EPIGENETIC REGULATION OF THE MELATONIN RECEPTOR

M.Sc. Thesis

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INHIBITION OF DNA METHYLTRANSFERASE INDUCES MELATONIN RECEPTOR EXPRESSION IN C6 GLIOMA CELLS

By

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirements for the Degree Master of Science

McMaster University

McMaster University MASTER OF SCIENCE (2019) Hamilton, Ontario (Medical Sciences)

TITLE: Inhibition of DNA Methyltransferase Induces Melatonin Receptor Expression in C6 Glioma Cells

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PAGES: xi, 77

LAY ABSTRACT

The hormone, melatonin, is involved in maintenance of the sleep cycle, and has many neuroprotective effects, initiated by its binding to specific proteins called receptors. Epigenetic or reversible chemical modifications which alter DNA, without changing its sequence, can alter the levels of these receptors. This process can be modulated by drugs, which can increase levels of the melatonin receptor. In this study, the drug 5-Azacytidine (AZA) was used to cause specific chemical changes to DNA, termed demethylation. This thesis shows for the first time, that AZA causes an increase in melatonin receptors. AZA's ability to cause demethylation was confirmed by observing decreased levels of the protein responsible for DNA methylation, DNA methyltransferase. Melatonin receptors in the brain exhibit changes in disorders such as Alzheimer's and Parkinson's disease. Understanding the mechanisms underlying the regulation of these receptors could provide avenues for enhancing the neuroprotective benefits of melatonin and related drugs.

ABSTRACT

The multiple physiological effects of the indoleamine hormone melatonin, are mediated primarily by its two G protein-coupled MT_1 and MT_2 receptors. Our group has shown an upregulation of melatonin receptors following treatment with histone deacetylase (HDAC) inhibitors, including valproic acid (VPA) and Trichostatin A, in cultured cells and/or in the rat brain. VPA increases histone H3 acetylation at the MT_1 gene promoter region in rat C6 glioma cells, indicating that this epigenetic mechanism underlies its upregulation of MT_1 expression. Since HDAC inhibitors can also alter DNA methylation, the possible involvement of this second major epigenetic mechanism in the regulation of MT_1 expression, was examined. C6 cells were treated with the DNA demethylating agent, azacytidine (AZA, 1 - 25 µM), for 24 or 48 hours. Treatment of C6 cells with AZA caused a significant upregulation of MT_1 mRNA expression, as compared with controls (DMSO 0.05%). Moreover, treatment with AZA (10 or 20 µM) for 24 or 48 hours, suppressed or abolished DNMT1 protein expression, and inhibited DNMT1 mRNA expression, which indicates inhibition of the DNMT1 enzyme activity. A combination of VPA and AZA caused a trend toward additive upregulation of the MT_1 receptor. These results show that DNA demethylation plays a role in the regulation of the MT_1 receptor, consistent with the well-known effects of this epigenetic mechanism on gene transcription. Epigenetic regulation of melatonin receptor expression could provide a novel strategy for modulating the therapeutic effects of this hormone and its clinically relevant agonists, such as agomelatine, and could also provide avenues for enhancing the antioxidant, neuroprotective, oncostatic and other benefits of this hormone and its agonists.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Lennard Niles, for his continued mentorship and support throughout the progress of this degree. His guidance has allowed me grow as a scientist, and I am grateful for this wonderful opportunity to pursue research. I would also like to express my thanks to my committee members, Dr. Ram Mishra and Dr. Derek Lobb for their participation and guidance over the past two years. My gratitude goes to Sumeya Mukhtar for all her support and encouragement through this project and her assistance with RT-qPCR experiments. Thank you to friends and family for your support and continuous expression of interest in my project.

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LIST OF ABBREVIATIONS & SYMBOLS

4P-PDOT	4-phenyl-2 propionamidotetralin
5caC	5-carboxycytosine
5-HT	serotonin
5fC	5-formylcytosine
5mC	5-methylcytosine
AA-NAT	arylalkylamine-N-acetyltransferase
AC	adenylyl cyclase
AD	Alzheimer's disease
Akt	protein kinase B (PKB)
ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
APP	amyloid precursor protein
APS	ammonium persulfate
AZA	azanucleosides 5-azacytidine
BDNF	brain-derived neurotrophic factor
BER	base excision repair
cAMP	cyclic adenosine monophosphate
cDNA	complimentary DNA
CNS	central nervous system
COX-2	cyclooxygenase-2
CREB	cAMP response element binding (protein)
DAC	2'-deoxy-5-azacytidine
DAG	diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
EDTA	ethylenediamine tetraacetic acid
ER-α	estrogen receptor-alpha
ERK	extracellular-signal-regulated kinase
FBS	fetal bovine serum
GPCR	G-protein coupled receptor
GSK-3β	glycogen synthase kinase 3 beta
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDACi	HDAC inhibitors
HIOMT	hydroxyindole-O-methyltransferase
M344	N-Hydroxy-7-(4-dimethylaminobenzoyl)-aminoheptanamide
MAPK	mitogen-activated protein kinase

MeCP	methyl-CpG-binding domain protein
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger RNA
NE	norepinephrine
NETs	neuroendocrine tumors
NMDA	N-methyl-D-aspartate
NP-40	nonylphenyl-polyethylene glycol
NTSR1	neurotensin receptor 1
OD	optical density
p21	a.k.a. cyclin dependent kinase inhibitor 1
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PD	Parkinson's disease
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PS1/PS2	presenilin 1 and 2
Raf	rapidly accelerated fibrosarcoma (serine/threonine-specific kinase)
RARB2	retinoic acid receptor beta 2
RHT	retinohypothalamic tract
RNA	ribonucleic acid
RNAPII	RNA polymerase II
RNS	reactive nitrogen species
ROS	reactive oxygen species
SAHA	suberanilohydroxamic acid
SAM	S-adenosylmethionine
SB	sodium butyrate
SCN	suprachiasmatic nuclei
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSRI	selective serotonin reuptake inhibitor
TBE	tris-borate EDTA
TBST	tris buffered saline/Tween 20
TDG	thymine DNA glycosylase
TEMED	N'N'N'N'-tetramethylethylenediamine
TET	ten-eleven translocation
TNBC	triple-negative breast cancer
TSA	Trichostatin A
VPA	valproic acid

DECLARATION OF ACADEMIC ACHIEVEMENT

My supervisor, Dr. Lennard Niles, designed and supervised the experiments for this project. I assisted in the design of the primers for PCR experiments. I was responsible for conducting all tissue culture, drug preparation, and treatments. I performed all RT-PCR experiments, and with assistance from Sumeya Mukhtar and Mahnoor Shah, RT-qPCR, and immunoblotting. I performed the statistical analyses, with the help of Dr. Lennard Niles, and Sumeya Mukhtar assisted with some of the ΔΔCT analyses.

CHAPTER 1: INTRODUCTION

1.1 Melatonin

1.1.1 Biosynthesis of Melatonin

Melatonin is an indoleamine hormone secreted primarily by the pineal gland, and it is widely known as a regulator of circadian rhythms and the sleep cycle. The synthesis of melatonin is therefore mainly regulated by light-dark cycles associated with daily and seasonal changes (Pevet, 2002). Melatonin exerts multiple neuroregulatory and neuroprotective effects, as it can easily cross the blood-brain barrier to reach its receptors in the central nervous system (CNS) (Ng et al., 2017).

The synthesis of melatonin in mammals is driven by multisynaptic neural pathways in the suprachiasmatic nuclei (SCN) of the hypothalamus, which is the location of the circadian clock (Pevet, 2002). The synthesis of this hormone starts with the amino acid tryptophan, that is converted into 5-hydroxytryptophan by tryptophan hydroxylase. It is then decarboxylated into serotonin (5-HT), from where two enzymatic reactions occur. N-acetylation by the enzyme arylalkylamine-N-acetyltransferase (AA-NAT) to generate N-acetylserotonin, then a methyl group is transferred from 5-adenosylmethionine to the 5-hydroxy group of N-acetylserotonin, catalyzed by the enzyme hydroxyindole-O-methyltransferase (HIOMT), finally yielding melatonin (Pevet, 2002) (see **Figure 1**).

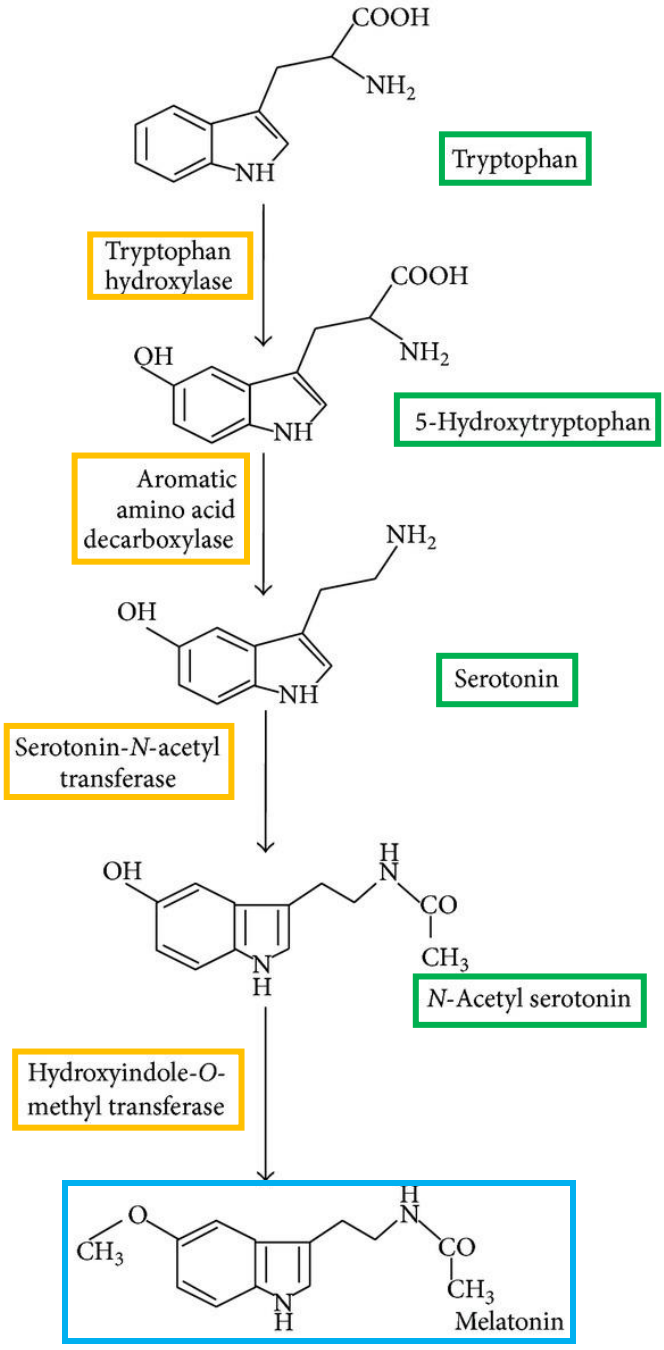

Figure 1. Biosynthesis of melatonin from dietary tryptophan. Adapted from Mas et al. (2014).

1.1.2 Circadian Control of Melatonin Production

The production of melatonin occurs in a daily rhythm, with high levels of production occurring at night. This cycle begins with extrinsic cues to the SCN, specifically the daily light-dark cycle which generates a 24-hour cycle of rhythmic neuronal activity. The retinohypothalamic tract (RHT) projects to the SCN, where retinal ganglion cells at the origin of the RHT contain the photopigment melanopsin, which is specifically sensitive to short-wave blue light. Light information is then transmitted from the RHT to the SCN via the release of excitatory neurotransmitter glutamate, which triggers influx of calcium and intracellular signaling cascades in the SCN (Gooley & Saper, 2017). As mentioned previously, melatonin is secreted from the pineal gland, which is regulated by the circadian clock in the SCN. Specifically, a subpopulation of SCN neurons project to the paraventricular nucleus of the hypothalamus, neurons originating here send excitatory projections to the upper thoracic spinal cord (Gooley & Saper, 2017) (**Figure 2**). This circuit is completed by sympathetic nerve fibers that innervate the pineal parenchyma and release norepinephrine (NE) in a rhythmic fashion, particularly at night (Schomerus, 2005). NE is important because it is the key neurotransmitter for the control of AA-NAT, and therefore melatonin synthesis.

