1	Histone H1
H2A	Histone H2A
H2B	Histone H2B
H3	Histone H3
H4	Histone H4
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase

HIOMT	Hydroxyindole-O-Methyltransferase
HP1	Heterochromatin Protein 1
IP_3	Inositol 1,4,5 –Triphosphate
JNK	C-Jun N-Terminal Kinase
K9/18	Lysine 9/18
Kd	Dissociation Constant
Keap1	Kelch-Like Ech-Associated Protein 1.
LiCl	Lithium Chloride
M344	4-(Dimethylamino)-N-[7-(Hydroxyamino)-7-Oxoheptyl] Benzamide
MANF	Mesencephalic Astrocyte-Derived Neurotrophic Factor
MAPK	Mitogen Activated Protein Kinase
MAPKK	Mitogen Activated Protein Kinase Kinase
MAPKKK	Mitogen Activated Protein Kinase Kinase Kinase
mRNA	Messenger Ribonucleic Acid
MT_1	Melatonin Receptor Subtype 1
MT_2	Melatonin Receptor Subtype 2
MeCP2	Methyl-CpG-Binding Protein 2
NaCl	Sodium Chloride
NAT	N-Acetyl Transferase
NGF	Nerve Growth Factor
NFκB	Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells
NMDA	N-Methyl-D-Aspartic Acid
Nrf2	Nuclear Factor Erythroid 2 [Nf-E2]-Related Factor 2
Nrf2-ARE	Nf-E2-Related Factor 2-Antioxidant Responsive Element
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction

pCREB	Phospho-CREB
PDK1	3-Phosphoinositide Dependent Protein Kinase-1
Per 1	Period 1
Per 2	Period 2
PI3K	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase
PIP_2	Phosphatidylinositol 4,5-Bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-Trisphosphate
Pitx1	Paired-Like Homeodomain Transcription Factor 1
PKA	Protein Kinase A
PKB/AKT	Protein Kinase B/Rac-Alpha Serine/Threonine-Protein Kinase
PKC	Protein Kinase C
PLC	Phospholipase C
PVDF	Polyvinylidene Fluoride
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SAHA	Suberoyl Anilide Hydroxamic Acid; Vorinostat
SCN	Suprachiasmatic Nucleus
SF1	Steroidogenic Factor 1
SMC	Structural Maintenance of Chromosomes
SP	Specificity Protein
TBS	Tris Buffered Saline
TPH	Tryptophan Hydroxylase
TSA	Trichostatin A
VPA	Valproic Acid
VPM	Valpromide

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DECLARATION OF ACADEMIC ACHIEVEMENT

My advisor, Dr. Lennard Niles, designed the experiments for this project. He also performed all animal sacrifices and tissue dissections for subsequent analyses. I was responsible for care of all animals, as well as all molecular experiments *in vitro* and *in vivo*, including drug treatments, RT-PCR, AcH3 ChIP-qPCR, and immunoblotting. Dr. Lennard Niles, Dr. Jane Foster, and I performed statistical analyses.

1 INTRODUCTION

1.1 THE PINEAL GLAND

The pineal gland is a circumventricular organ located deep in the center of the brain (Macchi and Bruce 2004). Development of the mammalian pineal gland from the diencephalon begins early in gestation, and maintains connections with the habenular and posterior commissures to form the pineal stock in the mature brain (Macchi and Bruce 2004). The mammalian pineal gland has both sympathetic and parasympathetic innervations, and is considered to be one of the most vascularized organs in the body (Goldman and Wurtman 1964). The pineal gland synthesizes and secretes various hormones and peptides, however its nocturnal production of melatonin is thought to be its main function (Macchi and Bruce 2004).

1.2 MELATONIN

Melatonin, 5-methoxy-N-acetyl-tryptamine, is the primary hormone of the pineal gland (Hardeland *et al.* 2011). It was discovered by its ability to promote the movement of melanin-containing chromatophores in various amphibian and fish species, which produced a reversible skin-lightening response (Lerner and Case 1959). The process of identifying the numerous biological targets of melatonin began following isolation of this hormone from the bovine pineal gland in the mid-

1900s (Lerner *et al.* 1960). Melatonin gained widespread attention upon the discovery that its secretion from the pineal gland occurred rhythmically, and was correlated with photoperiodic measurements and circadian rhythms (Ellison *et al.* 1972). The ensuing bioassays gave rise to a great deal of its currently known biochemical and molecular characteristics.

1.2.1 MOLECULAR STRUCTURE

Melatonin is an indoleamine hormone with a backbone comprised of two highly stable heteroaromatic rings, which are characteristic of indoleamines secreted by the pineal gland (Poeggeler *et al.* 2002). Its two substituents, the N-acetyl side chain, and the methoxy-substitution on the indole rings, form the basis of its binding specificity and amphiphilicity, allowing it to enter many organs and intracellular compartments (Sugden 1994).

1.2.2 PINEAL MELATONIN BIOSYNTHESIS

Melatonin synthesis and secretion follow a rhythmic pattern synchronized to the 24-hour circadian system, in which circulating melatonin levels peak nocturnally and deplete during the day (Hardeland *et al.* 2011). The biosynthetic pathway of melatonin synthesis is outlined in Figure 1. Melatonin is synthesized from dietary tryptophan and serotonin. Tryptophan is hydroxylated by tryptophan hydroxylase

(TPH) to form 5-hydroxytryptophan (5-HTP). This product is then decarboxylated by aromatic L-amino acid decarboxylase (AADC) to form 5-hydroxytryptamine (5-HT), which is also known as serotonin. Serotonin is then acetylated by serotonin N-acetyltransferase (NAT) to form N-acetylserotonin. Methylation by hydroxyindole-O-methyltransferase (HIOMT) converts N-acetylserotonin into the final product, melatonin. NAT expression follows a diurnal pattern of expression similar to that of melatonin, and is suggested to be the rate-limiting step in the overall mechanism (Ackermann and Stehle 2006).

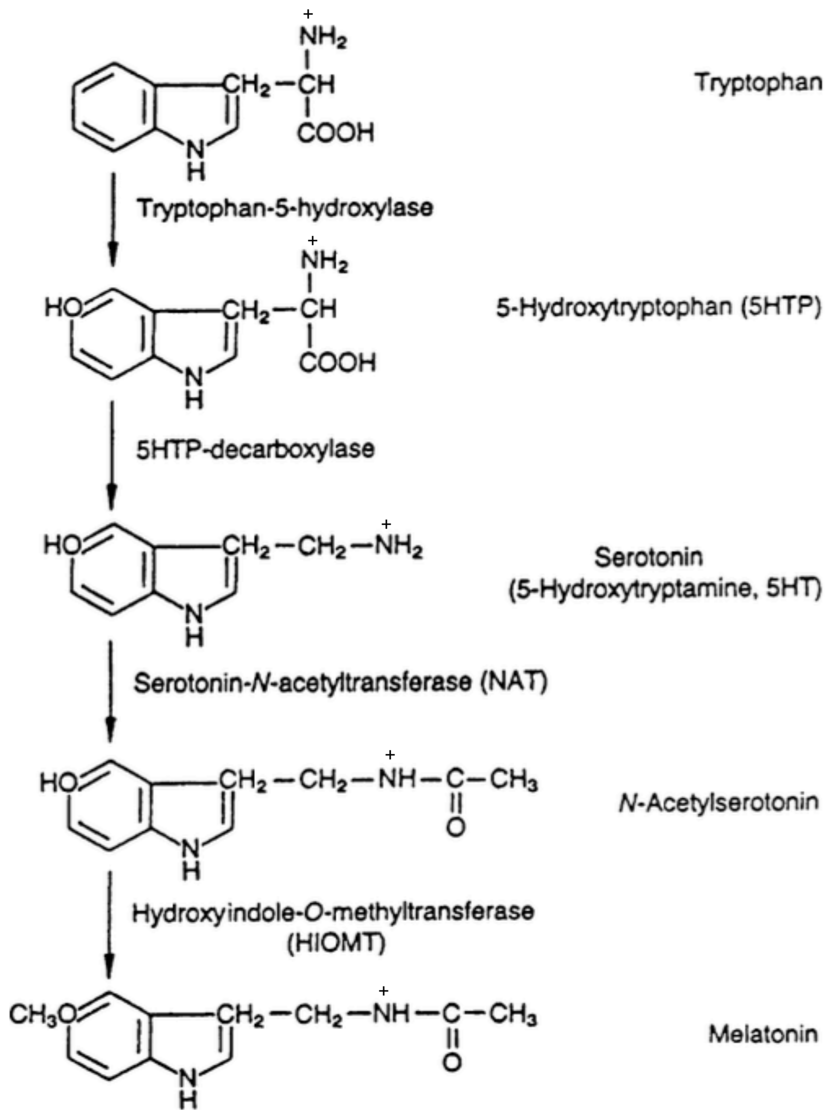


Figure 1: Summary of melatonin synthesis in the mammalian pineal gland.

The pathway of pineal melatonin synthesis is outlined above. This figure was taken from Macchi and Bruce 2004.

1.2.3 REGULATION OF PINEAL MELATONIN BIOSYNTHESIS

1.2.3.1 LIGHT

Many non-mammalian vertebrate pinealocytes are light sensitive and possess an internal physiological clock that allows them to independently synchronize with their surroundings in response to photoperiodic cues (Vigh *et al.* 2002). This earned the pineal gland in these organisms the title, "the third eye" (Mano and Fukada 2007). Mammalian pinealocytes are not light sensitive on their own, but rather are dependent on light cues transmitted from the retina (Macchi and Bruce 2004). As illustrated in Figure 2, photoperiodic cues are generated by melanopsin-producing retinal ganglion cells, which are transmitted via glutamatergic neurotransmissions in the retinohypothalamic tract to the suprachiasmatic nucleus (SCN) of the hypothalamus (Ebadi and Govitrapong 1986). Although SCN lesions have revealed that the retina is capable of generating circadian oscillations (Sakamoto *et al.* 2000), the SCN is thought to be the master regulator of circadian rhythmicity in mammals (Herzog *et al.* 1998). Melatonin synthesis and secretion in response to the relayed light/dark cues occurs rhythmically, with peak levels occurring during the dark phase of the circadian rhythm (Foulkes *et al.* 1997; Perreau-Lenz *et al.* 2005).

1.2.3.2 SUPRACHIASMATIC NUCLEUS

The SCN relays photoperiodic information through a multi-synaptic pathway to control the sympathetic release of melatonin from the pineal gland (Perreau-Lenz *et al.* 2003), as outlined in Figure 2, SCN neurons innervate the hypothalamic paraventricular nucleus, which descend into the intermediolateral cell column of the spinal cord (Perreau-Lenz *et al.* 2003). Gamma aminobutyric acid-ergic (GABAergic) projections from the intermediolateral cell column modulate excitability of the superior cervical ganglion, which innervates the pineal gland via postganglionic sympathetic axons (Perreau-Lenz *et al.* 2003). SCN lesions were found to cause an increase in daytime melatonin secretion, revealing that the SCN produces inhibitory signals during the light phase that prevents melatonin production (Perreau-Lenz *et al.* 2003).

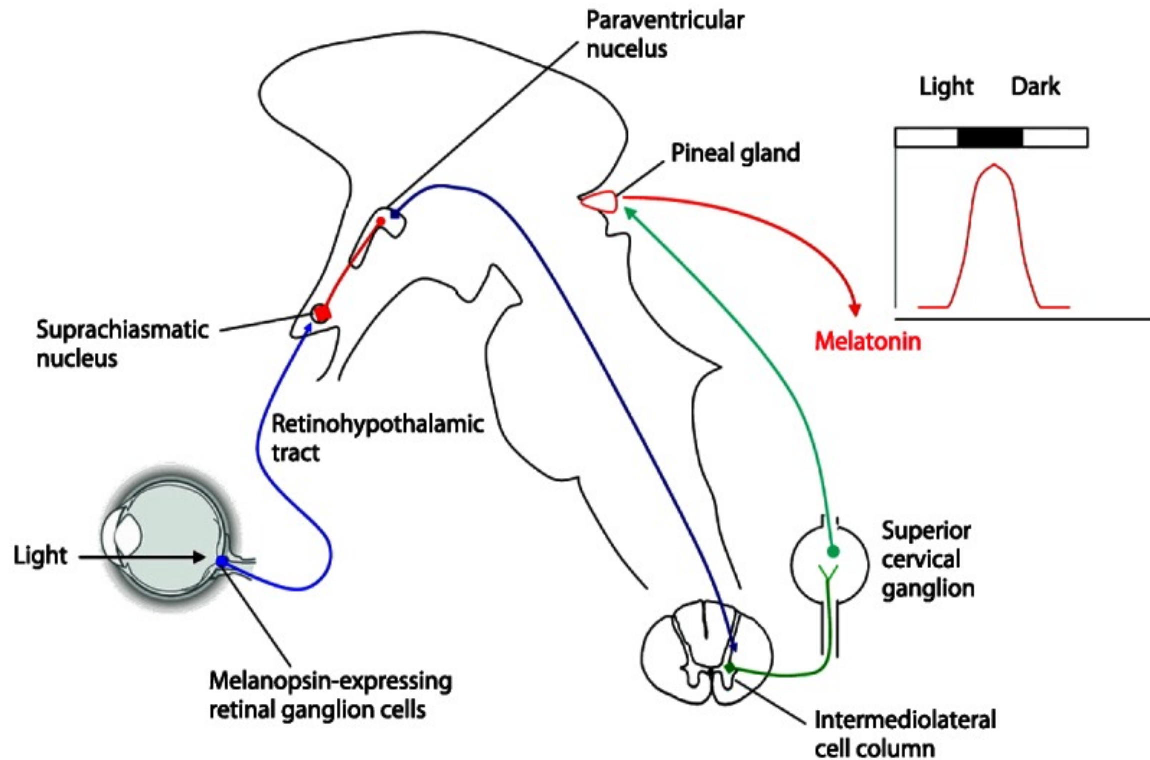


Figure 2: Representation of the regulation of mammalian melatonin secretion.

Light cues are transmitted from the retina to the SCN through the retinohypothalamic tract. Photoperiodic information is relayed from the SCN to the pineal gland through a multi-synaptic pathway, which releases melatonin nocturnally. This figure was taken from Zee *et al.* 2013.

1.2.4 MOLECULAR CONTROL OF MELATONIN BIOSYNTHESIS

NAT phosphorylation, which activates the rate-limiting enzyme for melatonin synthesis (Ackermann and Stehle 2006), is under the control of the adenylate cyclase (AC)/cyclic adenosine monophosphate (cAMP)/cAMP-response element binding protein (CREB) pathway (Schomerus and Korf 2005). Norepinephrine is released from postganglionic neuronal projections from the superior cervical ganglion, and acts via α and β adrenergic receptors in the pineal gland, to activate this pathway (Roseboom and Klein 1995). The nocturnal elevation in cAMP levels trigger NAT phosphorylation by protein kinase A (PKA), to initiate the production of melatonin (Ackermann and Stehle 2006; Roseboom and Klein 1995).

1.2.5 EXTRA PINEAL MELATONIN BIOSYNTHESIS

Although the pineal gland is the primary site of melatonin production, melatonin is also synthesized in several sites outside of this structure (Acuña-Castroviejo *et al.* 2014). The earliest indications that melatonin might not be exclusively made in the pineal gland was observed in animals with lesioned pineal glands that continued to produce melatonin (Reiter *et al.* 1983), as well as the production of melatonin in other organisms that lack pineal glands entirely (Vernadakis *et al.* 1998). Melatonin-synthesizing enzymes, HIOMT and NAT, were also detected in many structures within the central nervous system (CNS) and the periphery (Bubenik *et al.* 1974),

proposing a similar pathway of melatonin synthesis in these areas, to that of the pineal gland (Acuña-Castroviejo *et al.* 2014). Extra-pineal melatonin is produced in the cerebral cortex, striatum, thymus, spleen, heart, stomach, and the gut at concentrations which sometimes exceed that of the pineal gland (Acuña-Castroviejo *et al.* 2014).

1.3 MELATONIN RECEPTORS

1.3.1 STRUCTURE AND FUNCTION

2-[125I]Iodomelatonin (125I-Mel) ligand binding assays and genomic deoxyribonucleic acid (DNA) molecular cloning experiments were the first to define the family of high-affinity G-protein coupled receptors (GPCRs) termed "the melatonin receptors". These propelled several studies investigating the signal transduction pathways, target effectors, and clinical effects of melatonin. The melatonin receptor family is comprised of two pharmacologically distinct isoforms, MT_1 (Reppert *et al.* 1994; Ebisawa *et al.* 1994) and MT_2 (Reppert *et al.* 1995). Both melatonin receptor subtypes bind to melatonin with high affinity, with dissociation constant (Kd) values in the low picomolar range (Hardeland 2009; Morgan *et al.* 1994). MT_1 and MT_2 are comprised of the seven-transmembrane domains that are characteristic of GPCRs (Reppert 1997), with some distinguishing features: melatonin receptors include an N-terminal glycosylation site, palmitoylatable

cysteine residues on the fourth intracellular loop, a lipid anchor, and carboxy-terminal phosphorylation sites for various kinases (Hardeland 2009). While these are not thought to contribute to the affinity of the receptor to its ligand, the lipid anchor and carboxy-terminal are necessary for its interactions with G-proteins and internalization processes (Sethi *et al.* 2008; Bondi *et al.* 2008).

1.3.2 LOCALIZATION

Melatonin receptors are transiently expressed in development, and subsequently throughout adulthood (Johnston *et al.* 2003a; Johnston *et al.* 2006; Johnston *et al.* 2007). MT_1 messenger ribonucleic acid (mRNA) is broadly detected in the rat foetal pituitary beginning on embryonic day 15.5, and declines rapidly over the first few weeks of postnatal life (Johnston *et al.* 2006; Johnston *et al.* 2003a). In adulthood, melatonin receptors display diurnal expression rhythms (Masana *et al.* 2000).

Melatonin receptors have been identified in multiple species, including amphibians, fish, rodents, and mammals (Dubocovich *et al.* 2003; Hardeland *et al.* 2011; Pandi-Perumal *et al.* 2008). In the mammalian CNS, melatonin receptors are localized on neuronal dendrites and somata in the hypothalamus, pituitary, cerebral cortex, basal forebrain, hippocampus, basal ganglia, epithalamus, thalamus, and in the ventral mesencephalon (Lacoste *et al.* 2015). In regions where both isoforms are

detected, MT_1 and MT_2 expression occurs in a complementary fashion, where relative increases in MT_1 expressions correspond with relative decreases in MT_2, or vice versa (Lacoste *et al.* 2015). This property may be indicative of their unique functional specializations (Lacoste *et al.* 2015). Melatonin receptors exhibit a wide distribution throughout the periphery. They have also been identified in the retina (Mennenga *et al.* 1991; Sengupta *et al.* 2011; Natesan and Cassone 2002; Wiechmann and Wirsig-Wiechmann 1991), Harderian gland (Coto-Montes *et al.* 1996), lungs (Kumar Kharwar and Haldar 2011), aorta (Schepelmann *et al.* 2011), gastrointestinal tract (Bubenik 2002; Poon *et al.* 1997; Jaworek *et al.* 2005), kidneys (Song *et al.* 1996; Drew *et al.* 1998), and the gonads (Ayre *et al.* 1994).

1.3.3 SIGNALING MECHANSIMS

Functional studies have revealed that both melatonin receptor isoforms are coupled to various pertussis-sensitive and insensitive G proteins (Morgan *et al.* 1990). As illustrated in Figure 3, melatonergic signaling is primarily mediated by the inhibition of AC activity, and attenuation of cAMP accumulation, PKA activity, and CREB phosphorylation (pCREB; Reppert 1997; Brydon *et al.* 1999; Hardeland 2009; Pandi-Perumal *et al.* 2008; Morgan *et al.* 1995; Morgan *et al.* 1996; Morgan *et al.* 1991). The involvement of other signal transduction pathways have also been

documented. For example, melatonin receptor activation is coupled to increased phospholipase C (PLC) activity, diacylglycerol (DAG) formation, and protein kinase C (PKC) activation. Additionally, MT_2 activation is associated with decreased guanylate cyclase (GC) activity, and cyclic guanosine monophosphate (cGMP) production (Hardeland 2009).

MT_1 and MT_2 are often expressed as homo and heterodimers (Maggio *et al.* 2005). Dimerization, which occurs independently of ligand binding, creates extraordinary diversity not only in receptor binding, but also in the biological and pharmacological effects of melatonin (Maggio *et al.* 2005). MT_1 homodimerization (Ayoub *et al.* 2002), or MT_1/MT_2 heterodimerization (Ayoub *et al.* 2004), occur at a higher propensity than MT_2 homodimerization.

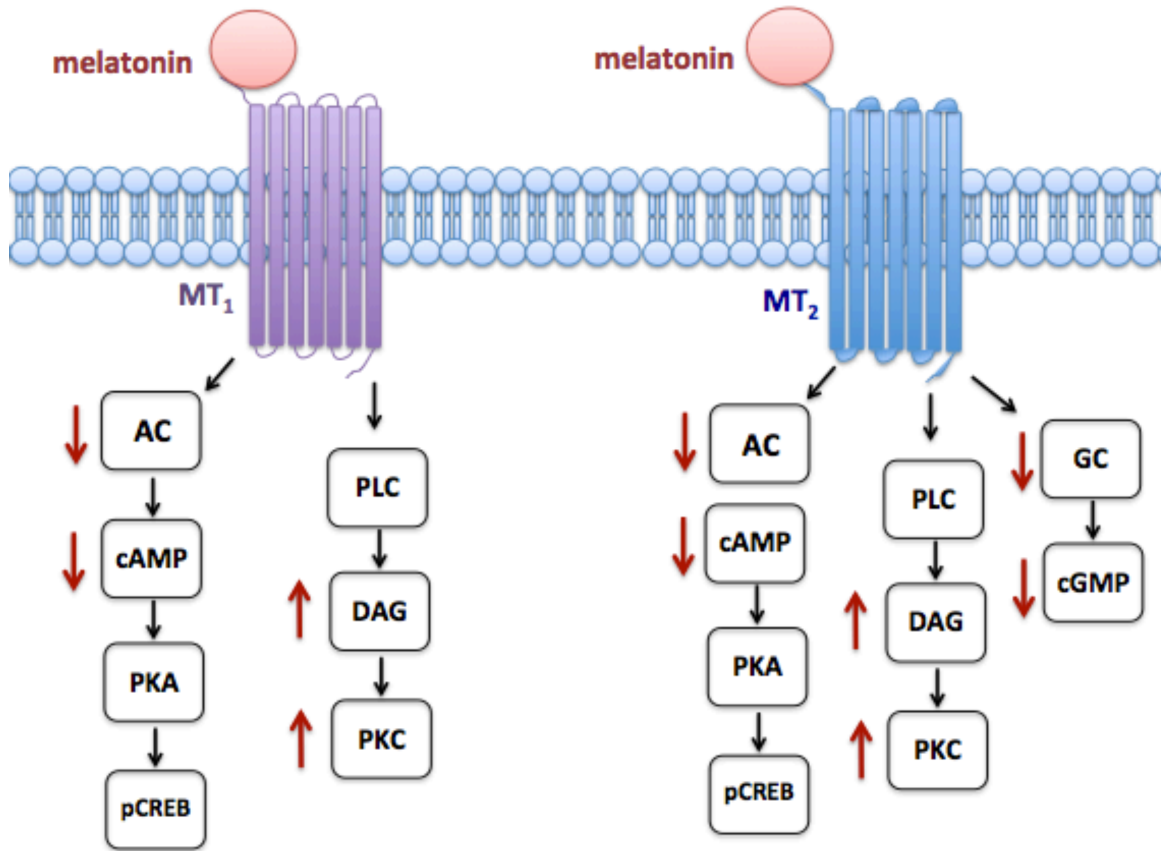


Figure 3: Overview of melatonergic signaling.

Melatonergic signaling is mediated by cAMP/PKA/CREB pathway. Other signaling pathways, including PKC and GC/cGMP have also been reported (Hardeland 2009).

1.3.4 RECEPTOR REGULATION

Studies addressing the regulation of the melatonin receptors have proven to be a challenge, on account of their rhythmic expression patterns (Masana *et al.* 2000). Melatonin receptors are regulated by circulating levels of melatonin, through a mechanism that is thought to involve its modulation of cAMP (Gauer *et al.* 1994; Gauer *et al.* 1993; Barrett *et al.* 1996; Maronde *et al.* 1999). Experiments conducted in the ovine pars tuberalis demonstrate that compounds which stimulate cAMP production, such as forskolin and the cholera toxin, cause an increase in MT_1 expression (Barrett *et al.* 1996). This is consistent with the effects of melatonin on MT_1, as melatonin antagonizes cAMP production, and prevents increases in MT_1 expression induced by forskolin (Barrett *et al.* 1996; Morgan *et al.* 1991). As cAMP levels regulate the transcription of many genes through the cAMP/PKA/CREB pathway, the transcription factor CREB has been highly implicated in the regulation of melatonin receptors (Barrett *et al.* 1996).

Cloning of the proximal 1.5kb region of the rat MT_1 promoter (GenBank AY228510) allowed for a more precise analysis of the regulation of MT_1 transcription. MT_1 promoter activity assays have identified Paired-like homeodomain transcription factor 1 (Pitx1), a transcription factor widely expressed in the rodent and ovine pituitary, as the chief regulator of basal MT_1 transcription (Johnston *et al.* 2003b; Johnston *et al.* 2003a; Johnston *et al.* 2006; Johnston *et al.*

2007). As Pitx1 is expressed widely throughout the pars tuberalis, the predominant hypothesis is that other transcription factors interact with Pitx1 to potentiate or suppress its induction of MT_1 transcription (Johnston *et al.* 2006). Characterization of the rat MT_1 promoter revealed putative binding sites for several transcription factors, as outlined in Figure 4, which lend support to this hypothesis (Johnston *et al.* 2003a). Promoter analysis of the interactions of steroidogenic factor 1 (SF1), early growth response 1 (Egr1), and GATA transcription factor 2 (GATA2) have revealed that, while Pitx1 alone can drive MT_1 transcription, its co-factors interact synergistically with Pitx1 to regulate its activity (Johnston *et al.* 2003b; Johnston *et al.* 2006). Specifically, SF1 or GATA2 augment Pitx1-mediated activation of MT_1 promoter activity, whereas Egr1 represses it (Johnston *et al.* 2003b).

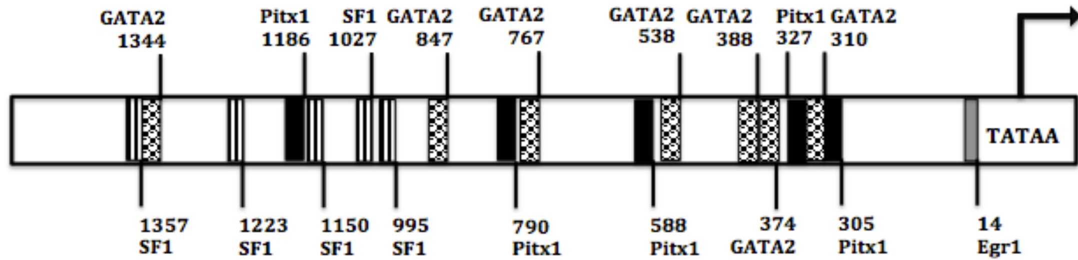


Figure 4: Schematic of MT_1-specific transcription factor binding sites along the rat MT_1 promoter.

Transcription factor binding sites for the rat MT_1 promoter (GenBank AY228510) were determined by Johnston *et al.* 2003a. The locations of the binding sites relative to the MT_1 transcriptional start are indicated (not to scale).

1.4 OTHER MELATONIN BINDING SITES

A third isoform, initially designated MT_3, was thought to be the third melatonin receptor, but was subsequently identified as the quinone reductase 2 enzyme, which is a cytosolic protein (Hardeland 2009). Several other melatonin binding sites have been established, such as the retinoic acid receptors ROR and RZR (Wiesenberg *et al.* 1998), the melatonin binding proteins calmodulin and calreticulin (Benítez-King *et al.* 1993; Macías *et al.* 2003), the orphan GPCR, GPR50 (Levoye *et al.* 2006) and various mitochondrial binding sites have been identified (Andrabi *et al.* 2004). The relevance of these sites to melatonin signalling is not well understood.

1.5 PHYSIOLOGICAL ROLE OF MELATONIN IN MAMMALS

1.5.1 CHRONOBIOLOGICAL REGULATION

The SCN functions within a network of peripheral oscillator genes, known as clock genes, to control mammalian circadian rhythmicity (Ko and Takahashi 2006). The fundamental genes involved are the circadian locomotor output cycles kaput (clock) period genes, *Per1* and *Per2*, the cryptochrome genes, *Cry1* and *Cry2*, and the aryl hydrocarbon receptor nuclear translocator-like (Bmal1) gene, which are expressed in an oscillating fashion (von Gall *et al*. 2005). Melatonin acts as an internal synchronizer of biological rhythms by conveying information regarding the duration of the dark phase from the SCN to its target organs within the CNS and the periphery (Stehle *et*

al. 2003). Melatonin inhibits the proteasomal degradation of clock genes (Vriend and Reiter 2015), and has effective phase shifting properties. Exogenous melatonin, administered at the appropriate time relative to the circadian clock, can be used to entrain or restore abnormal circadian rhythms in conditions such as blindness, and phase-shifts associated with neuropsychiatric disorders, shift work, or jet lag (Cardinali, Furio, Reyes, & Brusco, 2006; Skene & Arendt, 2007).

Interruptions in the chronobiological functions of melatonin have been linked to numerous disorders (Hardeland *et al.* 2011). For example, aberrant melatonergic signaling has been associated with accelerated senescence and other metabolic, cardiovascular, psychiatric, and neurodegenerative disorders (Jenwitheesuk *et al.* 2014; Bonmati-Carrion *et al.* 2014).

1.5.2 CELL PROTECTION

Melatonin has a widely protective role in the CNS, and as such, is highly implicated in mitigating the onset and/or progression of the previously mentioned conditions (Pandi-Perumal *et al.* 2012). Although there is no evidence of melatonin extending lifespans (Hardeland 2013), safeguarding melatonergic rhythms is vital to healthy aging and disease attenuation (Bonmati-Carrion *et al.* 2014).

1.5.2.1 DIRECT ANTIOXIDANT RESPONSES

Pharmacological dosages of melatonin are defined as those greater than the picomolar to low nanomolar ranges found physiologically (Reiter and Tan 2003). Melatonin, as well as its metabolites, have direct free radical scavenging capabilities (Luchetti *et al.* 2010; Reiter *et al.* 2002). As such, a single melatonin molecule can forage and neutralize multiple highly toxic reactive oxygen, nitrogen, and hydroxyl radicals at a time (Rosen *et al.* 2006). Melatonin was also found to enhance the actions of other antioxidants, causing a synergistic potentiation of radical clearance *in vitro* (Gitto *et al.* 2001).

Although free radical scavenging and avoidance is a widely beneficial property, it is especially relevant for mitochondrial support, as this is an organelle with increased susceptibility to oxidative damage. Melatonin readily enters the mitochondrial matrix, where it limits electron leakage from the electron transport chain and free radical production (Srinivasan *et al.* 2011; Reiter *et al.* 2003).

1.5.2.2 INDIRECT ANTIOXIDANT RESPONSES

Melatonin regulates the expression and activity of several antioxidant and detoxification genes at physiological concentrations at all stages of life (Reiter *et al.* 2003; Rodriguez *et al.* 2004), including in utero in the foetal brain (Okatani *et al.* 2000). Melatonin induces the expression and activity of several antioxidant

enzymes, including glutathione peroxidase and superoxide dismutase (Hardeland *et al.* 2011; Reiter *et al.* 2003). Antioxidant enzymes under the control of melatonin exhibit diurnal rhythms similar to that of melatonin, with expression levels peaking nocturnally and depleting in the light (Pablos *et al.* 1998; Martín *et al.* 2003). Melatonin modulates various intracellular pathways to regulate the antioxidant enzyme activity (Luchetti *et al.* 2010). It has been suggested that the indirect antioxidant effects of melatonin are receptor-mediated (Rodriguez *et al.* 2004).

1.5.2.3 INDUCTION OF NEUROTROPHIC FACTORS

Neurotrophic factors are a family of proteins that promote neuronal development, differentiation, and survival in the nervous system (Barbacid 1995). Early developmental synaptic pruning is guided by neurotrophic factors, which regulate the quantity of surviving neurons by preventing apoptosis (Henderson 1996). Neurotrophic factors also play a role in the regulation of synaptic plasticity (Lo 1995), and neurogenesis (Lee and Son 2009) throughout adulthood. Neurotrophic factors have specialized roles, and affect specific subsets of neuronal populations (Barbacid 1995). Melatonin provides neuronal support in the form of influencing neurotrophin induction and release, which is advantageous in the case of neuronal damage caused by injury or disease. For example, melatonin, upregulates the neurotrophic factors glial-derived neurotrophic factor (GDNF; Armstrong and

Niles 2002; Kong *et al.* 2008a; Kong *et al.* 2008b; Tasset *et al.* 2011), and nerve growth factor (NGF; Pongsa-Asawapaiboon *et al.* 1998; Erşahin *et al.* 2012). Additionally, the melatonin analogue, agomelatine, induces expression of brain-derived neurotrophic factor (BDNF; Païzanis *et al.* 2010; Soumier *et al.* 2009; Molteni *et al.* 2010; Boulle *et al.* 2014).

1.6 VALPROIC ACID

Valproic acid (VPA), 2-propylpentanoic acid, is a branched carboxylic acid derivative of valeric acid, a naturally occurring compound responsible for the odour of the *Valeriana officinalis* plant (Eadie 2004). VPA forms a transparent, colourless, and odourless solution at room temperature.

1.6.1 PHARMACOLOGICAL EFFECTS

VPA was initially synthesized to be an inert solvent for organic compounds being screened for their potential anti-convulsive activity, when it was discovered that it is a powerful antiepileptic agent on its own (Peterson and Naunton 2005). It has since sustained its position among the most potent antiepileptic drugs to date. Since its approval for use in the clinical setting, VPA has been demonstrated to have therapeutic potential against a diversity of pathologies. In addition to its anticonvulsive capabilities, VPA has widespread neuroprotective effects that make it

a beneficial therapeutic agent against, migraines and neuropathies, as well as psychiatric, neurodegenerative, and immunological diseases (Peterson and Naunton 2005; Chiu *et al.* 2013).