The rhythmic changes in circulating melatonin concentration are due to the changing rhythm of melatonin synthesis from serotonin in the pineal gland. Specifically, the rhythmic activity of the AA-NAT enzyme, wherein there is an increase in enzyme activity at night, therefore increasing the concentration of N-acetylserotonin (Klein, 1997). Exposure to light at night dramatically decreases AA-NAT activity, resulting in a decrease in melatonin. Pineal activation is initiated by NE binding to adrenergic receptors on

pinealocytes. When NE activates β_1-adrenergic receptors it causes an increase in intracellular cAMP levels, followed by activation of protein kinase A (PKA), which is required for activation of AA-NAT (Schomerus, 2005). PKA phosphorylates AA-NAT, leading to an interaction with regulatory proteins, which protect the enzyme from degradation. In primates, pinealocytes constantly generate AA-NAT at night, due to β-adrenergic stimulation by NE (**Figure 2**). However, in the day in the absence of β-adrenergic stimulation, AA-NAT is immediately degraded (Schomerus, 2005). Therefore, AA-NAT activity is the central player in melatonin production because all the regulatory mechanisms converge at this enzyme (Schomerus, 2005).

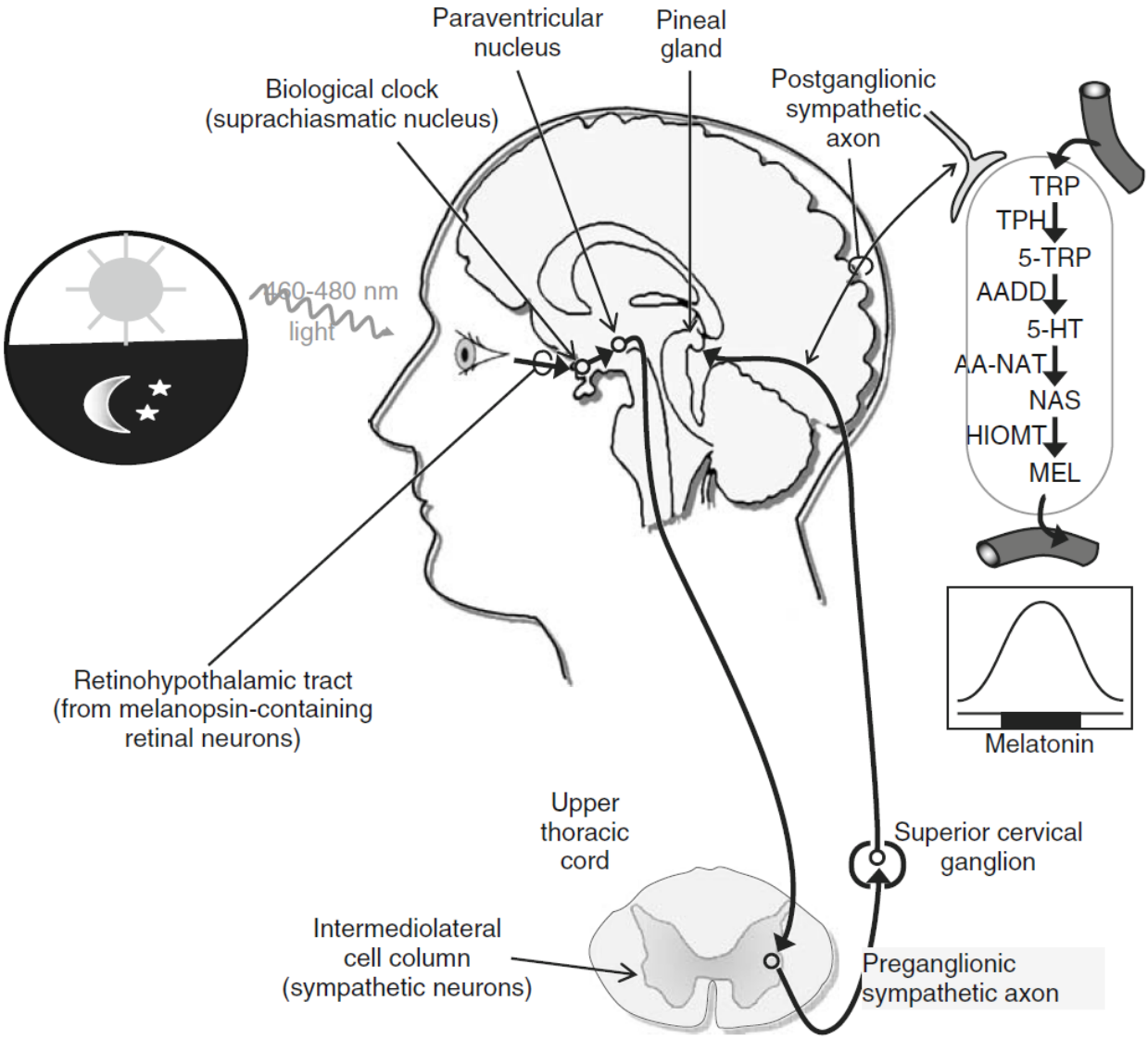

Figure 2. Outline of the retinohypothalamic tract activated or inhibited by light level, leading to production of melatonin in the pineal gland. Adapted from Korkmaz et al. (2009).

1.1.3 Antioxidant Effects of Melatonin

Free radicals are generated during metabolic reactions and are generally derived from oxygen and are therefore termed reactive oxygen species (ROS). These radicals contain at least one unpaired electron, some examples include superoxide, hydroxyl, and peroxyl radical (Phaniendra, 2015). The source of most intracellular ROS is the mitochondria, where superoxide radicals are produced during the electron transport chain, in which an unstable intermediate form of coenzyme Q transfers electrons to molecular oxygen, generating the superoxide anion. Therefore, an increased metabolic rate will lead to an increased production of ROS. The cell has the ability to detoxify superoxide anion via conversion to hydrogen peroxide by mitochondrial superoxide dismutase, and subsequent action of catalase and glutathione peroxidase (Phaniendra, 2015). However, oxidative stress can occur when there is an imbalanced state between the production of ROS, and antioxidant defenses in the cell or tissue (Burton, 2011).

A phenomenon termed selective neuronal vulnerability has been implicated in etiology of several neurodegenerative conditions, in which specific populations of neurons have differential sensitivity to stressful conditions leading to cell injury or death (Wang & Michaelis, 2010). The nervous system may be particularly vulnerable to ROS-mediated injury for several reasons including: high rate of oxidative metabolic activity, high concentration of readily oxidizable membrane lipid polyunsaturated fatty acids, low level of antioxidant enzymes, endogenous generation of ROS in neurochemical reactions such as dopamine oxidation, axonal morphology prone to injury, and non-replicating cells present (Evans, 1993). Specifically, in the CNS excessive production of ROS and reactive nitrogen species (RNS) have been identified as a mechanism of the

pathophysiology of neurodegeneration in several instances, such as insults to neurons in hypoxia, Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (Wang & Michaelis, 2010).

In 1993, melatonin was identified as a potent free radical scavenger and has been shown to detoxify many ROS, including hydrogen peroxide, hydroxyl radicals, peroxyl radicals, RNS, and nitric oxide radicals (Tan, 2002). As mentioned above, melatonin is an indoleamine hormone, and therefore contains an indole heterocycle (an electron-rich moiety with high stability and electroreactivity), which is key to melatonin's radical scavenging ability (Tan, 2002). Specifically, melatonin can scavenge the hydroxyl radical and hydrogen peroxide, generating two products that can be excreted by the urinary system (Reiter, 2002). Oxidative stress is likely a large contributor to neurodegeneration in general and it has been implicated in the loss of dopaminergic neurons in Parkinson's disease. Previous research has found that melatonin is neuroprotective in various *in vivo* models of Parkinson's disease, as it prevents the loss of dopaminergic neurons, via mechanisms which are thought to include its actions as a free radical scavenger (Carriere et al., 2016).

1.2 The Melatonin Receptor

1.2.1. Receptor Structure and Localization

In mammals, melatonin signaling is mediated by two G protein-coupled receptors (GPCRs), consisting of melatonin receptor subtypes MT_1 and MT_2. This was determined using the radioligand 2-[^{125}I]-iodomelatonin for melatonin immunoassays. The radioligand is able to bind to melatonin binding sites with high affinity and label them,

therefore autoradiographic methods were key to identifying the anatomical localization of melatonin binding sites, or melatonin receptors (von Gall, 2002). Subsequently, the binding sites were classified on the basis of high- or low-affinity binding into two groups, ML1 and ML2, respectively. After success in cloning the receptor from *Xenopus* cDNA, it was determined that the high-affinity binding sites contained the classical 7 transmembrane regions consistent with the GPCR superfamily (von Gall, 2002). Homology studies using the *Xenopus* receptor allowed for identification of two receptor subtypes with high affinity, termed Mel1a and Mel1b, and a third was cloned from an avian source (Mel1c), but no mammalian homolog was characterized. Therefore, there are three melatonin receptors in vertebrates, and two in mammals. Since, receptor nomenclature was updated and Mel1a became MT-1, Mel1b became MT-2, and the original low-affinity ML2 was renamed MT-3. However, MT-3 is not a GPCR, it represents a binding site for melatonin present on the quinone reductase enzyme (von Gall, 2002). This thesis deals with the two mammalian melatonin receptors, MT_1 and MT_2, with a focus on the MT_1.

Cloning techniques have identified the melatonin receptors as GPCRs, however, there are motifs present in all previously characterized GPCRs that are not found in the melatonin receptors (Barrett, 2003). For example, the highly conserved D/ERY motif at the interface of transmembrane domain 3 and the second intracellular loop is substituted with an NRY motif, and the sequence CXXCH, in the melatonin receptor (**Figure 3**). Furthermore, a substitution of the commonly found proline residue in transmembrane domain 7, resulting in an NPXXY motif, for an alanine residue was identified, causing an NAXXY motif (Barrett, 2003). Site-directed mutagenesis studies of the MT_1 receptor have

elucidated the function of the NRY motif, interestingly, mutations at this site do not affect binding affinity, but affect the downstream ability to inhibit cAMP. Therefore, this area promotes interactions with G proteins (Barrett, 2003). In addition, mutations to Gly258 in transmembrane domain 6 dramatically decreased binding affinity, therefore this residue is implicated in the formation of the binding pocket (Conway, 2000). These receptors are generally located on neuronal dendrites and somata in various CNS areas, including the cerebral cortex, basal forebrain, hippocampus, hypothalamus and basal ganglia (Lacoste et al., 2015). MT_1 has been identified in several peripheral tissues including the cardiovascular system, gastrointestinal tract, immune system and kidney (Ng et al., 2017), which can be applied to its many downstream signaling functions which will be discussed later.

MT_1 and MT_2 have high potential to homo- and heterodimerize, more specifically, MT_1/MT_2 heterodimers and MT_1 homodimers occur at relatively the same rate, whereas MT_2 homodimer formation is up to 4-fold lower. This indicates that MT_2 favorably occurs in the heterodimeric complex with MT_1 (Baba, 2013). It was also determined that the formation of heterodimers is crucial for the effects of melatonin on rod photoreceptor light sensitivity (Baba, 2013). As mentioned previously, melatonin is derived from dietary tryptophan, during which serotonin is produced as an intermediate. This link between serotonin and melatonin is strengthened by the discovery that crosstalk between these two hormones is mediated by heterodimers of MT_1 or MT_2 and the 5-HT_{2C} receptors (Kamal, 2015).