1.6.2 MECHANISMS OF ACTION

1.6.2.1 NEUROTRANSMITTER CONTROL

The fundamental characteristic of VPA that makes it a suitable therapeutic agent for a variety of CNS and peripheral disorders is its large capacity to influence the actions of a variety of biochemical and molecular targets (Monti *et al.* 2009). The antiepileptic and mood-stabilizing properties of VPA have traditionally been attributed to its ability to control GABAergic and glutamatergic transmissions in the CNS, although more recently other neurotransmitters have been identified as well (Chapman *et al.* 1982; Wu and Shih 2011; Schank *et al.* 2005). VPA raises CNS GABA concentrations by inactivating α-ketoglutarate dehydrogenase to increase levels of the GABA precursor, α-ketoglutarate (Luder *et al.* 1990), and by potentiating the effects of the GABA synthesizing enzyme, glutamate decarboxylase (El Hage *et al.* 2012). VPA enhances GABAergic activity by inhibiting GABA transaminase and succinate semialdehyde dehydrogenase-mediated GABA catabolism (Löscher 1993; Whittle and Turner 1978) to increase the availability of GABA at the synapse. Moreover, VPA interacts with postsynaptic $GABA_A$ and $GABA_B$ receptors to extend

their inhibitory responses (Cunningham *et al.* 2003; Motohashi 1992). The modulation of excitatory neurotransmissions by VPA is attributed to its lowering of CNS glutamate levels and uptake by Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid (AMPA) and N-Methyl-D-Aspartic Acid (NMDA) receptors (El Hage *et al.* 2012; Gean *et al.* 1994; Künig *et al.* 1998; Ko *et al.* 1997). VPA also reduces neuronal excitability by acting on sodium and potassium conductances, through their corresponding voltage-gated ion channels (VanDongen *et al.* 1986).

1.6.2.1 KINASE PATHWAY MODULATION

Clinical administration of VPA follows a pattern of delayed onset and reversal following drug administration and discontinuation, emphasizing the importance of molecular regulation in the therapeutic actions of VPA (Monti *et al.* 2009). VPA also exerts many of its neuroprotective and neurotrophic effects through its influence on the post-translational modifications of various intracellular kinases, which regulate transcription factor activation and subsequent gene expression. In particular, the Mitogen Activated Protein Kinases (MAPK), Phosphatidylinositol-3-Kinase and Protein Kinase B (PI3K/AKT), PKC, and the Glycogen Synthase Kinase 3 Beta (GSK3β) pathways are signaling targets of VPA that are implicated in its molecular regulation of gene expression (Monti *et al.* 2009). Recently, the adenosine monophosphate-activated protein kinase (AMPK) signaling pathway was also

discovered to be a target of VPA, though the specifics of this interaction are not yet known (Avery and Bumpus 2014).

1.6.2.1.1 MAPK

The MAPK signaling pathway is an evolutionarily conserved family of serine/threonine protein kinases that convert extracellular stimuli into intracellular responses essential for basic cell biology processes, such as cell growth, survival, stress responses, motility and differentiation (Plotnikov *et al.* 2011; Cargnello and Roux 2011). In general, MAPK signaling involves activation of mitogen-activated protein kinase kinase kinase (MAPKKK), which activates a mitogen-activated protein kinase kinase (MAPKK), which in turn activates a MAPK to initiate an intracellular response (Cargnello and Roux 2011). VPA increases the phosphorylation of the MAPK proteins, p38 MAPK (Xie *et al.* 2010), Extracellular-Signal-Regulated Kinase Proteins 1 and 2 (ERK1/2; Jung *et al.* 2008; Wu *et al.* 2012; Bitman *et al.* 2014; Boeckeler *et al.* 2006) and c-Jun-N-terminal Kinase (JNK) (Kim *et al.* 2013; Yamauchi *et al.* 2010), as shown in Figure 5. Phosphorylaton of these MAPK proteins initiates various transcription factors, including the activator protein 1 (AP1) and CREB (Cargnello and Roux 2011; Plotnikov *et al.* 2011), which affect subsequent gene expression. VPA has been shown to modulate expression of various genes via MAPK activation both *in vitro* and *in vivo* (Monti *et al.* 2009).

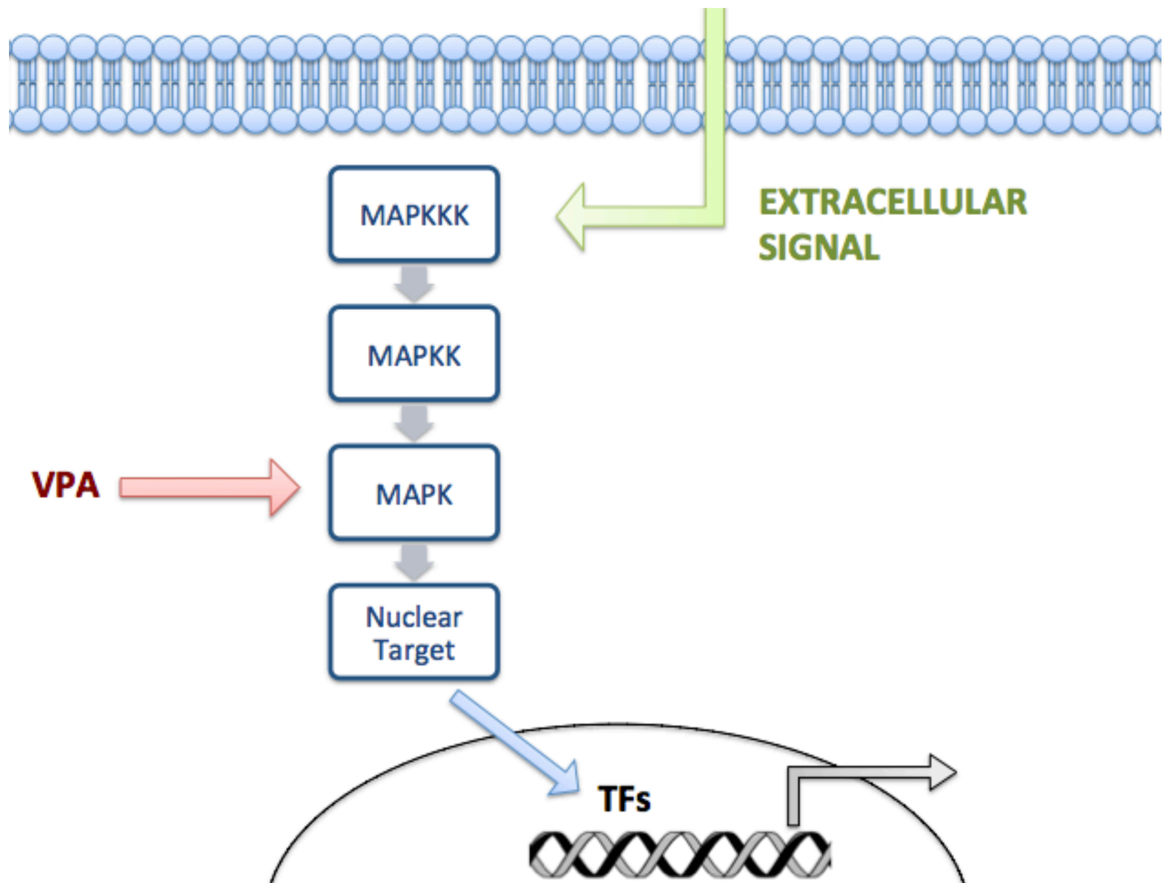


Figure 5: Overview of the effect of VPA on MAPK signaling.

VPA promotes the phosphorylation of various MAPK proteins. MAPK signaling controls the activity of various nuclear transcription factors, which are involved in the regulation of numerous genes.

1.6.2.1.2 PKC

PKC is a serine/threonine kinase that is regulated by the C1-C2 regions on its amino-terminal domain (Slater *et al.* 2002). PKC signaling is mediated by GPCR activation, which is arbitrated by PLC activity, as depicted in Figure 6. PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to DAG and inositol 1,4,5–triphosphate (IP_3), which stimulates the influx of intracellular calcium (Ca^{2+}). DAG and calcium are cofactors required in the activation of PKC (Nishizuka 1995; Mellor and Parker 1998). VPA antagonizes PKC activity (Monti *et al.* 2009; Chen *et al.* 1994), by reducing intracellular calcium release (Kurita *et al.* 2007).

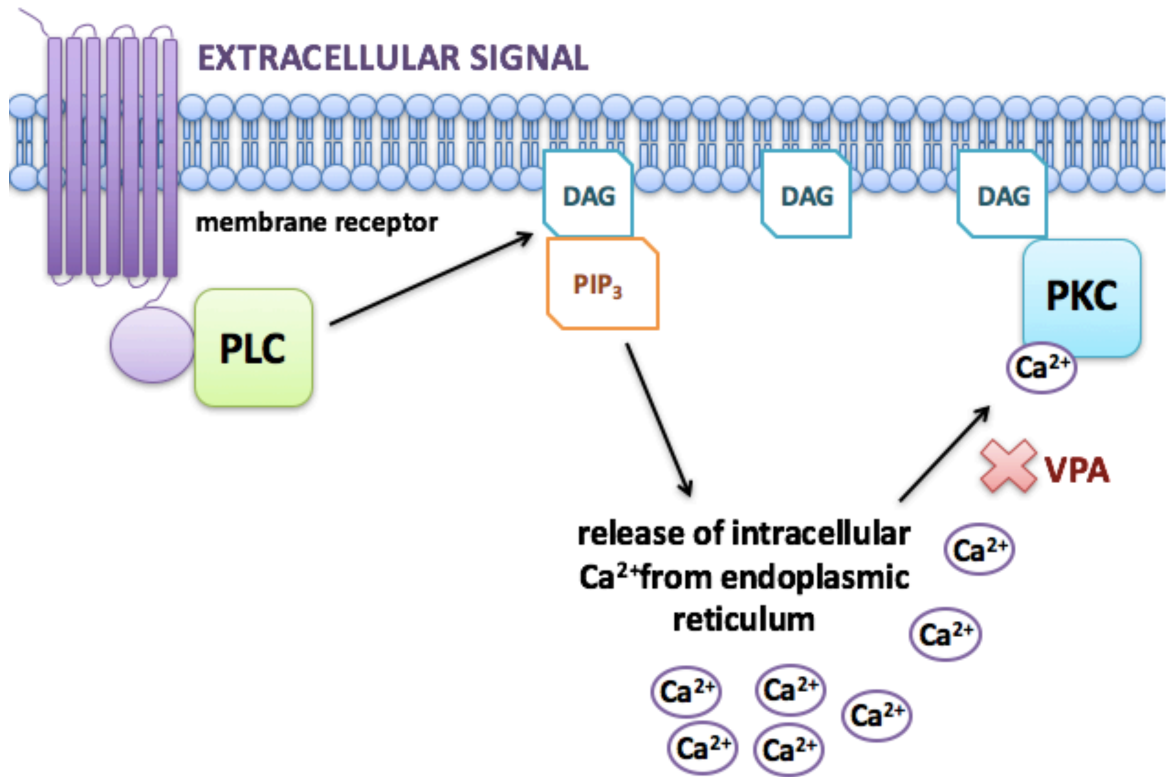


Figure 6: Overview of the effect of VPA on PKC signaling.

VPA antagonizes the expression and activity of PKC, through a mechanism that involves a decrease in intracellular calcium concentrations.

1.6.2.1.3 PI3K/AKT

Phosphoinositide 3-kinase (PI3K) is a kinase for phosphatidylinositol lipids. It controls this signaling pathway by phosphorylating PIP_2 into phosphatidylinositol 3,4,5–triphosphate (PIP_3) which creates an anchor for downstream proteins (Cantley 2002). 3-phosphoinositide dependent protein kinase-1 (PDK1), which activates Protein kinase B (PKB/AKT), binds to PIP_3 (Chen *et al.* 2001). Protein kinase B (PKB/AKT) is a serine/threonine kinase that has been widely studied for its role in various cancers as a proto-oncogene (Bozulic and Hemmings 2009). PKB/AKT signaling functions to regulate cellular metabolism, survival and proliferation, in response to extracellular signals transmitted through receptor tyrosine kinases (Manning and Cantley 2007; Bozulic and Hemmings 2009). AKT is a serine/threonine kinase, which upon activation, modulates various downstream intracellular proteins and transcription factors (Manning and Cantley 2007). AKT signaling is regulated by PIP_3 production and depletion. As illustrated in Figure 7, VPA promotes PI3K/AKT signaling (Gurpur *et al.* 2009; Teng *et al.* 2014; Wu and Shih 2011), through a mechanism which is thought to involve increased PIP_3 production (Chang *et al.* 2014).

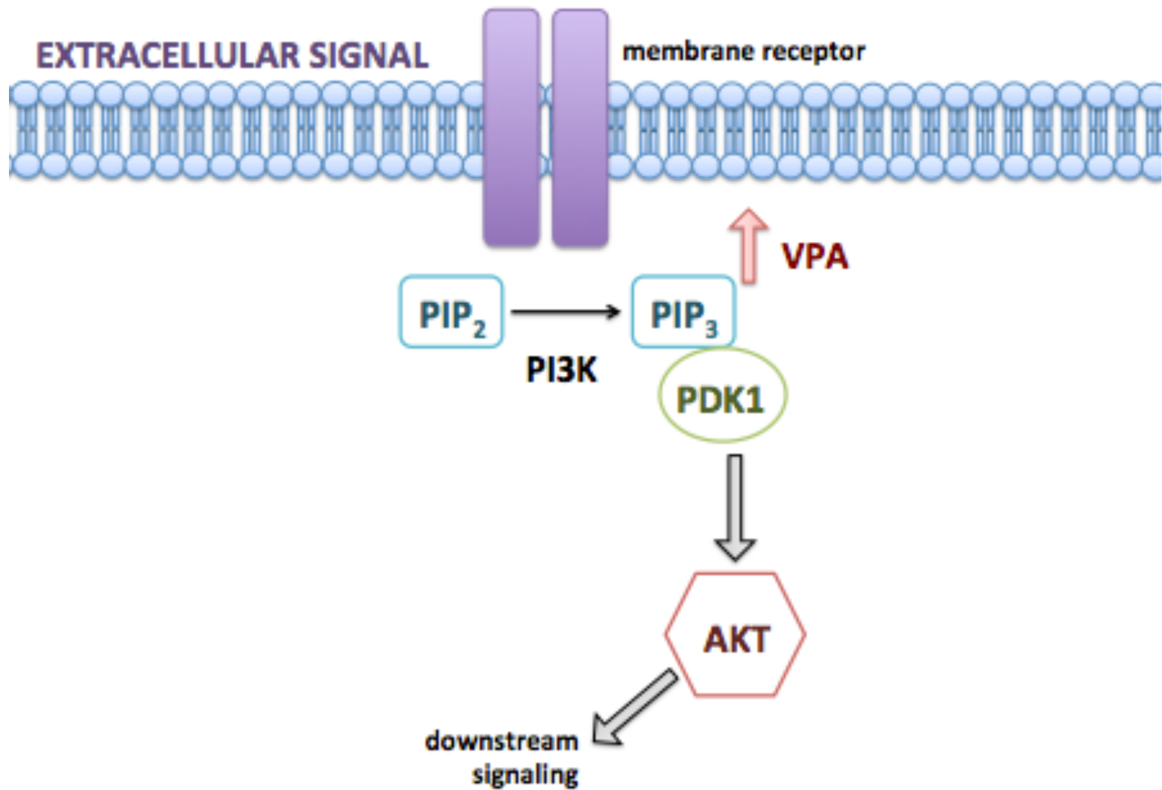


Figure 7: Overview of the effect of VPA on PI3K/AKT signaling.

VPA increases PIP_3 production, creating an increase in the availability of PDK1 docking sites. As PDK1 proteins activate AKT signaling, VPA augments the PI3K/AKT pathway.

1.6.2.1.4 GSK3β

Glycogen synthase kinase 3 beta (GSK3β) comes from a family of serine/threonine kinases that were initially discovered for their role in the regulation of glycogen synthesis (Woodgett 2001). A more widespread role emerged for GSK3β signaling, as it was also discovered to have a role in microtubule stabilization, cytoskeletal organization, and cell cycle regulation (Wakefield *et al.* 2003). GSK3β signaling occurs downstream of many molecular pathways, including the MAPK, PKC, PKA, and PI3K/AKT signaling cascades (Harwood 2001). Unlike other protein kinases, GSK3β is active in the resting state (Woodgett 2001). GSK3β couples with a series of proteins to form a destruction box complex that terminates intracellular signals by phosphorylating signaling molecules and targeting them for proteasomal degradation (Doble and Woodgett 2003). Inactivation of GSK3β signaling results in the stabilization of signaling molecules, which translocate into the nucleus and interact with diverse transcription factor complexes to initiate transcription (Doble and Woodgett 2003). As illustrated in Figure 8, PI3K-mediated AKT activation is a dominant negative regulator of GSK3β signaling (Mitsiades *et al.* 2004; Clodfelder-Miller *et al.* 2005). The VPA-mediated enhancement of the PI3K/AKT signaling cascade will inhibit the downstream effects of GSK3β signaling (Gurpur *et al.* 2009; Teng *et al.* 2014; Wu and Shih 2011). VPA is also thought to antagonize GSK3β activity independent of the PI3K/AKT pathway (Chen *et al.*

1999b), however the mechanism underlying this inhibition it is not yet clear (Monti *et al.* 2009).

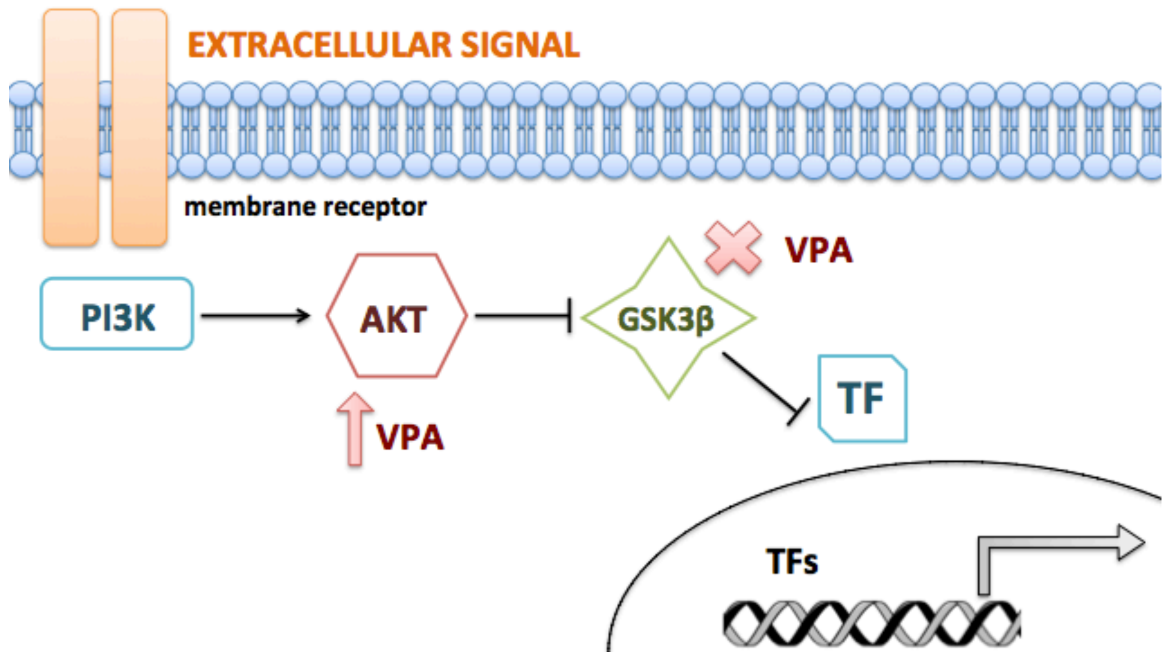


Figure 8: Overview of the effect of VPA on GSK3β signaling.

VPA antagonizes GSK3β activity, which prevents degradation of transcription factors by GSK3β. Inhibition of GSK3β may be a result of the activation of AKT by VPA. TF=transcription factor.

1.6.2.2 TRANSCRIPTION FACTOR MODULATION

Regulation of intracellular signal transduction pathways is coupled with the modulation of various transcription factors that affect subsequent gene expression. VPA influences the expression and binding affinities of various transcription factors in cultured cells, as well as in the brain (Monti *et al.* 2009).

A fundamental transcription factor regulated by VPA is activator protein 1 (AP1), which is a heterodimer composed of proteins from the Jun and Fos transcription factor families, and is important for neuronal development, differentiation, and proliferation (Hess *et al.* 2004). VPA induces expression of c-Fos, c-Jun (Asghari *et al.* 1998), and AP1 (Chen *et al.* 1999c), as well as several genes controlled by AP1 (Monti *et al.* 2009). As GSK3β attenuates expression of AP1, the induction of AP1 by VPA may therefore be the result of its inhibition of GSK3β activity (Chen *et al.* 1999b).

Another broad-spectrum transcription factor regulated by VPA is CREB (Chen *et al.* 1999a), however the nature of this interaction is not clear. VPA increases CREB phosphorylation, binding affinity, and cAMP-mediated gene expression in certain contexts (Biermann *et al.* 2010; Einat *et al.* 2003), but not others (Chen et al. 1999a; Chen et al. 1997).

VPA has also been shown to modulate the expression of genes controlled by the Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells (NFKβ; Ichiyama *et*

al. 2000; Rao *et al.* 2007) and Specificity Protein (SP; Arinze and Kawai 2003) transcription factor families.

1.6.2.3 EPIGENETIC REGULATION

Epigenetic regulation is defined as modifications that alter gene expression without changing the DNA sequence of the gene (Dupont *et al.* 2009). Epigenetic changes use covalent modifications to alter chromatin structure (Richards and Elgin 2002). The fundamental unit of DNA packaging is the nucleosome, which is composed of of an octamer consisting of two copies each of the four histone core proteins, H2A, H2B, H3, and H4 wrapped around approximately 150 base pairs (1.75 turns) of double-stranded DNA, to form supercoils (Hansen 2012). Nucleosomes are held together by histone H1, which acts as a linker protein, creating the "beads on a string" appearance of DNA (Hansen 2012). The histone core proteins interact with the negatively charged DNA backbone using their positively charged amino terminal tails to maintain chromatin in a highly condensed state, called heterochromatin (Horvath *et al.* 2001).

Enzymes that alter the electrostatic interactions between the negatively charged DNA and the positively charged histone core, regulate gene transcription. For example, histone acetylation, catalyzed by the histone acetyltransferase (HAT) enzyme, neutralizes the positive charge on the histone proteins. This reduces

histone affinity to the DNA strand, and the associated chromatin is maintained in a loosened structure called euchromatin (Marmorstein and Roth 2001). The loosened structure exposes regulatory genetic sequences, and permits the binding of transcriptional machinery to the appropriate regulatory genetic sequences, which then activate transcription (Morse 2007). The reverse process, deacetylation, is catalyzed by the histone deacetylase (HDAC) enzyme. The associated chromatin recondenses, and transcriptional activity is terminated (Kimura *et al.* 2005; Lennartsson and Ekwall 2009; Dokmanovic *et al.* 2007; Bannister and Kouzarides 2011). Histone hyperacetylation is linked to increased gene expression (Grunstein 1997), while extreme histone hypoacetylation transcriptionally silences genes (Kristjuhan *et al.* 2002). As such, it is clear that maintaining the balance between histone acetylation and deacetylation is essential for the regulation of transcriptional activity and ultimately, gene expression.

1.6.2.3.1 HDAC INHIBITION

HDAC inhibitors are a class of drugs that antagonize HDAC activity. As depicted in Figure 9, HDAC inhibitors maintain chromatin in an acetylated state, and therefore, a transcriptionally active conformation. They are organized according to their structural classifications, which include hydroxamates, cyclic peptides, short-

chain fatty acids, and benzamides (de Ruijter *et al.* 2003). The chemical structure of VPA classifies it as a short-chain fatty acid HDAC inhibitor (Phiel *et al.* 2001).

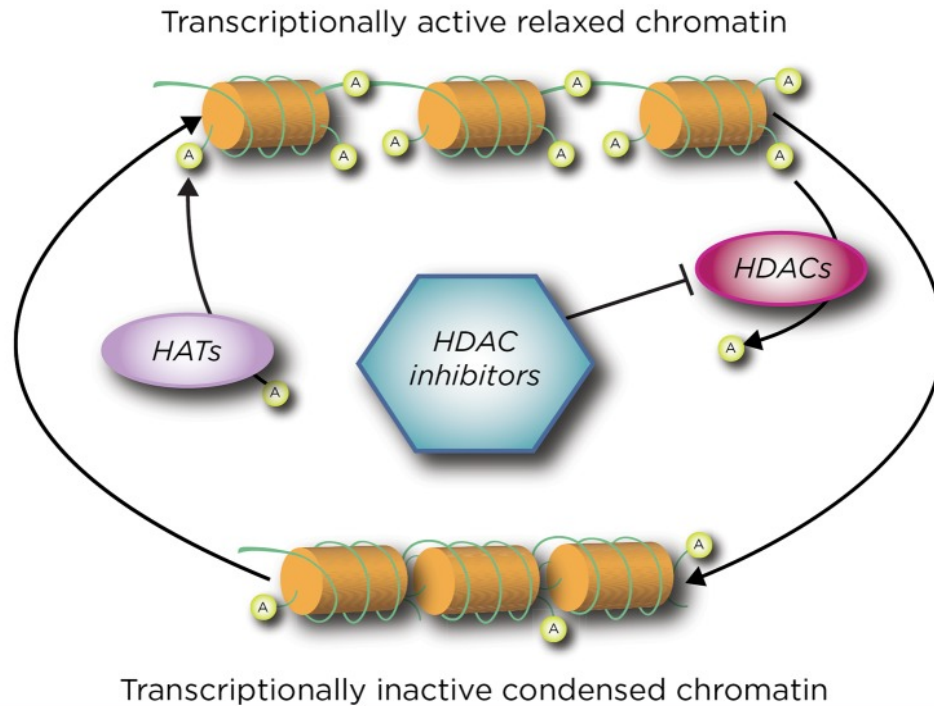


Figure 9: Chromatin remodelling by HDAC inhibitors.

Histone acetylation is catalyzed by HAT enzymes, which promote the unravelling of the chromatin fibre into a relaxed, transcriptionally active state. The removal of acetyl groups by HDAC enzymes reverts the chromatin into its condensed, transcriptionally inactive form. HDAC inhibitors sustain the chromatin in a transcriptionally active state by preventing the removal of acetyl groups by HDAC enzymes (de Ruijter *et al.* 2003). This figure taken from Marsoni *et al.* 2008.

1.7 VALPROIC ACID AND MELATONIN RECEPTORS

Initial studies on the effects of VPA on melatonin receptor expression were completed in rat C6 glioma cells (Castro *et al.* 2005; Kim *et al.* 2008) and in human MCF-7 breast cancer cells (Jawed *et al.* 2007). In these studies, a dose-dependent relationship was observed in which VPA caused a robust induction of MT_1 mRNA and protein (Castro *et al.* 2005; Kim *et al.* 2008; Jawed *et al.* 2007). These experiments set the groundwork for the *in vivo* studies that ensued, which examined the effects of VPA on melatonin receptor expression in the CNS. Chronic VPA administration was found to cause a robust increase in the levels of both melatonin receptor isoforms, MT_1 and MT_2 in the rat hippocampus (Niles *et al.* 2012).

Melatonin receptor expression is reduced during normal senescence, however this depletion is accelerated in the diseased state (Hardeland *et al.* 2011). Impairments in melatonergic signaling related to aberrant MT_1 and/or MT_2 expression profiles are thought to contribute to the overall deterioration of the nervous system. This has been demonstrated in several neurodegenerative conditions, including Alzheimer's disease (Brunner et al., 2006; E Savaskan, 2006; Egemen Savaskan et al., 2005, 2007), Parkinson's disease (Adi et al., 2010), amyotrophic lateral sclerosis (Zhang et al., 2013), and multiple sclerosis (Natarajan et al., 2012). Further scrutiny of the effects of VPA on melatonin receptors revealed that the MT_2 subtype is upregulated within the neurogenic region of the hippocampus (Bahna *et al.* 2014). As VPA is thought to be a promising reagent for

the management of many disorders, the upregulation of the melatonin receptors by VPA proposes a role for the melatonergic system in the therapeutic actions of VPA.

The safety and efficacy of VPA and melatonin co-treatments have been demonstrated in various models of pediatric epilepsy (Gupta *et al.* 2005; Gupta *et al.* 2004a; Gupta *et al.* 2004b), which suggest that VPA and melatonin might be administered in combination to augment melatonergic signaling by means of melatonin receptor overexpression. VPA-mediated enhancement of melatonin receptor expression may be beneficial to augment the efficiency of systemic melatonin, and/or offset deficiencies in receptor densities in areas where it is depleted as a consequence of normal aging or disease progression. This may represent a possible therapeutic strategy in the management of neurological/neurodegenerative diseases.

1.8 STUDY AIMS

1.8.1 OVERALL AIM

The aim of the present study is to determine the mechanism(s) underlying the transcriptional upregulation of the melatonin MT_1 receptor by VPA. Furthermore, the potential functional implications of VPA-mediated melatonin receptor upregulation will be addressed, using combination treatments involving VPA and melatonin.

1.8.2 SPECIFIC AIMS, RATIONALES, AND HYPOTHESES

1: To investigate the involvement of epigenetic modifications in the upregulation of the melatonin MT_1 receptor by VPA as follows: (A) To compare the effect of VPA with that of other HDAC inhibitors with structural classifications distinct from VPA, and (B) To examine the effects of valpromide (VPM), a VPA analogue lacking HDAC activity. Additionally, the relationship between increased melatonin receptor expression and MT_1 promoter histone acetylation by VPA will be examined.

Rationale: The induction of melatonin receptor expression may be the result of any of the mechanisms by which VPA is known to function, as outlined earlier (Monti *et al.* 2009). Blockade of the $GABA_A$ receptor by bicuculline methiodide did not prevent the induction of MT_1 by VPA *in vitro*, suggesting that the well-characterized modulation of GABAergic signaling by VPA does not have a role in its transcriptional upregulation of the melatonin receptors (Castro *et al.* 2005). Trichostatin A (TSA), an HDAC inhibitor that is structurally distinct from VPA, mimicked the inductive effect of VPA on the melatonin receptor, suggesting that an epigenetic mechanism may underlie the upregulation of this receptor (Kim *et al.* 2008).

Hypothesis: VPA-induced melatonin MT_1 receptor upregulation is mediated by an epigenetic mechanism involving chromatin remodelling and increased gene

expression. As such, we hypothesize that other HDAC inhibitors will parallel the effects of VPA on MT_1 induction *in vitro*, and that the melatonin MT_1 promoter will be hyperacetylated following treatment with VPA.

2: To determine the role of VPA in the molecular regulation of MT_1 expression. The effects of VPA on CBP expression will be examined, and a pharmacological blockade of CREB will be conducted to assess the role of this transcription factor in the upregulation of MT_1 by VPA. Specifically, KG501, which blocks the interaction of CREB to its nuclear co-factor, CBP, will be used. Moreover, the effects of VPA on the expression of Pitx1, Egr1, SF1 and GATA2, which are transcription factors known to regulate MT_1 promoter activity in vitro, will be examined.

Rationale: CREB is highly implicated in the regulation of MT_1 expression (Barrett et al. 1996). CREB, which is activated by phosphorylation, has several phosphorylation sites and is targeted by various kinases (Mayr and Montminy 2001). VPA has also been shown to have a direct influence on CREB phosphorylation and activity (Biermann et al. 2010; Einat et al. 2003). VPA regulates the expression of many genes via a CREB-sensitive mechanism (Monti *et al.* 2009; Creson *et al.* 2009), suggesting it may also induce MT_1 expression via CREB.

Moreover, rat MT_1-specific promoter assays have confirmed that Pitx1 initiates transcriptional activity of the rat MT_1 promoter, and that its effects are regulated by its co-factors, Egr1, SF1 and GATA2 (Johnston *et al.* 2003b). VPA modulates the expression of the co-regulators of Pitx1-driven transcription: Egr1 (Daigle *et al.* 2011; Almutawaa *et al.* 2014; Zhou *et al.* 2011), SF1 (Glister *et al.* 2012; Chen *et al.* 2007), and GATA2 (Liu *et al.* 2010) have been demonstrated to be targets of VPA in various cell and animal models. However, these co-regulators cannot drive MT_1 transcription in the absence of Pitx1 (Johnston *et al.* 2003b). While the effects of VPA on Pitx1 expression are not currently known, the robust induction of MT_1, and modulation of Egr1, SF1, and GATA2 suggests that VPA may modulate expression of Pitx1 and its co-regulators to affect MT_1 expression.

Hypothesis: CREB and Pitx1 have a prominent role in the induction of MT_1 expression. We hypothesize that the inhibition of CREB will attenuate VPA-mediated MT_1 upregulation, and that the induction of MT_1 will be matched with an increase in Pitx1 expression by VPA.

3: To study the involvement of intracellular kinases regulated by VPA in the induction of MT_1. Pharmacological inhibitors of PKC, PI3K/AKT, and GSK3β signaling will be combined with VPA to assess the roles of these signaling pathways in MT_1 expression.

Rationale: VPA modulates activity of the MAPK, PKC, PI3K/AKT, and GSK3β signaling cascades (Monti *et al.* 2009). As these pathways act as upstream regulators of many transcription factors that control subsequent gene expression, they may have a role in the upregulation of MT_1 expression by VPA. Pharmacological inhibition of MAPK/ERK signaling using PD98059 did not prevent upregulation of MT_1 by VPA, indicating that the mechanism underlying transcriptional induction of MT_1 by VPA is independent of MAPK/ERK signaling (Castro *et al.* 2005). The involvement of the PKC, PI3K/AKT, or GSK3β pathways in this interaction is not known.

Hypothesis: As the PKC, PI3K/AKT, and GSK3β signaling cascades regulate transcriptional factors that control gene expression, we hypothesize that inhibition of one or more of these pathways will influence the induction of MT_1 by VPA.

4: To test the regulation of melatonin receptor expression by chronic VPA treatment in the rat striatum and ventral midbrain.

Rationale: VPA was shown to cause a dose-time dependent induction of MT_1 expression in cultured cells (Castro *et al.* 2005; Kim *et al.* 2008; Jawed *et al.* 2007), as well as both MT_1 and MT_2 in the rat hippocampus (Niles *et al.* 2012). The induction of melatonin receptor expression in the hippocampus is thought to

contribute to the neuroprotective effects of VPA in this brain region (Bahna *et al.* 2014). VPA had been recently demonstrated to exert neuroprotective effects in the striatum and substantia nigra in a rodent model of Parkinson's disease (Carriere *et al.* 2014). Both of these regions are known to express one or both melatonin receptor isoforms (Lacoste *et al.* 2015), however the effect of VPA on melatonin receptor expression in these areas has not been investigated.

Hypothesis: VPA causes a robust transcriptional upregulation of melatonin receptor expression in various cell types and tissues. Thus, we hypothesize that VPA will influence melatonin receptor expression in the rat striatum and ventral midbrain.

5. To examine the significance of increased melatonin receptor mRNA expression, and presumably increased receptor density, following VPA treatment, as reflected by the modulation of Kelch-Like ECH-Associated Protein 1 (Keap1) and nuclear factor erythroid 2 [NF-E2]-related factor 2 (Nrf2) expression in the rat brain.

Rationale: The NF-E2-related factor 2-Antioxidant responsive element (Nrf2-ARE) signaling cascade, illustrated in Figure 10, is one of the intracellular pathways arbitrating the induction of antioxidant gene expression by melatonin. Under normal conditions, Keap1 appears to have a role in the surveillance of oxidative conditions

in the cell. It is bound to an E3 ubiquitin ligase, which restrains Nrf2 entry into the nucleus by targeting it for proteasomal degradation. Oxidative stressors modify Keap1 and inactivate the E3 ubiquitin ligase complex. Thus under oxidative stress conditions, Nrf2 is free to translocate into the nucleus, where it couples with a Maf protein to induce transcription of several antioxidant genes (Katsuoka *et al.* 2005). Endogenous dysregulation of Nrf2-ARE signaling in the CNS is thought to contribute to the overall deterioration of the nervous system, particularly in conditions involving neurodegeneration (Yang *et al.* 2015). Melatonin induces Nrf2 expression and downstream Nrf2-ARE enzymes in multiple models of peripheral oxidative stress (Jung *et al.* 2009; Jung *et al.* 2010; Tripathi and Jena 2010). Importantly, these effects were mirrored in the models of cerebral oxidative stress in rat C6 glioma cells (Jumnongprakhon *et al.* 2015), as well as in the rat brain (Wang *et al.* 2012). The Nrf2-related antioxidant actions of melatonin are thought to be mediated by melatonin receptor activation (Shin *et al.* 2015; O'Neal-Moffitt *et al.* 2015).

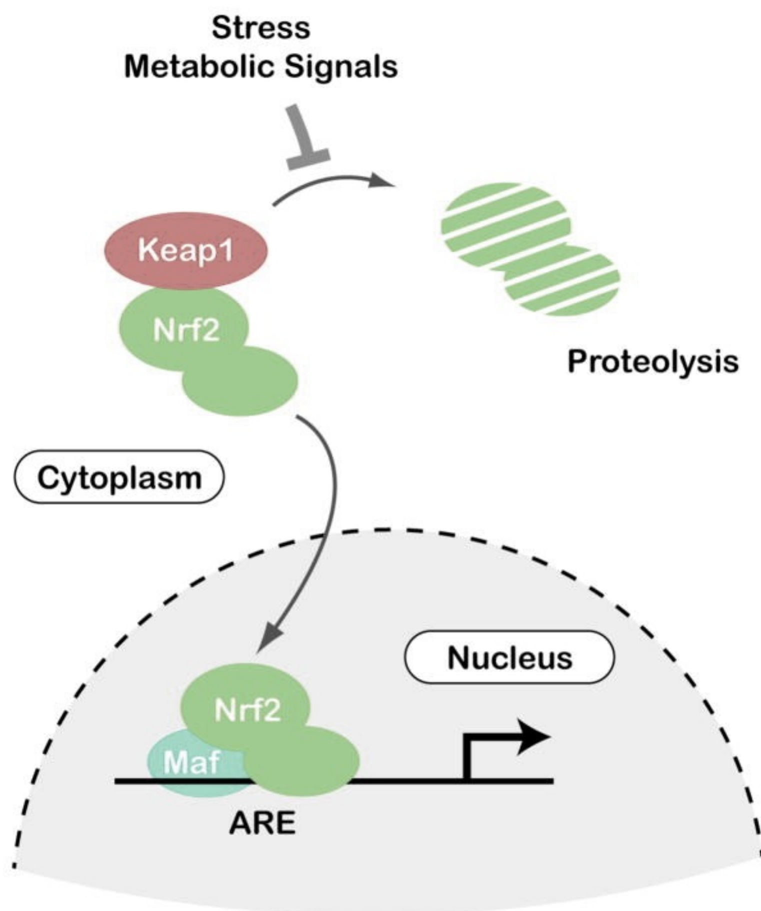


Figure 10: Overview of Nrf2-ARE Signaling.

Nrf2 is a nuclear transcription factor, which is targeted for proteolysis by Keap1 under normal conditions. Oxidative stressors inhibit Nrf2 degradation by Keap1. Nrf2 translocation into the nucleus binds to the ARE sequence to initiate transcription of antioxidant response element genes (Katsuoka et al. 2005). This figure was taken from Sykiotis *et al.* 2011.

Hypothesis: As melatonin receptor activation is thought to underlie the initiation of the Nrf2-ARE pathway, we hypothesize that the increase in melatonin receptor density by VPA, accompanied by exogenous melatonin administration, will augment the activation of Nrf2-ARE signaling by melatonin in the rat brain.

6. To explore the significance of increased melatonin receptor expression, and presumably increased receptor density, following VPA treatment, as reflected by the modulation of cerebral dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF) expression in the rat brain.

Rationale: Melatonin induces GDNF expression in cultured astrocytes under normal conditions (Kong *et al.* 2008b; Armstrong and Niles 2002), as well as in conditions of mild oxidative stress (Chen *et al.* 2003; Lee *et al.* 2006). In models of severe neurodegeneration, melatonin was shown to prevent GDNF overexpression in response to injury and/or disease (Sharma *et al.* 2006; Olivieri *et al.* 2003). The upregulation of GDNF occurs in cells which express MT_1 and/or MT_2 (Kong *et al.* 2008b; Armstrong and Niles 2002; Niles *et al.* 2004). Pharmacological agents that increase melatonin receptor expression were found to simultaneously increase GDNF expression, suggesting a possible correlation between melatonin receptors

and GDNF induction may exist (Castro *et al.* 2005; Niles *et al.* 2012; Hardeland *et al.* 2011). Recently, two novel neurotrophic factors, CDNF and MANF, were characterized (Lindholm *et al.* 2007; Voutilainen *et al.* 2009). Similar to GDNF, CDNF and MANF are important in the maintenance and survival of midbrain dopaminergic neurons (Voutilainen *et al.* 2011; Voutilainen *et al.* 2015; Cordero-Llana *et al.* 2015). Intra-striatal administration of CDNF and MANF have been demonstrated to be potent neuroprotective and neurorestorative agents in models of severe neurodegeneration (Airavaara *et al.* 2012; Lindholm *et al.* 2007; Voutilainen *et al.* 2009). Also similar to GDNF, VPA causes a transcriptional upregulation of CDNF and/or MANF expression in paradigms which correspond with increased MT_1 and/or MT_2 levels (Niles *et al.* 2012). The effects of melatonin on CDNF and MANF are not known.

Hypothesis: Melatonin is thought to induce neurotrophic factor expression via activation of its receptor(s). As such, we hypothesize that melatonin will increase the expression of CDNF and MANF. Furthermore, the VPA-mediated increase in melatonin receptor expression, supplemented with exogenous melatonin treatments, will cause a greater induction of CDNF and/or MANF expression, than that of melatonin alone.

2 METHODS

2.1 Study Overview

This project was divided into four parts covering the mechanisms underlying the induction of MT_1 by VPA, and the consequences of melatonin receptor upregulation by this drug. The first study scrutinized the involvement of histone acetylation in the upregulation of MT_1 by VPA; the second study assessed the role of transcription factors and intracellular kinases in this induction; the third study explored other regions of the rat brain where melatonin receptor expression might be sensitive to VPA treatments; the fourth study gauged the relevance of VPA-mediated melatonin receptor upregulation in the rat brain. Studies 1 and 2, which assessed the mechanisms underlying the induction of MT_1 expression, were conducted *in vitro*. The rat C6 glioma cell line, which has been previously demonstrated to express MT_1 was used as a model of VPA-mediated transcriptional induction of the melatonin receptor (Kim *et al.* 2008; Castro *et al.* 2005). Studies 3 and 4 were conducted *in vivo* in the adult rat brain, as we have reported previously (Niles *et al.,* 2012).

2.1.1 Experimental Design: Study 1

Early studies revealed that TSA, an HDAC inhibitor that is structurally distinct from VPA, induces MT_1 expression (Kim *et al.* 2008). In order to further understand the mechanism underlying the robust upregulation of MT_1 by VPA, the first study

examined the effects of various other compounds in comparison to VPA on the transcriptional induction of MT_1: Suberanilohydroxamic Acid (SAHA) and 4-(Dimethylamino)-N-[7-(Hydroxyamino)-7-Oxoheptyl] Benzamide (M344), which are HDAC inhibitors that are not related to VPA or each other (Dokmanovic *et al.* 2007; Riessland *et al.* 2006), as well as VPM, a VPA analogue lacking HDAC inhibitory activities (Phiel *et al.* 2001). The effects of SAHA and VPA on the levels of global acetyl H3 (AcH3) lysine residues 9 and 18 (K9/18) were examined, and subsequently the effect of VPA on the AcH3 K9/18 sites localized across the MT_1 promoter was examined (Figure 11).

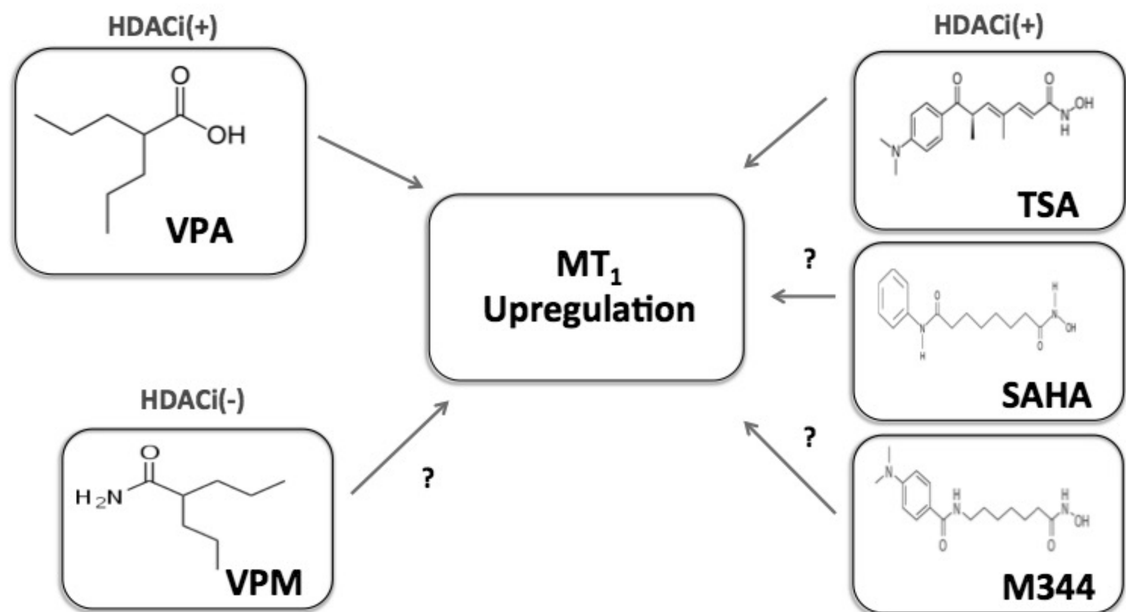


Figure 11: Experimental design of Study 1.

The relevance of HDAC inhibition on the induction of MT_1 by VPA was assessed in comparison to the HDAC inhibitors TSA (Kim *et al.* 2008), SAHA, and M344, as well as the VPA analogue, VPM. The effects of VPA or SAHA on global histone H3 acetylation, and the effect of VPA on the acetylation statuses of histones along the MT_1 promoter were examined subsequently.

2.1.2 Experimental Design: Study 2

As CREB is thought to be involved in the regulation of MT_1 (Barrett *et al.* 1996), the role of CREB in VPA-mediated MT_1 induction was examined. The effect of VPA on CBP expression was assessed initially. KG501, a direct antagonist of CREB-mediated transcription (Best *et al.* 2004), was then used to study the role of CREB on the induction of MT_1 expression by VPA.

The effects of intracellular signaling pathways known to be targeted in the molecular regulation of gene expression by VPA, were also examined using pharmacological kinase inhibitors (Monti *et al.* 2009). Previously, we showed that the MAPK pathway was not involved in the transcriptional regulation of MT_1 by VPA (Castro *et al.* 2005). In this study, Bisindolymaleimide 1 (BIM1), LY294002, and AR-A014418 were used to block PKC, PI3K/AKT, and GSK3β signaling respectively. Further molecular analysis was focused on the effects of VPA on the mRNA expression of Pitx1, Egr1, SF1, and GATA2 to examine the role of VPA in the transcriptional regulation of MT_1 (Figure 12).

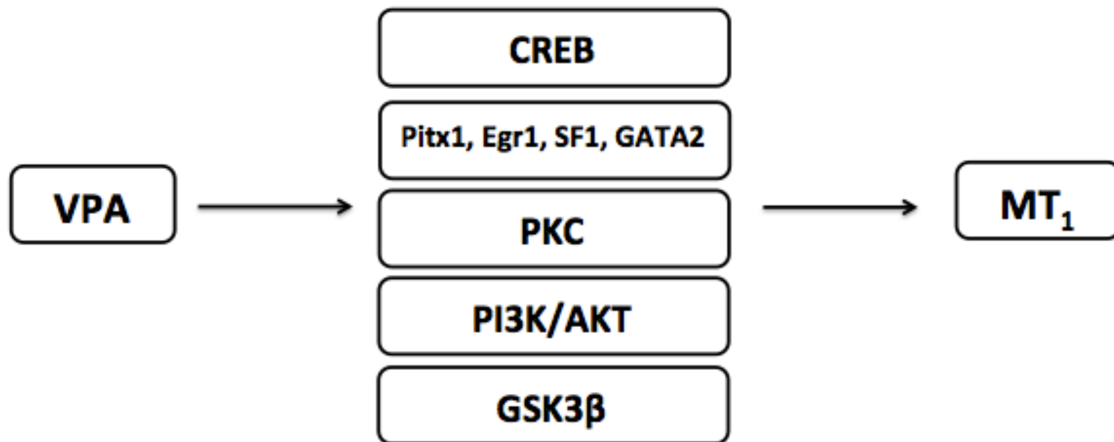


Figure 12: Experimental design of Study 2.

The role of CREB, MT_1 promoter-specific transcription factors (Pitx1, Egr1, SF1, GATA2), and intracellular kinases in the induction of MT_1 by VPA were evaluated.

2.1.3 Experimental Design: Study 3

We previously showed that VPA upregulates MT_1 and MT_2 in the rat hippocampus (Niles *et al.* 2012). In this study, we aimed to determine whether the melatonin receptors are sensitive to VPA treatments in other brain regions. The effects of chronic VPA treatment on melatonin receptor expression on the rat striatum and ventral midbrain were examined.

2.1.4 Experimental Design: Study 4

The mRNA expression of Nrf2 and Keap1 of the Nrf2-ARE signaling pathway, as well as the neurotrophic factors CDNF and MANF in the rat brain were measured following chronic co-administration of VPA and melatonin. This was done to determine whether the VPA-mediated increases in melatonin receptor expression could enhance melatonergic signaling (Figure 13).

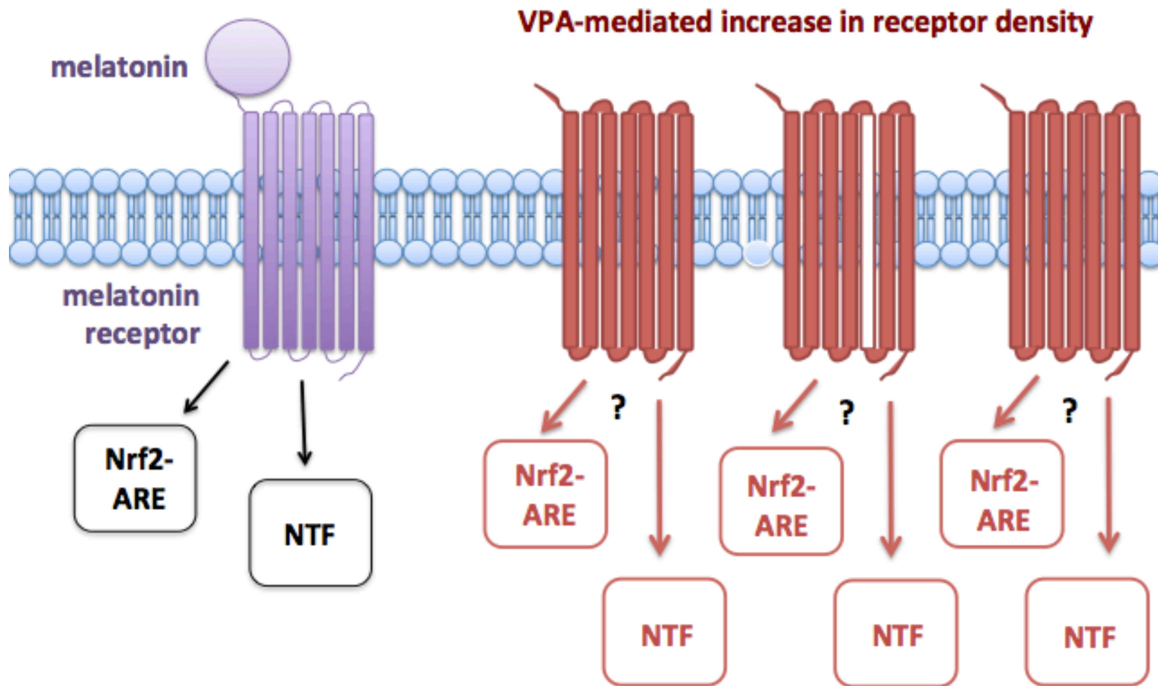


Figure 13: Experimental design of Study 4.

VPA and melatonin combination treatments were used to study the therapeutic implications of VPA-mediated melatonin receptor upregulation, as reflected by the mRNA expression levels of the Nrf2-ARE signaling cascade genes, Nrf2 and Keap1, and the neurotrophic factors (NTF), CDNF and MANF.

2.2 ANIMALS

Adult male Sprague-Dawley rats (500-600g; Charles River) were housed in a 12h light/dark cycle, with lights on at 7 am. Animals had free access to a standard diet and water. All experiments were carried out according to the guidelines set by the McMaster University Animal Research Ethics Board (AREB).

2.2.1 DRUG PREPARATION

2.2.1.1 VPA

VPA treatments were prepared from VPA sodium salt (Sigma-Aldrich) in regular drinking water as a solution of 4mg/ml. Controls for VPA treatments received only drinking water. Treatments were administered via drinking water for 21 days, and the water was changed twice per week.

2.2.1.2 MELATONIN

Melatonin (Sigma-Aldrich) treatments were prepared in ethanol and diluted in non-sterilized water to a final concentration of 4μg/ml. Controls for melatonin treatments received 0.4% ethanol. Treatments were administered via drinking water for 21 days, and the water was changed twice per week.

2.2.2 DRUG TREATMENTS

2.2.2.1 VPA

Animals were randomly divided into 2 groups: one treatment group receiving chronic VPA (4mg/mL) and one control group receiving vehicle (water). This dose of VPA has been shown to induce melatonin receptor expression in the rat brain (Niles *et al.* 2012).

2.2.2.2 VPA + MELATONIN

Animals were randomly divided into 5 groups: the first treatment group receiving chronic treatment of VPA (4mg/mL), a second treatment group receiving melatonin (4μg/mL), a third treatment group receiving VPA+ melatonin (4 mg/mL VPA plus 4μg/mL melatonin), and two control groups receiving water (VPA) or 0.4% ethanol (VPA + melatonin).

2.2.3 TISSUE COLLECTION

Animals were decapitated between the hours of 11 am and 3 pm. The striata, hippocampi, and ventral midbrains were dissected rapidly on ice and stored in RNALater (Sigma-Aldrich) at 4°C until used for RNA isolation, complimentary DNA (cDNA) synthesis and polymerase chain reaction (PCR) amplification.

2.3 CELL CULTURE

Rat C6 glioma cells from passages 8-18 were cultured in Dulbecco's modified Eagle's Medium (DMEM; Invitrogen) with 10% Fetal bovine serum (FBS; Invitrogen), penicillin/streptomycin (10, 000 U/mL; Invitrogen), and fungizone (2.50 μg/mL; Invitrogen), at 37°C under 5% CO2/air.

2.3.1 DRUG PREPARATION

2.3.1.1 VPA

A working solution of 50mM VPA sodium salt was prepared in sterile DMEM. Controls for VPA treatments were maintained in DMEM/1% FBS. For 72h treatments, both drug and medium were replaced on the second day. VPA concentrations (1-3mM) that have been previously reported to induce MT_1 expression in this cell line were held used for this study (Castro *et al.* 2005; Kim *et al.* 2008).

2.3.1.2 SAHA

A working solution of 10mM SAHA (Santa Cruz Biotechnology) was prepared in 100% DMSO. Controls for SAHA treatments were maintained in DMEM/1% FBS and 0.05% dimethyl sulfoxide (DMSO). For 72h treatments, both drug and medium were replaced on the second day. The dosages of SAHA used were determined

empirically. A range of SAHA treatments (1-10μM) were tested. Low dosages (1μM, 3μM and 5μM) were chosen to minimize DMSO-associated cytotoxicity.

2.3.1.3 M344

A working solution of 10mM M344 (Tocris Bioscience) was prepared in 100% DMSO. Controls for M344 treatments were maintained in DMEM/1% FBS and 0.03% DMSO. The dosage of M344 used was determined empirically. A range of M344 concentrations (1-5μM) were tested for their induction of MT_1 in rat C6 cells. No differences in MT_1 induction were detected between the different M344 dosages.

2.3.1.4 VPM

A working solution of 500mM VPM (Sigma-Aldrich) was prepared in 100% DMSO, and 20μL or 60μL VPM were added to cells to give final concentrations of 1mM or 3mM VPM. Controls for VPM treatments were maintained in DMEM/1% FBS and 0.2% or 0.6% DMSO. The dosages of VPM chosen matched those of VPA for comparison.

2.3.1.5 AR-A014418

For GSK3β pathway blockade, AR-A014418 (GSK3β Inhibitor VIII, Cayman Chemical) was prepared as a 50mM stock solution in 100% DMSO, and diluted into a

10mM working solution. Controls for AR-A014418 treatments were maintained in DMEM/1% FBS and 0.2% DMSO. AR-A014418 dosage and pre-treatment times were selected based on previous reports of successful GSK3β pathway inhibition in C6 glioma cells, with minimal DMSO-associated cytotoxicity (Chikano *et al.* 2015).

2.3.1.6 BIM1

For PKC pathway blockade, BIM1 (Cayman Chemical) was prepared as a 50mM stock solution in 100% DMSO, and diluted into a 10mM working solution. Controls for BIM1 treatments were maintained in DMEM/1% FBS and 0.01% DMSO. BIM1 dosage and pre-treatment times were selected based on previous reports of successful PKC pathway inhibition in C6 glioma cells, with minimal DMSO-associated cytotoxicity (Adornetto *et al.* 2013).

2.3.1.7 KG501

For blockade of the interaction between CREB and CBP, KG501 (Sigma-Aldrich) was prepared as a 50mM stock solution in 100% DMSO. Controls for KG501 treatments were maintained in DMEM/1% FBS and 0.05% DMSO. KG-501 dosages were selected based on successful CREB inhibition in C6 cells (Morioka *et al.* 2010). A range of pre-treatment times (0.5-3h) was tested on the effects of this drug on VPA-mediated MT_1 induction. No differences were observed between pre-treatment

times tested. The dosage of KG501 was selected based on previous reports of successful inhibition of the CREB to CBP interaction in C6 cells, with minimal cytotoxicity (Morioka *et al.* 2010).

2.3.1.8 LiCl

A working solution of 1M Lithium chloride salt (LiCl; Sigma-Aldrich) was prepared in DMEM. Controls for LiCl treatments were maintained in DMEM/1% FBS. LiCl dosage and pre-treatment times were selected based on previous reports of successful GSK3β pathway inhibition in C6 glioma cells, with minimal cytotoxicity (Fu *et al.* 2014).

2.3.1.9 LY294002

For PI3K/AKT pathway blockade, LY294002 (Cayman Chemical) was prepared as a 50mM stock solution in 100% DMSO, and diluted into a 10mM working solution. Controls for LY294002 treatments were maintained in DMEM/1% FBS and 0.1% DMSO. LY294002 dosage and pre-treatment times were selected based on previous reports of successful PI3K/AKT pathway inhibition in C6 glioma cells, with minimal DMSO-associated cytotoxicity (Li *et al.* 2011).

2.3.2 DRUG TREATMENTS

Treatment outlines for all reagents used in epigenetic and molecular analyses are summarized in Tables 1 and 2.

Table 1: Reagents for Epigenetic Analysis

COMPOUND	HDAC ACTIVITY	STOCK	VOLUME	FINAL
VPA	(+)	50mM	100μL	0.5mM
			200μL	1mM
			600μL	3mM
SAHA	(+)	10mM	1μL	1μM
			3μL	3μM
			5μL	5μM
M344	(+)	10mM	3μL	3μM
VPM	(-)	500mM	20μL	1mM
			60μL	3mM

Table 2: Reagents for Molecular Analysis

COMPOUND	TARGET	STOCK	VOLUME	FINAL	PREINCUBATION TIME
BIM-1	PKC	10mM	1µL	1µM	1h
AR-A014418	GSK3β	20mM	20µL	20µM	1h
KG501	CREB:CBP	50mM	5µL	25µM	1h
LiCl	GSK3β	1M	200µL	20mM	1h
LY294002	PI3K	10mM	10µL	10µM	1h

2.3.3 RT-PCR

2.3.3.1 RNA ISOLATION AND QUANTIFICATION

Total RNA was isolated from C6 cells with TRIzol Reagent (Invitrogen), and precipitated using isopropyl alcohol. RNA pellets were washed in 75% ethanol and solubilized into nuclease-free water. Optical densities were measured at 260nm using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific) and stored at -80°C.

2.3.3.2 DNASE TREATMENT AND DNA SYNTHESIS

5-15µg of RNA was subject to DNAse treatment (Qiagen). 2.2µL of DNAse-treated RNA was used for complimentary DNA (cDNA) synthesis using the

Omniscript Reverse Transcription PCR (RT-PCR) kit (Qiagen) and oligo(dT) primers (Thermo Fisher Scientific).

2.3.3.3 PCR

PCR amplifications were conducted using the GeneAmp PCR System 2400 Thermal Cycler (Perkin Elmer). Amplifications began with heat activation of the HotStarTaq DNA Polymerase (Qiagen) at 95^{0}C for 5m and ended with a final incubation at 72^{0}C for 10m. MT_1 or MT_2 were detected by amplifying 10μl RT product with appropriate primers using the following parameters: 40 cycles of 94^{0}C for 30s, 57^{0}C for 30s, 72^{0}C for 1m. CDNF or MANF were detected by amplifying 2μl RT product with appropriate primers using the following parameters: 33 cycles of 94^{0}C for 30s, 55^{0}C for 30s, 72^{0}C for 1m. Nrf2 or Keap1 were detected by amplifying 2μl RT product with appropriate primers using the following parameters: 28 cycles of 94^{0}C for 30s, 55^{0}C for 30s, 72^{0}C for 1m. Glyceraldehyde-3-Phosphate (GAPDH) and 18S ribosomal RNA (18S) were included as internal controls, and amplified using 2μl RT product with the appropriate primers using the following parameters: 28 cycles of 94^{0}C for 30s, 55^{0}C for 30s, 72^{0}C for 1m. MT_1, MT_2, CDNF, MANF, Nrf2, Keap1, GAPDH, and 18S primers are provided in Table 3.

Table 3: PCR Primers

Gene	Primer Sequence (5'- 3')	Target Size (base pairs)
MT_1	*Fwd:* TTGTGGCGAGTTTAGCTGTG *Rev:* GACACTCAGGCCCATTAGGA	140
MT_2	*Fwd:* TACATCAGCCTCATCTGGCTT *Rev:* CACAAACACTGCGAACATGGT	297
CDNF	*Fwd:* AAAGAAAACCGCCTGTGCTA *Rev:* TCATTTTCCACAGGTCCACA	199
MANF	*Fwd:* GCAAGAGGCAAAGAAAATCG *Rev:* AGAGATGGCAGAAGGCACAT	204
Nrf2	*Fwd:* TGTCAGCTACTCCCAGGTTG *Rev*: ATCAGGGGTGGTGAAGACTG	195
Keap1	*Fwd:* TGAAATCCATGGGCCCTTCT *Rev:* GTGTGGGTGGTAGGAGTTCA	116
GAPDH	*Fwd:* TTCACCACCATGGAGAAGGC *Rev:* GGCATGGACTGGGTCATGA	237
18S RNA	*Fwd:* CGTTCTTAGTTGGTGGAGCG *Rev:* AACGCCACTTGTCCCTCTAA	127

2.3.3.4 AGAROSE GEL ELECTROPHORESIS

Amplified PCR products were separated using 1.5% agarose gel stained with ethidium bromide and run at 85V for 1h at room temperature.

2.3.3.5 PCR DATA ANALYSIS

PCR products were digitally scanned using the AlphaImager 2200 program (Alpha Innotech Corp.). The optical density values for target genes were normalized against those of GAPDH or 18S RNA for semi-quantitative analysis, and represented as a relative change expressed as a percentage of control. The data was analyzed by a paired Student's t-test, or by one-way ANOVA, with $p<0.05$. Holm-Sidak post-hoc testing of ANOVAs was conducted to correct for multiple comparisons. The data is expressed as the mean ± SEM and represent three biological replicates.

2.3.3.6 qPCR

Quantitative PCR (qPCR) was conducted using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad), in a final volume of 25µl containing 12.5µl SsoAdvanced™ Universal Inhibitor-Tolerant SYBR Green Supermix (BioRad), 1.25µl each of forward primer and reverse primers (10µM), and 1µl DNA template. PCR was conducted under the following conditions: the template was first denatured at 98°C for 2m, followed by 40 cycles of 98°C for 15s and 63°C (MT_1, 18S RNA), 65°C (Egr1, CBP), 67°C (Pitx1, SF1, GATA2) for 60s. 18S was included as an internal control, and amplified using 2µl RT product with the appropriate primers using the following parameters: 40 cycles of 98°C for 15s and 63°C for 30s. A thermal gradient was run to determine the optimal annealing temperature for each primer set. MT_1, PITX1, CBP, Egr1, GATA2, SF2 and 18S primers are provided in Table 4.

Table 4: qPCR Primers

Gene	Promoter Sequence (5'- 3')	Target Size (base pairs)
MT_1	*Fwd:* GAGGAAATAAGATCGCGGCC *Rev:* CTGCGTTCCTGAGCTTCTTG	136
PITX1	*Fwd:* GCTCCATCTCTTCCATGACC *Rev:* CCGTGAGGTTGTTGATGTTG	96
CBP	*Fwd:* TGCTACCATGGAGAAGCACA *Rev:* AGGAAGAGAACTCCCAGTGC	176
Egr1	*Fwd:* GTTGCCTCCCATCACCTATAC *Rev:* GCTCACAAGGCCACTGACTA	109
GATA2	*Fwd:* TCAGCAGGGGGTAGTGTAGC *Rev:* CTGCCACCTTCCATCTTCAT	92
SF1	*Fwd:* CTATTGTGCCTGGTGGAGGT *Rev:* CTCGTTGCCCAAATGCTTAT	78
18S RNA	*Fwd:* CGTTCTTAGTTGGTGGAGCG *Rev:* AACGCCACTTGTCCCTCTAA	127

2.3.3.7 QPCR DATA ANALYSIS

qPCR cycle threshold (Ct) values were obtained from the CFX96 Touch Real-time PCR Detection System (Bio-Rad). Statistical analysis was calculated using the Relative Expression Software Tool (REST) 2009 (Pfaffl *et al.* 2002; Qiagen), with PCR efficiency corrections. The Ct values from treated samples were compared to the Ct values from their respective controls using randomization algorithms for resampling

(bootstrapping). Normal hypothesis testing was used to evaluate significance, with $p<0.05$ taken as the level of significance. The data is expressed as the ratio of treated/untreated samples, and represent three biological replicates. The data from qPCR experiments is expressed as the $\log_2$ of the expression ratios obtained from REST analysis to equally depict up- and down- regulation patterns in the data. Log_2 transformation was done for data presentation only.

2.3.4 ACETYL-H3 CHROMATIN IMMUNOPRECIPITATION

2.3.4.1 CROSS-LINKING, CELL LYSIS AND DNA SHEARING

DNA was cross-linked in 1 % formaldehyde for 10m at room temperature. Glycine was added to a final concentration of 1.25M, and incubated for 5m at room temperature to stop the crosslinking reaction. The cells were washed in cold phosphate-buffered saline (PBS) once, and centrifuged at 2500rpm for 5m at 4°C. The pellet was then collected and re-suspended in homogenizing buffer (ChIP-IT Express Chromatin Immunoprecipitation Kit; Active Motif) using a dounce homogenizer by 10–20 strokes. The homogenate was again centrifuged at 2500 rpm for 5m at 4°C. Lysates were sonicated on ice at 30% power for 10s X 7 times with a 10s rest period in between each cycle to yield 200–1000bp DNA fragments.

2.3.4.2 CHROMATIN IMMUNOPRECIPITATION

25μg of sheared chromatin was immunoprecipitated using 5μg of anti-acetyl-histone H3 (K9/18) polyclonal antibody (EMD Millipore), or 5μg of normal rabbit IgG (Santa Cruz Biotechnology) as a negative control. The bound chromatin-histone complexes were purified using Protein G Magnetic beads (ChIP-IT Express Chromatin Immunoprecipitation Kit; Active Motif). After immunoprecipitation, the samples were treated with Proteinase K (Sigma-Aldrich), incubated 60°C overnight for reverse-crosslinking, and column purified using the QIAquick MinElute PCR Purification Kit (Qiagen). Purified DNA was eluted to a final volume of 20μl. After ChIP, the samples were stored at -80°C.

2.3.4.3 QPCR

The immunoprecipitated DNA was amplified using a nested PCR approach to detect the MT_1 promoter. 2μl of immunoprecipitated chromatin was used for preamplification using conventional PCR on the GeneAmp PCR System 2400 Thermal Cycler (Perkin Elmer). Large segments of the MT_1 promoter (~500bp) were preamplified with HotStarTaq DNA Polymerase (Qiagen) using the GeneAmp PCR System 2400 Thermal Cycler (Perkin Elmer, according to the following parameters: 95^0C for 5m, followed by 15 cycles of 94^0C for 30s, 55^0C for 30s, 72^0C for 1m, and a final incubation at 72^0C for 10m. Pre-amplified chromatin was diluted 1:9, and 4μl was used for qPCR amplification using primers detecting shorter segments of the

MT_1 promoter (~200bp). qPCR was conducted using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad), in a final volume of 25µl containing 12.5µl SsoAdvanced™ Universal Inhibitor-Tolerant SYBR Green Supermix (BioRad), 1.25µl each of forward primer and reverse primers (10µM), and 4µl DNA template under the following conditions: the template was first denatured at 98°C for 3m, followed by 40 cycles of 98°C for 15s and 60°C for 60s. Amplification of BDNF P1 and the β-globin gene promoter were amplified using qPCR only, and were included as positive and negative controls respectively. All promoter primers are provided in Table 5. Primers for the BDNF and β-globin promoters were obtained from Wu *et al,.* 2008.