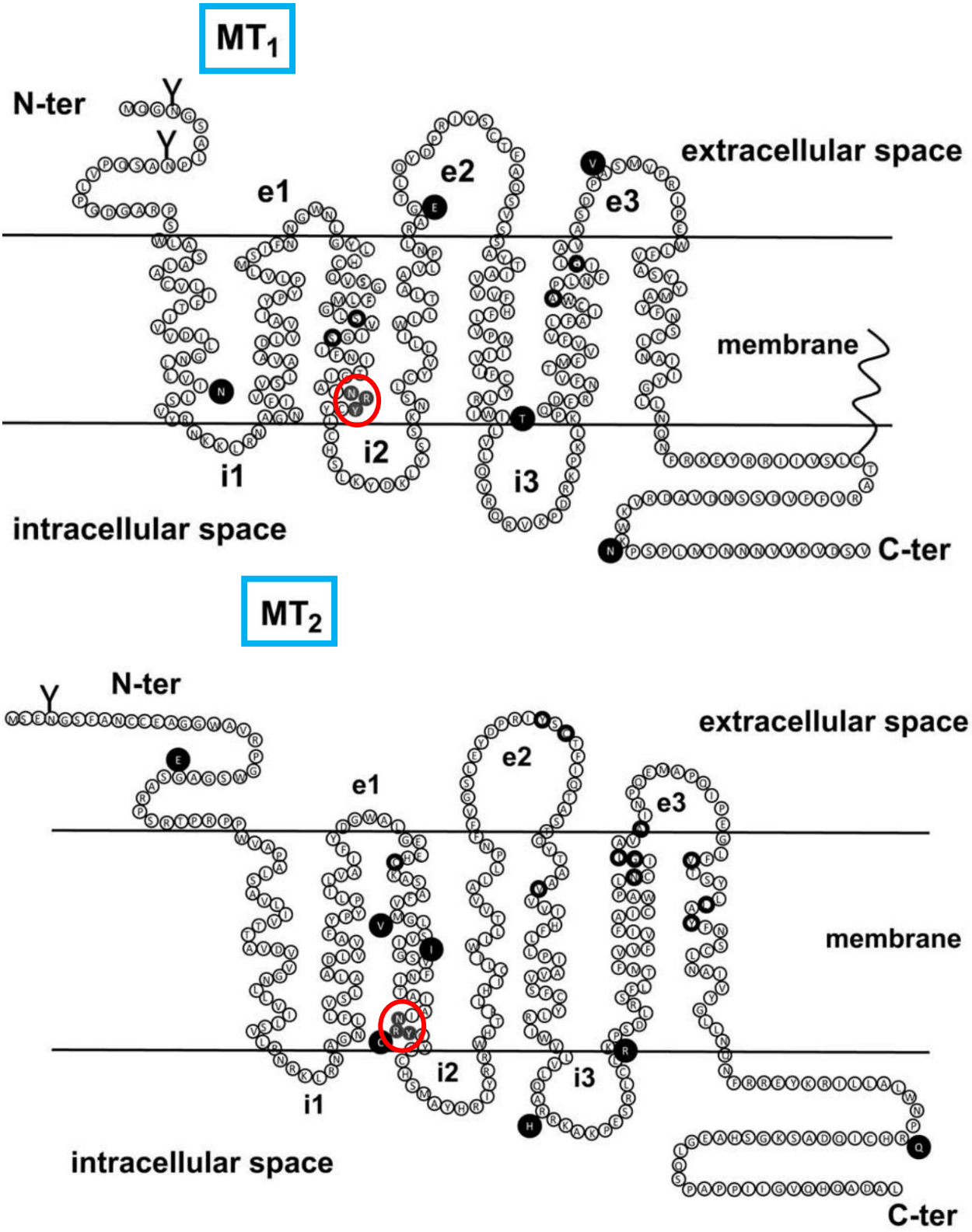

Figure 3. MT_1 and MT_2 amino acid sequences. Residues involved in melatonin binding are circled in black, NRY motif circled in red. Modified from Chaste et al. (2010).

1.2.2. Melatonin Receptor Agonists

There are several melatonin receptor agonists that were developed during the time of radioligand characterization of the melatonin receptors (**Figure 4**). Specifically, generation of naphthalene derivatives of melatonin, gave rise to the potent agonist agomelatine, which has high affinity for melatonin receptors and is able to suppress cAMP generation (de Bodinat, 2010). Several *in vivo* studies showed that agomelatine had the ability to reinstate circadian rhythmicity. However, pinealectomy in rodents does not affect the influence of agomelatine on circadian rhythms, which suggests that agomelatine acts in the suprachiasmatic nuclei, not the pineal gland (Redman, 1998). It was discovered that agomelatine interacts with 5-HT_{2C} receptors, and agomelatine competitively antagonizes the downstream activation of G proteins by serotonin, such as $G\alpha_i$ activation of phospholipase C (PLC), activation of protein kinase C (PKC), and downstream protein phosphorylation (de Bodinat, 2010). In addition, PD-6735 (TIK-301) is a MT_1/MT_2 agonist that antagonizes 5-HT_{2C} and 5-HT_{2B} more powerfully than agomelatine (Landolt & Wehrle, 2009). The implications of agomelatine and/or PD-6735 activity on 5-HT receptors and depression, which is of particular interest due to the apparent heterodimerization of melatonin and 5-HT_{2C} receptors (Kamal, 2015), will be discussed later. Melatonin analogues are readily available on the market for treatment of sleep disorders such as insomnia and jet lag. One of which is Ramelteon, a non-selective MT_1/MT_2 agonist, which has a longer half-life and higher binding affinity than endogenous melatonin (Kato, 2005). Another non-selective agonist is Tasimelteon, which was recently approved for treatment of non-24-h sleep-wake disorder in patients with total blindness (Zlotos, 2013).

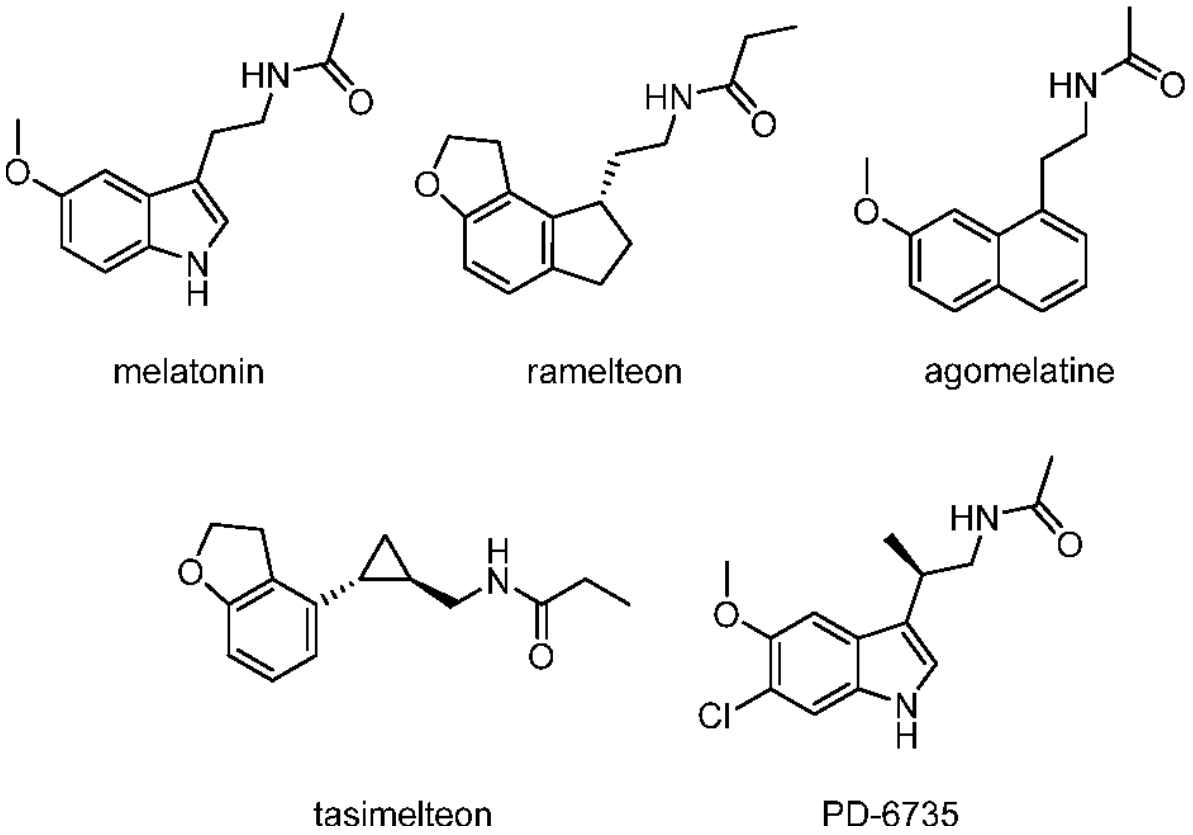

Figure 4. Chemical structures of melatonin and select melatonergic agonists. Adapted from Pala et al. (2013).

1.3 Melatonin Signaling Pathways

Previous studies have elucidated the downstream signalling pathways modulated by the melatonin receptors. Firstly, both receptors are linked to pertussis toxin-sensitive guanine nucleotide-binding proteins (G proteins), specifically interacting with $G\alpha_{i2}$, $G\alpha_{i3}$, that mediate inhibition of the adenylyl cyclase-cAMP pathway, leading to a decrease in both PKA activity and phosphorylation of cAMP response element binding protein (CREB) (Ng et al., 2017). The coupling of MT_1 to $G\alpha_{q/11}$ protein stimulates the mobilization of calcium from intracellular stores via PLC, leading to downstream activation of PKC, which may be a cell-specific function of this specific G-protein coupling (Brydon, 1999).

Interestingly, MT_1/MT_2 heterodimers amplify the $G\alpha_q$ pathway significantly greater than cells expressing MT_1 in isolation, and MT_2 was not involved in this pathway (Jockers, 2008). This indicates that MT_2 could be involved in the positive allosteric regulation of MT_1, which could explain the amplification of signalling through the heterodimer.

Other research has shown that melatonin improves cell survival and exerts neuroprotective effects via the activation of the Raf-MAPK/ERK signaling pathway (Koh, 2008). Furthermore, melatonin is known to activate the PI3K/Akt signalling pathway via phosphorylation of Akt, which also plays a key role in controlling cell growth, proliferation and survival (Pan & Niles, 2015). As the transcription of many genes is regulated by the cAMP/CREB pathway, the transcription factor CREB has been suggested as a potential regulator of melatonin receptor expression (Barrett, 1996). Some of the central pathways involved in melatonin signalling via its receptor monomers or heterodimers, which also couple to $G\alpha_i$/cAMP, are shown in **Figure 5**.

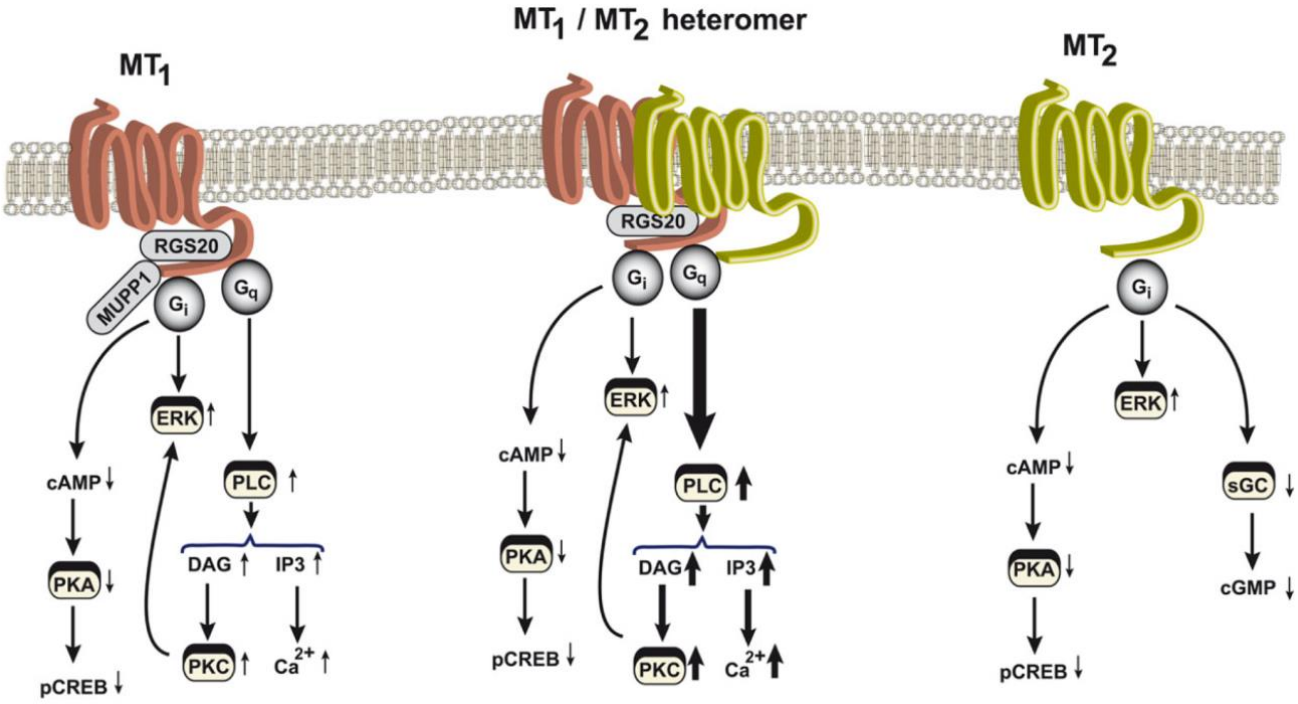

Figure 5. Signaling pathways downstream of MT_1 and MT_2, which include inhibition of cAMP and increased ERK through $G\alpha_i$ signaling, and heterodimers which primarily signal through the $G\alpha_q$ pathway to modulate PLC. Adapted from Tosini et al. (2014).