Table 5: Promoter Primers

Promoter	Primer Sequence (5'- 3')	Target Size (base pairs)
MT_1 P1	*Fwd:* TGGCCTTGAACTTCTGATCC	223
	Rev: CATGCTGACACCTTGACGAT	
MT_1 P2	*Fwd:* CCCAAAGTGGCATTGATTCT	182
	Rev: CATTTCTTCCAGAGTCCCTTTG	
MT_1 P3	*Fwd:* TGGCTAATCCACTTCCCAGA	166
	Rev: TAAAGGCTGTGCTGGATGCT	
MT_1 P4	*Fwd:* TCATCCTCATTTTGCCGATA	122
	Rev: GTCAAGTGCAGGGGAAACTT	
BDNF P1	*Fwd:* TGATCATCACTCACGACCACG	134
	Rev: CAGCCTCTCTGAGCCAGTTACG	
β-GLOBIN	*Fwd:* TGACCAATAGTCTCGGAGTCCTG	81
	Rev: AGGCTGAAGGCCTGTCCTTT	

2.3.4.4 AGAROSE GEL ELECTROPHORESIS

Amplified PCR products were separated using 1.5% agarose gel stained with ethidium bromide and run at 85V for 1h at room temperature.

2.3.4.5 CHIP DATA ANALYSIS

AcH3 and IgG ChIP, and input DNA Ct values were obtained from the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). AcH3 or IgG ChIP samples were expressed as the percentage of input DNA recovered following immunoprecipitation. 1% of the starting chromatin was used for input DNA, which was adjusted to 100% by subtracting 6.644 cycles ($\log_2$ of 100) from the input Ct values. The percent of input recovered was calculated as $2^{[(\text{Ct Input} - 6.644) - (\text{Ct Immunoprecipitated})]}$ X 100% (Haring *et al.* 2007). The data was analyzed by a paired Student's t-test, with $p<0.05$. The data are expressed as the mean ± SEM, and represent three separate ChIP experiments.

2.3.5 IMMUNOBLOTTING

2.3.5.1 HISTONE ISOLATION

Nuclear proteins were extracted using 20mM Tris pH 8.0, 150mM sodium chloride (NaCl), 1mM Ethylenediaminetetraacetic Acid (EDTA), 1% Triton X-100 with Complete Protein Inhibitor Cocktail (Roche Diagnostics), as reported by Pan and Niles 2015.

2.3.5.2 MT_1 PROTEIN ISOLATION

Melatonin MT_1 receptors were isolated using 20mM Tris pH 8.0, 150mM NaCl, 1mM EDTA, 1% Triton X-100 with Complete Protein Inhibitor Cocktail (Roche Diagnostics) as reported by Castro *et al.* 2005.

2.3.6 PROTEIN QUANTIFICATION

Protein concentrations were measured in a Bradford Assay using the Protein Assay Reagent (Bio-Rad), and CU-640 spectrophotometer (Beckman-Coulter). Optical densities were measured at 595nm and protein samples were stored at -80°C.

2.3.7 IMMUNOBLOTTING

Proteins were separated using acrylamide gel electrophoresis run at 200V for 1h at 4^0C. Proteins were transferred from the acrylamide gel to Polyvinylidene Fluoride (PDVF) membranes overnight using 25V and kept in 4^0C. The blots were blocked for 1h at room temperature with 5% skim milk in tris-buffered saline (TBS) buffer (50mM Tris-HCl, 150mM NaCl; pH 8.5), and subsequently incubated in rabbit anti-acetyl-histone H3 (Lys9/18) (1:2500 dilution, 4^0C/24h; EMD Millipore) or rabbit anti-MT_1 (1:200 dilution, 4^0C/72h; Abbiotech). The blots were then incubated in 5% skim milk–TBS containing anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:1000; Santa Cruz Biotechnology) for 2h at room temperature,

illuminated with enhanced chemiluminescence reagents (Amersham Biosciences) for 5m, and detected by film autoradiography. The blots were stripped using a mild stripping buffer (0.2M glycine, 3.47mM SDS, and 0.01% Tween20, at pH 2.2), and re-probed with a 1:10000 dilution of anti-total H3 or anti-β-actin serum (Sigma-Aldrich).

3 RESULTS

3.1 EPIGENETIC REGULATION

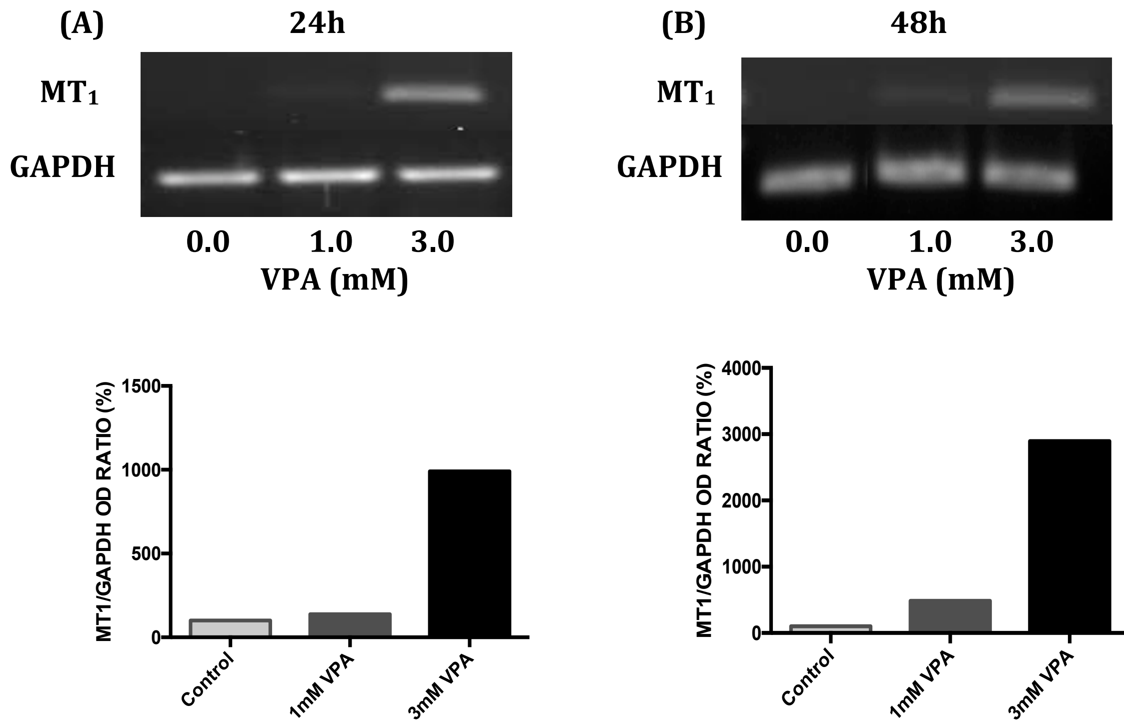
3.1.1 INDUCTION OF MT_1 mRNA EXPRESSION BY THE SHORT-CHAIN FATTY ACID HDAC INHIBITOR, VPA

Rat C6 glioma cells were chosen for this study because of the preceding work from our lab which showed that 1mM and 3mM VPA administered for 24 - 72h robustly induce MT_1 mRNA expression in this cell line (Castro *et al.* 2005; Kim *et al.* 2008). On account of the variations in C6 passage numbers between those used in the earlier work and this study, the responsiveness of MT_1 to VPA in these cells was tested once for qualitative assessment only, prior to any experimentation (Figure 14). A dose-dependent induction of MT_1 mRNA expression by VPA was observed, as was reported previously (Castro *et al.* 2005; Kim *et al.* 2008). The MT_2 isoform was not examined because it is not consistently detected in this cell line (Castro *et al.* 2005).

Similarly, as previous studies showed an induction of MT_1 protein by VPA in rat C6 glioma cells and in human MCF-7 breast cancer cells (Castro *et al.* 2005; Jawed *et al.* 2007), the effects of VPA on MT_1 protein expression were also examined in this cell line prior to any experimentation. Various efforts were made to replicate these findings following the methods reported in both studies (Castro *et al.* 2005; Jawed *et al.* 2007), although none were successful. Other attempts, in which increased

amounts of primary MT_1 antibody and incubation times (up to 1:200, for up to 72h) were tested, however these also did not yield any results.

While our group has had success in earlier melatonin receptor protein studies (Castro *et al.* 2005; Jawed *et al.* 2007), using anti-MT_1 antibodies from CIDTech (which is no longer in business), at present, acquiring suitable antibodies to detect either MT_1 or MT_2 has proven to be a challenge. In recent years, we have tried antibodies from several companies, but were not able to produce any reliable data. Following troubleshooting which did not resolve this problem, examination of MT_1 protein levels were excluded from this study.


(A)
24h
MT1
GAPDH
0.0
1.0
3.0
VPA (mM)
(B)
48h
MT1
GAPDH
0.0
1.0
3.0
VPA (mM)
MT1/GAPDH OD RATIO (%)
1500
1000
500
0
Control
1mM VPA
3mM VPA
MT1/GAPDH OD RATIO (%)
4000
3000
2000
1000
0
Control
1mM VPA
3mM VPA

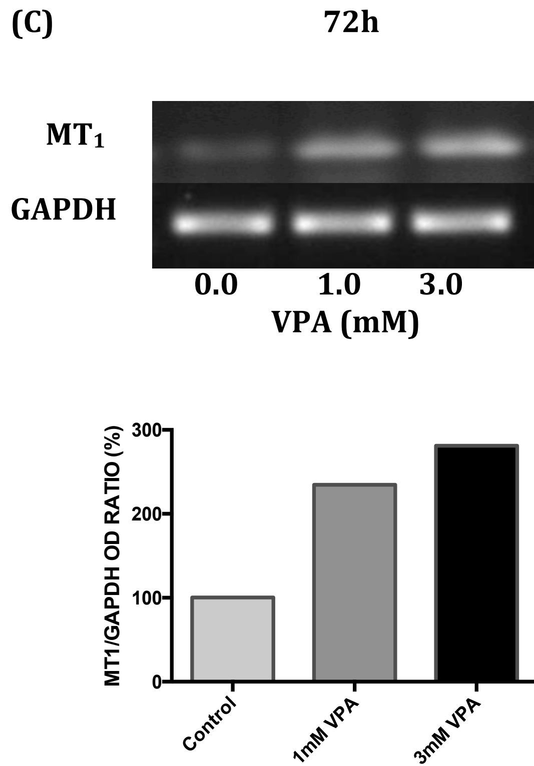


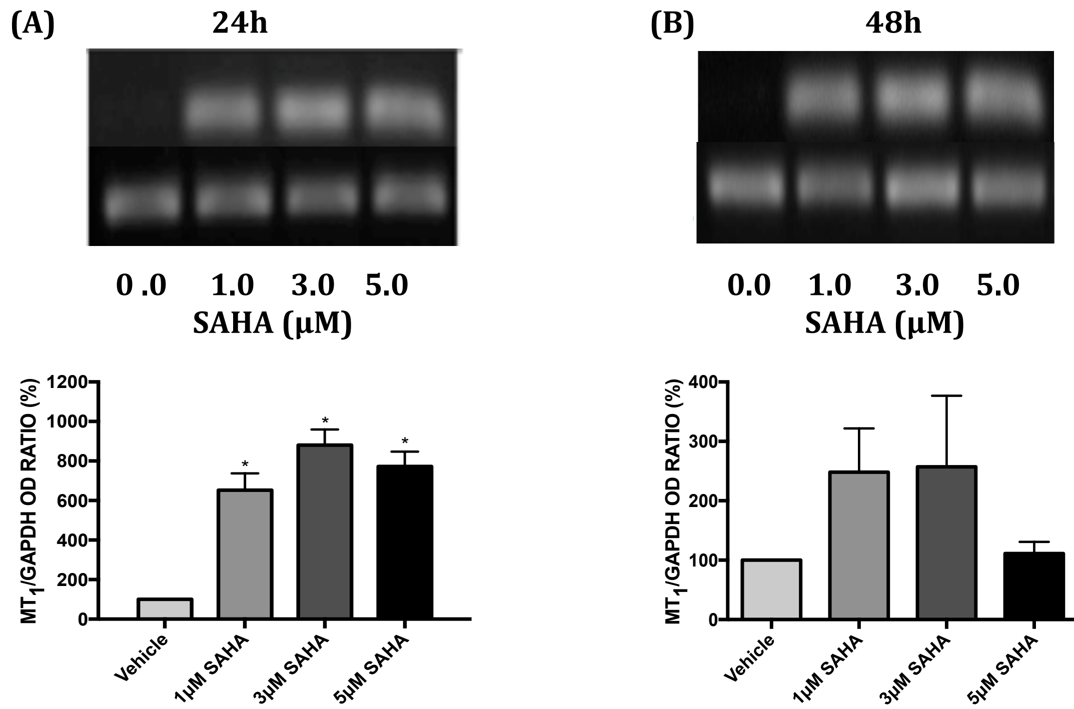
Figure 14: Effect of VPA on MT_1 mRNA expression in rat C6 glioma cells. Representative gel images of RT-PCR amplification of MT_1 following VPA treatments for (A) 24h, (B) 48h, or (C) 72h. Histograms represent the percentage values of MT_1/GAPDH optical density ratios as a function VPA treatments. Bars represent means of MT_1/GAPDH expressed as percentages of control. n=1.

3.1.2 INDUCTION OF MT_1 EXPRESSION BY THE HYDROXAMIC ACID HDAC INHIBITOR, SAHA

The induction of MT_1 mRNA by VPA was compared with a structurally distinct HDAC inhibitor, SAHA. SAHA was administered in a range of concentrations and treatment times to examine its effects on MT_1 expression. 24h SAHA treatments were shown to upregulate MT_1 mRNA expression ($p<0.05$; Figure 15). A tendency towards increased MT_1 expression was detected following 48h ($p=0.3823$) and 72h ($p=0.0891$) SAHA treatments (Figure 15).

MT1 MT1

PDH GAPDH



MT₁

ɅPDH

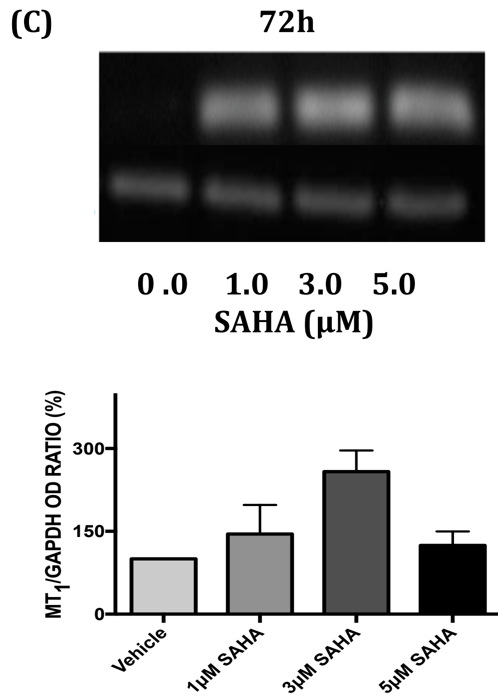


Figure 15: Effect of SAHA treatments on MT_1 mRNA expression in rat C6 glioma cells.

Representative gel images of RT-PCR amplification of MT_1 following SAHA treatments for (A) 24h, (B) 48h, or (C) 72h. Histograms represent means ± S.E.M. of MT_1/GAPDH expressed as percentages of control. Statistical significance was determined using a one-way ANOVA and the Holm-Sidak multiple comparisons test. n=3, *$p<0.05$ vs. vehicle.

3.1.3 M344, BUT NOT VPM, INDUCE MT_1 mRNA EXPRESSION

VPA concentrations previously reported to induce MT_1 expression in the C6 cell line were held constant for this study. 1mM and 3mM VPA treatments were shown to upregulate MT_1 expression in comparison to the control group by a mean factor of 3.379 ($p<0.05$) and 19.272 ($p<0.05$) respectively. VPM, a VPA analogue deficient in HDAC inhibitory properties, was administered at dosages that matched those of VPA for comparison. 1mM and 3mM VPM treatments caused a 1.939 and 1.242 increase in MT_1 expression relative to controls, however neither group reached significance. In contrast, the benzamide HDAC inhibitor, M344, caused an increase in MT_1 expression relative to its control by a mean factor of 19.682 ($p<0.05$), highlighting the importance of HDAC inhibition in this upregulation (Figure 16).

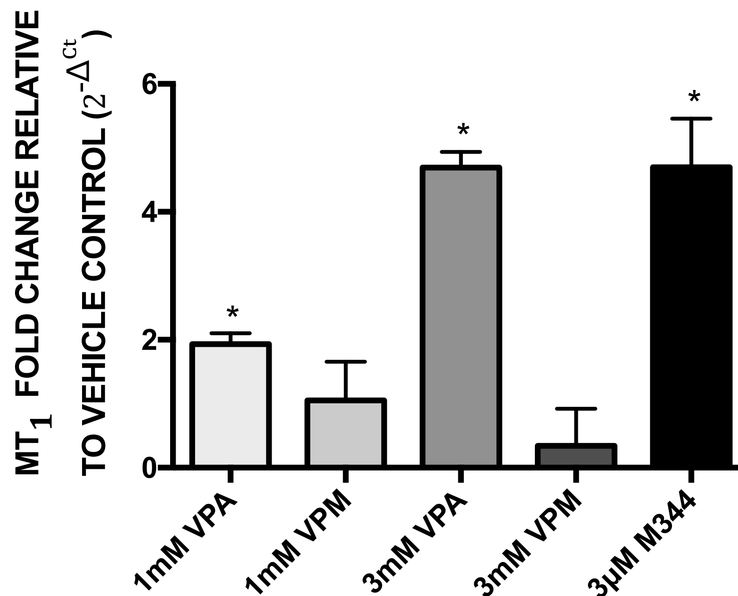


Figure 16: Effect of 24h VPA, VPM, and M344 treatments on MT_1 expression in rat C6 glioma cells.

Expression ratios of the Ct values for MT_1 transcripts in VPA, VPM, or M344 treated cells were normalized to the Ct values of their respective controls (DMEM or DMSO), and calculated using REST. Statistical significance was determined using randomization tests. The data is presented as the $\log_2$ fold change of MT_1 expression between treated samples and untreated samples. The histogram represents the means ± S.E.M. n=3, *$p<0.05$ vs. vehicle.

3.1.4 HDAC INHIBITORS, VPA AND SAHA, CAUSE HISTONE H3 HYPERACETYLATION

To further investigate the role of HDAC inhibition in the induction of MT_1, the effects of VPA and SAHA on global histone acetylation were examined. This was tested once for each drug, for qualitative assessment only. H3K9/18 sites are highly targeted by HDAC inhibitors, and are frequently associated with H3-regulated transcriptional activation (Richards and Elgin 2002; Kristjuhan *et al.* 2002). VPA (Figure 17) and SAHA (Figure 18) caused global histone H3K9/18 hyperacetylation in C6 glioma cells. Importantly, this occurred at treatment dosages and times that match the observed induction of MT_1 by both of these drugs.

AcH3 **AcH3**

Total H3 **Total H3**

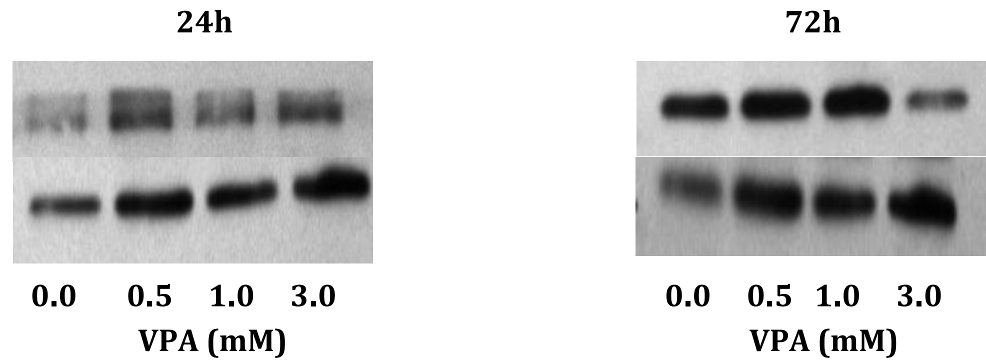


Figure 17: Effect of VPA treatments on histone H3 acetylation in rat C6 glioma cells.

Immunoblots of acetylated histone H3K9/18 following treatment with VPA for 24h or 72h.

Total H3

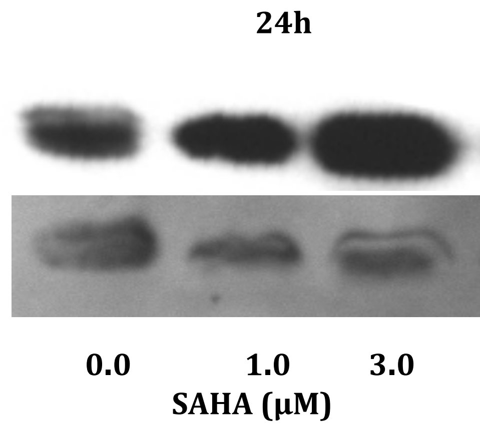


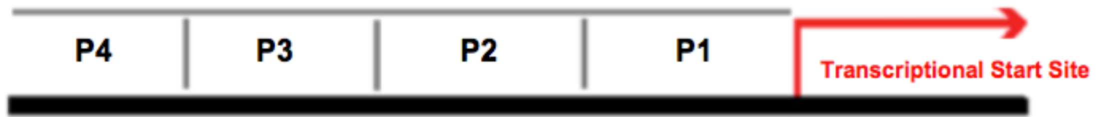
Figure 18: Effect of 24h SAHA treatments on histone H3 acetylation in rat C6 glioma cells.

Immunoblot of acetylated histone H3K9/18 following treatment with SAHA for 24h.

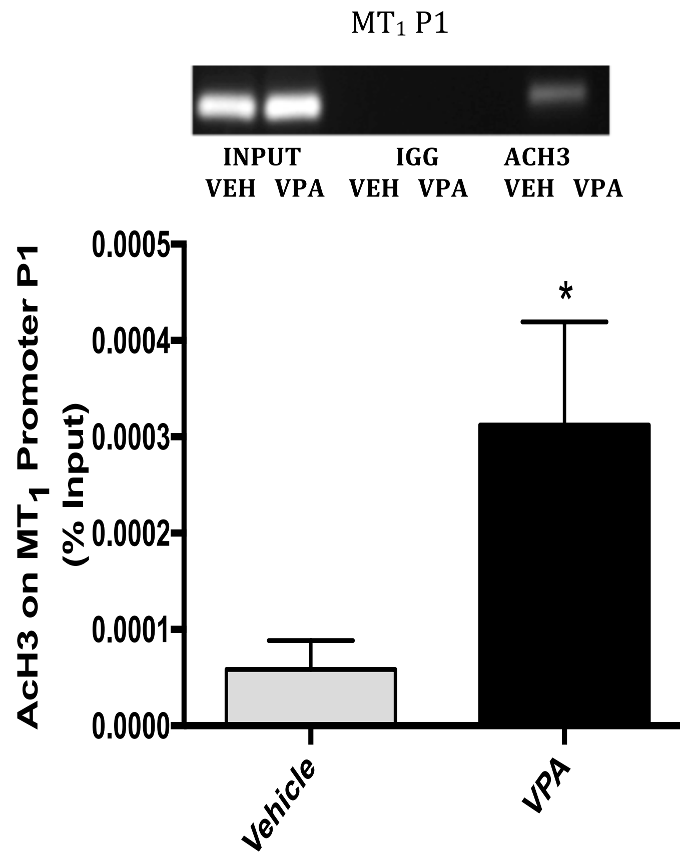
3.1.5 VPA CAUSES MT_1 PROMOTER-ASSOCIATED HISTONE H3 K9/18 HYPERACETYLATION

To examine the relationship between increased MT_1 mRNA expression and H3 hyperacetylation by VPA, the effect of VPA on the acetylation statuses of histone H3K9/18 sites across various regions of the melatonin receptor promoter (GenBank AY228510) were studied using ChIP-qPCR. DNA was sheared by sonication to create fragments of 250-1000bp for ChIP. The immunoprecipitation results demonstrate that VPA induces higher levels of H3K9/18 acetylation along the MT_1 promoter (Figure 19). The acetylation levels of histones associated with the BDNF P1 and β-Globin promoters (Figure 20) were used to confirm the accuracy of the ChIP-qPCR procedure used.

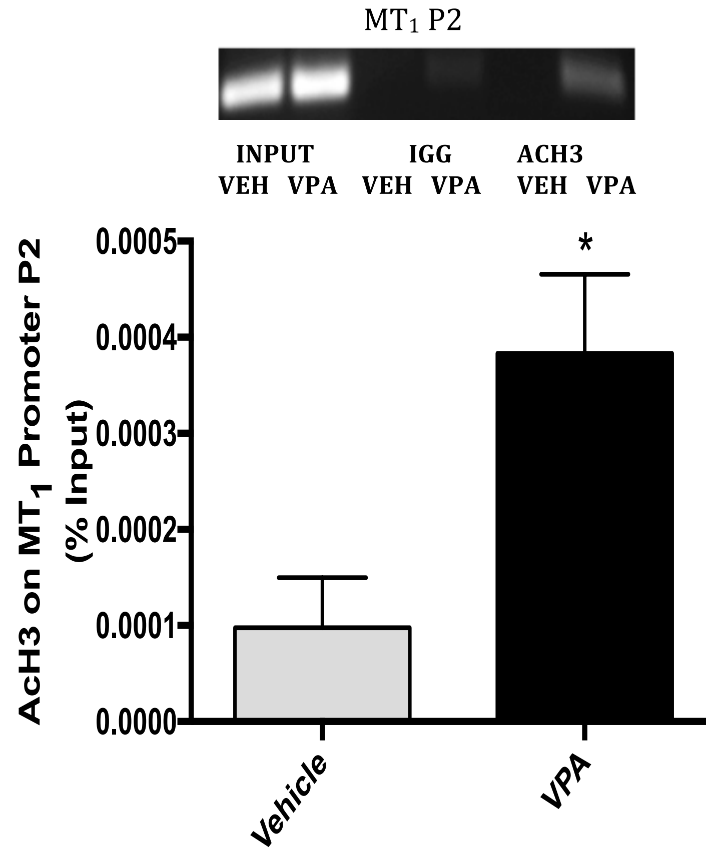
(A)



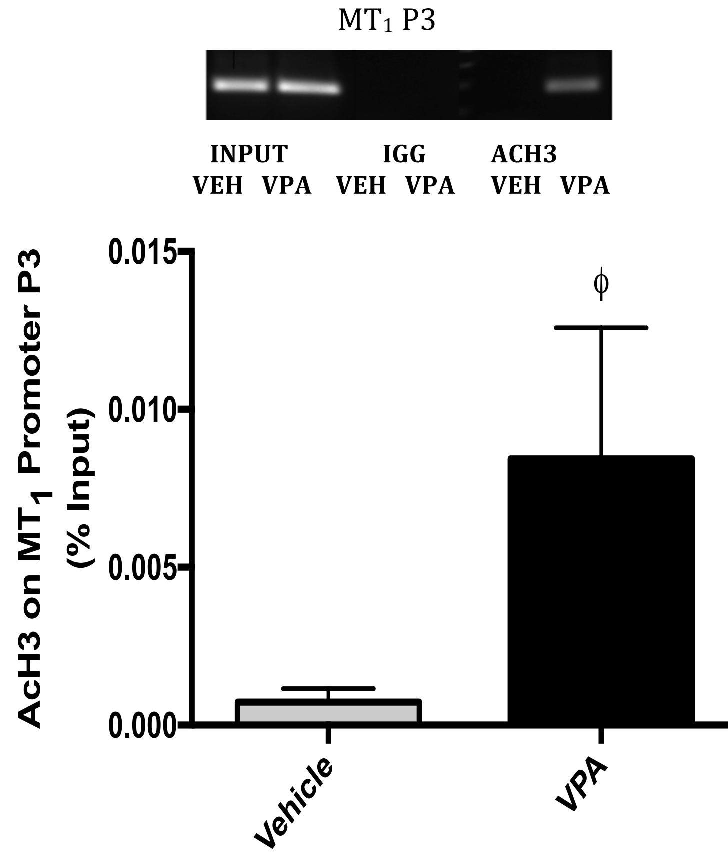
(B)



(C)



(D)



(E)

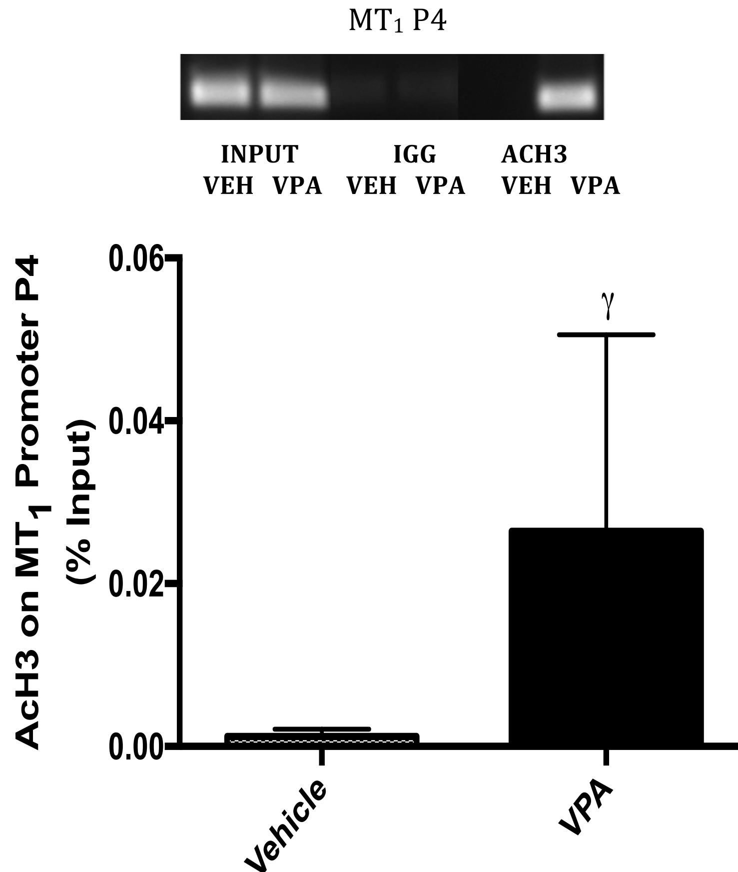


Figure 19: VPA causes MT_1 promoter-associated histone H3 K9/18 acetylation in rat C6 glioma cells

(A) The locations of rat MT_1 promoter primers are indicated. Each primer pair amplifies a ~200bp fragment of the promoter. Representative agarose gel images for MT_1 (B) P1, (C) P2, (D) P3, and (E) P4 following ChIP. The amount of immunoprecipitated (AcH3 ChIP DNA) or non-immunoprecipitated genomic DNA (input DNA) following 1mM VPA treatment for 72h were measured by qPCR with MT_1 promoter primers. Normal IgG was included as a negative control. AcH3 ChIP DNA was normalized to the input DNA, and the data is expressed as the percentage

of input chromatin. Bars represent means ± S.E.M. Statistical significance was determined using a Student's t-test. n=3, *$p<0.05$ vs. vehicle; ᶲ$p=0.0947$ vs. vehicle; $^{\gamma}$ $p=0.3384$ vs. vehicle.

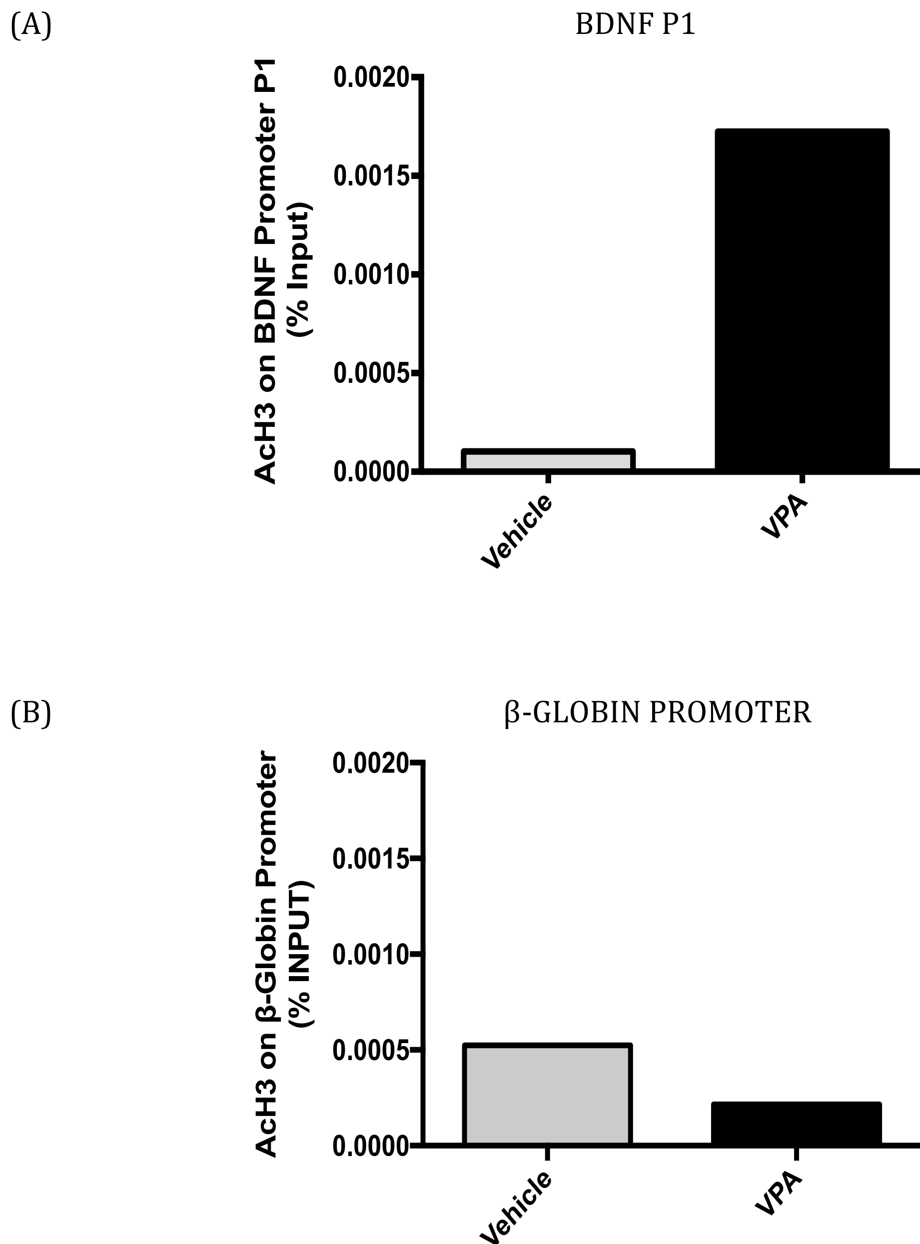


Figure 20: Effect of VPA on BDNF P1 and β-globin promoter-associated histone H3 K9/18 acetylation following VPA treatments.

The amount of immunoprecipitated (AcH3 ChIP DNA) or non- immunoprecipitated genomic DNA (input DNA) following 1mM VPA treatment for 72h were measured by

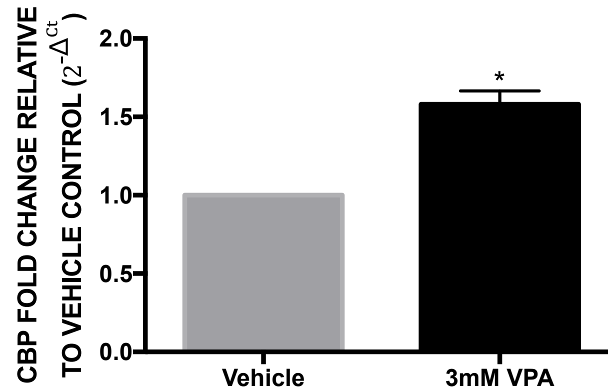
qPCR with (A) BDNF P1 or (B) β-globin primers. Normal IgG was included as a negative control. ChIP DNA was normalized to the input DNA, and the data are expressed as the % of input DNA retrieved following immunoprecipitation. n=1.

3.2 MOLECULAR REGULATION

3.2.1 CREB/CBP-MEDIATED TRANSCRIPTION IS NOT INVOLVED IN VPA-MEDIATED MT_1 UPREGULATION

As mentioned previously, CREB is a transcription factor that is implicated in the regulation of MT_1 (Barrett *et al.* 1996). As CREB is also targeted by VPA (Monti *et al.* 2009), we assessed the role of CREB in VPA-mediated MT_1 induction. VPA increased expression of the CREB adaptor protein, CBP by a factor of 1.505 ($p<0.05$) relative to control, through its influence on the PKC, PI3K/AKT, and GSK3β pathways (Figure 21). The effects of VPA on MT_1 expression were then tested in the presence of KG501, which is a compound that interferes with the interaction of CREB to CBP (Best *et al.* 2004). MT_1 transcripts were upregulated by a mean factor of 33.211 ($p<0.05$) by VPA alone, and 31.977 ($p<0.05$) by VPA and KG501. Although CBP levels are raised by VPA, the blockade of CREB-mediated transcription by KG501 did not prevent the upregulation of MT_1 expression by VPA (Figure 22).

(A)



(B)

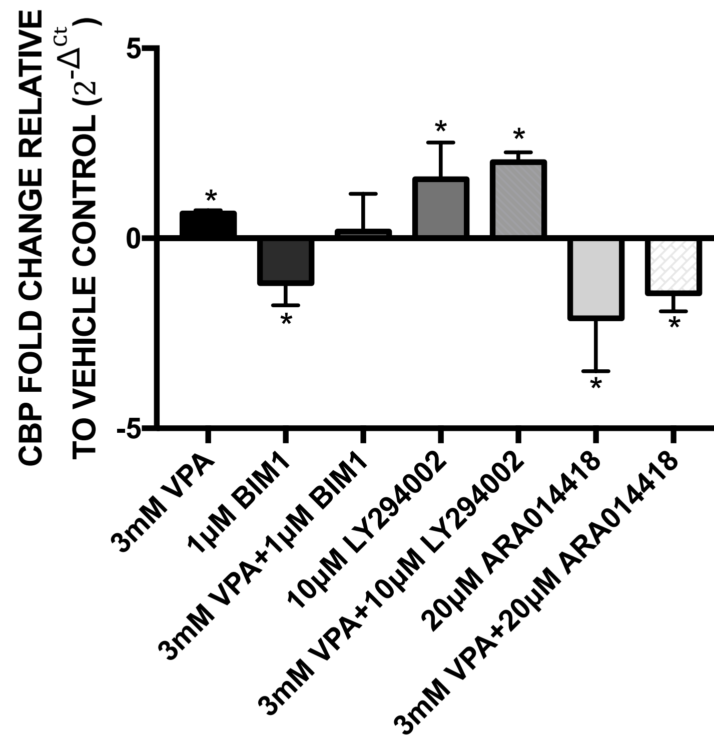


Figure 21: Effect of 24h VPA treatment on CBP expression in rat C6 glioma cells.

(A) Expression ratios of CBP transcripts in VPA treated cells were normalized to their respective controls (DMEM) and calculated using REST. (B) Expression ratios

of CBP transcripts in VPA, BIM1, LY294002, or AR-A014418 treated cells were normalized to their respective controls. For combination treatments, the inhibitor was added for 1 hour prior to the addition of VPA for 24 hours. Statistical significance was determined using randomization tests. The data is presented as the fold change of CBP expression between treated samples and untreated samples. The histogram represents the means ± S.E.M. n=3, *$p<0.05$ vs. vehicle.

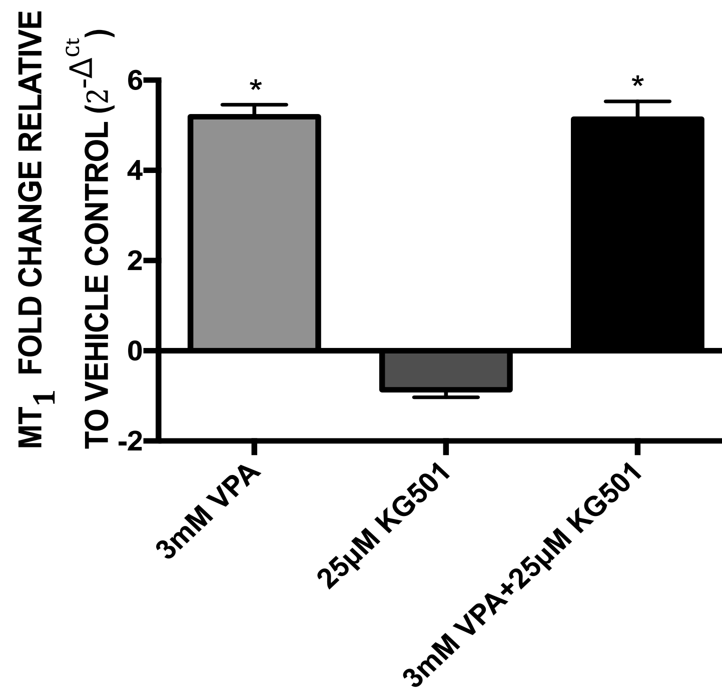


Figure 22: Effect of KG501-mediated CREB/CBP blockade on MT_1 induction by 24h VPA treatments in rat C6 glioma cells.

Expression ratios of MT_1 transcripts VPA, KG501, or VPA + KG501 treated cells, normalized to their respective controls (DMEM or DMSO), and calculated using REST. For combination treatments, the inhibitor was added for 1 hour prior to the addition of VPA for 24 hours. Statistical significance was determined using randomization tests. The data is presented as the $\log_2$ fold change of MT_1 expression between treated samples and untreated samples. The histogram represents the means ± S.E.M. n=3, *$p<0.05$ vs. vehicle.

3.2.2 INHIBITION OF GSK3β SIGNALING, BUT NOT PKC OR PI3K/AKT, BLOCKS INDUCTION OF MT_1 BY VPA

Pharmacological blockades of the PKC, PI3K/AKT, or GSK3β signaling cascades were conducted to assess the involvement of these pathways on the transcriptional induction of MT_1 by VPA. VPA was observed to raise MT_1 transcript levels by a factor of 19.394 ($p<0.05$) relative to controls. Treatment with the PKC inhibitor, BIM1, did not change MT_1 transcript levels relative to controls. Co-administration of BIM1 and VPA raised MT_1 expression by a mean factor of 11.635 ($p<0.05$) in comparison to controls (Figure 23).

Similarly, treatment with the PI3K/AKT inhibitor, LY294002, did not induce MT_1 expression in comparison to controls. Treatment of LY294002 and VPA raised MT_1 levels by a mean factor of 17.385 ($p<0.05$) relative to controls (Figure 23). These results suggest that PKC and PI3K/AKT signaling are not involved in the induction of MT_1 by VPA.

The addition of AR-A014418, which inhibits GSK3β, also did not cause any notable changes in MT_1 expression relative to control. Co-treatment of AR-AO14418 and VPA caused a reduction in MT_1 expression by a mean factor of 12.195 ($p<0.05$) in comparison to control (Figure 23), suggesting VPA might induce MT_1 expression via a GSK3β-sensitive mechanism.

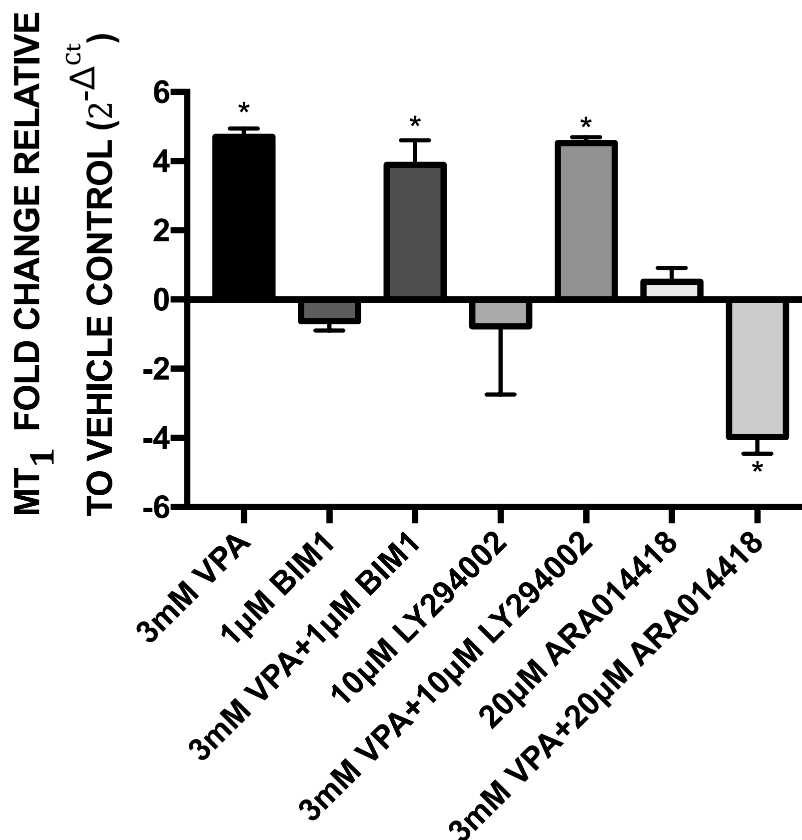


Figure 23: Effect of PKC, PI3K/AKT, and GSK3β pathway blockades on MT_1 induction by 24h VPA treatments in rat C6 glioma cells.

Expression ratios of MT_1 transcripts in VPA, BIM1, LY294002, or AR-A014418 treated cells were normalized to their respective controls (DMEM or DMSO) and calculated using REST. For combination treatments, the inhibitor was added for 1 hour prior to the addition of VPA for 24 hours. Statistical significance was determined using randomization tests. The data is presented as the log_2 fold change of MT_1 expression between treated samples and untreated samples. The histogram represents the means ± S.E.M. n=3, *$p<0.05$ vs. vehicle.

3.2.3 VPA MODULATES MT_1 PROMOTER ASSOCIATED TRANSCRIPTION FACTORS

Pitx1, in combination with Egr1, SF1, or GATA2 regulate transcriptional activity of the rat MT_1 promoter (Johnston *et al.* 2006; Johnston *et al.* 2003b). The effects of VPA on the mRNA expression of these genes were tested to assess their potential involvement in the induction of MT_1 by this drug. VPA negatively affected Pitx1 expression in comparison to control, but increased Egr1, SF1, and GATA2 transcript levels by mean factors of 5.009 ($p<0.05$), 9.099 ($p=0.066$) and 15.163 ($p<0.05$) respectively, in comparison to their control groups (Figure 24).

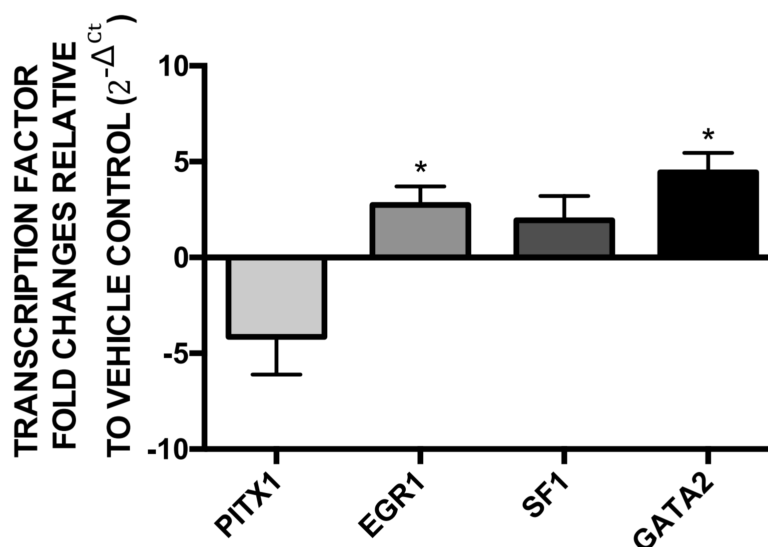


Figure 24: Effect of 24h VPA treatments on Pitx1, Egr1, SF1, and GATA2 expression in rat C6 glioma cells.

Expression ratios of Pitx1, EGR1, SF1, and GATA2 transcripts in 3mM VPA treated cells were normalized to their controls (DMEM) and calculated using REST. Statistical significance was determined using randomization tests. The data is presented as the $\log_2$ fold change of gene expression between treated samples and untreated samples. The histogram represents the means ± S.E.M. n=3, *p<0.05 vs. vehicle.

3.2.4 VPA MODULATES MT_1 PROMOTER ASSOCIATED TRANSCRIPTION FACTORS VIA PKC, PI3K/AKT, AND GSK3β SIGNALING

The effects of PKC, PI3K/AKT, and GSK3β pathway blockades were examined on the modulation of Pitx1, Egr1, SF1 and GATA2 by VPA. As shown previously, VPA treatment had a negative effect on Pitx1 transcript levels (Figure 24). Treatment with BIM1 alone did not change Pitx1 expression in comparison to its vehicle, however co-administration of VPA and BIM1 increased Pitx1 expression by a mean factor of 29.647 ($p<0.05$) in comparison to control. Blockade of the PI3K/AKT pathway reversed this effect, as Pitx1 transcript levels were raised following treatment with VPA and LY294002 by a mean factor of 28.750 relative to control, however this effect did not reach significance. Treatment with AR-A014418 did not affect Pitx1 expression. The reversal in Pitx1 inhibition by VPA in the presence of BIM1, LY294002, or AR-A014418, suggest that the PKC or GSK3β signaling pathways may be involved in the negative regulation of Pitx1 by VPA (Figure 25).

Interestingly, the transcript levels of Egr1, which antagonizes Pitx1-driven MT_1 promoter activity (Johnston *et al.* 2003b), were raised by VPA by a mean factor of 5.009 ($p<0.05$). Co-administration of BIM1 with VPA raised Egr1 transcript levels by a mean factor of 1.684 ($p<0.05$) in comparison to control. Treatment with LY294002 raised Egr1 transcripts by a mean factor of 2.826 ($p<0.05$) relative to controls, however this was not affected by the addition of VPA, as the relative

expression levels of Egr1 remained at 2.343 ($p<0.05$) in comparison to control. Treatment with AR-A014418 did not affect Egr1 expression. Co-treatment of VPA with AR-A014418 had a negative influence Egr1 expression in comparison to controls, though no significant differences were detected. These results suggest that the PKC pathway may have a positive regulatory role in the induction of Egr1 by VPA, and the mechanism occurs independently of the PI3K/AKT pathway. The role of GSK3β signaling on this transcriptional regulation is not clear (Figure 26).

VPA caused an upward trend in SF1 expression by a mean factor of 9.099 ($p=0.066$) relative to controls. SF1 transcript levels were reduced by a mean factor of 4.386 ($p<0.05$) by BIM1 in comparison to controls. Co-treatment of BIM1 with VPA also reduced SF1 transcripts relative to controls, though no significant differences were detected. Administration of LY294002 alone did not affect SF1 expression. The addition of VPA to LY294004 treatments had a positive effect on SF1 transcript levels relative to controls, but no significant differences were detected. Treatment with AR-A014418 did not affect SF1 expression. Co-administration of VPA and AR-A014418 resulted in a robust decrease SF1 expression ($p<0.05$) in comparison to controls. These results suggest that PI3K/AKT or GSK3β signaling pathways may be involved in the positive regulation of SF1 expression by VPA (Figure 27).

Finally, VPA caused an increase in GATA2 transcript levels by a mean factor of 15.163 ($p<0.05$) relative to control. While BIM1 or LY294002 alone did not affect GATA2 expression, co-administration of VPA with BIM1 or LY294002 raised GATA2

expression by a mean factor of 6.406 ($p<0.05$) or 10.056 ($p<0.05$), respectively. Treatment with AR-A014418 did not affect GATA2 expression in presence or absence of VPA. These results suggest that the PKC or PI3K/AKT signaling cascades may be involved in the regulation of GATA2 by VPA (Figure 28).

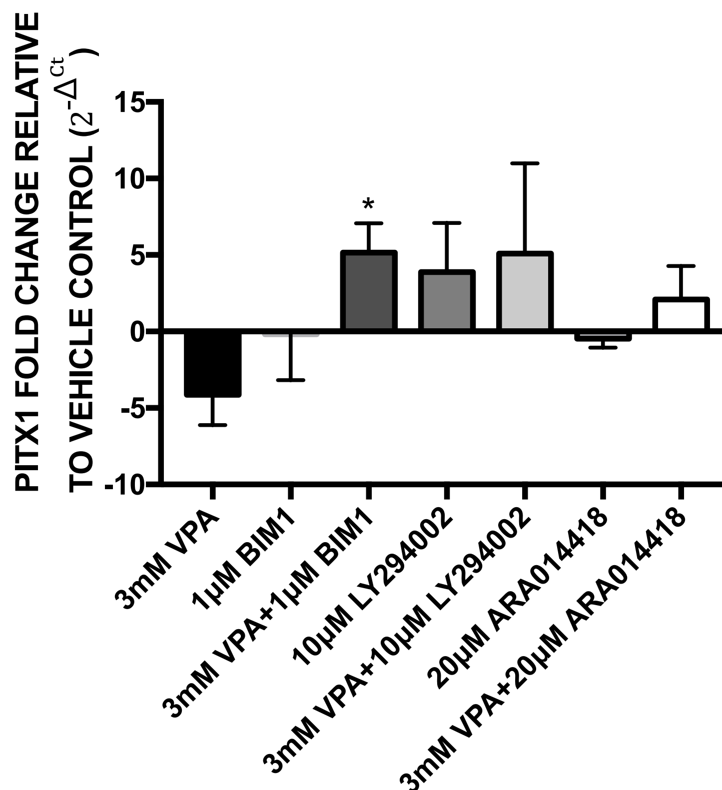


Figure 25: Effect of PKC, PI3K/AKT, and GSK3β pathway blockades on the modulation of Pitx1 expression by 24h VPA treatments in rat C6 glioma cells.

Expression ratios of Pitx1 transcripts in VPA, BIM1, LY294002, or AR-A014418 treated cells were normalized to their respective controls (DMEM or DMSO) and calculated using REST. For combination treatments, the inhibitor was added for 1 hour prior to the addition of VPA for 24 hours. Statistical significance was determined using randomization tests. The data is presented as the $\log_2$ fold change of gene expression between treated samples and untreated samples. The histogram represents the means ± S.E.M. n=3, *$p<0.05$ vs. vehicle.

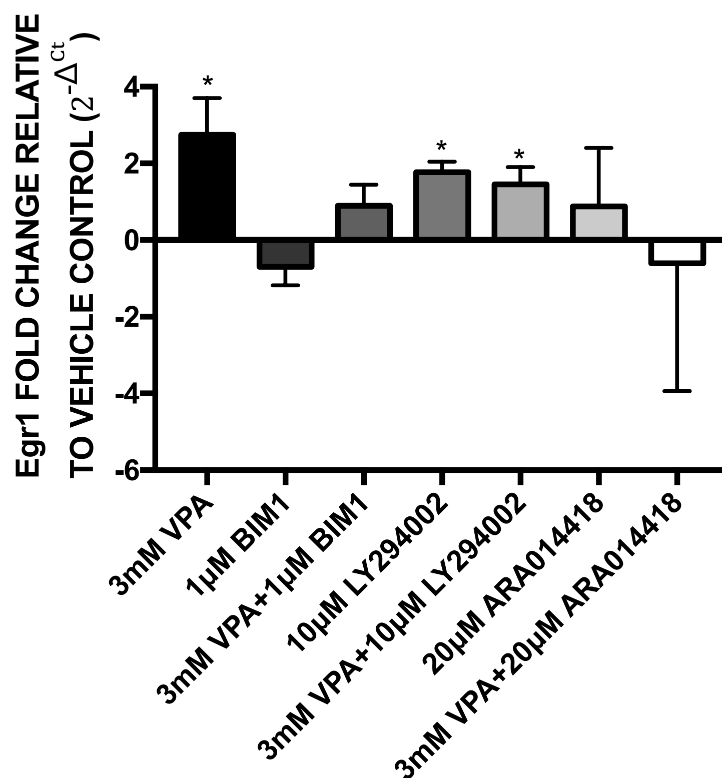


Figure 26: Effect of PKC, PI3K/AKT, and GSK3β pathway blockades on the modulation of Egr1 expression by 24h VPA treatments in rat C6 glioma cells.

Expression ratios of Egr1 transcripts in VPA, BIM1, LY294002, or AR-A014418 treated cells were normalized to their respective controls (DMEM or DMSO) and calculated using REST. For combination treatments, the inhibitor was added for 1 hour prior to the addition of VPA for 24 hours. Statistical significance was determined using randomization tests. The data is presented as the log_2 fold change of Egr1 expression between treated samples and untreated samples. The histogram represents the means ± S.E.M. n=3, *p<0.05 vs. vehicle.

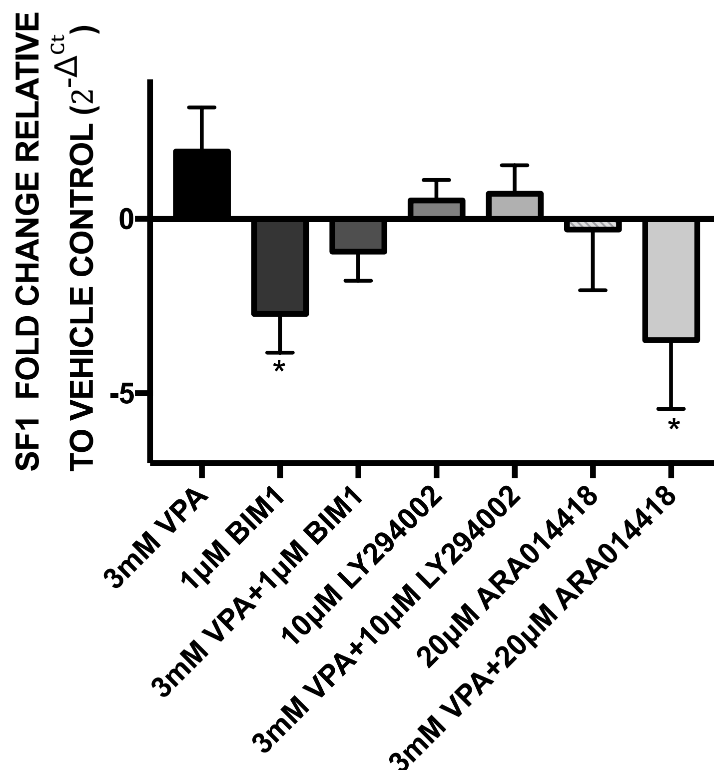


Figure 27: Effect of PKC, PI3K/AKT, and GSK3β pathway blockades on the modulation of SF1 expression by 24h VPA in rat C6 glioma cells.

Expression ratios of SF1 transcripts in VPA, BIM1, LY294002, or AR-A014418 treated cells were normalized to their respective controls (DMEM or DMSO) and calculated using REST. For combination treatments, the inhibitor was added for 1 hour prior to the addition of VPA for 24 hours. Statistical significance was determined using randomization tests. The data is presented as the $\log_2$ fold change of SF1 expression between treated samples and untreated samples. The histogram represents the means ± S.E.M. n=3, *$p<0.05$ vs. vehicle.

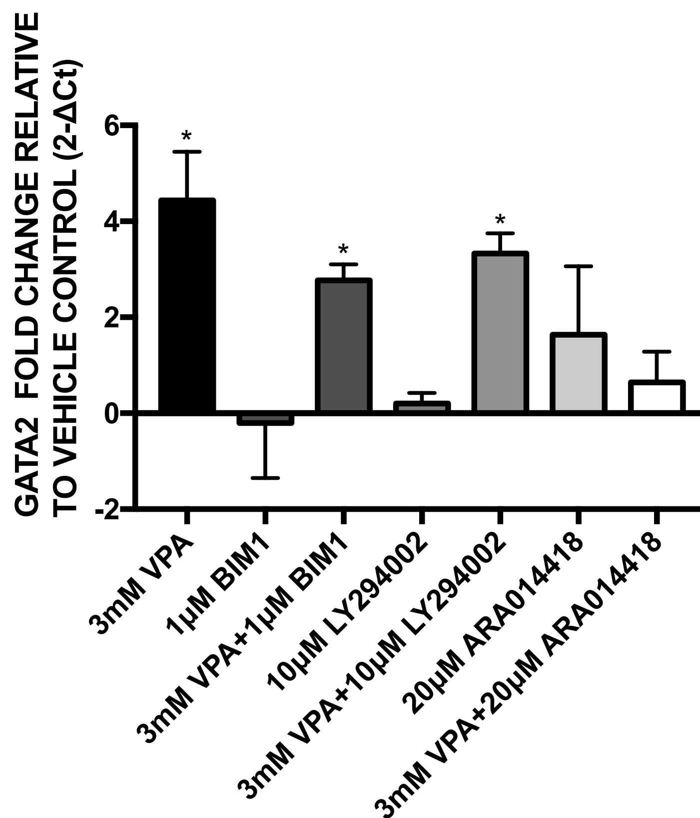


Figure 28: Effect of PKC, PI3K/AKT, and GSK3β pathway blockades on the modulation of GATA2 expression by 24h VPA in rat C6 glioma cells.

Expression ratios of GATA2 transcripts in VPA, BIM1, LY294002, or AR-A014418 treated cells were normalized to their respective controls (DMEM or DMSO) and calculated using REST. For combination treatments, the inhibitor was added for 1 hour prior to the addition of VPA for 24 hours. Statistical significance was determined using randomization tests. The data is presented as the log_2 fold change of GATA2 expression between treated samples and untreated samples. The histogram represents the means ± S.E.M. n=3, *p<0.05 vs. vehicle.

3.2.5 LICL DOES NOT BLOCK INDUCTION OF MT_1 BY VPA

Treatment with lithium, a second antagonist of GSK3β signaling that is not related to AR-A014418 (Stambolic *et al.* 1996), was used to further examine the role of GSK3β signaling in the mechanism underlying the upregulation of MT_1 by VPA suggested by AR-A014418 (Figure 23). While treatment with lithium did not induce MT_1 expression, co-administration of VPA and lithium caused an upregulation of MT_1 transcript levels by a mean factor of 9.155 ($p<0.05$) relative to control. These results suggest that GSK3β signaling is not be involved in the induction of MT_1 by VPA (Figure 29).

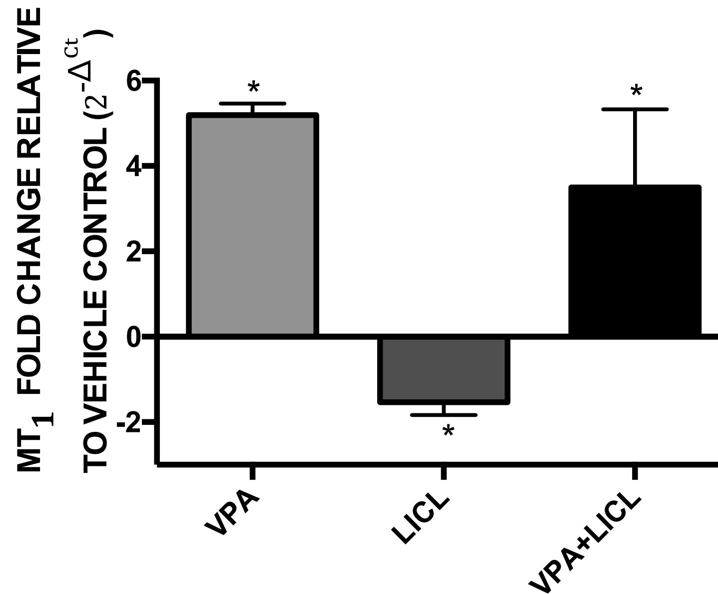


Figure 29: Effect of LiCl on MT_1 induction by 24h VPA.

Expression ratios of MT_1 transcripts in 3mM VPA, 20mM LiCl, or 3mM VPA + 20mM LiCl treated cells were normalized to their respective controls (DMEM) and calculated using REST. For combination treatments, the inhibitor was added for 1 hour prior to the addition of VPA for 24 hours. Statistical significance was determined using randomization tests. The data is presented as the log_2 fold change of MT_1 expression between treated samples and untreated samples. The histogram represents the means ± S.E.M. n=3, *p<0.05 vs. vehicle.

3.3 MELATONIN RECEPTOR UPREGULATION IN THE RAT BRAIN

Chronic VPA treatment was previously observed to cause a robust induction of MT_1 and MT_2 expression in the rat hippocampus (Niles *et al.* 2012). This study was replicated in order to examine other therapeutically relevant brain regions where the upregulation in melatonin receptor expression might occur. The striatum and ventral midbrain were selected because VPA was recently demonstrated to exert neuroprotective effects in these brain regions in a rodent model of Parkinson's disease (Carriere *et al.* 2014).

Chronic VPA treatments induced MT_2 expression ($p<0.05$), and caused an upward trend in MT_1 transcript levels ($p=0.0965$) in the rat striatum (Figure 30). VPA did not induce MT_1 expression in the ventral midbrain, but was observed to cause an upward trend in MT_2 expression ($p=0.0824$; Figure 31).

3.3.1 CHRONIC VPA TREATMENT INDUCES MT_2 EXPRESSION IN THE RAT STRIATUM

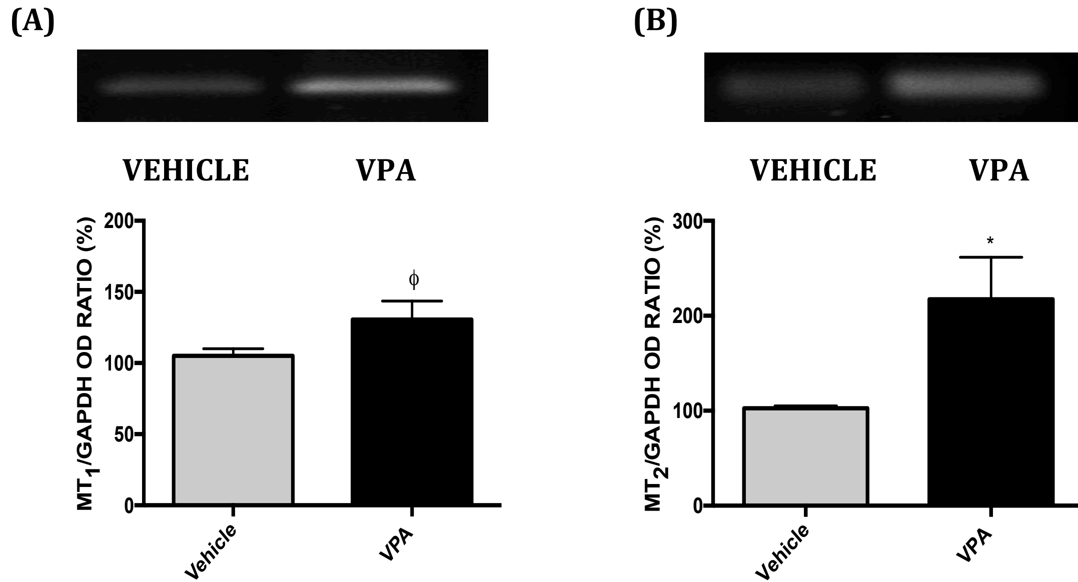


Figure 30: Effect of chronic VPA administration on melatonin receptor mRNA expression in the rat striatum.

A representative agarose gel image of RT-PCR amplification of (A) MT_1 or (B) MT_2 following chronic VPA treatment (4mg/ml). Histograms represent the percentage values of MT_1/GAPDH optical density ratios as a function VPA treatment. Bars represent means ± S.E.M. of MT_1/GAPDH expressed as percentages of control. Statistical significance was determined using an unpaired Student's t-test. n=3, $^*p<0.05$; $^{\Phi}p=0.0965$ vs. vehicle.

3.3.2 CHRONIC VPA TREATMENT INDUCES MT_2 EXPRESSION IN THE RAT VENTRAL MIDBRAIN

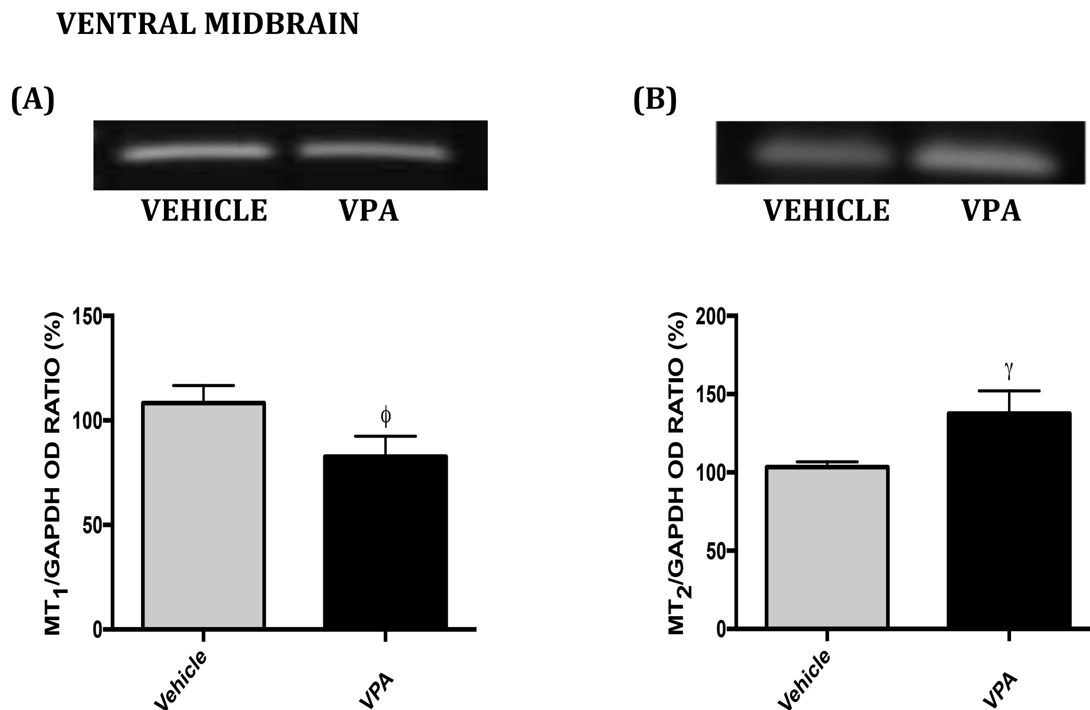


Figure 31: Effect of chronic VPA administration on melatonin receptor mRNA expression in the rat ventral midbrain.

A representative agarose gel image of RT-PCR amplification of (A) MT_1 or (B) MT_2 following chronic VPA treatment. Histograms represent the percentage values of MT_1/GAPDH optical density ratios as a function VPA treatment. Bars represent means ± S.E.M. of MT_1/GAPDH expressed as percentages of control. Statistical significance was determined using an unpaired Student's t-test. n=3, $^{\Phi}p=0.1169$ vs. vehicle; $^{\gamma}$ p=0.0824 vs. vehicle.