1.4 Melatonin Signaling in Neurodegenerative Diseases

Disruptions in circadian rhythmicity and sleep are often observed in neurological disorders, therefore implicating melatonin, and its downstream signaling pathways, in the etiology of these disorders (Pevet, 2002). Furthermore, serum levels and circadian amplitude of blood melatonin concentrations decrease with age *in vivo* (Pang, 1990; Lahiri, 2004). It was found that the expression of melatonin receptors MT_1 and MT_2 decrease with age in rats, indicating that the reduction in response to melatonin with age could be due to a decline in melatonin receptors (Sanchez-Hidalgo, 2009). Therefore, a decrease in signaling through the melatonin receptors, and the loss of free-radical

scavenging due to a decrease in the hormone itself, can both contribute to the development of age-related neurodegenerative diseases.

1.4.1 Amyotrophic lateral sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS) is characterized by progressive loss of motor neurons, resulting in gradual paralysis and ultimately death. The death of these motor neurons is associated with the activation of caspase-mediated cell death pathways, in which cytochrome *c* is released from the mitochondria into the cytoplasm, leading to downstream caspase activation, and eventually triggering apoptosis (Wang et al., 2008). Specifically, damage from ROS can initiate cytochrome *c* release (Friedlander, 2003). Furthermore, the involvement of apoptosis-related pathways is implicated with disease progression. A screen of over 1000 cytochrome *c* inhibitors identified melatonin as an anti-apoptotic agent, as it is one of the most effective agents at inhibiting cytochrome *c* release, and therefore preventing neuronal death via inhibition of apoptosis (Wang et al., 2008). It was found that melatonin delays disease progression and inhibits motor-neuron loss in an ALS mouse model. Additionally, MT_1 expression levels were significantly reduced in the spinal cord of ALS patients, and ALS mice (Zhang, 2013). Therefore, MT_1 and its downstream signaling may be a promising target for the treatment of ALS.

1.4.2 Alzheimer's Disease (AD)

Alzheimer's disease (AD) is an age-related neurodegenerative disease, described by progressive loss of cognition and memory. Some hallmarks of the disease include generation of extracellular senile plaques, specifically comprising β-amyloid and intracellular neurofibrillary tangles (Lin, 2013). Many mechanisms have been proposed

to explain the etiology of AD; however, the cause is largely still unknown. β-amyloid peptide is derived from the membrane-bound amyloid precursor protein (APP), and mutations in APP, and the presenilin 1 and 2 (PS1/PS2) genes are associated with familial early onset AD (Dragicevic, 2011). The loss of diurnal circadian rhythmicity and decreased levels of melatonin in the serum and cerebrospinal fluid are age-related and are pronounced in AD patients, and melatonin levels continue to decrease with disease progression (Lin, 2013). Furthermore, the levels of the MT_1 and MT_2 receptors are decreased in the pineal and occipital cortex of AD patients (Brunner, 2006). As previously discussed, mitochondrial dysfunction and apoptosis are involved in etiology of ALS, and this has also been observed in the brains of AD patients, observed using post-mortem samples from the hippocampus and primary motor cortex (Bosetti, 2002). In AD, the mitochondrial dysfunction is characterized by increased ROS generation, and decreased cytochrome *c* oxidase activity. This was determined using mitochondria isolated from AD or control brain samples versus platelets, and enzymatic activity of cytochrome *c* oxidase activity was assessed spectrophotometrically following oxidation of reduced cytochrome *c* (Bosetti, 2002). Additionally, experiments using the aged APP/PS1 transgenic mouse model of AD, and in a neuroblastoma cell line (N2a) transfected with APP, found that treatment with melatonin reversed mitochondrial dysfunction in the cortex, hippocampus and striatum, assessed by mitochondrial respiratory function and membrane potential, and ROS generation (Dragicevic, 2011). This effect was dependent on melatonin signaling, as administration of a non-specific melatonin receptor antagonist (luzindole) blocked the restorative effect. This effect appears to be more dependent on MT_2, as when a specific MT_2 inhibitor was used (4-phenyl-2 propionamidotetralin; 4P-PDOT), it

blocked the protective effects of melatonin to a higher degree than luzindole (Dragicevic, 2011). Therefore, melatonin and signaling through its receptors appears to be protective against onset or progression of AD via inhibition of mitochondrial dysfunction.

1.4.3 Parkinson's Disease (PD)

There is similar evidence for implications of melatonin, and melatonin receptors in Parkinson's disease (PD), which is a multisystem neurological disorder with a diverse clinical phenotype. It is characterized by steadily progressing neuronal dysfunction and cell death, leading to loss of dopaminergic neurons, and therefore depletion of the neurotransmitter dopamine in the striatum (Aldi, 2010). The striatum is the central hub of the basal ganglia, which is responsible for the initiation and control of movements, directly correlating to the observable motor symptoms associated with PD. The exact mechanism for neuronal loss is unknown, but is thought to be caused by apoptosis, aligning with the theories surrounding the neurodegenerative diseases previously discussed. Therefore, oxidative stress appears to be involved in the initiation and progression of PD.

Melatonin has been previously shown to be involved in the modulation of dopamine release and receptor activation (Sheih, 1997; Hamdi, 1998). Several lines of experimental evidence show that administration of melatonin in rodent models of PD has neuroprotective effects. Specifically, in rats given intrastriatal rotenone lesions, melatonin prevented dopamine depletion in the striatum and shielded against loss of dopaminergic neurons as observed via maintenance of tyrosine-hydroxylase immunoreactivity (Bassani, 2014; Carriere, 2016). Similarly, in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) rodent model of PD, melatonin had the same effect and reduced hypolocomotion (Capitelli, 2008). Development of Lewy Bodies, which are

composed of the protein α-synuclein, is associated with pathological lesioning of dopaminergic neurons in the substantia nigra and other areas in PD. It was shown that administration of melatonin inhibited formation of α-synuclein assembly, a preliminary step in Lewy Body formation. Melatonin also inhibited α-synuclein-induced cytotoxicity (Ono, 2012). Furthermore, it was determined that the MT_1 and MT_2 receptor expression was significantly decreased in PD patients versus healthy controls in the substantia nigra and amygdala (Aldi, 2010). Therefore, the depletion of the melatonin receptors in the brain of PD patients, and similarly AD patients, may be involved in the pathogenesis of these neurodegenerative diseases. Therefore, the neuroprotective effects of melatonin are mediated by its two receptors, and these effects could be exploited and targeted as a potential treatment avenue.

1.5 Melatonin Signaling in Other Diseases

1.5.1. Depression

There are many strong links between circadian disturbance and symptoms of clinical depression. These can include delayed sleep onset, non-restful sleep, daytime fatigue, and others. Commonly administered antidepressants generally do not treat any sleep disturbances or seasonal rhythmicity dysfunctions associated with depression (Liu, 2016). The link between melatonin and depression originated from rodent studies which identified anti-depressant effects of melatonin in conditions of learned helplessness and during the forced swim test (Liu, 2016). Furthermore, mice with deletion of MT_1 receptor showed higher depressive-like symptoms. Interestingly, there is upregulation of MT_1 in patients with major depressive disorder, implicating the receptor as a potential target for treatment (Liu, 2016).

Neurotrophins are a class of proteins that support growth and survival of neurons in the CNS. Specifically, brain-derived neurotrophic factor (BDNF) is involved in the maintenance of dopaminergic neurons, and studies indicate that patients with depressive disorder have significant reduction in levels of BDNF (Yoshida, 2012). It has been shown in *in vivo* and human studies in depressed patients that treatment with agomelatine increases serum BDNF levels, and subsequently improves depressive symptoms (Martinotti, 2016). Moreover, *in vivo*, this effect appears to be caused by synergistic activity of the MT_1/MT_2 agonism and 5-HT_{2C} receptor antagonism by agomelatine, as melatonin itself, and a selective 5-HT_{2C} antagonist did not induce neurotrophic factor expression (Molteni, 2010). This is supported by findings of melatonin and serotonin receptor heterodimerization as shown by Kamal et al. (2015). Moreover, there are several lines of experimental evidence that agomelatine has antidepressant effects and is effective in treating sleep disorders. Ongoing non-inferiority studies to demonstrate the antidepressant effects of agomelatine compared to standard treatment (SSRIs) indicate that agomelatine is effective and better tolerated, it is promising as it improves mood, neurochemical imbalance and anxiety, while also inducing the known positive effects on circadian disruption (Liu, 2016). Therefore, future clinical trials guided by the knowledge generated about the MT_1/MT_2 receptors, and their interactions with serotonergic or other mood-regulating systems, could lead to improved treatment of depressive disorders.

1.5.2. Breast Cancer

In the 1960's some research focused around light effects on mammary tumorigenesis in rodents. Additionally, in the early 1980's researchers determined the ability of melatonin to hinder mammary carcinogenesis and investigated the impact of a

constant light environment on mammary tissue development. The effects of constant light on mammary tumor development was highly similar to that of the tumor-promoting results of pinealectomies, and it was thought that perhaps light-induced suppression of melatonin could be a key player in enhancing mammary carcinogenesis (Stevens, 2014). Since then, several experiments utilizing human breast cancer xenografts, have observed a dose-dependent suppression of blood melatonin levels by fluorescent lighting at night, which was directly related to increased tumor growth rate and size (Blask, 2003). This was even shown when blood from women under three conditions of light was administered to human xenografts in nude rats, where blood taken in darkness, rich with melatonin, was tumor suppressive. Alternatively, blood taken from the same women exposed to light at night (low melatonin) did not inhibit tumor growth (Blask, 2005). In the mid-2000s several epidemiological studies found results indicating a carcinogenic effect of circadian disruption in humans occurring during shift work, and an increased risk of breast cancer among women working night shifts (Truong, 2014).

Human breast cancers can be classified generally depending on expression of the estrogen receptor-alpha (ER-α), and breast cancer cells expressing the ER-α receptor are less aggressive and often responsive to treatment (Yuan, 2002). The ER-α receptor is also sensitive to melatonin, and previous research has indicated that physiological concentrations of melatonin directly inhibit ER-α-containing breast cancer cells *in vitro*. Furthermore, researchers have demonstrated that MT_1-overexpression in MCF-7 breast cancer cells (ER-α positive) have significantly enhanced growth-suppression, and this response was inhibited using an MT_1/MT_2 antagonist (Yuan, 2002). In addition, cells overexpressing MT_1, subsequently implanted into nude mice had 60% mammary tumor

reduction, and tumor incidence in response to exogenous melatonin treatment was decreased by 80% (Collins, 2003). These results indicate that the anti-tumor effects of melatonin in breast cancer involve the MT_1 receptor. The MT_1 receptor can also act as a biomarker in certain breast cancers. For instance, expression of the MT_1 correlated with ER-α expression, and it is an independent prognostic marker for overall survival and event-free survival in ER-α-positive breast tumors (Jablonska et al., 2013). Additionally, it was found that there was a higher incidence of MT_1-negative tumors in African American women, compared to Caucasian women in a cohort of triple-negative breast cancer (TNBC); and MT_1 expression is associated with lower cancer stage, and smaller tumor size, where MT_1 negativity correlated to higher risk of disease progression, shorter progression free survival and disease-related death (Oprea-Ilies et al., 2013). Therefore, the melatonin receptor is a key player in protection from development and progression of various breast cancer pathologies.

1.6 Epigenetic Regulation of Gene Expression

The concept of epigenetics refers to covalent chemical modifications made to the DNA, or histone proteins, that do not change the DNA sequence itself. Furthermore, epigenetics is concerned with the mechanisms by which these chemical changes influence the chromatin structure, and subsequently gene expression. DNA is tightly wound around four core histones (H2A, H2B, H3 and H4), forming the nucleosome. Chromatin exists in two states, heterochromatin or euchromatin. Heterochromatin corresponds to silenced regions of DNA and euchromatin describes active regions undergoing transcription. Covalent chemical changes to the histones and DNA itself can remodel the chromatin between the two different states. There are various epigenetic

mechanisms by which gene expression can be altered via chromatin remodelling. More specifically, these mechanisms include DNA methylation and histone modification, which will be discussed. Euchromatin is identified by increased levels of histone acetylation, catalyzed by histone acetyltransferase (HAT) enzymes, resulting in a “loose” chromatin structure allowing for transcription factors and other proteins to reach the DNA. The alternative process of deacetylation is modulated by histone deacetylase (HDAC) enzymes that result in a closed chromatin structure and halting of transcription (Bannister & Kouzarides, 2011). There are four classes of HDAC enzymes: I, II, III, and IV. In general, Classes I and II are involved with cellular growth and development, Class III is involved with metabolic processes, and Class IV HDACs are relatively novel and lack research (Heerboth, 2014).