3.4 RELEVANCE OF MELATONIN RECEPTOR UPREGULATION IN THE RAT BRAIN

The *in vivo* effects of VPA and melatonin treatments were examined to assess the relevance of VPA-mediated melatonin receptor upregulation in the rat brain. Oxidative stress, and the lack of neurotrophic factor support are highly implicated in the etiology of many neurodegenerative diseases (Mytilineou *et al.* 2002; Stewart and Appel 1988). The effects of VPA and melatonin co-treatments were tested on the expression of Nrf2 and Keap1 from the inducible Nrf2-ARE signaling pathway, and the expression of the neurotrophic factors, CDNF and MANF. These were examined in the rat hippocampus and striatum, as these are the brain regions where we have seen the strongest modulation of melatonin receptor expression by VPA (Niles *et al.* 2012; Figure 30).

3.4.1 VPA AND MELATONIN CO-TREATMENTS MODULATE NRF2-ARE SIGNALING IN THE RAT BRAIN

Co-treatments of VPA and melatonin did not change hippocampal Nrf2 or Keap1 levels relative to controls (Table 6). This may indicate that the Nrf2-ARE signaling pathway is not activated by VPA and melatonin in the rat hippocampus (Kansanen *et al.* 2013; Pan *et al.* 2013; Zhang *et al.* 2012).

In the striatum, VPA treatment alone was observed to upregulate Nrf2 transcripts relative to controls ($p<0.05$). No significant changes in Nrf2 or Keap1

expression were observed following treatment with VPA and melatonin (Table 7). These results suggest that the VPA and melatonin combination treatment may not affect Nrf2-ARE signaling in this brain region.

Table 6: Nrf2 mRNA levels following chronic VPA and melatonin treatments in the rat brain.

Results are expressed as the percentage values of Nrf2/GAPDH optical density ratios as a function drug treatment, and are the means ± S.E.M. n=3, *p<0.05.

Nrf2	VPA	Melatonin	VPA + Melatonin
Hippocampus	120.7 ± 20.3	110.3 ± 2.8	107.7 ± 19.4
Striatum	**183.3 ± 26.3***	112.7 ± 9.0	151.0 ± 6.0

Table 7: Keap1 mRNA levels following chronic VPA and melatonin treatments in the rat brain.

Results are expressed as the percentage values of Keap1/GAPDH optical density ratios as a function drug treatment, and are the means ± S.E.M. n=3.

Keap1	VPA	Melatonin	VPA + Melatonin
Hippocampus	112.7 ± 18.9	121.0 ± 15.5	182.3 ± 42.9
Striatum	98.3 ± 30.8	103.0 ± 14.7	93.3 ± 2.7

3.4.2 VPA AND MELATONIN CO-TREATMENTS MODULATE NEUROTROPHIC FACTOR EXPRESSION IN THE RAT BRAIN

The effects of chronic VPA and melatonin combination treatments on the neurotrophic factors, CDNF and MANF, were also examined in the rat hippocampus and striatum. While VPA and melatonin co-treatments appeared to have a positive influence on CDNF expression relative to controls in the rat hippocampus and striatum, there were no significant differences. A similar effect was observed in MANF expression by VPA and melatonin in the rat striatum and in the hippocampus.

Table 8: CDNF mRNA levels following chronic VPA and melatonin treatments in the rat brain.

Results are expressed as the percentage values of CDNF/GAPDH optical density ratios as a function drug treatment, and are the means ± S.E.M. n=3.

CDNF	VPA	Melatonin	VPA + Melatonin
Hippocampus	123.0 ± 59.0	93.0 ± 21.0	220.7 ± 97.7
Striatum	148.7 ± 83.1	116.7 ± 7.5	213.3 ± 78.5

Table 9: MANF mRNA levels following chronic VPA and melatonin treatments in the rat brain.

Results are expressed as the percentage values of MANF/GAPDH optical density ratios as a function drug treatment, and are the means ± S.E.M. n=3.

MANF	VPA	Melatonin	VPA + Melatonin
Hippocampus	117.0 ± 25.9	94.0 ± 14.7	117.0 ± 25.5
Striatum	128.7 ± 34.5	171.0 ± 13.0	175.0 ± 58.2

4 DISCUSSION

4.1 SUMMARY OF RESULTS

Earlier work from our group showed that VPA causes a robust upregulation of melatonin receptor expression in cultured rat C6 glioma cells (Kim *et al.* 2008; Castro *et al.* 2005), and in the rat hippocampus (Niles *et al.* 2012; Bahna *et al.* 2014). This study assessed the epigenetic and molecular mechanisms underlying MT_1 induction by VPA in rat C6 glioma cells, explored other brain regions where VPA may upregulate melatonin receptor expression, and examined the functional implications of VPA-mediated melatonin receptor upregulation in the rat brain.

Epigenetic analyses were conducted by comparing the effects of VPA on MT_1 induction to other compounds with varying epigenetic capacities. SAHA (Figure 15) and M344 (Figure 16), which resemble VPA in their aptitude for HDAC inhibition, parallel the effect of VPA on MT_1 expression. VPM, which lacks HDAC inhibition capabilities, does not induce MT_1 expression (Figure 16). This provides evidence that HDAC inhibition is an essential component of the mechanism underlying this induction. An increase in global H3K9/18 acetylation by VPA (Figure 17) or SAHA (Figure 18) was observed at treatment dosages and times that paralleled the observed increase in MT_1 expression, providing further support that an epigenetic mechanism underlies this induction. AcH3 ChIP-qPCR conducted across the MT_1 promoter revealed higher levels of H3K9/18 acetylation on the MT_1 promoter following treatment with VPA (Figure 19), suggesting that H3K9/18 acetylation is involved in the transcriptional activation of MT_1 by VPA.

Molecular analysis revealed that VPA elevates expression of CBP mRNA (Figure 21). Inhibition of the CBP to CREB interaction using KG501 (Best *et al.* 2004), did not block the induction of MT_1 by VPA (Figure 22), implying that VPA induces MT_1 expression independent of the transcription factor CREB. Further molecular analysis was carried out using VPA treatments administered in combination with inhibitors of the PKC, PI3K/AKT or GSK3β signaling cascades, to determine whether these pathways had a role in the induction of MT_1 by VPA. Pharmacological blockades of the PKC or PI3K/AKT signaling cascades did not block MT_1 upregulation by VPA (Figure 23). Inhibition of GSK3β signaling using AR-A014418 prevented MT_1 upregulation by VPA (Figure 23). A supplementary investigation into the role of GSK3β signaling in the induction of MT_1 by VPA was conducted using LiCl, which is a GSK3β antagonist unrelated to AR-A014418 (Stambolic *et al.* 1996). MT_1 transcripts were upregulated following LiCl and VPA co-treatments, suggesting that the inhibition of GSK3β may not interfere with VPA-mediated melatonin receptor upregulation (Figure 29). The prevention of MT_1 induction by VPA by AR-A014418 may indicate that there are non-specific side effects of AR-A014418 that are silencing the influence of VPA on the expression of this gene.

VPA appears to target the transcription factors that positively/negatively regulate MT_1 promoter activity. As mentioned previously, under normal biological circumstances, Pitx1 initiates transcriptional activity of the rat MT_1 promoter (Johnston *et al.* 2003b). The effects of Pitx1 are regulated via its interactions with

other co-factors, however these transcription factors alone cannot drive MT_1 promoter activity (Johnston *et al.* 2006; Johnston *et al.* 2007; Johnston *et al.* 2003b). Positive regulators of Pitx1-mediated MT_1 transcription are SF1 and GATA2, and a negative regulator is Egr1 (Johnston *et al.* 2003b). VPA decreased expression of Pitx1, but increased expression of its co-regulators Egr1, SF1 and GATA2 (Figure 24). These results suggest that VPA may also be involved in regulation of transcriptional activity of the rat MT_1 promoter by influencing the expression of these transcription factors. The cumulative changes in Pitx1, Egr1, SF1, and GATA2 (Figures 25 - 28) observed following blockade of the PKC, PI3K/AKT, or GSK3β signaling cascades suggest that VPA mediates these effects via multiple signaling pathways, rather than by a single kinase target.

This study also showed that VPA influences melatonin receptor expression in the rat striatum and ventral midbrain. VPA upregulated MT_2 expression, and caused an upward trend in MT_1 expression in the rat striatum (Figure 30). VPA did not raise MT_1 transcript levels in the rat ventral midbrain, but caused an upward trend in MT_2 expression in this area (Figure 31).

The implications of VPA-mediated melatonin receptor upregulation were studied in the rat hippocampus and striatum because robust responses in melatonin receptor expression to VPA treatments were detected in these brain regions (Niles *et al.* 2012; Figure 30). The functional relevance of melatonin receptor upregulation was addressed using *in vivo* VPA and melatonin co-treatments. The enhancement of melatonin receptor expression in the hippocampus (Niles *et al.* 2012), was matched

with higher CDNF transcript levels that were greater than those observed following treatments with VPA or melatonin alone (Table 8). VPA and melatonin co-treatments were also observed to drive CDNF and MANF expression upwards in the rat striatum (Tables 8, 9).

VPA treatment alone induced expression of Nrf2 in the striatum, and VPA and melatonin co-treatments appeared to have a positive effect on Nrf2 expression in this brain region (Table 6). Based on the view that increases in Nrf2 mRNA levels correlate with Nrf2-ARE signaling activation (Kansanen *et al.* 2013; Pan *et al.* 2013; Zhang *et al.* 2012), these results suggest that VPA may also initiate Nrf2-ARE signaling in the striatum by means of Nrf2 upregulation.

4.2 ROLE OF HDAC INHIBITION IN VPA-MEDIATED MT_1 UPREGULATION

Early studies investigating the mechanism underlying the upregulation of melatonin receptor induction by VPA, hinted that an epigenetic mechanism related to histone acetylation and chromatin remodelling may underlie this interaction (Castro *et al.* 2005; Kim *et al.* 2008). In support of this view, VPM, which is an aliphatic acid derivative of VPA with no HDAC inhibitory activities (Phiel *et al.* 2001), did not replicate the induction of melatonin receptor expression by VPA (Figure 16). To evaluate the significance of HDAC inhibition in the upregulation of melatonin receptors by VPA, the effect of various HDAC inhibitors on melatonin receptor expression were tested in comparison to VPA. HDAC inhibitors are organized

according to their structural classifications, which include hydroxamic acids, aromatic acids, benzamides, and cyclic peptides (de Ruijter *et al.* 2003; Riessland *et al.* 2006). VPA is a short-chain fatty acid, which directly inhibits the HDAC enzyme (Phiel *et al.* 2001). TSA and SAHA are related hydroxamate HDAC inhibitors (de Ruijter *et al.* 2003), and M344 is a benzamide HDAC inhibitor (Riessland *et al.* 2006). Distinct mechanisms of intracellular regulation and HDAC inhibition have been documented for each class of HDAC inhibitor (de Ruijter *et al.* 2003). The upregulation of melatonin receptor expression by a diversity of compounds suggests that this effect is not exclusive to VPA or its general classification of short-chain fatty acids, but is more broadly associated to compounds with the ability to antagonize HDAC activity.

Melatonin receptor expression is often diminished in the diseased state, however the mechanisms driving this depletion are not known (Hardeland *et al.* 2011). Accruing evidence proposes a role for dysregulated epigenetic processes, including imbalances in HAT/HDAC activity, in the pathogenesis of many neurological and neurodegenerative diseases (Saha and Pahan 2006; Coppedè 2014). This implies that the reduction in melatonin receptor expression in the diseased state may be a maladaptive response caused by an imbalance in HAT/HDAC activity associated with disease progression. Strong evidence is presented in this study to show that the inhibition of HDAC activity by various HDAC inhibitors enhances MT_1 expression by increasing histone acetylation. Specifically, VPA causes H3 hyperacetylation along

the length of the MT_1 promoter (Figure 19), indicating that chromatin remodelling induced by VPA facilitates transcription of MT_1.

4.3 VPA-MEDIATED EPIGENETIC REGULATION OF GENE EXPRESSION THROUGH DNA METHYLATION

DNA methylation is an epigenetic process that involves the conversion of cytosine nucleotides into 5-methylcytosine (Lim and Maher 2010). The cytosine residues receiving this covalent modification are typically located directly next to a guanine residue, a conformation known as a CpG island (Lim and Maher 2010). DNA methylation is catalyzed by the DNA methyltransferase (DNMT) enzyme, in association with the methyl-CpG-binding protein 2 (MeCP2; Kimura and Shiota 2003). Several CpG islands are located within the promoter regions of many genes, and function to control their expression. Methylated CpG islands suppress transcription by restricting transcription factor binding, and by recruiting transcriptional repressor complexes (Cedar and Bergman 2009).

VPA also exerts an epigenetic influence on gene expression via the regulation of DNA methylation (Detich *et al.* 2003). VPA (Reid *et al.* 2005), and other HDAC inhibitors (Arzenani *et al.* 2011), promote the demethylation of promoter regions by impeding the nuclear dynamics of the DMNT and MeCP2 interaction. Similar to the results of histone hyperacetylation, DNA hypomethylation is associated with increased gene expression (Kia *et al.* 2013). The inhibition of HDAC activity and the

attenuation of DNA methylation by VPA are not mutually exclusive, but rather are thought to be in a dynamic correlation, and can impose compounded effects on gene transcription (Milutinovic *et al.* 2007).

4.4 INFLUENCE OF VPA ON CHROMATIN STRUCTURE

Conformational changes in chromatin structure induced by covalent modifications, including acetylation and methylation, influence the access of regulatory proteins to their target sites (Bannister and Kouzarides 2011). Several factors are involved in the maintenance of chromatin structure. For example, the structural maintenance of chromosomes (SMC) family of proteins are required for the general stabilization of chromosome conformation (Yokomori 2003). More specifically, heterochromatin protein 1 (HP1) maintains DNA in the heterochromatin state (Maison and Almouzni 2004). VPA treatments stimulate a conformational change in chromatin structure, from a highly condensed state to a more loosened structure (Felisbino *et al.* 2014). These changes induced by VPA correspond with decreased expression of proteins which promote heterochromatin stability (Marchion *et al.* 2005; Felisbino *et al.* 2014). It is interesting to note that histone acetylation is thought to precede the modulation of HP1 and SMC protein expression by VPA (Marchion *et al.* 2005). This implies that the conformational changes in chromatin structure induced by VPA are sustained by its modulation of chromatin

maintenance proteins, and may be related to the kinetics of downstream transcriptional events.

4.5 ROLE OF CREB IN MT_1 INDUCTION BY VPA

The overall loosening of the chromatin structure by VPA exposes the MT_1 promoter sequence to a number of regulatory elements, which in turn may affect expression of MT_1. The MT_1 gene has been highly implicated to be a transcriptional target of CREB, as its expression was shown to correspond with activation of cAMP/PKA/CREB signaling (Barrett *et al.* 1996). The binding of CREB, and its nuclear adaptor protein, CBP, to the cAMP response element (CRE) sequence activates CREB-directed transcription. Inhibition of CREB-mediated transcription by KG501 (Best *et al.* 2004), did not block MT_1 induction by VPA, indicating that the mechanism of MT_1 induction by VPA does not depend on CREB (Figure 22).

The lack of CREB involvement in this upregulation is further supported by kinase blockade experiments. The PKC, PI3K/AKT and GSK3β pathways have distinct effects on the regulation of CREB activation phosphorylation and downstream signaling (Mayr and Montminy 2001). PKC (Mao *et al.* 2007; Martín *et al.* 2011) and PI3K/AKT (Du and Montminy 1998) activation correlate with increased CREB phosphorylation and signaling, whereas GSK3β antagonizes CREB (Grimes and Jope 2001). The upregulation of MT_1 by VPA withstood the inhibition of these pathways (Figures 34-36), despite the distinct effects of these kinases on CREB (Mayr and

Montminy 2001), supporting the idea that CREB in not included in the mechanism underlying melatonin receptor upregulation by VPA.

Nevertheless, VPA was shown to raise CBP mRNA levels, which is an effect which may be mediated by its influence on intracellular kinase pathways (Figure 21). In addition to acting as a transcriptional co-activator to CREB, CBP, pairs with p300 proteins to form a structure that has inherent HAT activity (Ogryzko *et al.* 1996). The p300/CBP complex acetylates lysine residues from all four histone protein subtypes (Ogryzko *et al.* 1996), and is regulated by many signaling molecules, including melatonin (Pan and Niles 2015). VPA does not have any known effects on HAT activity to date. Nonetheless, the observed enrichment of CBP levels supports the epigenetic hypothesis of MT_1 induction by this drug, and further suggests that enhancement of HAT expression and/or activity by VPA may be involved in its upregulation of MT_1.

4.6 ROLE OF MT_1-SPECIFIC TRANSCRIPTION FACTORS IN MT_1 INDUCTION BY VPA

Although the effect of VPA on Pitx1 expression has not been previously documented, the modulation of Egr1 (Almutawaa *et al.* 2014; Zhou *et al.* 2011; Daigle *et al.* 2011), SF1 (Chen *et al.* 2007), and GATA2 (Liu *et al.* 2010) by VPA have been reported many times. The data presented in this dissertation indicate that VPA negatively regulates Pitx1 mRNA expression, and positively regulates Egr1, SF1 and

GATA2 mRNA transcript levels (Figure 24). The observed decrease in Pitx1 expression, and corresponding increase in Egr1 expression is interesting as Egr1 is a repressor of Pitx1-mediated MT_1 promoter activity (Johnston *et al.* 2003b). Although these results may represent a compensatory intracellular mechanism initiated to offset the large induction of MT_1 transcript levels by VPA, they are in agreement with other reports of VPA elevating Egr1 transcript levels in other paradigms. This effect appears to be related to the neurogenic and/or immunomodulatory properties of VPA (Daigle *et al.* 2011; Almutawaa *et al.* 2014; Zhou *et al.* 2011). Nonetheless, the transcriptional consequences of decreased Pitx1 and increased Egr1 levels are muted, likely due to the effects of VPA on histone acetylation on the MT_1 promoter. Consistent with these findings, VPA is also known to increase the mRNA expression of other genes which can antagonize its histone acetylating effect, including various HDAC or MeCP2 protein isoforms (Castro *et. al.,* 2005; Kim *et. al.,* 2008). There is a decrease in the transcriptional effects of VPA on MT_1 expression at treatment times that exceed 24h (Kim *et. al.,* 2008) that could be related to its induction of these negative regulators. The delay in transcriptional inhibition may be a result of the rate of mRNA turnover or post-translational processing of these negative regulators.

4.7 ROLE OF PKC AND PI3K/AKT SIGNALING IN MT_1 INDUCTION BY VPA

Inhibition of the PKC and PI3K/AKT (Figure 23) signaling pathways by BIM1 or LY294002 respectively, did not block MT_1 upregulation by VPA. Although PKC signaling has been reported to antagonize MT_1 expression (Barrett *et al.* 1998), the

blockade of PKC did not strengthen the upregulation of MT_1 induction by VPA. The cumulative changes observed following inhibition of intracellular kinases (specifically, PKC, PI3K/AKT, or GSK3β) suggest that VPA may act via signaling cascades to regulate the expression, and presumably the availability, of transcription factors that control MT_1 promoter activity. For example, the suppression of Pitx1 by VPA was reversed by inhibition of PKC and PI3K/AKT signaling (Figure 25), suggesting that the effect of VPA on Pitx1 expression is under the control of these pathways. In a similar manner, the induction of Egr1, SF1, and GATA2 (Figures 26 - 28) by VPA was moderated by PKC, PI3K/AKT, and GSK3β blockades. Thus, while the PKC and PI3K/AKT pathways do not appear to have a direct role in the upregulation of MT_1 by VPA, the effects of these signaling cascades on transcription factor modulation suggest that they may regulate the kinetics of MT_1 transcription, following chromatin remodelling by VPA.

4.8 ROLE OF GSK3β SIGNALING IN MT_1 INDUCTION BY VPA

Inhibition of GSK3β signaling by AR-A014418 blocked the upregulation of MT_1 by VPA (Figure 23). Such a prominent role in the induction of MT_1, as suggested by the effects of VPA co-administered with AR-A014418 was surprising. Further scrutiny of this drug revealed that, in addition to inhibiting GSK3β signaling, a non-specific effect of its administration is H3K9 methylation, which causes epigenetic silencing of gene expression (Ougolkov *et al.* 2007). Similar to DNA methylation on CpG islands, histone methylation suppresses gene expression (Cedar and Bergman

2009). In support of this view, lysine residues on histones are thought to be particularly receptive to epigenetic cross-talk resulting from different types of modifications, such as acetylation and methylation (Bannister and Kouzarides 2011). As this site is the same target for histone acetylation by VPA along the MT_1 promoter shown in this study, the complete inhibition of VPA-mediated MT_1 induction may be the result of competitive epigenetic modifications mediated by these drugs along the MT_1 promoter.

The involvement of GSK3β signaling in the mechanism underlying MT_1 upregulation by VPA was reassessed using lithium, a related psychotherapeutic agent with several overlapping, and contradictory, mechanisms to that of VPA (Monti *et al.* 2009). Lithium was chosen because it is widely known to be a potent inhibitor of GSK3β signaling (Stambolic *et al.* 1996; Shastry 1997), with no known histone methylating capabilities. While lithium does not induce MT_1 expression, co-treatment of lithium with VPA raised MT_1 mRNA levels similar to those observed following VPA treatment alone (Figure 29). The exclusion of GSK3β in the mechanism driving the transcriptional upregulation of MT_1 by VPA is further supported by the effects observed following treatment with VPM, which unlike VPA, does not affect GSK3β signaling (Phiel *et al.* 2001). As such, GSK3β signaling does not appear to be involved in the regulation of melatonin receptor expression by VPA.

4.9 PROPOSED MECHANISM OF TRANSCRIPTIONAL INDUCTION OF MT_1 BY VPA

The proposed mechanism of MT_1 induction by VPA is illustrated in Figure 32. Evidence from this study suggests that an epigenetic mechanism, specifically that of promoter histone acetylation mediated by HDAC inhibition, underlies the transcriptional activation of the melatonin receptor gene by VPA. This is supported by the observation that structurally diverse drugs that parallel the acetylation of histones by VPA also induce MT_1, and those that prevent histone acetylation hinder this induction. VPA is also involved in several aspects of chromatin dynamics, including the promotion and stabilization of the euchromatin state (Marchion *et al.* 2005). Chromatin decondensation surrounding the MT_1 promoter region allows transcription factors and transcriptional machinery to access regulatory sequences along the MT_1 promoter to initiate transcription of the gene. Results from this study indicate that VPA influences the expression of MT_1 promoter-associated transcription factors via several signaling cascades, including the PKC and PI3K/AKT pathways. While the effects of VPA on these transcription factors do not appear to be sufficient to drive MT_1 expression alone, the ability of VPA to prevent heterochromatin formation following histone acetylation (Marchion *et al.* 2005), suggests that VPA may have a role in the transcriptional kinetics of MT_1.

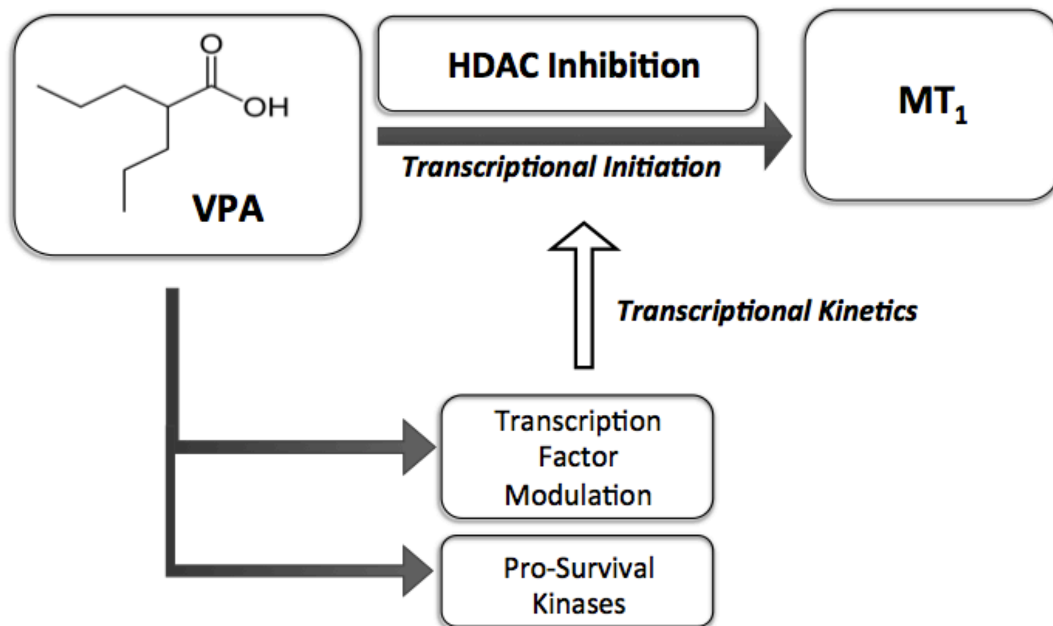


Figure 32: Proposed mechanism of MT_1 induction by VPA.

VPA activates MT_1 transcription by causing chromatin remodelling, mediated by HDAC inhibition and MT_1 promoter hyperacetylation. VPA also acts via intracellular kinases to regulate the expression of transcription factors that control activity of the MT_1 promoter, proposing a role for this drug in the transcriptional kinetics of MT_1. The ability of VPA to maintain chromatin structure in the transcriptionally active state further supports this notion.

4.10 INDUCTION OF MELATONIN RECEPTOR EXPRESSION IN THE RAT STRIATUM AND VENTRAL MIDBRAIN BY VPA

MT_1 and MT_2 are differentially regulated, and exhibit a wide distribution in the mammalian CNS (Lacoste *et al.* 2015). In the adult rat brain, both melatonin receptor isoforms are present in the ventral midbrain, however the MT_1 subtype is relatively enriched in the substantia nigra *pars compacta*, and MT_2 in the substantia nigra *pars reticulata* (Lacoste *et al.* 2015). In the striatum, both isoforms are detected in the nucleus accumbens, caudate nucleus and the putamen, though to a much lesser extent than that of the ventral midbrain (Lacoste *et al.* 2015). Therapeutic dosages of VPA chronically administered caused an upward trend in MT_1 expression, and a robust upregulation of the MT_2 subtype in the rat striatum (Figure 30). An upward trend in MT_2 expression was also detected in the ventral midbrain (Figure 31). Melatonin receptors are expressed diurnally, and are regulated by a number of environmental and physiological factors, including photoperiodicity and circulating levels of melatonin (Gauer *et al.* 1994; Masana *et al.* 2000). Given the robust effects of VPA on melatonin receptor induction in other paradigms (Castro *et al.* 2005; Kim *et al.* 2008; Jawed *et al.* 2007; Niles *et al.* 2012), the lack of significance of VPA on MT_1, or MT_1 and MT_2 induction in the striatum and ventral midbrain respectively, may be related to the daily fluctuations in receptor expression.

Melatonin has been shown to exert protective effects on the nigrostriatal pathway in various rodent models of Parkinson's disease. For example, melatonin

attenuates dopaminergic cell loss (Capitelli *et al.* 2008; Carriere *et al.* 2015; Ma *et al.* 2009), and restores motor function deficits associated with the disease (Carriere *et al.* 2015; Capitelli *et al.* 2008). Although it is possible that neuroprotection by melatonin in the striatum and ventral midbrain may be carried out independently of its receptors, it is interesting to note that VPA was also demonstrated to exert neuroprotective effects in these brain areas (Carriere *et al.* 2014). As melatonin receptors are depleted in the Parkinson's disease brain (Adi *et al.* 2010), evidence that VPA induces melatonin receptor expression in the striatum and/or ventral midbrain might be useful for the enhancement of melatonergic signaling, which could be beneficial in the treatment of Parkinson's disease.

4.11 IN VIVO EFFECTS OF VPA-MEDIATED MELATONIN RECEPTOR UPREGULATION

Two major factors which contribute to neuronal degeneration in various CNS disorders are inefficient oxidative stress responses, and the lack of trophic support (Mytilineou *et al.* 2002). The CNS is more vulnerable to damage from oxidative stress than other areas of the body because of its large composition of lipids, high demands for oxidative metabolism and relatedly, production of toxic metabolites (Wang and Michaelis 2010). Areas most predisposed to oxidative damage may have a higher production of harmful by-products, and/or inefficient antioxidant responses (Candelario-Jalil *et al.* 2001; Vandresen-Filho *et al.* 2015). Similarly,

neurotrophic support is essential for the survival and maintenance of the mammalian nervous system (Barbacid 1995). The Nrf2/ARE signaling pathway, and CDNF/MANF expression were used in this study to assess the functional effects of melatonin receptor upregulation in the CNS.

In the hippocampus, co-administration of VPA and melatonin did not appear to activate Nrf2/ARE signaling. In the striatum, VPA treatment alone increased Nrf2 expression (Table 6), suggesting that VPA can act independently of melatonin to induce the Nrf2/ARE pathway. The observation that VPA and melatonin combination treatments have a positive influence on Nrf2 expression lends support to the hypothesis that melatonin acts via its receptors to activate Nrf2/ARE signaling (Shin *et al.* 2015; O'Neal-Moffitt *et al.* 2015), and therefore, that increased melatonin receptor expression by VPA may be beneficial in augmenting melatonergic signaling. The induction of Nrf2 transcripts by VPA is important, as Nrf2 is protective in the CNS: deficiencies in Nrf2 expression exacerbate dopaminergic degeneration in the striatum (Granado *et al.* 2011), whereas neuronal damage can be prevented by Nrf2 overexpression (Calkins *et al.* 2005; Shih *et al.* 2005).

VPA was previously shown to increase expression of CDNF and MANF in cultured neural stem cells (Almutawaa *et al.* 2014), as well as in both the rat hippocampus and the striatum (Niles *et al.* 2012), however the effects of melatonin on the expression of these neurotrophic factors was not known. Co-administration of VPA and melatonin were observed to drive hippocampal and striatal CDNF

expression higher than that observed for either drug alone (Table 8). A similar effect was also detected in MANF expression in the striatum (Table 9). CDNF and MANF are widely expressed in the CNS, and are thought to be especially relevant for the protection of dopaminergic neurons (Lindholm *et al.* 2008; Lindholm and Saarma 2010). CDNF and MANF have shown potential in attenuating CNS damage in several rodent models of Parkinson's disease (Lindholm *et al.* 2007; Voutilainen *et al.* 2009; Voutilainen *et al.* 2011; Airavaara *et al.* 2012). The neurorestorative effects of CDNF and MANF are thought to be mediated by the regulation of endoplasmic reticulum stress and clearance of protein aggregates that promote disease progression (Voutilainen *et al.* 2015). Although the molecular structures of these neurotrophic factors suggest that they are regulated in a similar manner (Norisada *et al.* 2016), differential regulation patterns of CDNF and MANF expression by VPA and melatonin were presented in this study. The differences may be a reflection of the complementarity of their effects on the protection of dopaminergic neurons, and repair of motor dysfunction (Cordero-Llana *et al.* 2015).

4.12 PHYSIOLOGICAL AND CLINICAL RELEVANCE OF VPA-MEDIATED MELATONIN RECEPTOR OVEREXPRESSION

The effects of melatonin receptor overexpression have been demonstrated using several *in vitro* models of glutamate toxicity. Melatonin treatments in cultured embryonic rat VSC4.1 motor neurons transfected with MT_1 and MT_2 were found to

have a large increase in cell survival markers, and a concurrent suppression in apoptotic and inflammatory markers (Das *et al.* 2013). Melatonin treatments alone could not replicate these findings, highlighting the importance of receptor density in the neuroprotective effects of this hormone (Das *et al.* 2013). The protective effects of melatonin receptor overexpression have also been explored in the periphery. In human MCF-7 breast cancer cells, MT_1-transfected cells treated with melatonin were found to have a greater antiproliferative effect than treatment with melatonin alone (González *et al.* 2007; Yuan *et al.* 2002).

A limitation to these studies is the means by which melatonin receptor overexpression is achieved. Gene therapy is a challenging approach, and may not be a clinically suitable treatment option for a large number of patients (Gonin *et al.* 2005). VPA-mediated melatonin receptor upregulation might be a way to bypass these challenges, while achieving comparable therapeutic benefits. In support of this view, a similar study assessing the effects of VPA and melatonin on MCF-7 cell proliferation showed that VPA and melatonin treatments have a combined antiproliferative effect, which surpasses that of either drug alone (Jawed *et al.* 2007). The collective protective effect is thought to be related to the increase in melatonin receptor density induced by VPA in these cells (Jawed *et al.* 2007).

The epigenetic reprogramming of gene expression by VPA to regain transcriptional control of the melatonin receptor gene can be a therapeutic strategy used to offset imbalances in receptor expression, and relatedly, melatonergic

signaling. To illustrate, melatonin receptor expression is depleted in the hippocampus of Alzheimer's patients (Savaskan *et al.* 2005). VPA causes an upregulation of melatonin receptor expression in the hippocampal CA1, CA2, CA3, and dentate gyrus, which are regions important in the regulation of cognitive function and neuronal proliferation (Bahna *et al.* 2014). The amelioration of melatonin receptor expression, and relatedly, melatonergic signaling, alleviates memory impairments in aged animals (Liu *et al.* 2013), and attenuates several pathologies which promote neurodegeneration in Alzheimer's disease (Lin *et al.* 2013). Melatonin acts via its receptors to augment adult hippocampal neurogenesis (Chern *et al.* 2012). As this is thought to be sufficient to improve cognitive function (Sahay *et al.* 2011), the upregulation of melatonin receptor expression by VPA has several therapeutic implications for the management of Alzheimer's disease, as well as other neurodegenerative conditions.

4.13 STUDY LIMITATIONS

1. RAT C6 GLIOMA CELLS AS A MODEL OF VPA-MEDIATED MT_1 UPREGULATION: The rat C6 glioma cell line was chosen for this study because of its ability to reliably model VPA-mediated MT_1 upregulation. While the use of C6 cells is convenient for the mechanistic screening of this interaction, the dynamics of *in vivo* transcriptional regulation are too complex to precisely replicate in a homogeneous glial cell culture. It is difficult to determine whether the controlled environment created in the cell culture dish discounts unknown factors involved in MT_1

upregulation by VPA, which may influence the overall interpretation of the results. Moreover, the MT_2 subtype is not consistently detected in C6 cells. As MT_1 and MT_2 are differentially regulated (Lacoste *et al.* 2015), it is important that the interactions regulating melatonin receptor expression by VPA proposed in this study are confirmed for both melatonin receptor isoforms in an *in vivo* model.

2. PROTEIN ANALYSIS OF MT_1 EXPRESSION: Although mRNA analysis gives insight into the molecular regulation of MT_1 induction by VPA, mRNA levels do not always correlate with protein levels (Vogel and Marcotte 2012). While previous studies have confirmed the dose-dependent induction of MT_1 protein expression by VPA (Castro *et al.* 2005; Jawed *et al.* 2007), the supplier for the antibodies used in these studies is no longer in service. Several attempts were made with MT_1 antibodies from other suppliers, however none were successful. As such, all MT_1 protein analysis was excluded from this study.