DNA methylation is one of the best understood epigenetic mechanisms and is a marker for silenced genes. It involves the addition of a methyl group to carbon 5 of cytosine, which is catalyzed by DNA methyltransferase (DNMT) enzymes. There are three isoforms of DNMT: DNMT1 maintains DNA methylation by methylating the complementary strand in hemi-methylated DNA before its replication, whereas DNMT3A and DNMT3B are involved in the *de novo* methylation of DNA (Bayraktar and Kreutz, 2017). The source of methyl groups is derived from dietary methionine, which is converted to the methyl cofactor S-adenosylmethionine (SAM), the primary biochemical methyl donor (see **Figure 6**) (Ulrey, 2005). DNMT1 contains several N-terminal domains, importantly the Replication Foci Targeting Sequence domain, the CxxC zinc binding domain which recognizes non-methylated regions, the proliferating cell nuclear antigen (PCNA) binding domain which allows recruitment to the DNA replication fork, and the

Nuclear Localization Signal (Long, 2013; Jeltsch, 2016). The C-terminal domain of DNMT1 is the catalytic methyl transferase domain, also containing a motif that binds SAM, which is brought into proximity to cytosine by DNMT1. The enzyme has a concave binding pocket for DNA, in which a cytosine base is extracted, and the methyltransferase catalyzes the addition of the methyl group from SAM to cytosine (Dhe-Paganon, 2011). DNA methylation most often occurs at CpG dinucleotides, or concentrated regions of methylation known as CpG islands. Methylation in these areas recruits methyl-CpG-binding domain proteins (MeCP), one of which, MeCP2, is abundantly expressed in the CNS. MeCP can silence transcription by recruiting HDAC, therefore inhibiting the arrival of transcription factors and RNA polymerase (Mellen et al., 2012).

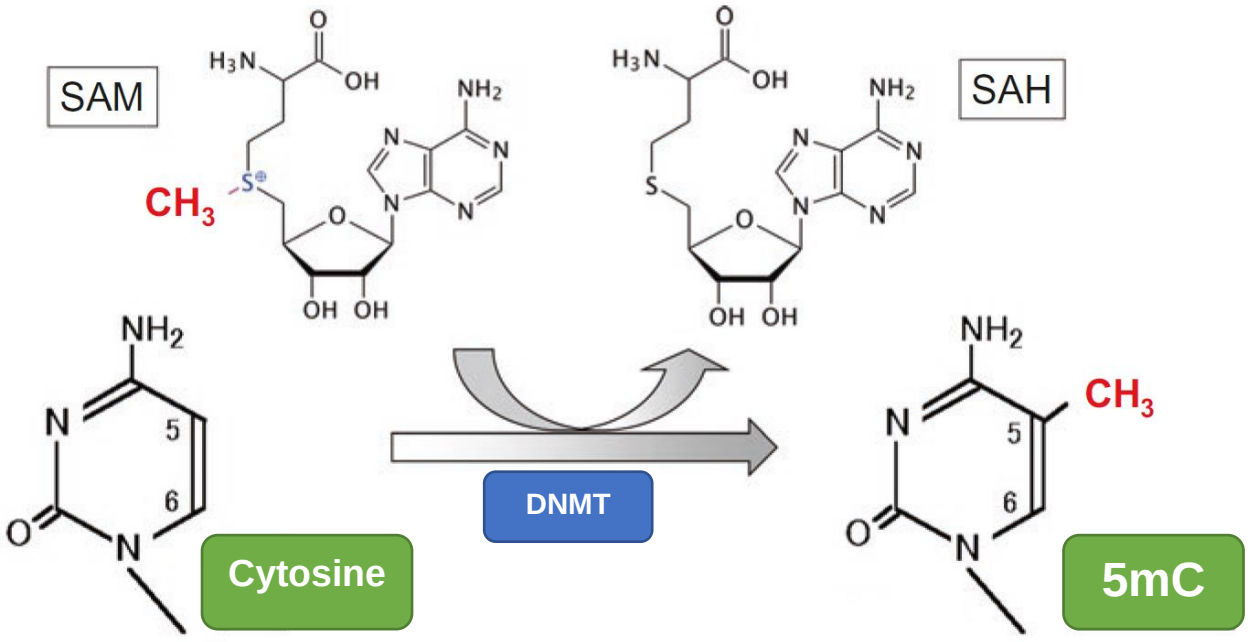

Figure 6. The transfer of methyl group from SAM to cytosine, catalyzed by DNMT. Modified from Suetake et al. (2017).

1.7 Modulation of DNA Methylation Status

As previously mentioned, DNA methylation plays a role in many biological processes through the regulation of gene expression. For many years the reverse process of DNA demethylation has been poorly understood. It was thought that DNA methylation was removed through passive processes involving DNA replication-dependent dilution of the methylation in the absence of DNMT1 (Yin & Xu, 2016). Due to the mounting evidence that the passive-demethylation model is not enough to explain the rapid loss of methylation in various stages of development, for example, active methods of demethylation were proposed (Sajadian et al., 2015). In recent years, the ten-eleven translocation (TET) proteins have been identified as potential demethylating enzymes as they have been shown to catalyze the oxidation of 5-methylcytosine (5mC) (Yin & Xu, 2016). It has since been suggested that DNA methylation is reversible, either through passive demethylation via DNA replication, or by active demethylation through base excision repair of modified nucleotides. This active demethylation pathway is mediated by the TET enzymes (TET1, 2, 3), as they can convert 5mC into 5-hydroxymethylcytosine (5hmC) and can further oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxycytosine (5caC) (Melamet, 2018). These final modified cytosines are quickly excised through thymine DNA glycosylase (TDG) and replaced by unmodified bases via base excision repair (**Figure 7**) (Melamet, 2018).

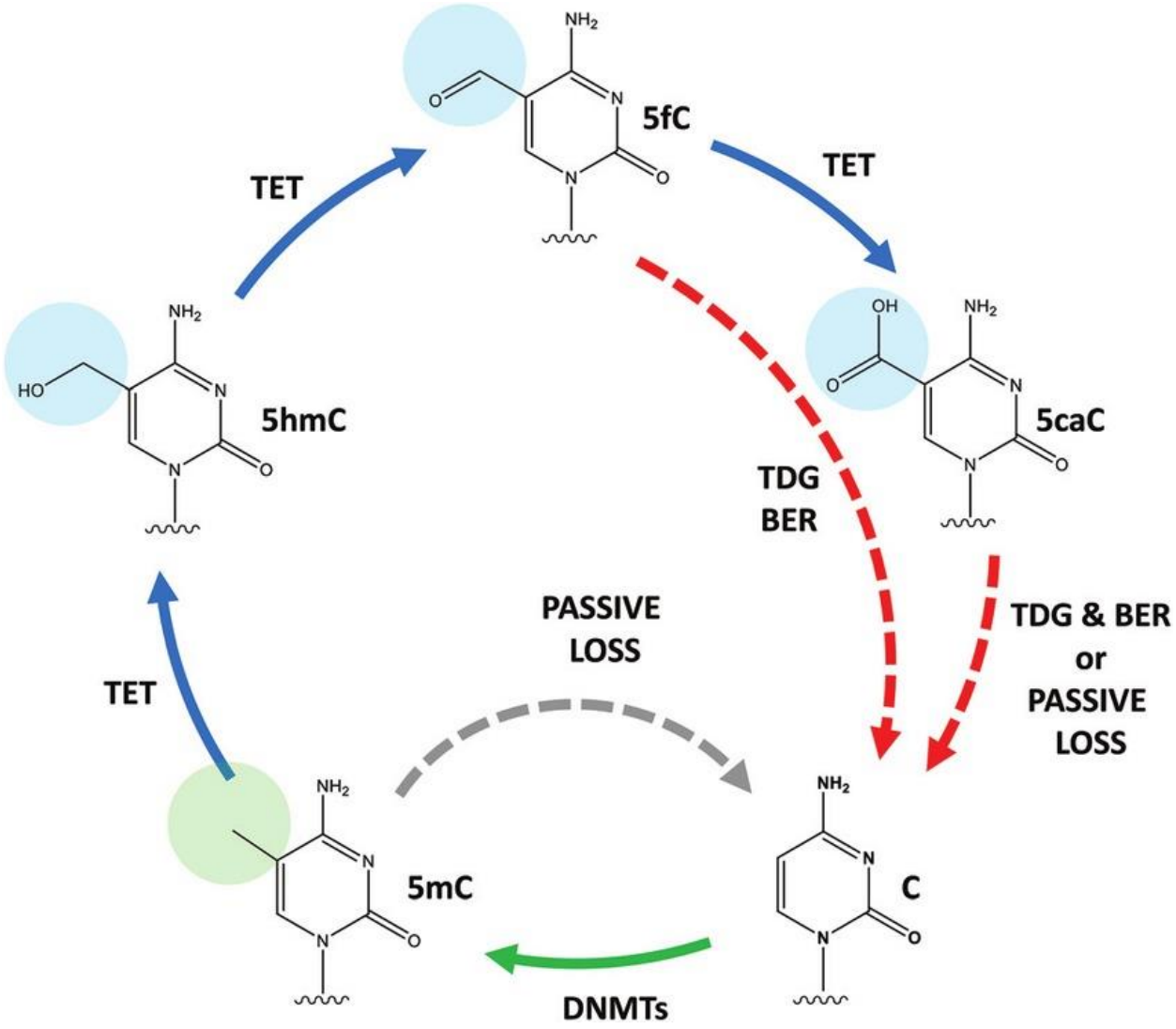

Figure 7. DNA methylation by DNMT, 5-methylcytosine oxidation via TET enzymes. Modified from Ravichandran (2017).

1.8 Epigenetic Drugs (Epi-Drugs)

1.8.1. Histone Deacetylase (HDAC) Inhibition

Regulation of epigenetic modifications of the genome is crucial to maintaining appropriate cell growth and gene expression. Dysregulation of these epigenetic patterns can therefore lead to the propagation of disease through inappropriate gene expression. As discussed above, the histone acetylation status is mediated by the balance of two enzymes, HAT and HDAC, which plays a crucial role in regulating gene expression. Therefore, HDACs represent a large target for treating diseases originating from abnormal gene expression such as inflammatory disorders, metabolic disorders such as diabetes, autoimmune disorders, and cancer (Nebbioso, 2012). As deacetylation of histones corresponds with reduced gene expression, inhibiting this process would lead to open chromatin conformation and increased gene expression. The generation of compounds that target this process, HDAC inhibitors (HDACi), affect multiple cellular processes, and several have been approved by the FDA for treatment of select diseases.

HDACi can be classified into categories based on their chemical structure, these structurally diverse groups include: hydroxamic acids, benzamides, and short-chain fatty acids (Nebbioso, 2012). The hydroxamic acids include inhibitors that target Class I and II HDACs. More precisely, the first natural hydroxamate identified to inhibit HDAC, Trichostatin A (TSA), is a pan-HDAC inhibitor (Kim & Bae, 2011). Furthermore, suberanilohydroxamic acid (SAHA, Vorinostat) is a structurally similar compound that inhibits HDACs and cell proliferation, it has been FDA approved for treatment of difficult cases of cutaneous T-cell lymphoma (Heerboth, 2014). The benzamides inhibit HDAC in the low micromolar range. Specifically, the amide analogs of TSA, such as M344 (N-

Hydroxy-7-(4-dimethylaminobenzoyl)-aminoheptanamide), have significant anti-cancer effects and are also potent HDACi (Jung, 1999; Li & Chen, 2009). Valproic acid (VPA) is a branched short-chain fatty acid, which is used clinically as an anticonvulsant and mood stabilizer (Monti et al., 2009). It exerts this function through reduction in neuron excitability via increased GABAergic activity, and inhibition of NMDA receptor-mediated glutaminergic excitatory activity. Similar to lithium, VPA exerts several functions on kinase pathways, including activation of pro-survival kinases PKB/Akt, the MAPKs, PI3K, and inhibition of GSK-3β, which support the neuroprotective effects of the drug. However, in the early 2000's it was discovered to be an effective HDAC inhibitor. VPA is able to inhibit the catalytic activity of Class I HDACs, and also induce proteasomal degradation of HDAC2 (Monti et al., 2009). Another short-chain fatty acid HDACi is butyrate, which is derived from fermentation of dietary fibres in the large intestine (Shankar & Srivastava, 2008). Derivatives of this compound, such as sodium butyrate (SB) have been used historically as anti-cancer drugs as these drugs can inhibit cell proliferation and DNA synthesis, however SB is also an inhibitor of Class I and II HDACs (Davie, 2003). Another butyrate derivative, phenylbutyrate, originated as an ammonia scavenger in urinary disorders, among this activity phenylbutyrate is also a pan-HDAC inhibitor, inhibits cell proliferation and a pro-apoptotic (Kusaczuk, 2016). The structural diagrams of each of the aforementioned HDACi compounds are depicted in **Figure 8**.

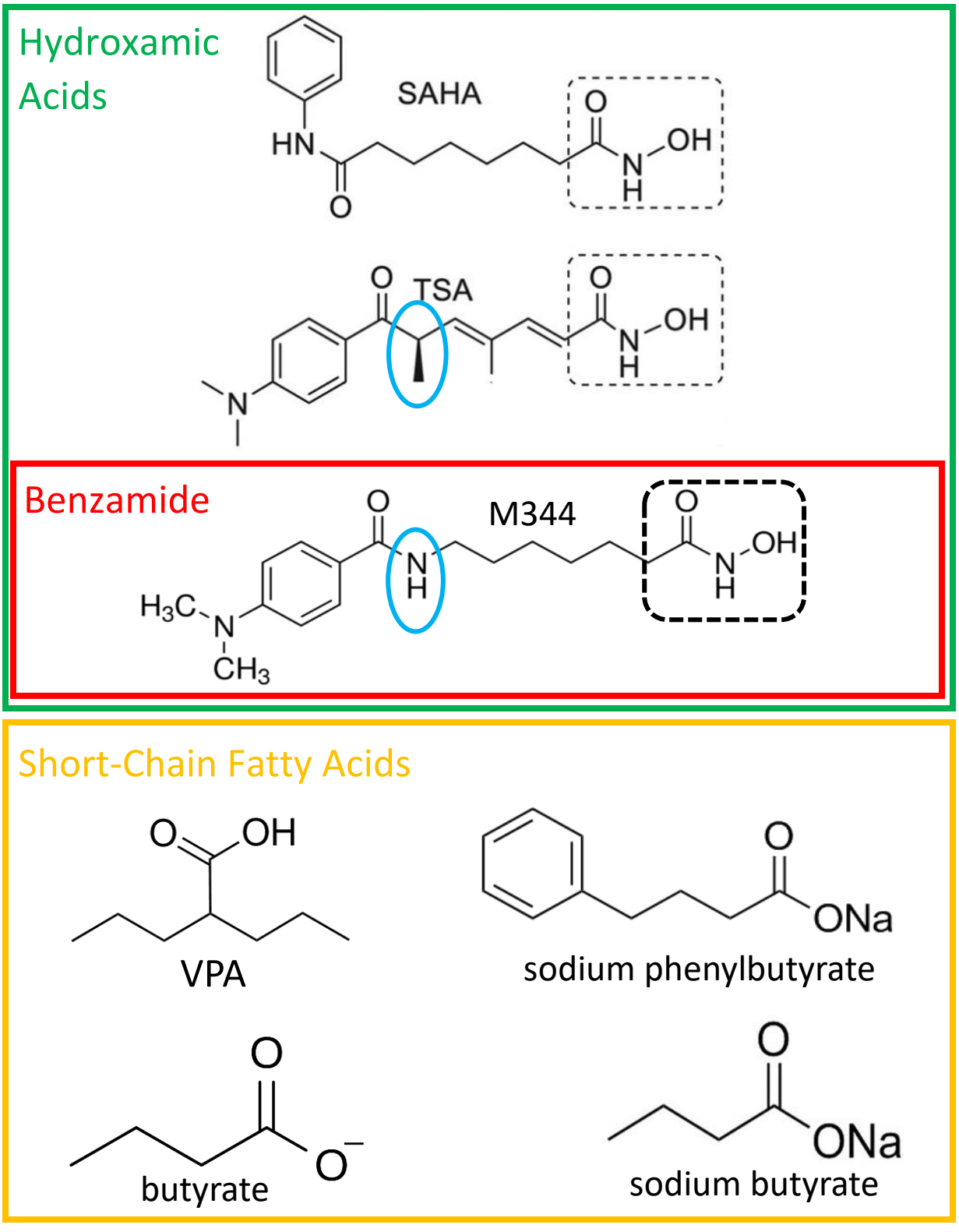

Figure 8. HDAC inhibitor compounds of interest, by type. The dotted lines represent the zinc-chelating domain of the compounds, crucial for HDACi function of these drugs. The blue circle highlights the amide group substituted in M344, as compared to TSA.

1.8.2. DNA Methyltransferase (DNMT) Inhibition: Azanucleosides

The azanucleosides 5-azacytidine (AZA) and 2'-deoxy-5-azacytidine (DAC) are cytosine analogs. They were originally developed as cytostatic agents and were later found to inhibit DNA methylation (Stresemann & Lyko, 2008). This observation sparked research into the azanucleosides as epigenetic drugs, which are currently FDA approved for treatment of specific cancers. Two transporter families are responsible for the uptake of natural nucleosides and their analogs, concentrative nucleoside transporter, and equilibrative nucleoside transporters (Pastor-Anglada, 2004). Once inside the cell, azanucleosides are metabolized into 5-aza-2'-deoxycytidine-5'-triphosphate. They then become substrates for the DNA replication machinery and are readily incorporated into DNA as a substitute for cytosine (Stresemann & Lyko, 2008). Azacytosine-guanine dinucleotides are recognized by DNMT enzymes, which become trapped due to formation of covalent bonds. The full reaction is visualized in **Figure 9**, where nucleophilic attack initiates the methylation reaction, resulting in a covalent bond between carbon 6 of the cytosine, and DNMT. The bond is normally resolved by β-elimination, but due to the substitution of nitrogen at carbon 5, the DNMT enzyme remains covalently bound to DNA, and methyltransferase function is blocked. The presence of the trapped enzyme triggers DNA damage signalling and degradation of DNMT, therefore methylation marks are lost due to passive demethylation (Stresemann & Lyko, 2008). However, a role for AZA in active demethylation has also been proposed, based on its induction of active demethylation via upregulation of TET enzyme expression (Sajadian et al., 2015). Consequently, AZA functions as a demethylating agent via both passive and active demethylation processes.

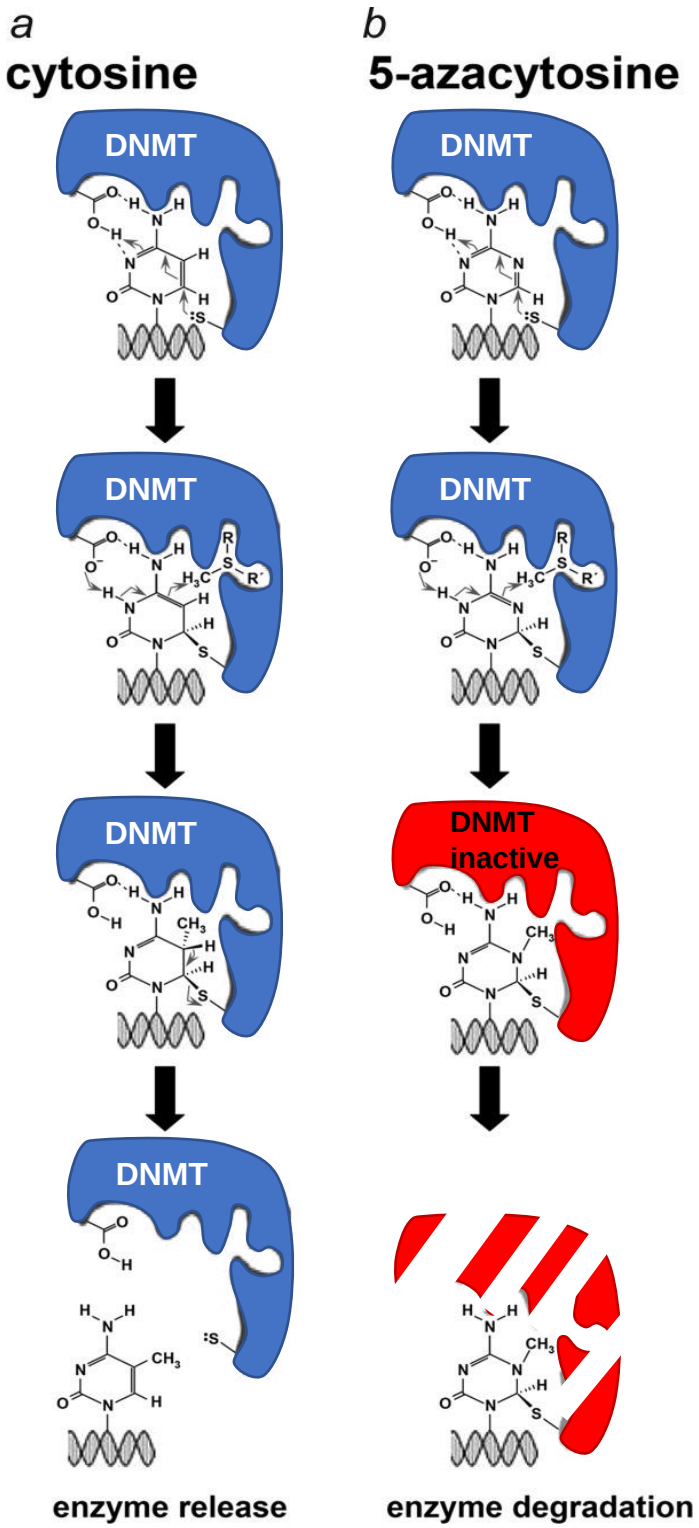

Figure 9. A) Normal methylation of cytosine by DNMT. **B)** DNMT trapping mechanism of AZA. Adapted from Stresemann & Lyko (2008).

1.9 HDAC Inhibition Affecting DNA Methylation

HDACi such as VPA, SB, or TSA are implicated in the methylation of both histones and DNA. As mentioned previously, DNA methylation and repressive histone marks (deacetylation) work together to silence gene transcription. This interaction is important in the area of cancer research, as it is well known that the silencing of tumor suppressor genes via DNA methylation plays a central role in cancer development. It has been demonstrated that HDACi, including TSA and phenylbutyrate, are able to enhance the activation of tumor suppressor genes initiated by DNA demethylation induced by DAC *in vitro* (Lemarie et al., 2004; Lemarie et al., 2005). Importantly, the DNA demethylating activity of VPA, which has been shown using ectopically methylated plasmid DNA transfected into HEK293 cells, was dependent on histone acetylation, as a VPA analog with no HDAC inhibition activity had no effect on demethylation (Detich et al., 2003). Others have shown that HDACi depletes DNMT1 protein expression via proteasomal pathway-mediated degradation (Zhou et al., 2008). It was also found that the levels of DNMT1 protein were decreased, after treatment with AZA followed by treatment with the HDAC inhibitor LBH589, to a higher degree than that each drug independently (Zhou et al., 2008). Furthermore, the combination of DAC (1 µM) and VPA (1 mM) has been shown to induce reactivation of the p57KIP2 and p21CIP1 (cyclin-dependent kinase inhibitors) genes in a leukemic cell line (MOLT4) in a synergistic manner, as compared to treatment with either drug alone (Yang et al., 2005). Therefore, a mechanism involving these two epigenetic events (histone acetylation and DNA methylation) may underlie the upregulation of the MT_1 receptor induced by VPA, as discussed in the following section.

1.10 Epigenetic Regulation of Melatonin Receptors

As mentioned above, VPA is an effective inhibitor of HDACs, and it can also activate multiple kinases such as PI3K, PKC, and MEK (Monti et al., 2009). Previous research supports the neuroprotective effects of VPA which can significantly upregulate the expression of diverse neurotrophic factors. Furthermore, previous research supports the epigenetic regulation of the MT_1 receptor by VPA. It was found that VPA increases the expression of MT_1 receptor mRNA in rat C6 glioma cells after treatment for 24 or 48 hours at a concentration of 3 mM; and this finding was corroborated at the protein level via western blot (Castro et al., 2005). Additionally, treatment of C6 cells with VPA (3 mM, 24 or 48 hrs) increased expression of HDAC1 mRNA, which suggested that histone deacetylation could be involved in the increase of MT_1 expression, as described below (Castro et al., 2005). The increased expression of MT_1 mRNA in a time-dependent manner was also observed at lower clinically relevant concentrations (0.5 mM – 1 mM) of VPA (Kim et al., 2008). These concentrations also induced significant increases in mRNA expression of Class 1 HDAC isoforms (HDAC 1, 2, 3) (Kim et al., 2008). Furthermore, an increase in MeCP2 mRNA expression was observed following VPA treatment in a time-dependent manner (Kim et al., 2008). As mentioned above, HDACs are recruited to areas of DNA methylation via binding to MeCP2, indicating that histone acetylation is linked to DNA demethylation, which can impair binding of MeCP2 to DNA. Therefore, the observed changes in MeCP2 and HDAC expression could involve a compensatory response linked to inhibition of HDAC activity by VPA (Kim et al., 2008).