3. DOSAGES OF VPA AND MELATONIN ADMINISTERED IN COMBINATION: A combination of two drugs can create a mixture that behaves like a new drug all together (Foucquier and Guedj 2015). As such, a true combination study should take into consideration the additional effects not seen by either drug alone. The dosages for VPA and melatonin administered were chosen based on previous reports of these drugs promoting neuroprotective effects individually (Niles *et al.* 2012; Carriere *et*

al. 2015). Using dosages known to exert neuroprotection creates a situation where it is difficult to separate the effects mediated by melatonin receptor upregulation, from other general effects of each drug. Various concentrations of each drug should be combined incrementally to determine the optimal dosages for administration in combination. Ideally, the combination dosages would allow the melatonin receptor to be upregulated, and would minimize other nonspecific effects associated with increased drug concentrations.

4. BASAL LEVELS OF ANTIOXIDANT GENE EXPRESSION: Nrf2-ARE signaling is an inducible antioxidant response, implying that an oxidative stress challenge should be conducted in order to initiate this pathway. The same animals used in the combination study were also used to measure neurotrophic factor modulation by VPA and melatonin co-treatments. As neurotrophic factors are responsive to various insults and injuries in the brain, we chose not to challenge the animals and measure basal levels of Nrf2-ARE signaling in response to VPA and melatonin.

4.14 CONCLUSIONS

This dissertation shows that the G-protein coupled melatonin MT_1 receptor is epigenetically regulated by the psychotherapeutic reagent, VPA. The findings of this study are consistent with earlier work suggesting that an epigenetic mechanism might underlie the induction of MT_1 by VPA (Kim et al. 2008). The results presented

in this study demonstrate that in addition to VPA, the HDAC inhibitors SAHA and M344 also induce melatonin MT_1 expression in rat C6 glioma cells. This effect could not be replicated using VPM, a VPA analogue that lacks HDAC inhibitory activity, emphasizing the importance of HDAC inhibition in this process. The epigenetic mechanism underlying the upregulation of MT_1 by VPA is proposed to proceed via increased acetylation of histone proteins localized on the MT_1 promoter by this drug.

Inhibition of several intracellular signaling pathways did not prevent VPA-mediated MT_1 upregulation, proposing that HDAC inhibition and promoter histone acetylation are a primary mechanism driving this interaction. Interestingly, blockade of GSK3β signaling by the histone-methylating reagent, AR-A014418, blocked MT_1 induction by VPA, while LiCl, a GSK3β antagonist that is not known to affect this epigenetic modification, did not. These findings further support the hypothesis that an epigenetic mechanism underlies the upregulation of MT_1 by VPA.

GPCRs act as molecular sensors that convert extracellular stimuli into intracellular responses. The expression of melatonin receptors, as well as other GPCRs, is often depleted in the aged and/or diseased state (Hardeland *et al.* 2011; Dogra *et al.* 2016). Although the mechanisms driving these depletions are not yet characterized, increasing evidence suggests that epigenetic processes influence the development of several CNS diseases (Saha and Pahan 2006; Coppedè 2014), which may also involve aberrant GPCR expression (Dogra *et al.* 2016). VPA has been shown to upregulate the expression of other GPCRs in addition to the melatonin receptors (Franko-Tobin *et al.* 2012), however the mechanism by which these interactions

occur are also not known. Evidence that melatonin receptor expression can be epigenetically controlled by VPA broadens our understanding of the regulation of the melatonin receptor, and provides insight into the regulation of other GPCRs by VPA. The finding that VPA modulates melatonin receptor expression by inducing promoter histone hyperacetylation has implications for future epigenetic therapies aimed at remedying abnormal GPCR expression associated with aging or disease.

4.15 FUTURE DIRECTIONS

Epigenetic changes targeting histones are typically detected using specific antibodies and chromatin immunoprecipitation. As histones are able to carry more than one covalent modification at a time (Bannister and Kouzarides 2011), it is important to consider other techniques that are more sensitive to multiple histone modifications, to understand their relevance to epigenetic gene regulation.

Furthermore, in this study, the regulation of transcription factors by VPA is proposed to influence the rate of MT_1 transcription. Evidence that the MT_1 promoter-specific transcription factors are targeted by VPA, provides the groundwork for new research addressing the transcriptional kinetics of MT_1 by this drug. In addition to examining the effects of VPA on the translational states of Pitx1, Egr1, SF1, and GATA2, electrophoretic mobile shift assays, as well as MT_1-promoter activity assays should be conducted to gain a better understanding of this interaction.

While melatonin receptor regulation studies have focused almost exclusively on the MT_1 isoform, MT_1 and MT_2 are differentially expressed (Lacoste *et al.* 2015). This indicates that these isoforms may also be subject to different regulatory mechanisms. As we have shown that VPA also induces MT_2 expression in the rat brain, the effects of VPA on the MT_2 promoter should also be examined to determine whether an epigenetic mechanism also underlies that induction.

Finally, data is presented to show that chronic VPA and melatonin combination treatments may augment melatonergic signaling, via VPA-mediated melatonin receptor upregulation. The effects of VPA and melatonin combination treatments should be re-examined in a neurodegenerative disease model, in order to determine whether these drugs can be therapeutically beneficial in conditions where melatonin receptor expression levels are significantly depleted.

5 REFERENCES

Ackermann K., Stehle J. H. (2006) Melatonin synthesis in the human pineal gland: advantages, implications, and difficulties. *Chronobiol. Int.* **23**, 369–79.

Acuña-Castroviejo D., Escames G., Venegas C., Díaz-Casado M. E., Lima-Cabello E., López L. C., Rosales-Corral S., Tan D.-X., Reiter R. J. (2014) Extrapineal melatonin: sources, regulation, and potential functions. *Cell. Mol. Life Sci.* **71**, 2997–3025.

Adi N., Mash D. C., Ali Y., Singer C., Shehadeh L., Papapetropoulos S. (2010) Melatonin MT1 and MT2 receptor expression in Parkinson's disease. *Med. Sci. Monit.* **16**, BR61–7.

Adornetto A., Pagliara V., Renzo G. Di, Arcone R. (2013) Polychlorinated biphenyls impair dibutyryl cAMP-induced astrocytic differentiation in rat C6 glial cell line. *FEBS Open Bio* **3**, 459–66.

Airavaara M., Harvey B. K., Voutilainen M. H., Shen H., Chou J., Lindholm P., Lindahl M., et al. (2012) CDNF protects the nigrostriatal dopamine system and promotes recovery after MPTP treatment in mice. *Cell Transplant.* **21**, 1213–23.

Almutawaa W., Kang N. H., Pan Y., Niles L. P. (2014) Induction of neurotrophic and differentiation factors in neural stem cells by valproic acid. *Basic Clin. Pharmacol. Toxicol.* **115**, 216–21.

Andrabi S. A., Sayeed I., Siemen D., Wolf G., Horn T. F. W. (2004) Direct inhibition of the mitochondrial permeability transition pore: a possible mechanism responsible for anti-apoptotic effects of melatonin. *FASEB J.* **18**, 869–71.

Arinze I. J., Kawai Y. (2003) Sp family of transcription factors is involved in valproic

acid-induced expression of Galphai2. *J. Biol. Chem.* **278**, 17785–91.

Armstrong K. J., Niles L. P. (2002) Induction of GDNF mRNA expression by melatonin in rat C6 glioma cells. *Neuroreport* **13**, 473–5.

Arzenani M. K., Zade A. E., Ming Y., Vijverberg S. J. H., Zhang Z., Khan Z., Sadique S., et al. (2011) Genomic DNA hypomethylation by histone deacetylase inhibition implicates DNMT1 nuclear dynamics. *Mol. Cell. Biol.* **31**, 4119–28.

Asghari V., Wang J. F., Reiach J. S., Young L. T. (1998) Differential effects of mood stabilizers on Fos/Jun proteins and AP-1 DNA binding activity in human neuroblastoma SH-SY5Y cells. *Brain Res. Mol. Brain Res.* **58**, 95–102.

Avery L. B., Bumpus N. N. (2014) Valproic acid is a novel activator of AMP-activated protein kinase and decreases liver mass, hepatic fat accumulation, and serum glucose in obese mice. *Mol. Pharmacol.* **85**, 1–10.

Ayoub M. A., Couturier C., Lucas-Meunier E., Angers S., Fossier P., Bouvier M., Jockers R. (2002) Monitoring of ligand-independent dimerization and ligand-induced conformational changes of melatonin receptors in living cells by bioluminescence resonance energy transfer. *J. Biol. Chem.* **277**, 21522–8.

Ayoub M. A., Levoye A., Delagrange P., Jockers R. (2004) Preferential formation of MT1/MT2 melatonin receptor heterodimers with distinct ligand interaction properties compared with MT2 homodimers. *Mol. Pharmacol.* **66**, 312–21.

Ayre E. A., Wang Z. P., Brown G. M., Pang S. F. (1994) Localization and characterization of [125I]iodomelatonin binding sites in duck gonads. *J. Pineal Res.* **17**, 39–47.

Bahna S. G., Sathiyapalan A., Foster J. A., Niles L. P. (2014) Regional upregulation of

hippocampal melatonin MT2 receptors by valproic acid: therapeutic implications for Alzheimer's disease. *Neurosci. Lett.* **576**, 84–7.

Bannister A. J., Kouzarides T. (2011) Regulation of chromatin by histone modifications. *Cell Res.* **21**, 381–95.

Barbacid M. (1995) Neurotrophic factors and their receptors. *Curr. Opin. Cell Biol.* **7**, 148–55.

Barrett P., Davidson G., Hazlerigg D. G., Morris M. A., Ross A. W., Morgan P. J. (1998) Mel 1a melatonin receptor expression is regulated by protein kinase C and an additional pathway addressed by the protein kinase C inhibitor Ro 31-8220 in ovine pars tuberalis cells. *Endocrinology* **139**, 163–71.

Barrett P., MacLean A., Davidson G., Morgan P. J. (1996) Regulation of the Mel 1a melatonin receptor mRNA and protein levels in the ovine pars tuberalis: evidence for a cyclic adenosine 3',5'-monophosphate-independent Mel 1a receptor coupling and an autoregulatory mechanism of expression. *Mol. Endocrinol.* **10**, 892–902.

Benítez-King G., Huerto-Delgadillo L., Antón-Tay F. (1993) Binding of 3H-melatonin to calmodulin. *Life Sci.* **53**, 201–7.

Best J. L., Amezcua C. A., Mayr B., Flechner L., Murawsky C. M., Emerson B., Zor T., Gardner K. H., Montminy M. (2004) Identification of small-molecule antagonists that inhibit an activator: coactivator interaction. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 17622–7.

Biermann J., Grieshaber P., Goebel U., Martin G., Thanos S., Giovanni S. Di, Lagrèze W. A. (2010) Valproic acid-mediated neuroprotection and regeneration in injured

retinal ganglion cells. *Invest. Ophthalmol. Vis. Sci.* **51**, 526–34.

Bitman M., Vrzal R., Dvorak Z., Pavek P. (2014) Valproate activates ERK signaling pathway in primary human hepatocytes. *Biomed. Pap. Med. Fac. Univ. Palacký, Olomouc, Czechoslov.* **158**, 39–43.

Boeckeler K., Adley K., Xu X., Jenkins A., Jin T., Williams R. S. B. (2006) The neuroprotective agent, valproic acid, regulates the mitogen-activated protein kinase pathway through modulation of protein kinase A signalling in Dictyostelium discoideum. *Eur. J. Cell Biol.* **85**, 1047–57.

Bondi C. D., McKeon R. M., Bennett J. M., Ignatius P. F., Brydon L., Jockers R., Melan M. A., Witt-Enderby P. A. (2008) MT1 melatonin receptor internalization underlies melatonin-induced morphologic changes in Chinese hamster ovary cells and these processes are dependent on Gi proteins, MEK 1/2 and microtubule modulation. *J. Pineal Res.* **44**, 288–298.

Bonmati-Carrion M., Arguelles-Prieto R., Martinez-Madrid M., Reiter R., Hardeland R., Rol M., Madrid J. (2014) Protecting the Melatonin Rhythm through Circadian Healthy Light Exposure. *Int. J. Mol. Sci.* **15**, 23448–23500.

Boulle F., Massart R., Stragier E., Païzanis E., Zaidan L., Marday S., Gabriel C., Mocaer E., Mongeau R., Lanfumey L. (2014) Hippocampal and behavioral dysfunctions in a mouse model of environmental stress: normalization by agomelatine. *Transl. Psychiatry* **4**, e485.

Bozulic L., Hemmings B. A. (2009) PIKKing on PKB: regulation of PKB activity by phosphorylation. *Curr. Opin. Cell Biol.* **21**, 256–61.

Brydon L., Roka F., Petit L., Coppet P. de, Tissot M., Barrett P., Morgan P. J., Nanoff C.,

Strosberg A. D., Jockers R. (1999) Dual Signaling of Human Mel1a Melatonin Receptors via G i2 , G i3 , and G q/11 Proteins. *Mol. Endocrinol.* **13**, 2025–2038.

Bubenik G. A. (2002) Gastrointestinal melatonin: localization, function, and clinical relevance. *Dig. Dis. Sci.* **47**, 2336–48.

Bubenik G. A., Brown G. M., Uhlir I., Grota L. J. (1974) Immunohistological localization of N-acetylindolealkylamines in pineal gland, retina and cerebellum. *Brain Res.* **81**, 233–42.

Calkins M. J., Jakel R. J., Johnson D. A., Chan K., Kan Y. W., Johnson J. A. (2005) Protection from mitochondrial complex II inhibition in vitro and in vivo by Nrf2-mediated transcription. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 244–9.

Candelario-Jalil E., Mhadu N. H., Al-Dalain S. M., Martínez G., León O. S. (2001) Time course of oxidative damage in different brain regions following transient cerebral ischemia in gerbils. *Neurosci. Res.* **41**, 233–41.

Cantley L. C. (2002) The phosphoinositide 3-kinase pathway. *Science* **296**, 1655–7.

Capitelli C., Sereniki A., Lima M. M. S., Reksidler A. B., Tufik S., Vital M. A. B. F. (2008) Melatonin attenuates tyrosine hydroxylase loss and hypolocomotion in MPTP-lesioned rats. *Eur. J. Pharmacol.* **594**, 101–8.

Cardinali D. P., Furio A. M., Reyes M. P., Brusco L. I. (2006) The Use of Chronobiotics in the Resynchronization of the Sleep–wake Cycle. *Cancer Causes Control* **17**, 601–609.

Cargnello M., Roux P. P. (2011) Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol. Mol. Biol. Rev.* **75**, 50–83.

Carriere C. H., Kang N. H., Niles L. P. (2014) Neuroprotection by valproic acid in an intrastriatal rotenone model of Parkinson's disease. *Neuroscience* **267**, 114–21.

Carriere C. H., Kang N. H., Niles L. P. (2015) Chronic low-dose melatonin treatment maintains nigrostriatal integrity in an intrastriatal rotenone model of Parkinson's disease. *Brain Res.* **1633**, 115–25.

Castro L. M. R., Gallant M., Niles L. P. (2005) Novel targets for valproic acid: up-regulation of melatonin receptors and neurotrophic factors in C6 glioma cells. *J. Neurochem.* **95**, 1227–36.

Cedar H., Bergman Y. (2009) Linking DNA methylation and histone modification: patterns and paradigms. *Nat. Rev. Genet.* **10**, 295–304.

Chang P., Walker M. C., Williams R. S. B. (2014) Seizure-induced reduction in PIP3 levels contributes to seizure-activity and is rescued by valproic acid. *Neurobiol. Dis.* **62**, 296–306.

Chapman A., Keane P. E., Meldrum B. S., Simiand J., Vernieres J. C. (1982) Mechanism of anticonvulsant action of valproate. *Prog. Neurobiol.* **19**, 315–359.

Chen B., Wang J. F., Hill B. C., Young L. T. (1999a) Lithium and valproate differentially regulate brain regional expression of phosphorylated CREB and c-Fos. *Brain Res. Mol. Brain Res.* **70**, 45–53.

Chen G., Huang L. D., Jiang Y. M., Manji H. K. (1999b) The mood-stabilizing agent valproate inhibits the activity of glycogen synthase kinase-3. *J. Neurochem.* **72**, 1327–30.

Chen G., Manji H. K., Hawver D. B., Wright C. B., Potter W. Z. (1994) Chronic sodium valproate selectively decreases protein kinase C alpha and epsilon in vitro. *J.*

Neurochem. **63**, 2361–4.

Chen G., Yuan P., Hawver D. B., Potter W. Z., Manji H. K. (1997) Increase in AP-1 transcription factor DNA binding activity by valproic acid. *Neuropsychopharmacology* **16**, 238–45.

Chen G., Yuan P. X., Jiang Y. M., Huang L. D., Manji H. K. (1999c) Valproate robustly enhances AP-1 mediated gene expression. *Brain Res. Mol. Brain Res.* **64**, 52–8.

Chen K.-B., Lin A. M.-Y., Chiu T.-H. (2003) Oxidative injury to the locus coeruleus of rat brain: neuroprotection by melatonin. *J. Pineal Res.* **35**, 109–17.

Chen R., Kim O., Yang J., Sato K., Eisenmann K. M., McCarthy J., Chen H., Qiu Y. (2001) Regulation of Akt/PKB activation by tyrosine phosphorylation. *J. Biol. Chem.* **276**, 31858–62.

Chen W.-Y., Weng J.-H., Huang C.-C., Chung B.-C. (2007) Histone deacetylase inhibitors reduce steroidogenesis through SCF-mediated ubiquitination and degradation of steroidogenic factor 1 (NR5A1). *Mol. Cell. Biol.* **27**, 7284–90.

Chern C.-M., Liao J.-F., Wang Y.-H., Shen Y.-C. (2012) Melatonin ameliorates neural function by promoting endogenous neurogenesis through the MT2 melatonin receptor in ischemic-stroke mice. *Free Radic. Biol. Med.* **52**, 1634–47.

Chikano Y., Domoto T., Furuta T., Sabit H., Kitano-Tamura A., Pyko I. V, Takino T., et al. (2015) Glycogen synthase kinase 3β sustains invasion of glioblastoma via the focal adhesion kinase, Rac1, and c-Jun N-terminal kinase-mediated pathway. *Mol. Cancer Ther.* **14**, 564–74.

Chiu C.-T., Wang Z., Hunsberger J. G., Chuang D.-M. (2013) Therapeutic potential of mood stabilizers lithium and valproic acid: beyond bipolar disorder. *Pharmacol.*

Rev. **65**, 105–42.

Clodfelder-Miller B., Sarno P. De, Zmijewska A. A., Song L., Jope R. S. (2005) Physiological and pathological changes in glucose regulate brain Akt and glycogen synthase kinase-3. *J. Biol. Chem.* **280**, 39723–31.

Coppedè F. (2014) The potential of epigenetic therapies in neurodegenerative diseases. *Front. Genet.* **5**, 220.

Cordero-Llana Ó., Houghton B. C., Rinaldi F., Taylor H., Yáñez-Muñoz R. J., Uney J. B., Wong L.-F., Caldwell M. A. (2015) Enhanced efficacy of the CDNF/MANF family by combined intranigral overexpression in the 6-OHDA rat model of Parkinson's disease. *Mol. Ther.* **23**, 244–54.

Coto-Montes A. M., Rodríguez-Colunga M. J., Tolivia D., Menéndez-Peláez A. (1996) Immunocytochemical localization of melatonin in the harderian gland of Syrian hamster. *Anat. Rec.* **245**, 13–6.

Creson T. K., Yuan P., Manji H. K., Chen G. (2009) Evidence for involvement of ERK, PI3K, and RSK in induction of Bcl-2 by valproate. *J. Mol. Neurosci.* **37**, 123–34.

Cunningham M. O., Woodhall G. L., Jones R. S. G. (2003) Valproate modifies spontaneous excitation and inhibition at cortical synapses in vitro. *Neuropharmacology* **45**, 907–17.

Daigle D., Gradoville L., Tuck D., Schulz V., Wang'ondu R., Ye J., Gorres K., Miller G. (2011) Valproic acid antagonizes the capacity of other histone deacetylase inhibitors to activate the Epstein-barr virus lytic cycle. *J. Virol.* **85**, 5628–43.

Das A., Wallace G., Reiter R. J., Varma A. K., Ray S. K., Banik N. L. (2013) Overexpression of melatonin membrane receptors increases calcium-binding

proteins and protects VSC4.1 motoneurons from glutamate toxicity through multiple mechanisms. *J. Pineal Res.* **54**, 58–68.

Detich N., Bovenzi V., Szyf M. (2003) Valproate induces replication-independent active DNA demethylation. *J. Biol. Chem.* **278**, 27586–92.

Doble B. W., Woodgett J. R. (2003) GSK-3: tricks of the trade for a multi-tasking kinase. *J. Cell Sci.* **116**, 1175–86.

Dogra S., Sona C., Kumar A., Yadav P. N. (2016) Epigenetic regulation of G protein coupled receptor signaling and its implications in psychiatric disorders. *Int. J. Biochem. Cell Biol.*

Dokmanovic M., Clarke C., Marks P. A. (2007) Histone deacetylase inhibitors: overview and perspectives. *Mol. Cancer Res.* **5**, 981–9.

Drew J. E., Williams L. M., Hannah L. T., Barrett P., Abramovich D. R. (1998) Melatonin receptors in the human fetal kidney: 2-[125I]iodomelatonin binding sites correlated with expression of Mel1a and Mel1b receptor genes. *J. Endocrinol.* **156**, 261–7.

Du K., Montminy M. (1998) CREB is a regulatory target for the protein kinase Akt/PKB. *J. Biol. Chem.* **273**, 32377–9.

Dubocovich M. L., Rivera-Bermudez M. A., Gerdin M. J., Masana M. I. (2003) Molecular pharmacology, regulation and function of mammalian melatonin receptors. *Front. Biosci.* **8**, d1093–108.

Dupont C., Armant D. R., Brenner C. A. (2009) Epigenetics: definition, mechanisms and clinical perspective. *Semin. Reprod. Med.* **27**, 351–7.

Eadie M. J. (2004) Could valerian have been the first anticonvulsant? *Epilepsia* **45**,

1338–43.

Ebadi M., Govitrapong P. (1986) Neural pathways and neurotransmitters affecting melatonin synthesis. *J. Neural Transm. Suppl.* **21**, 125–55.

Ebisawa T., Karne S., Lerner M. R., Reppert S. M. (1994) Expression cloning of a high-affinity melatonin receptor from Xenopus dermal melanophores. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 6133–7.

Einat H., Yuan P., Gould T. D., Li J., Du J., Zhang L., Manji H. K., Chen G. (2003) The role of the extracellular signal-regulated kinase signaling pathway in mood modulation. *J. Neurosci.* **23**, 7311–6.

Ellison N., Weller J. L., Klein D. C. (1972) Development of a circadian rhythm in the activity of pineal serotonin N-acetyltransferase. *J. Neurochem.* **19**, 1335–41.

Erşahin M., Özdemir Z., Özsavcı D., Akakın D., Yeğen B. Ç., Reiter R. J., Sener G. (2012) Melatonin treatment protects against spinal cord injury induced functional and biochemical changes in rat urinary bladder. *J. Pineal Res.* **52**, 340–8.

Felisbino M. B., Gatti M. S. V., Mello M. L. S. (2014) Changes in chromatin structure in NIH 3T3 cells induced by valproic acid and trichostatin A. *J. Cell. Biochem.* **115**, 1937–47.

Foucquier J., Guedj M. (2015) Analysis of drug combinations: current methodological landscape. *Pharmacol. Res. Perspect.* **3**, e00149.

Foulkes N. S., Borjigin J., Snyder S. H., Sassone-Corsi P. (1997) Rhythmic transcription: the molecular basis of circadian melatonin synthesis. *Trends Neurosci.* **20**, 487–92.

Franko-Tobin L. G., Mackey L. V., Huang W., Song X., Jin B., Luo J., Morris L. M., et al.

(2012) Notch1-mediated tumor suppression in cervical cancer with the involvement of SST signaling and its application in enhanced SSTR-targeted therapeutics. *Oncologist* **17**, 220–32.

Fu Y., Zheng Y., Chan K.-G., Liang A., Hu F. (2014) Lithium chloride decreases proliferation and migration of C6 glioma cells harboring isocitrate dehydrogenase 2 mutant via GSK-3β. *Mol. Biol. Rep.* **41**, 3907–13.

Gall C. von, Weaver D. R., Moek J., Jilg A., Stehle J. H., Korf H.-W. (2005) Melatonin plays a crucial role in the regulation of rhythmic clock gene expression in the mouse pars tuberalis. *Ann. N. Y. Acad. Sci.* **1040**, 508–11.

Gauer F., Masson-Pévet M., Skene D. J., Vivien-Roels B., Pévet P. (1993) Daily rhythms of melatonin binding sites in the rat pars tuberalis and suprachiasmatic nuclei; evidence for a regulation of melatonin receptors by melatonin itself. *Neuroendocrinology* **57**, 120–6.

Gauer F., Masson-Pevet M., Stehle J., Pevet P. (1994) Daily variations in melatonin receptor density of rat pars tuberalis and suprachiasmatic nuclei are distinctly regulated. *Brain Res.* **641**, 92–8.

Gean P. W., Huang C. C., Hung C. R., Tsai J. J. (1994) Valproic acid suppresses the synaptic response mediated by the NMDA receptors in rat amygdalar slices. *Brain Res. Bull.* **33**, 333–6.

Gitto E., Tan D. X., Reiter R. J., Karbownik M., Manchester L. C., Cuzzocrea S., Fulia F., Barberi I. (2001) Individual and synergistic antioxidative actions of melatonin: studies with vitamin E, vitamin C, glutathione and desferrioxamine (desferoxamine) in rat liver homogenates. *J. Pharm. Pharmacol.* **53**, 1393–401.

Glister C., Satchell L., Michael A. E., Bicknell A. B., Knight P. G. (2012) The anti-epileptic drug valproic acid (VPA) inhibits steroidogenesis in bovine theca and granulosa cells in vitro. *PLoS One* **7**, e49553.

Goldman H., Wurtman R. J. (1964) Flow of blood to the pineal body of the rat. *Nature* **203**, 87–8.

Gonin P., Buchholz C. J., Pallardy M., Mezzina M. (2005) Gene therapy bio-safety: scientific and regulatory issues. *Gene Ther.* **12 Suppl 1**, S146–52.

González A., Martínez-Campa C., Mediavilla M. D., Alonso-González C., Sánchez-Mateos S., Hill S. M., Sánchez-Barceló E. J., Cos S. (2007) Effects of MT1 melatonin receptor overexpression on the aromatase-suppressive effect of melatonin in MCF-7 human breast cancer cells. *Oncol. Rep.* **17**, 947–53.

Granado N., Lastres-Becker I., Ares-Santos S., Oliva I., Martin E., Cuadrado A., Moratalla R. (2011) Nrf2 deficiency potentiates methamphetamine-induced dopaminergic axonal damage and gliosis in the striatum. *Glia* **59**, 1850–63.

Grimes C. A., Jope R. S. (2001) CREB DNA binding activity is inhibited by glycogen synthase kinase-3 beta and facilitated by lithium. *J. Neurochem.* **78**, 1219–32.

Grunstein M. (1997) Histone acetylation in chromatin structure and transcription. *Nature* **389**, 349–52.

Gupta M., Aneja S., Kohli K. (2004a) Add-on melatonin improves quality of life in epileptic children on valproate monotherapy: a randomized, double-blind, placebo-controlled trial. *Epilepsy Behav.* **5**, 316–21.

Gupta M., Aneja S., Kohli K. (2005) Add-on melatonin improves sleep behavior in children with epilepsy: randomized, double-blind, placebo-controlled trial. *J.*

Child Neurol. **20**, 112–5.

Gupta M., Gupta Y. K., Agarwal S., Aneja S., Kohli K. (2004b) A randomized, double-blind, placebo controlled trial of melatonin add-on therapy in epileptic children on valproate monotherapy: effect on glutathione peroxidase and glutathione reductase enzymes. *Br. J. Clin. Pharmacol.* **58**, 542–7.

Gurpur P. B., Liu J., Burkin D. J., Kaufman S. J. (2009) Valproic acid activates the PI3K/Akt/mTOR pathway in muscle and ameliorates pathology in a mouse model of Duchenne muscular dystrophy. *Am. J. Pathol.* **174**, 999–1008.

Hage M. El, Baverel G., Martin G. (2012) Effects of valproate on glutamate metabolism in rat brain slices: a (13)C NMR study. *Epilepsy Res.* **99**, 94–100.

Hansen J. C. (2012) Human mitotic chromosome structure: what happened to the 30-nm fibre? *EMBO J.* **31**, 1621–3.

Hardeland R. (2009) Melatonin: Signaling mechanisms of a pleiotropic agent. *BioFactors* **35**, 183–192.

Hardeland R. (2013) Melatonin and the theories of aging: a critical appraisal of melatonin's role in antiaging mechanisms. *J. Pineal Res.* **55**, 325–56.

Hardeland R., Cardinali D. P., Srinivasan V., Spence D. W., Brown G. M., Pandi-Perumal S. R. (2011) Melatonin—A pleiotropic, orchestrating regulator molecule. *Prog. Neurobiol.* **93**, 350–384.

Haring M., Offermann S., Danker T., Horst I., Peterhansel C., Stam M. (2007) Chromatin immunoprecipitation: optimization, quantitative analysis and data normalization. *Plant Methods* **3**, 11.

Harwood A. J. (2001) Regulation of GSK-3: a cellular multiprocessor. *Cell* **105**, 821–4.

Henderson C. E. (1996) Role of neurotrophic factors in neuronal development. *Curr. Opin. Neurobiol.* **6**, 64–70.

Herzog E. D., Takahashi J. S., Block G. D. (1998) Clock controls circadian period in isolated suprachiasmatic nucleus neurons. *Nat. Neurosci.* **1**, 708–13.

Hess J., Angel P., Schorpp-Kistner M. (2004) AP-1 subunits: quarrel and harmony among siblings. *J. Cell Sci.* **117**, 5965–73.

Horvath J. E., Bailey J. A., Locke D. P., Eichler E. E. (2001) Lessons from the human genome: transitions between euchromatin and heterochromatin. *Hum. Mol. Genet.* **10**, 2215–23.

Ichiyama T., Okada K., Lipton J. M., Matsubara T., Hayashi T., Furukawa S. (2000) Sodium valproate inhibits production of TNF-alpha and IL-6 and activation of NF-kappaB. *Brain Res.* **857**, 246–51.

Jawed S., Kim B., Ottenhof T., Brown G. M., Werstiuk E. S., Niles L. P. (2007) Human melatonin MT1 receptor induction by valproic acid and its effects in combination with melatonin on MCF-7 breast cancer cell proliferation. *Eur. J. Pharmacol.* **560**, 17–22.

Jaworek J., Brzozowski T., Konturek S. J. (2005) Melatonin as an organoprotector in the stomach and the pancreas. *J. Pineal Res.* **38**, 73–83.

Jenwitheesuk A., Nopparat C., Mukda S., Wongchitrat P., Govitrapong P. (2014) Melatonin regulates aging and neurodegeneration through energy metabolism, epigenetics, autophagy and circadian rhythm pathways. *Int. J. Mol. Sci.* **15**, 16848–84.

Johnston J. D., Klosen P., Barrett P., Hazlerigg D. G. (2006) Regulation of MT1

Melatonin Receptor Expression in the Foetal Rat Pituitary. *J. Neuroendocrinol.* **18**, 50–56.

Johnston J. D., Messager S., Barrett P., Hazlerigg D. G. (2003a) Melatonin action in the pituitary: neuroendocrine synchronizer and developmental modulator? *J. Neuroendocrinol.* **15**, 405–8.

Johnston J. D., Messager S., Ebling F. J. P., Williams L. M., Barrett P., Hazlerigg D. G. (2003b) Gonadotrophin-releasing hormone drives melatonin receptor down-regulation in the developing pituitary gland. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 2831–5.

Johnston J. D., Schuster C., Barrett P., Hazlerigg D. G. (2007) Regulation of the ovine MT1 melatonin receptor promoter: interaction between multiple pituitary transcription at different phases of development. *Mol. Cell. Endocrinol.* **268**, 59–66.

Jumnongprakhon P., Govitrapong P., Tocharus C., Pinkaew D., Tocharus J. (2015) Melatonin Protects Methamphetamine-Induced Neuroinflammation Through NF-κB and Nrf2 Pathways in Glioma Cell Line. *Neurochem. Res.* **40**, 1448–56.

Jung G.-A., Yoon J.-Y., Moon B.-S., Yang D.-H., Kim H.-Y., Lee S.-H., Bryja V., Arenas E., Choi K.-Y. (2008) Valproic acid induces differentiation and inhibition of proliferation in neural progenitor cells via the beta-catenin-Ras-ERK-p21Cip/WAF1 pathway. *BMC Cell Biol.* **9**, 66.

Jung K. H., Hong S.-W., Zheng H.-M., Lee D.-H., Hong S.-S. (2009) Melatonin downregulates nuclear erythroid 2-related factor 2 and nuclear factor-kappaB during prevention of oxidative liver injury in a dimethylnitrosamine model. *J.*

Pineal Res. **47**, 173–83.

Jung K. H., Hong S.-W., Zheng H.-M., Lee H.-S., Lee H., Lee D.-H., Lee S. Y., Hong S.-S. (2010) Melatonin ameliorates cerulein-induced pancreatitis by the modulation of nuclear erythroid 2-related factor 2 and nuclear factor-kappaB in rats. *J. Pineal Res.* **48**, 239–50.

Kansanen E., Kuosmanen S. M., Leinonen H., Levonen A.-L. (2013) The Keap1-Nrf2 pathway: Mechanisms of activation and dysregulation in cancer. *Redox Biol.* **1**, 45–9.

Katsuoka F., Motohashi H., Ishii T., Aburatani H., Engel J. D., Yamamoto M. (2005) Genetic evidence that small maf proteins are essential for the activation of antioxidant response element-dependent genes. *Mol. Cell. Biol.* **25**, 8044–51.

Kia A., Yata T., Hajji N., Hajitou A. (2013) Inhibition of histone deacetylation and DNA methylation improves gene expression mediated by the adeno-associated virus/phage in cancer cells. *Viruses* **5**, 2561–72.

Kim B., Rincón Castro L. M., Jawed S., Niles L. P. (2008) Clinically relevant concentrations of valproic acid modulate melatonin MT(1) receptor, HDAC and MeCP2 mRNA expression in C6 glioma cells. *Eur. J. Pharmacol.* **589**, 45–8.

Kim J. N., Kim M. K., Cho K. S., Choi C. S., Park S. H., Yang S.-I., Joo S. H., et al. (2013) Valproic Acid Regulates α-Synuclein Expression through JNK Pathway in Rat Primary Astrocytes. *Biomol. Ther. (Seoul).* **21**, 222–8.

Kimura A., Matsubara K., Horikoshi M. (2005) A decade of histone acetylation: marking eukaryotic chromosomes with specific codes. *J. Biochem.* **138**, 647–62.