To support the notion of epigenetic regulation of MT_1 receptor expression, C6 cells were treated with TSA, which is a different type and structurally distinct HDACi as

compared to VPA. It was observed that TSA also induced significant concentration-dependent induction of MT_1 receptor mRNA (Kim et al., 2008). In addition, *in vivo* administration of VPA has been shown to upregulate melatonin receptors in the CNS, which supports the pharmacological relevance of the foregoing (Niles et al., 2012; Bahna et al., 2014). Recently, it was determined that VPA induces histone H3 hyperacetylation along the length of the MT_1 receptor promoter region, showing that an epigenetic mechanism involving histone acetylation is tied to the induction of MT_1 receptor expression after VPA treatment (Bahna & Niles, 2017). This was observed in C6 cells after treatment with 1 mM VPA for 72 hrs, and the acetylation along the MT_1 promoter was studied using ChIP-qPCR (Bahna & Niles, 2017). In view of the interaction between histone acetylation and DNA demethylation, a mechanism involving both of these processes may be of importance to the epigenetic regulation of the MT_1 receptor, and this could potentially be exploited by epi-drugs.

1.11 Hypothesis and Rationale of Specific Aims

1.11.1 Rationale

It is clear that melatonin and the melatonin receptors, MT_1 and MT_2, have several important roles in maintaining homeostasis through circadian rhythmicity, but are also key players in the etiology and pathogenesis of several diseases that widely effect the population, namely, neurodegenerative diseases, depression, and cancer. As mentioned previously, signaling through MT_1 and MT_2 have downstream neuroprotective effects via the ERK and PI3K pathways. In addition, these receptors have decreased expression levels in aged individuals, and patients with ALS, AD, and PD. Interestingly, MT_1 appears to be an important marker for breast cancer, and presence of MT_1 has anti-cancer effects. However, not much is known about the pharmacological regulation of these receptors, due to their rhythmic nature of up- and down-expression, in response to endogenous melatonin, and their generally low basal expression.

The involvement of epigenetics in the control of chromatin conformation, and subsequently gene expression has been at the forefront of novel research. Specifically, the generation of “epi-drugs” to target silenced tumor suppressor genes, such as HDAC inhibitors and azanucleosides. Furthermore, it has been shown that HDACi can also affect the DNA methylation status, and this could potentially involve the depletion of the DNMT1 enzyme (Detich, 2003; Zhou, 2008). In addition, demethylating drugs such as DAC or AZA, in combination with HDACi can act synergistically to increase transcription of specific genes (Lemarie, 2005; Yang, 2005). More specifically, previous research has also demonstrated that epi-drugs, such as VPA and TSA, can induce MT_1 receptor expression in a neuronal cell line, and *in vivo* (Kim et al., 2008; Niles et al., 2012; Bahna

et al., 2014). Recent evidence that VPA specifically causes hyperacetylation at the MT_1 promoter region, together with the notion that VPA (and other HDACi) have DNA demethylating ability, indicates that an epigenetic mechanism involving histone acetylation and DNA demethylation could underlie the epigenetic control of the MT_1 receptor (**Figure 10**). This thesis will assess the ability of demethylating drugs to cause upregulation of the MT_1 receptor and modulate DNMT1 expression in a rat glioma cell line, which expresses melatonin receptors.

1.11.2 Hypothesis

Treatment of C6 glioma cells with 5-azacytidine (AZA), which demethylates DNA by inhibition of DNA methyltransferase activity, will upregulate the MT_1 receptor.

1.11.3 Specific Aims

1. Examine the concentration- and time-dependent effects of AZA on MT_1 mRNA expression in C6 cells.
2. Examine the concentration- and time-dependent effects of AZA on DNMT mRNA expression in C6 cells.
3. Determine DNMT protein expression levels after treatment of C6 cells with AZA.
4. Assess the effects of combination treatment with AZA and VPA on MT_1 mRNA expression in C6 cells.

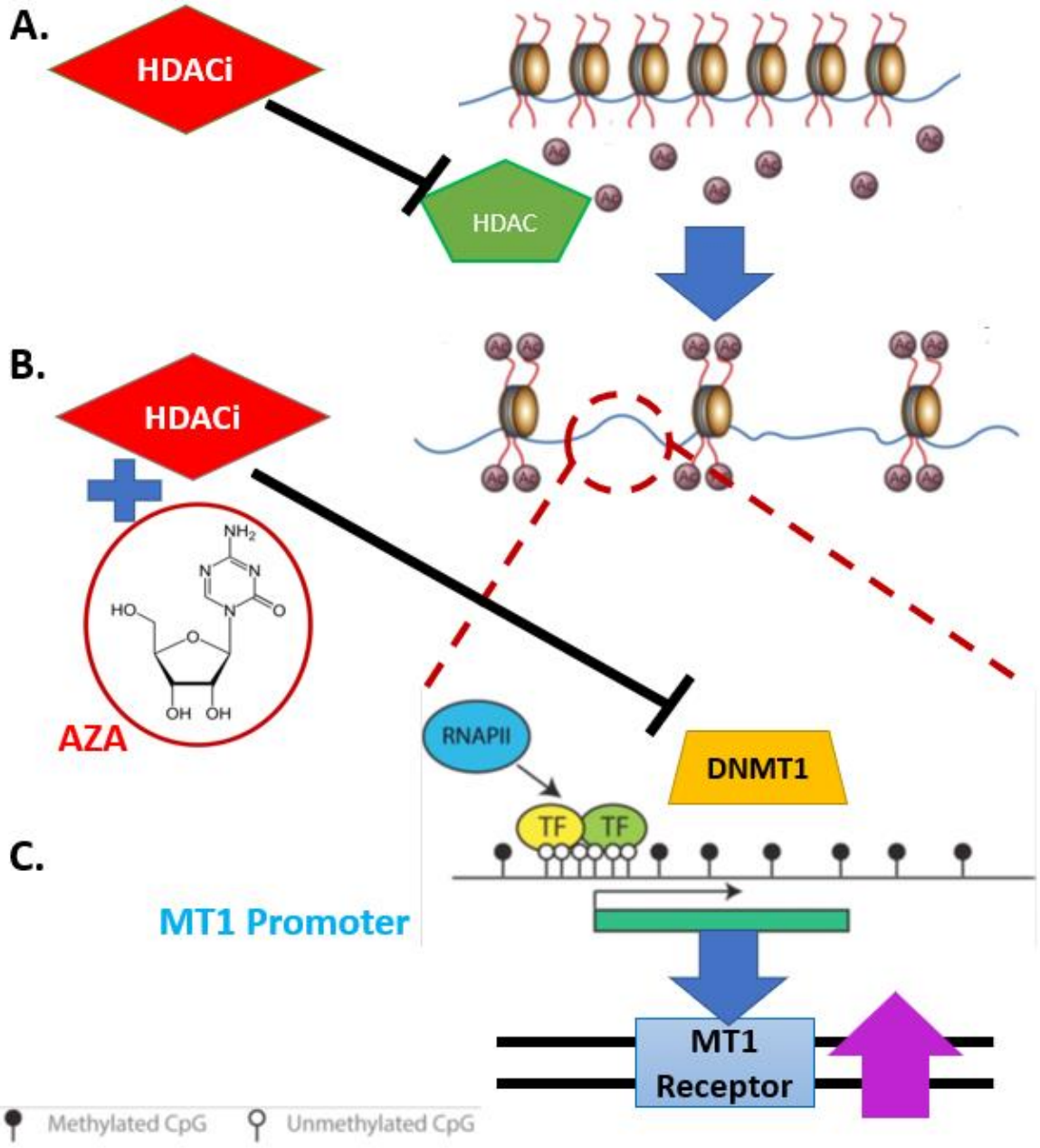

Figure 10. Overview of thesis rationale. **A)** HDAC inhibition attenuates the removal of acetyl groups from histones, leading to open chromatin conformation. **B)** HDACi and/or DNA demethylation (caused by AZA, or HDACi itself), inhibits DNMT1. **C)** This allows transcription factors and RNA Polymerase II (RNAPII) to access the DNA, specifically at the MT_1 promoter region, causing upregulation of the MT_1 receptor.

CHAPTER 2: MATERIALS & METHODS

2.1 Cell Culture

In vitro experimentation using a rat C6 glioma cell line was used to investigate the specific aims. This cell line was chosen because it is easily grown in culture, and it expresses the melatonin MT_1 and MT_2 receptors. The receptors are functional in transducing downstream effects, as was observed in the ability of physiological doses of melatonin to induce GDNF expression (Armstrong & Niles, 2002). In addition, the ability of HDACi to induce MT_1 has been shown previously in C6 cells (Castro et al., 2005; Kim et al., 2008).

C6 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), streptomycin and fungizone as reported previously (Castro et al., 2005; Pan & Niles, 2015). Cells at passages ranging from about 10 – 25, were seeded at a density of $10^4/cm^2$ on 10 cm Corning culture dishes (Fisher Scientific Ltd., Nepean, ON, CA), and maintained at 37^{o}C in a humidified incubator with 5% CO_2/air. Subsequently, at a confluency of approximately 40-50%, media was changed to DMEM plus 1% FBS for 24 hours, followed by drug treatment at a confluency of 60 – 70% as previously reported (Castro et al., 2005; Pan & Niles, 2015).

2.2 Drug Preparation and Treatments

Initially, to confirm that current C6 cells were responsive to HDAC inhibition, as reported previously (Bahna & Niles, 2017), the HDAC inhibitor, M344 (N-Hydroxy-7-(4-dimethylaminobenzoyl)-aminoheptanamide) was used. The drug was prepared in a

working solution of 20mM in 100% DMSO. M344 was then administered at a range of concentrations including 0.5, 1, 3, and 5 µM, or vehicle (DMSO 0.04%) for 24 hours.

Subsequently, following confirmation that the above treatment induced MT_1 receptor expression, the effects of AZA were examined. AZA was prepared in a stock solution of 100mM in 100% DMSO, then working solutions of 50 mM and 10 mM were prepared in a serial dilution using 100% DMSO. Cells were treated with AZA at a concentration of 1, 5, 10, 20, 25 µM or vehicle (DMSO 0.05%) for either 24 or 48 hours.

To assess any synergistic or additive effects of HDAC inhibition and DNA demethylation on MT_1 expression, combination experiments were conducted. For drug combination treatments, VPA was freshly prepared from powder form for each experiment, to generate a 50 mM working stock in plain DMEM, and AZA working stocks were prepared as described above, then the drugs were both added to plates of C6 cells at concentrations of 1 or 3 mM VPA, and AZA 1, 5, 10 µM. VPA was added to the cells in a dropwise fashion due to the larger volume for the treatment, AZA was added directly to the plate and the pipette was flushed out with media to ensure full volume was added (see **Table 1**). Each plate was subsequently swirled gently before being incubated for 24 hours.

Compound	Activity	Stock	Volume	Final
M344	HDACi	20 mM	2.5 µL	5 µM
			1.5 µL	3 µM
			0.5 µL	1 µM
			0.25 µL	0.5 µM
5 -Azacytidine	DNMT Inhibitor	50 mM	5 µL	25 µM
			4 µL	20 µM
		10 mM	2 µL	10 µM
			5 µL	5 µM
			1 µL	1 µM
Valproic Acid	HDACi	50 mM	600 µL	3 mM
			200 µL	1 mM

Table 1. Concentrations and volumes used of each drug for treatment in C6 cells.

2.3 RNA Extraction and cDNA Preparation

Total RNA was isolated from cells homogenized with a 21G needle, using Trizol as described by the supplier (Invitrogen). For the higher concentrations of AZA used (20 and 25 µM) two plates were pooled with the Trizol to ensure that an adequate yield of RNA was obtained, as some cells were lost due to cytotoxicity of AZA. Cell homogenates were transferred to PhaseMaker centrifuge tubes (Thermo Fisher Scientific), in which a gel is used to separate the aqueous layer to ensure efficient phase separation. Total RNA was precipitated using 100% isopropanol, the resulting RNA pellets were washed twice in 75% ethanol and solubilized into nuclease-free water. The solubilized RNA was then incubated for 15 minutes at approximately 57^{o}C. The optical densities of the RNA samples were measured at 260 nm using a Nanodrop 2000 or a Nanodrop One (Thermo

Fisher Scientific). All RNA samples were in a range of acceptable purities assessed by the 260/230 and 260/280 ratios.

To remove residual DNA, 25 µg of the RNA samples were then DNase treated, and complimentary DNA (cDNA)was synthesized from 2 µg of the DNase-treated total RNA using the Omniscript reverse transcriptase kit (Qiagen), including an RNase inhibitor and Oligo(dT) primers (Thermo Fisher Scientific). Assessment of RNA purity was conducted using a formaldehyde gel, which included denaturing approximately 2 µg of the RNA samples at 65 – 70^{o}C for approximately 10 minutes. The gel was run at 60V for 1 hour and the 28S and 18S bands were visualized using an AlphaImager 2200 system (Alpha Innotech Corp).