Kimura H., Shiota K. (2003) Methyl-CpG-binding protein, MeCP2, is a target molecule

for maintenance DNA methyltransferase, Dnmt1. *J. Biol. Chem.* **278**, 4806–12.

Ko C. H., Takahashi J. S. (2006) Molecular components of the mammalian circadian clock. *Hum. Mol. Genet.* **15 Spec No**, R271–7.

Ko G. Y., Brown-Croyts L. M., Teyler T. J. (1997) The effects of anticonvulsant drugs on NMDA-EPSP, AMPA-EPSP, and GABA-IPSP in the rat hippocampus. *Brain Res. Bull.* **42**, 297–302.

Kong P.-J., Byun J.-S., Lim S.-Y., Lee J.-J., Hong S.-J., Kwon K.-J., Kim S.-S. (2008a) Melatonin Induces Akt Phosphorylation through Melatonin Receptor- and PI3K-Dependent Pathways in Primary Astrocytes. *Korean J. Physiol. Pharmacol.* **12**, 37–41.

Kong X., Li X., Cai Z., Yang N., Liu Y., Shu J., Pan L., Zuo P. (2008b) Melatonin regulates the viability and differentiation of rat midbrain neural stem cells. *Cell. Mol. Neurobiol.* **28**, 569–79.

Kristjuhan A., Walker J., Suka N., Grunstein M., Roberts D., Cairns B. R., Svejstrup J. Q. (2002) Transcriptional inhibition of genes with severe histone h3 hypoacetylation in the coding region. *Mol. Cell* **10**, 925–33.

Kumar Kharwar R., Haldar C. (2011) Anatomical and histological profile of bronchus-associated lymphoid tissue and localization of melatonin receptor types (Mel 1a and Mel 1b) in the lung-associated immune system of a tropical bird, Perdicula asiatica. *Acta Histochem.* **113**, 333–9.

Künig G., Niedermeyer B., Deckert J., Gsell W., Ransmayr G., Riederer P. (1998) Inhibition of [3H]alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid [AMPA] binding by the anticonvulsant valproate in clinically relevant

concentrations: an autoradiographic investigation in human hippocampus. *Epilepsy Res.* **31**, 153–7.

Kurita M., Nishino S., Ohtomo K., Rai M., Shirakawa H., Mashiko H., Niwa S.-I., Nakahata N. (2007) Sodium valproate at therapeutic concentrations changes Ca2+ response accompanied with its weak inhibition of protein kinase C in human astrocytoma cells. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **31**, 600–4.

Lacoste B., Angeloni D., Dominguez-Lopez S., Calderoni S., Mauro A., Fraschini F., Descarries L., Gobbi G. (2015) Anatomical and cellular localization of melatonin MT1 and MT2 receptors in the adult rat brain. *J. Pineal Res.* **58**, 397–417.

Lee E., Son H. (2009) Adult hippocampal neurogenesis and related neurotrophic factors. *BMB Rep.* **42**, 239–44.

Lee S.-H., Chun W., Kong P.-J., Han J. A., Cho B. P., Kwon O.-Y., Lee H. J., Kim S.-S. (2006) Sustained activation of Akt by melatonin contributes to the protection against kainic acid-induced neuronal death in hippocampus. *J. Pineal Res.* **40**, 79–85.

Lennartsson A., Ekwall K. (2009) Histone modification patterns and epigenetic codes. *Biochim. Biophys. Acta* **1790**, 863–8.

Lerner A. B., Case J. D. (1959) Pigment cell regulatory factors. *J. Invest. Dermatol.* **32**, 211–21.

Lerner A. B., Case J. D., Takahashi Y. (1960) Isolation of melatonin and 5-methoxyindole-3-acetic acid from bovine pineal glands. *J. Biol. Chem.* **235**, 1992–7.

Levoye A., Dam J., Ayoub M. A., Guillaume J.-L., Couturier C., Delagrange P., Jockers R. (2006) The orphan GPR50 receptor specifically inhibits MT1 melatonin receptor function through heterodimerization. *EMBO J.* **25**, 3012–3023.

Li C., Zhou C., Wang S., Feng Y., Lin W., Lin S., Wang Y., et al. (2011) Sensitization of glioma cells to tamoxifen-induced apoptosis by Pl3-kinase inhibitor through the GSK-3β/β-catenin signaling pathway. *PLoS One* **6**, e27053.

Lim D. H. K., Maher E. R. (2010) DNA methylation: a form of epigenetic control of gene expression. *Obstet. Gynaecol.* **12**, 37–42.

Lin L., Huang Q.-X., Yang S.-S., Chu J., Wang J.-Z., Tian Q. (2013) Melatonin in Alzheimer's disease. *Int. J. Mol. Sci.* **14**, 14575–93.

Lindholm P., Peränen J., Andressoo J.-O., Kalkkinen N., Kokaia Z., Lindvall O., Timmusk T., Saarma M. (2008) MANF is widely expressed in mammalian tissues and differently regulated after ischemic and epileptic insults in rodent brain. *Mol. Cell. Neurosci.* **39**, 356–71.

Lindholm P., Saarma M. (2010) Novel CDNF/MANF family of neurotrophic factors. *Dev. Neurobiol.* **70**, 360–71.

Lindholm P., Voutilainen M. H., Laurén J., Peränen J., Leppänen V.-M., Andressoo J.-O., Lindahl M., et al. (2007) Novel neurotrophic factor CDNF protects and rescues midbrain dopamine neurons in vivo. *Nature* **448**, 73–7.

Liu B., Ohishi K., Yamamura K., Suzuki K., Monma F., Ino K., Nishii K., et al. (2010) A potential activity of valproic acid in the stimulation of interleukin-3-mediated megakaryopoiesis and erythropoiesis. *Exp. Hematol.* **38**, 685–95.

Liu Y., Ni C., Tang Y., Tian X., Zhou Y., Qian M., Li Z., Chui D., Guo X. (2013) Melatonin

attenuates isoflurane-induced acute memory impairments in aged rats. *Basic Clin. Pharmacol. Toxicol.* **113**, 215–20.

Lo D. C. (1995) Neurotrophic factors and synaptic plasticity. *Neuron* **15**, 979–981.

Löscher W. (1993) In vivo administration of valproate reduces the nerve terminal (synaptosomal) activity of GABA aminotransferase in discrete brain areas of rats. *Neurosci. Lett.* **160**, 177–80.

Luchetti F., Canonico B., Betti M., Arcangeletti M., Pilolli F., Piroddi M., Canesi L., Papa S., Galli F. (2010) Melatonin signaling and cell protection function. *FASEB J.* **24**, 3603–3624.

Luder A. S., Parks J. K., Frerman F., Parker W. D. (1990) Inactivation of beef brain alpha-ketoglutarate dehydrogenase complex by valproic acid and valproic acid metabolites. Possible mechanism of anticonvulsant and toxic actions. *J. Clin. Invest.* **86**, 1574–81.

Ma J., Shaw V. E., Mitrofanis J. (2009) Does melatonin help save dopaminergic cells in MPTP-treated mice? *Parkinsonism Relat. Disord.* **15**, 307–14.

Macchi M. M., Bruce J. N. (2004) Human pineal physiology and functional significance of melatonin. *Front. Neuroendocrinol.* **25**, 177–95.

Macías M., Escames G., Leon J., Coto A., Sbihi Y., Osuna A., Acuña-Castroviejo D. (2003) Calreticulin-melatonin. An unexpected relationship. *Eur. J. Biochem.* **270**, 832–40.

Maggio R., Novi F., Scarselli M., Corsini G. U. (2005) The impact of G-protein-coupled receptor hetero-oligomerization on function and pharmacology. *FEBS J.* **272**, 2939–46.

Maison C., Almouzni G. (2004) HP1 and the dynamics of heterochromatin maintenance. *Nat. Rev. Mol. Cell Biol.* **5**, 296–304.

Manning B. D., Cantley L. C. (2007) AKT/PKB signaling: navigating downstream. *Cell* **129**, 1261–74.

Mano H., Fukada Y. (2007) A median third eye: pineal gland retraces evolution of vertebrate photoreceptive organs. *Photochem. Photobiol.* **83**, 11–8.

Mao L.-M., Tang Q., Wang J. Q. (2007) Protein kinase C-regulated cAMP response element-binding protein phosphorylation in cultured rat striatal neurons. *Brain Res. Bull.* **72**, 302–8.

Marchion D. C., Bicaku E., Daud A. I., Sullivan D. M., Munster P. N. (2005) Valproic acid alters chromatin structure by regulation of chromatin modulation proteins. *Cancer Res.* **65**, 3815–22.

Marmorstein R., Roth S. Y. (2001) Histone acetyltransferases: function, structure, and catalysis. *Curr. Opin. Genet. Dev.* **11**, 155–61.

Maronde E., Pfeffer M., Olcese J., Molina C. A., Schlotter F., Dehghani F., Korf H. W., Stehle J. H. (1999) Transcription factors in neuroendocrine regulation: rhythmic changes in pCREB and ICER levels frame melatonin synthesis. *J. Neurosci.* **19**, 3326–36.

Marsoni S., Damia G., Camboni G. (2008) A work in progress: the clinical development of histone deacetylase inhibitors. *Epigenetics* **3**, 164–71.

Martín F., Mora L., Laorden M., Milanés M. (2011) Protein kinase C phosphorylates the cAMP response element binding protein in the hypothalamic paraventricular nucleus during morphine withdrawal. *Br. J. Pharmacol.* **163**,

857–75.

Martín V., Sainz R. M., Mayo J. C., Antolín I., Herrera F., Rodríguez C. (2003) Daily rhythm of gene expression in rat superoxide dismutases. *Endocr. Res.* **29**, 83–95.

Masana M. I., Benloucif S., Dubocovich M. L. (2000) Circadian rhythm of mt1 melatonin receptor expression in the suprachiasmatic nucleus of the C3H/HeN mouse. *J. Pineal Res.* **28**, 185–92.

Mayr B., Montminy M. (2001) Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat. Rev. Mol. Cell Biol.* **2**, 599–609.

Mellor H., Parker P. J. (1998) The extended protein kinase C superfamily. *Biochem. J.* **332 (Pt 2**, 281–92.

Mennenga K., Ueck M., Reiter R. J. (1991) Immunohistological localization of melatonin in the pineal gland and retina of the rat. *J. Pineal Res.* **10**, 159–64.

Milutinovic S., D'Alessio A. C., Detich N., Szyf M. (2007) Valproate induces widespread epigenetic reprogramming which involves demethylation of specific genes. *Carcinogenesis* **28**, 560–71.

Mitsiades C. S., Mitsiades N., Koutsilieris M. (2004) The Akt pathway: molecular targets for anti-cancer drug development. *Curr. Cancer Drug Targets* **4**, 235–56.

Molteni R., Calabrese F., Pisoni S., Gabriel C., Mocaer E., Racagni G., Riva M. A. (2010) Synergistic mechanisms in the modulation of the neurotrophin BDNF in the rat prefrontal cortex following acute agomelatine administration. *World J. Biol. Psychiatry* **11**, 148–53.

Monti B., Polazzi E., Contestabile A. (2009) Biochemical, molecular and epigenetic mechanisms of valproic acid neuroprotection. *Curr. Mol. Pharmacol.* **2**, 95–109.

Morgan P. J., Barrett P., Hazlerigg D., Milligan G., Lawson W., MacLean A., Davidson G. (1995) Melatonin receptors couple through a cholera toxin-sensitive mechanism to inhibit cyclic AMP in the ovine pituitary. *J. Neuroendocrinol.* **7**, 361–9.

Morgan P. J., Barrett P., Howell H. E., Helliwell R. (1994) Melatonin receptors: localization, molecular pharmacology and physiological significance. *Neurochem. Int.* **24**, 101–46.

Morgan P. J., Davidson G., Lawson W., Barrett P. (1990) Both pertussis toxin-sensitive and insensitive g-proteins link melatonin receptor to inhibition of adenylate cyclase in the ovine pars tuberalis. *J. Neuroendocrinol.* **2**, 773–6.

Morgan P. J., Lawson W., Davidson G. (1991) Interaction of Forskolin and Melatonin on Cyclic AMP Generation in Pars Tuberalis Cells of Ovine Pituitary. *J. Neuroendocrinol.* **3**, 497–501.

Morgan P. J., Williams L. M., Barrett P., Lawson W., Davidson G., Hannah L., MacLean A. (1996) Differential regulation of melatonin receptors in sheep, chicken and lizard brains by cholera and pertussis toxins and guanine nucleotides. *Neurochem. Int.* **28**, 259–69.

Morioka N., Sugimoto T., Tokuhara M., Dohi T., Nakata Y. (2010) Noradrenaline induces clock gene Per1 mRNA expression in C6 glioma cells through beta(2)-adrenergic receptor coupled with protein kinase A - cAMP response element binding protein (PKA-CREB) and Src-tyrosine kinase - glycogen synthase kinase-3beta (Src-GSK. *J. Pharmacol. Sci.* **113**, 234–45.

Morse R. H. (2007) Transcription factor access to promoter elements. *J. Cell.*

Biochem. **102**, 560–70.

Motohashi N. (1992) GABA receptor alterations after chronic lithium administration. Comparison with carbamazepine and sodium valproate. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **16**, 571–9.

Mytilineou C., Kramer B. C., Yabut J. A. (2002) Glutathione depletion and oxidative stress. *Parkinsonism Relat. Disord.* **8**, 385–7.

Natesan A. K., Cassone V. M. (2002) Melatonin receptor mRNA localization and rhythmicity in the retina of the domestic chick, Gallus domesticus. *Vis. Neurosci.* **19**, 265–74.

Niles L. P., Armstrong K. J., Rincón Castro L. M., Dao C. V, Sharma R., McMillan C. R., Doering L. C., Kirkham D. L. (2004) Neural stem cells express melatonin receptors and neurotrophic factors: colocalization of the MT1 receptor with neuronal and glial markers. *BMC Neurosci.* **5**, 41.

Niles L. P., Sathiyapalan A., Bahna S., Kang N. H., Pan Y. (2012) Valproic acid up-regulates melatonin MT1 and MT2 receptors and neurotrophic factors CDNF and MANF in the rat brain. *Int. J. Neuropsychopharmacol.* **15**, 1343–50.

Nishizuka Y. (1995) Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J.* **9**, 484–96.

Norisada J., Hirata Y., Amaya F., Kiuchi K., Oh-Hashi K. (2016) A Comparative Analysis of the Molecular Features of MANF and CDNF. *PLoS One* **11**, e0146923.

O'Neal-Moffitt G., Delic V., Bradshaw P. C., Olcese J. (2015) Prophylactic melatonin significantly reduces Alzheimer's neuropathology and associated cognitive deficits independent of antioxidant pathways in AβPP(swe)/PS1 mice. *Mol.*

Neurodegener. **10**, 27.

Ogryzko V. V, Schiltz R. L., Russanova V., Howard B. H., Nakatani Y. (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* **87**, 953–9.

Okatani Y., Wakatsuki A., Kaneda C. (2000) Melatonin increases activities of glutathione peroxidase and superoxide dismutase in fetal rat brain. *J. Pineal Res.* **28**, 89–96.

Olivieri G., Otten U., Meier F., Baysang G., Dimitriades-Schmutz B., Müller-Spahn F., Savaskan E. (2003) Beta-amyloid modulates tyrosine kinase B receptor expression in SHSY5Y neuroblastoma cells: influence of the antioxidant melatonin. *Neuroscience* **120**, 659–65.

Ougolkov A. V, Bone N. D., Fernandez-Zapico M. E., Kay N. E., Billadeau D. D. (2007) Inhibition of glycogen synthase kinase-3 activity leads to epigenetic silencing of nuclear factor kappaB target genes and induction of apoptosis in chronic lymphocytic leukemia B cells. *Blood* **110**, 735–42.

Pablos M. I., Reiter R. J., Ortiz G. G., Guerrero J. M., Agapito M. T., Chuang J. I., Sewerynek E. (1998) Rhythms of glutathione peroxidase and glutathione reductase in brain of chick and their inhibition by light. *Neurochem. Int.* **32**, 69–75.

Païzanis E., Renoir T., Lelievre V., Saurini F., Melfort M., Gabriel C., Barden N., Mocaër E., Hamon M., Lanfumey L. (2010) Behavioural and neuroplastic effects of the new-generation antidepressant agomelatine compared to fluoxetine in glucocorticoid receptor-impaired mice. *Int. J. Neuropsychopharmacol.* **13**, 759–

74.

Pan H., Wang H., Zhu L., Mao L., Qiao L., Su X. (2013) The role of Nrf2 in migration and invasion of human glioma cell U251. *World Neurosurg.* **80**, 363–70.

Pan Y., Niles L. P. (2015) Epigenetic mechanisms of melatonin action in human SH-SY5Y neuroblastoma cells. *Mol. Cell. Endocrinol.* **402**, 57–63.

Pandi-Perumal S. R., BaHammam A. S., Brown G. M., Spence D. W., Bharti V. K., Kaur C., Hardeland R., Cardinali D. P. (2012) Melatonin Antioxidative Defense: Therapeutical Implications for Aging and Neurodegenerative Processes. *Neurotox. Res.* **23**, 267–300.

Pandi-Perumal S. R., Trakht I., Srinivasan V., Spence D. W., Maestroni G. J. M., Zisapel N., Cardinali D. P. (2008) Physiological effects of melatonin: role of melatonin receptors and signal transduction pathways. *Prog. Neurobiol.* **85**, 335–53.

Perreau-Lenz S., Kalsbeek A., Garidou M.-L., Wortel J., Vliet J. van der, Heijningen C. van, Simonneaux V., Pévet P., Buijs R. M. (2003) Suprachiasmatic control of melatonin synthesis in rats: inhibitory and stimulatory mechanisms. *Eur. J. Neurosci.* **17**, 221–8.

Perreau-Lenz S., Kalsbeek A., Vliet J. Van Der, Pévet P., Buijs R. M. (2005) In vivo evidence for a controlled offset of melatonin synthesis at dawn by the suprachiasmatic nucleus in the rat. *Neuroscience* **130**, 797–803.

Peterson G. M., Naunton M. (2005) Valproate: a simple chemical with so much to offer. *J. Clin. Pharm. Ther.* **30**, 417–21.

Pfaffl M. W., Horgan G. W., Dempfle L. (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression

results in real-time PCR. *Nucleic Acids Res.* **30**, e36.

Phiel C. J., Zhang F., Huang E. Y., Guenther M. G., Lazar M. A., Klein P. S. (2001) Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. *J. Biol. Chem.* **276**, 36734–41.

Plotnikov A., Zehorai E., Procaccia S., Seger R. (2011) The MAPK cascades: signaling components, nuclear roles and mechanisms of nuclear translocation. *Biochim. Biophys. Acta* **1813**, 1619–33.

Poeggeler B., Thuermann S., Dose A., Schoenke M., Burkhardt S., Hardeland R. (2002) Melatonin's unique radical scavenging properties - roles of its functional substituents as revealed by a comparison with its structural analogs. *J. Pineal Res.* **33**, 20–30.

Pongsa-Asawapaiboon A., Asavaritikrai P., Withyachumnarnkul B., Sumridthong A. (1998) Melatonin increases nerve growth factor in mouse submandibular gland. *J. Pineal Res.* **24**, 73–7.

Poon A. M., Chow P. H., Mak A. S., Pang S. F. (1997) Autoradiographic localization of 2[125I]iodomelatonin binding sites in the gastrointestinal tract of mammals including humans and birds. *J. Pineal Res.* **23**, 5–14.

Rao J. S., Bazinet R. P., Rapoport S. I., Lee H.-J. (2007) Chronic treatment of rats with sodium valproate downregulates frontal cortex NF-kappaB DNA binding activity and COX-2 mRNA. *Bipolar Disord.* **9**, 513–20.

Reid G., Métivier R., Lin C.-Y., Denger S., Ibberson D., Ivacevic T., Brand H., Benes V., Liu E. T., Gannon F. (2005) Multiple mechanisms induce transcriptional silencing of a subset of genes, including oestrogen receptor alpha, in response to

deacetylase inhibition by valproic acid and trichostatin A. *Oncogene* **24**, 4894–907.

Reiter R. J., Richardson B. A., Matthews S. A., Lane S. J., Ferguson B. N. (1983) Rhythms in immunoreactive melatonin in the retina and Harderian gland of rats: persistence after pinealectomy. *Life Sci.* **32**, 1229–36.

Reiter R. J., Tan D., Burkhardt S. (2002) Reactive oxygen and nitrogen species and cellular and organismal decline: amelioration with melatonin. *Mech. Ageing Dev.* **123**, 1007–19.

Reiter R. J., Tan D.-X. (2003) What constitutes a physiological concentration of melatonin? *J. Pineal Res.* **34**, 79–80.

Reiter R. J., Tan D.-X., Mayo J. C., Sainz R. M., Leon J., Czarnocki Z. (2003) Melatonin as an antioxidant: biochemical mechanisms and pathophysiological implications in humans. *Acta Biochim. Pol.* **50**, 1129–46.

Reppert S. M. (1997) Melatonin receptors: molecular biology of a new family of G protein-coupled receptors. *J. Biol. Rhythms* **12**, 528–31.

Reppert S. M., Godson C., Mahle C. D., Weaver D. R., Slaugenhaupt S. A., Gusella J. F. (1995) Molecular characterization of a second melatonin receptor expressed in human retina and brain: the Mel1b melatonin receptor. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 8734–8.

Reppert S. M., Weaver D. R., Ebisawa T. (1994) Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. *Neuron* **13**, 1177–85.

Richards E. J., Elgin S. C. R. (2002) Epigenetic codes for heterochromatin formation

and silencing: rounding up the usual suspects. *Cell* **108**, 489–500.

Riessland M., Brichta L., Hahnen E., Wirth B. (2006) The benzamide M344, a novel histone deacetylase inhibitor, significantly increases SMN2 RNA/protein levels in spinal muscular atrophy cells. *Hum. Genet.* **120**, 101–10.

Rodriguez C., Mayo J. C., Sainz R. M., Antolín I., Herrera F., Martín V., Reiter R. J. (2004) Regulation of antioxidant enzymes: a significant role for melatonin. *J. Pineal Res.* **36**, 1–9.

Roseboom P. H., Klein D. C. (1995) Norepinephrine stimulation of pineal cyclic AMP response element-binding protein phosphorylation: primary role of a beta-adrenergic receptor/cyclic AMP mechanism. *Mol. Pharmacol.* **47**, 439–49.

Rosen J., Than N. N., Koch D., Poeggeler B., Laatsch H., Hardeland R. (2006) Interactions of melatonin and its metabolites with the ABTS cation radical: extension of the radical scavenger cascade and formation of a novel class of oxidation products, C2-substituted 3-indolinones. *J. Pineal Res.* **41**, 374–81.

Ruijter A. J. M. de, Gennip A. H. van, Caron H. N., Kemp S., Kuilenburg A. B. P. van (2003) Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem. J.* **370**, 737–49.

Saha R. N., Pahan K. (2006) HATs and HDACs in neurodegeneration: a tale of disconcerted acetylation homeostasis. *Cell Death Differ.* **13**, 539–50.

Sahay A., Scobie K. N., Hill A. S., O'Carroll C. M., Kheirbek M. A., Burghardt N. S., Fenton A. A., Dranovsky A., Hen R. (2011) Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. *Nature* **472**, 466–470.

Sakamoto K., Oishi K., Shiraishi M., Hamano S., Otsuka H., Miyake Y., Ishida N. (2000)

Two circadian oscillatory mechanisms in the mammalian retina. *Neuroreport* **11**, 3995–7.

Savaskan E., Ayoub M. A., Ravid R., Angeloni D., Fraschini F., Meier F., Eckert A., Müller-Spahn F., Jockers R. (2005) Reduced hippocampal MT2 melatonin receptor expression in Alzheimer's disease. *J. Pineal Res.* **38**, 10–6.

Schank J. R., Liles L. C., Weinshenker D. (2005) Reduced anticonvulsant efficacy of valproic acid in dopamine beta-hydroxylase knockout mice. *Epilepsy Res.* **65**, 23–31.

Schepelmann M., Molcan L., Uhrova H., Zeman M., Ellinger I. (2011) The Presence and Localization of Melatonin Receptors in the Rat Aorta. *Cell. Mol. Neurobiol.* **31**, 1257–1265.

Schomerus C., Korf H.-W. (2005) Mechanisms regulating melatonin synthesis in the mammalian pineal organ. *Ann. N. Y. Acad. Sci.* **1057**, 372–83.

Sengupta A., Baba K., Mazzoni F., Pozdeyev N. V., Strettoi E., Iuvone P. M., Tosini G. (2011) Localization of Melatonin Receptor 1 in Mouse Retina and Its Role in the Circadian Regulation of the Electroretinogram and Dopamine Levels. *PLoS One* **6**, e24483.

Sethi S., Adams W., Pollock J., Witt-Enderby P. A. (2008) C-terminal domains within human MT 1 and MT 2 melatonin receptors are involved in internalization processes. *J. Pineal Res.* **45**, 212–218.

Sharma R., McMillan C. R., Tenn C. C., Niles L. P. (2006) Physiological neuroprotection by melatonin in a 6-hydroxydopamine model of Parkinson's disease. *Brain Res.* **1068**, 230–6.

Shastry B. S. (1997) On the functions of lithium: the mood stabilizer. *Bioessays* **19**, 199–200.

Shih A. Y., Imbeault S., Barakauskas V., Erb H., Jiang L., Li P., Murphy T. H. (2005) Induction of the Nrf2-driven antioxidant response confers neuroprotection during mitochondrial stress in vivo. *J. Biol. Chem.* **280**, 22925–36.

Shin E.-J., Chung Y. H., Le H.-L. T., Jeong J. H., Dang D.-K., Nam Y., Wie M. B., et al. (2015) Melatonin attenuates memory impairment induced by Klotho gene deficiency via interactive signaling between MT2 receptor, ERK, and Nrf2-related antioxidant potential. *Int. J. Neuropsychopharmacol.* **18**.

Skene D. J., Arendt J. (2007) Circadian rhythm sleep disorders in the blind and their treatment with melatonin. *Sleep Med.* **8**, 651–5.

Slater S. J., Seiz J. L., Cook A. C., Buzas C. J., Malinowski S. A., Kershner J. L., Stagliano B. A., Stubbs C. D. (2002) Regulation of PKC alpha activity by C1-C2 domain interactions. *J. Biol. Chem.* **277**, 15277–85.

Song Y., Pang C. S., Ayre E. A., Brown G. M., Pang S. F. (1996) Melatonin receptors in the chicken kidney are up-regulated by pinealectomy and linked to adenylate cyclase. *Eur. J. Endocrinol.* **135**, 128–33.

Soumier A., Banasr M., Lortet S., Masmejean F., Bernard N., Kerkerian-Le-Goff L., Gabriel C., Millan M. J., Mocaer E., Daszuta A. (2009) Mechanisms contributing to the phase-dependent regulation of neurogenesis by the novel antidepressant, agomelatine, in the adult rat hippocampus. *Neuropsychopharmacology* **34**, 2390–403.

Srinivasan V., Spence D. W., Pandi-Perumal S. R., Brown G. M., Cardinali D. P. (2011)

Melatonin in Mitochondrial Dysfunction and Related Disorders. *Int. J. Alzheimers. Dis.* **2011**, 1–16.

Stambolic V., Ruel L., Woodgett J. R. (1996) Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. *Curr. Biol.* **6**, 1664–8.

Stehle J. H., Gall C. von, Korf H.-W. (2003) Melatonin: a clock-output, a clock-input. *J. Neuroendocrinol.* **15**, 383–9.

Stewart S. S., Appel S. H. (1988) Trophic factors in neurologic disease. *Annu. Rev. Med.* **39**, 193–201.

Sugden D. (1994) Melatonin: binding site characteristics and biochemical and cellular responses. *Neurochem. Int.* **24**, 147–57.

Sykiotis G. P., Habeos I. G., Samuelson A. V, Bohmann D. (2011) The role of the antioxidant and longevity-promoting Nrf2 pathway in metabolic regulation. *Curr. Opin. Clin. Nutr. Metab. Care* **14**, 41–8.

Tasset I., Agüera E., Olmo-Camacho R., Escribano B., Sánchez-López F., Delgado M. J., Cruz A. H., et al. (2011) Melatonin improves 3-nitropropionic acid induced behavioral alterations and neurotrophic factors levels. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **35**, 1944–9.

Teng H. F., Li P. N., Hou D. R., Liu S. W., Lin C. T., Loo M. R., Kao C. H., Lin K. H., Chen S. L. (2014) Valproic acid enhances Oct4 promoter activity through PI3K/Akt/mTOR pathway activated nuclear receptors. *Mol. Cell. Endocrinol.* **383**, 147–58.

Tripathi D. N., Jena G. B. (2010) Effect of melatonin on the expression of Nrf2 and NF-

kappaB during cyclophosphamide-induced urinary bladder injury in rat. *J. Pineal Res.* **48**, 324–31.

VanDongen A. M., VanErp M. G., Voskuyl R. A. (1986) Valproate reduces excitability by blockage of sodium and potassium conductance. *Epilepsia* **27**, 177–82.

Vandresen-Filho S., Martins W. C., Bertoldo D. B., Mancini G., Bem A. F. De, Tasca C. I. (2015) Cerebral cortex, hippocampus, striatum and cerebellum show differential susceptibility to quinolinic acid-induced oxidative stress. *Neurol. Sci.* **36**, 1449–56.

Vernadakis A. J., Bemis W. E., Bittman E. L. (1998) Localization and partial characterization of melatonin receptors in amphioxus, hagfish, lamprey, and skate. *Gen. Comp. Endocrinol.* **110**, 67–78.

Vigh B., Manzano M. J., Zádori A., Frank C. L., Lukáts A., Röhlich P., Szél A., Dávid C. (2002) Nonvisual photoreceptors of the deep brain, pineal organs and retina. *Histol. Histopathol.* **17**, 555–90.

Vogel C., Marcotte E. M. (2012) Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat. Rev. Genet.* **13**, 227–32.

Voutilainen M. H., Arumäe U., Airavaara M., Saarma M. (2015) Therapeutic potential of the endoplasmic reticulum located and secreted CDNF/MANF family of neurotrophic factors in Parkinson's disease. *FEBS Lett.* **589**, 3739–48.

Voutilainen M. H., Bäck S., Peränen J., Lindholm P., Raasmaja A., Männistö P. T., Saarma M., Tuominen R. K. (2011) Chronic infusion of CDNF prevents 6-OHDA-induced deficits in a rat model of Parkinson's disease. *Exp. Neurol.* **228**, 99–108.

Voutilainen M. H., Bäck S., Pörsti E., Toppinen L., Lindgren L., Lindholm P., Peränen J.,

Saarma M., Tuominen R. K. (2009) Mesencephalic astrocyte-derived neurotrophic factor is neurorestorative in rat model of Parkinson's disease. *J. Neurosci.* **29**, 9651–9.

Vriend J., Reiter R. J. (2015) Melatonin feedback on clock genes: a theory involving the proteasome. *J. Pineal Res.* **58**, 1–11.

Wakefield J. G., Stephens D. J., Tavaré J. M. (2003) A role for glycogen synthase kinase-3 in mitotic spindle dynamics and chromosome alignment. *J. Cell Sci.* **116**, 637–46.

Wang X., Michaelis E. K. (2010) Selective neuronal vulnerability to oxidative stress in the brain. *Front. Aging Neurosci.* **2**, 12.

Wang Z., Ma C., Meng C.-J., Zhu G.-Q., Sun X.-B., Huo L., Zhang J., et al. (2012) Melatonin activates the Nrf2-ARE pathway when it protects against early brain injury in a subarachnoid hemorrhage model. *J. Pineal Res.* **53**, 129–37.

Whittle S. R., Turner A. J. (1978) Effects of the anticonvulsant sodium valproate on gamma-aminobutyrate and aldehyde metabolism in ox brain. *J. Neurochem.* **31**, 1453–9.

Wiechmann A. F., Wirsig-Wiechmann C. R. (1991) Localization and quantification of high-affinity melatonin binding sites in Rana pipiens retina. *J. Pineal Res.* **10**, 174–9.

Wiesenberg I., Missbach M., Carlberg C. (1998) The potential role of the transcription factor RZR/ROR as a mediator of nuclear melatonin signaling. *Restor. Neurol. Neurosci.* **12**, 143–50.

Woodgett J. R. (2001) Judging a protein by more than its name: GSK-3. *Sci. STKE*

2001, re12.

Wu J. B., Shih J. C. (2011) Valproic acid induces monoamine oxidase A via Akt/forkhead box O1 activation. *Mol. Pharmacol.* **80**, 714–23.

Wu X., Chen P. S., Dallas S., Wilson B., Block M. L., Wang C.-C., Kinyamu H., et al. (2008) Histone deacetylase inhibitors up-regulate astrocyte GDNF and BDNF gene transcription and protect dopaminergic neurons. *Int. J. Neuropsychopharmacol.* **11**, 1123–34.

Wu X., Tao Y., Hou J., Meng X., Shi J. (2012) Valproic acid upregulates NKG2D ligand expression through an ERK-dependent mechanism and potentially enhances NK cell-mediated lysis of myeloma. *Neoplasia* **14**, 1178–89.

Xie N., Wang C., Lin Y., Li H., Chen L., Zhang T., Sun Y., Zhang Y., Yin D., Chi Z. (2010) The role of p38 MAPK in valproic acid induced microglia apoptosis. *Neurosci. Lett.* **482**, 51–6.

Yamauchi J., Torii T., Kusakawa S., Sanbe A., Nakamura K., Takashima S., Hamasaki H., Kawaguchi S., Miyamoto Y., Tanoue A. (2010) The mood stabilizer valproic acid improves defective neurite formation caused by Charcot-Marie-Tooth disease-associated mutant Rab7 through the JNK signaling pathway. *J. Neurosci. Res.* **88**, 3189–97.

Yang Y., Jiang S., Yan J., Li Y., Xin Z., Lin Y., Qu Y. (2015) An overview of the molecular mechanisms and novel roles of Nrf2 in neurodegenerative disorders. *Cytokine Growth Factor Rev.* **26**, 47–57.

Yokomori K. (2003) SMC protein complexes and the maintenance of chromosome integrity. *Curr. Top. Microbiol. Immunol.* **274**, 79–112.

Yuan L., Collins A. R., Dai J., Dubocovich M. L., Hill S. M. (2002) MT(1) melatonin receptor overexpression enhances the growth suppressive effect of melatonin in human breast cancer cells. *Mol. Cell. Endocrinol.* **192**, 147–56.

Zee P. C., Attarian H., Videnovic A. (2013) Circadian rhythm abnormalities. *Continuum (Minneap. Minn).* **19**, 132–47.

Zhang L., Wang N., Zhou S., Ye W., Jing G., Zhang M. (2012) Propofol induces proliferation and invasion of gallbladder cancer cells through activation of Nrf2. *J. Exp. Clin. Cancer Res.* **31**, 66.

Zhou Q., Dalgard C. L., Wynder C., Doughty M. L. (2011) Valproic acid inhibits neurosphere formation by adult subventricular cells by a lithium-sensitive mechanism. *Neurosci. Lett.* **500**, 202–6.