2.4 RT-PCR

After optimizing RT-PCR conditions for MT_1-344 and MT_1-132, (**Table 2**), such as cycle number, annealing temperature and template concentration, samples were amplified using a HotStarTaq Master Mix Kit (Qiagen) and the GeneAmp PCR System 2400 Thermal Cycler (Perkin Elmer) as reported previously (Castro et al. 2005; Kim et al. 2008; Sharma et al. 2008). The internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was included in each experiment (**Table 2**). In brief, following initial heat inactivation of the HotStarTaq DNA polymerase, at 95^{o}C for 5 minutes, PCR proceeded at 94^{o}C for 30 seconds, 55^{o}C for 30 seconds, 72^{o}C for 1 minute, cycling for 30 – 40 cycles, followed by a final incubation period of 72^{o}C for 10 minutes.

Gene	Primers (5' → 3')	Nucleotides	Size (bp)
MT_1-82	CATAGCCATCATGCCCAACC GACGGACTGGGTGAAGGTAC	154 – 173 235 – 216	82
MT_1-132	CCTGTACCTTCACCCAGTCC TCCGTCTGACCTGAAGAACC	213 – 232 344 – 325	132
MT_1-344	CCTAATGGGCCTGAGTGTCA TCCGTCTGACCTGAAGAACC	529 – 548 872 – 853	344
DNMT1	CCTGGAGAACGGAACACTCT CATGGTCTCACTGTCCGACT	113 – 132 275 – 256	163
GAPDH	TTCACCACCATGGAGAAGGC GGCATGGACTGTGGTCATGA	1147 – 1166 1383 – 1364	237
18S RNA	CGTTCTTAGTTGGTGGAGCG AACGCCACTTGTCCCTCTAA	1341 – 1360 1468 – 1449	127

Table 2. Nucleotide sequences of all primers used for RT-PCR and RT-qPCR.

Due to the low basal expression of melatonin receptors in the current C6 cells, a nested PCR strategy was used, as previously detailed for MT_1 (Jawed et al., 2007). In this protocol, a first-round amplification of MT_1 with a product size of 344 base pairs (bp) (MT_1-344) was generated from 8µL of cDNA after cycling 40 times through the PCR cycling conditions as described above. Subsequently, products from the 344 bp reaction were diluted 1:10 in nuclease-free water and then 1 µL of the diluted product was amplified again for 30 cycles using different MT_1 primers resulting in a product size of 132 bp (MT_1-132) (**Figure 11**). GAPDH was amplified using the same PCR cycling conditions, and a cycle number of 35.

Negative controls containing RNA without reverse transcription or no template cDNA were processed to confirm RT-PCR specificity and the absence of DNA contamination. Amplified RT-PCR products were separated on a 2% agarose gel, stained with RedSafe and run at 90V for 25-30 minutes at room temperature.

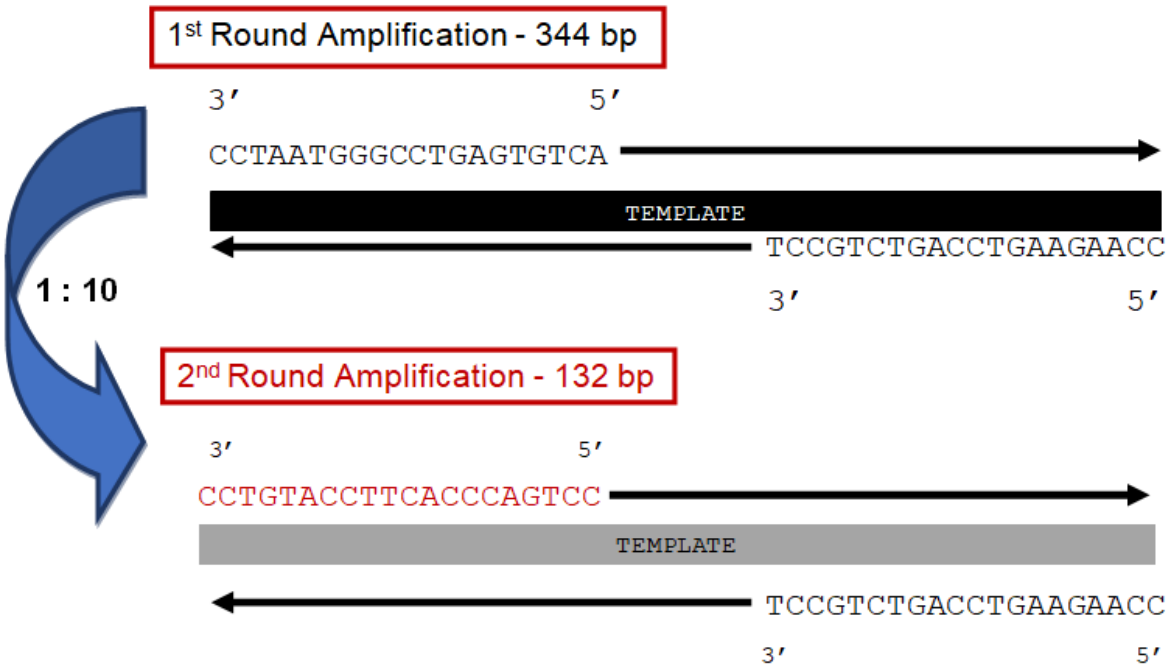

Figure 11. Nested PCR Schematic

2.5 RT-qPCR

The effects of drug treatment on expression of MT_1 and DNMT1 were confirmed and quantified by Real-Time PCR (qPCR) using an appropriate mastermix (eg. SsoAdvanced Universal SYBR Green Supermix-Biorad), as previously reported (Bahna & Niles, 2017; Niles et al., 2012). The internal control genes such as 18S ribosomal RNA (18S rRNA) or GAPDH were included in all RT-qPCR experiments. Thermal gradient testing was done to identify appropriate annealing temperatures for the target genes MT_1-82 and DNMT1 (**Table 2**). This was done using temperatures ranging from 55.5 – 62.5^{o}C, and 55 – 60^{o}C respectively. Master mixes were prepared with primers at a concentration of 500 nM, and template cDNA in the amount of 100 ng for DNMT1,18S and GAPDH. A template concentration of 100 or 200 ng was used for MT_1-82. RT-qPCR experiments were generally conducted in duplicate as follows for DNMT1 and reference gene 18S:

initial activation step at 95°C for 30 sec, then cycling between 95°C for 15 sec, and 60°C for 30 sec for 40 cycles. For MT_1-82, an annealing temperature of 57 was used, with the same cycling conditions. This was followed by a melt curve analysis consisting of 95°C for 10 sec, 65°C for 5 sec, and finally 95°C for 5 sec. qPCR experiments were conducted using the CFX96 Touch Real-Time PCR Detection System (BioRad). PCR controls (including no reverse transcription, and no template) were included in all experiments, which were replicated at least 3 – 4 times.

2.6 Data Analysis for RT-PCR and RT-qPCR Experiments

After separating the RT-PCR products on an agarose gel, as described above, the gels were then digitally scanned using an AlphaImager 2200 system (Alpha Innotech Corp). Optical density (OD) ratios of each target gene over the internal control, GAPDH, was used for data normalization and semi-quantitative analysis. After normalization of OD data, one-way analysis of variance (ANOVA) was used for statistical assessment of data. The level of significance is $P<0.05$ for all experiments. Post hoc analysis (Newman-Keuls) was conducted to determine significant differences between treatments (Castro et al., 2005; Kim et al., 2008; Sharma et al., 2008).

For experiments involving qPCR, cycle threshold (Ct) values were obtained from the CFX96 Touch Real-Time PCR Detection System (BioRad). Statistical analysis was conducted using the ΔΔCT method as reported previously (Niles et al., 2012; Bahna & Niles, 2017). In which, the average Ct values for each sample were calculated, followed by obtaining the ΔCT values by subtracting the average Ct value of the housekeeping gene from the target gene. The ΔΔCT was then calculated by subtracting the ΔCT value of the control sample from the ΔCT value of the treated sample ($\Delta CT_{treated} - \Delta CT_{control} =$

ΔΔCT). Finally, the relative fold change in target gene expression for each sample was then calculated using the equation $2^{(-\Delta\Delta CT)}$.

2.7 Sequencing of PCR Products

To confirm the sequences for the target genes of interest, PCR products were submitted to the Mobix sequencing facility at McMaster University. PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen), where the column was washed twice with PE buffer (ethanol added), and DNA was eluted using 30 µL of warmed water. Purity of resulting DNA was confirmed using a NanoDrop 2000 (Thermo Fisher Scientific), before samples were sent to Mobix.

2.8 Nuclear Protein Extraction

Western blotting was used to assess the effect of AZA on the expression of DNMT1 protein. Nuclear extraction techniques were used to purify the nuclear fraction of the cell lysates. This was done by harvesting cells in lysis buffer containing 0.1% NP-40, followed by centrifugation at 3000 rpm for 10 min. After removing the supernatant which contains the cytosolic fraction, nuclear pellets were resuspended in a buffer containing glycerol and NaCl, which lyses plasma membranes and forces DNA into solution. Samples were then homogenized with a glass tissue homogenizer, followed by a 21G needle, incubated on ice for up to 1 hour and centrifuged at high speed (14 000 rpm) for 35 min. at 4^{o}C.

2.9 Protein Quantification

The supernatant containing the nuclear protein fraction was then quantified using a DC Assay (BioRad), as done previously (Castro et al., 2005). A standard curve was generated using bovine serum albumin (BSA), to determine the concentration of the sample proteins for each experiment. The unknown protein concentrations were calculated using the equation of the line of a standard curve, generated from absorbances from a spectrophotometer at 750 nm versus a range of known protein concentrations (0.2 – 1.4 mg/mL BSA).

2.10 Western Blotting

Nuclear protein (15 µg) was prepared in 2xSDS buffer and boiled for 5 minutes. The samples were then loaded onto a 10% acrylamide gel, run initially at 60V for 30 min, then 100V for approximately 1.5 hours until the ladder was well separated in the high molecular weight range. The proteins were transferred onto PVDF membranes activated in methanol, at 25V for at least 20 hours at 4^{o}C. Membranes were then blocked for 1 hour in 5% milk in Tris-Buffered Saline plus Tween (TBST) and incubated with DNMT1 (1:500, Cell Signaling) or β-Actin (1:10000, Sigma-Aldrich) primary antibodies for 48 hours at 4^{o}C. Membranes were then probed with the secondary antibodies for one hour at room temperature: anti-mouse IgG-HRP was used at a dilution of 1:20 000 for the β-Actin blots (Sigma-Aldrich), and anti-rabbit IgG-HRP was used at 1:5000 for the DNMT1 blots (Santa Cruz Biotechnology). Enhanced chemiluminescence (ECL) reagent was then pipetted onto each blot and films were then used to visualize the proteins in a dark room by film autoradiography, as reported previously (Castro et al., 2005).

2.11 Data Analysis for Western Blots

The films for protein visualization were scanned using a digital printer, the resulting images were then digitally scanned using an AlphaImager 2200 system (Alpha Innotech Corp). OD ratios of the resulting bands for DNMT1 over the OD ratios for the internal control, β-Actin, were used for data normalization and semi-quantitative analysis. After normalization of OD data, one-way ANOVA was used for statistical analysis. The level of significance is $P<0.05$ for all protein experiments. Post hoc analysis (Newman-Keuls) was conducted to determine significant differences between treatments (Castro et al., 2005; Kim et al., 2008; Sharma et al., 2008).

CHAPTER 3: RESULTS

3.1 HDAC inhibition via M344 induces MT_1 mRNA upregulation

RT-PCR:

In keeping with previous observations with other HDAC inhibitors, treatment of C6 cells with the HDAC inhibitor M344 (0.5, 1, 3,5 µM) for 24 hours caused a significant concentration-dependent increase in relative MT_1 mRNA levels, whereas the control gene GAPDH was unchanged by this drug (**Figure 12**). One-way ANOVA conducted on optical density (OD) values obtained from gel images, of which a representative image is included in **Figure 12**, indicated a significant treatment effect ($F_{(4, 10)} = 13.88, P < 0.0004$) compared to cells treated with the vehicle (DMSO 0.04%). A Newman-Keuls post-hoc test indicated that the relative levels of MT_1 mRNA were significantly higher in cells treated with as low as 0.5 µM M344 ($P < 0.001$) (**Figure 12**). Sanger sequencing with the MT_1-132 primer confirmed specificity for RT-PCR amplification of MT_1.

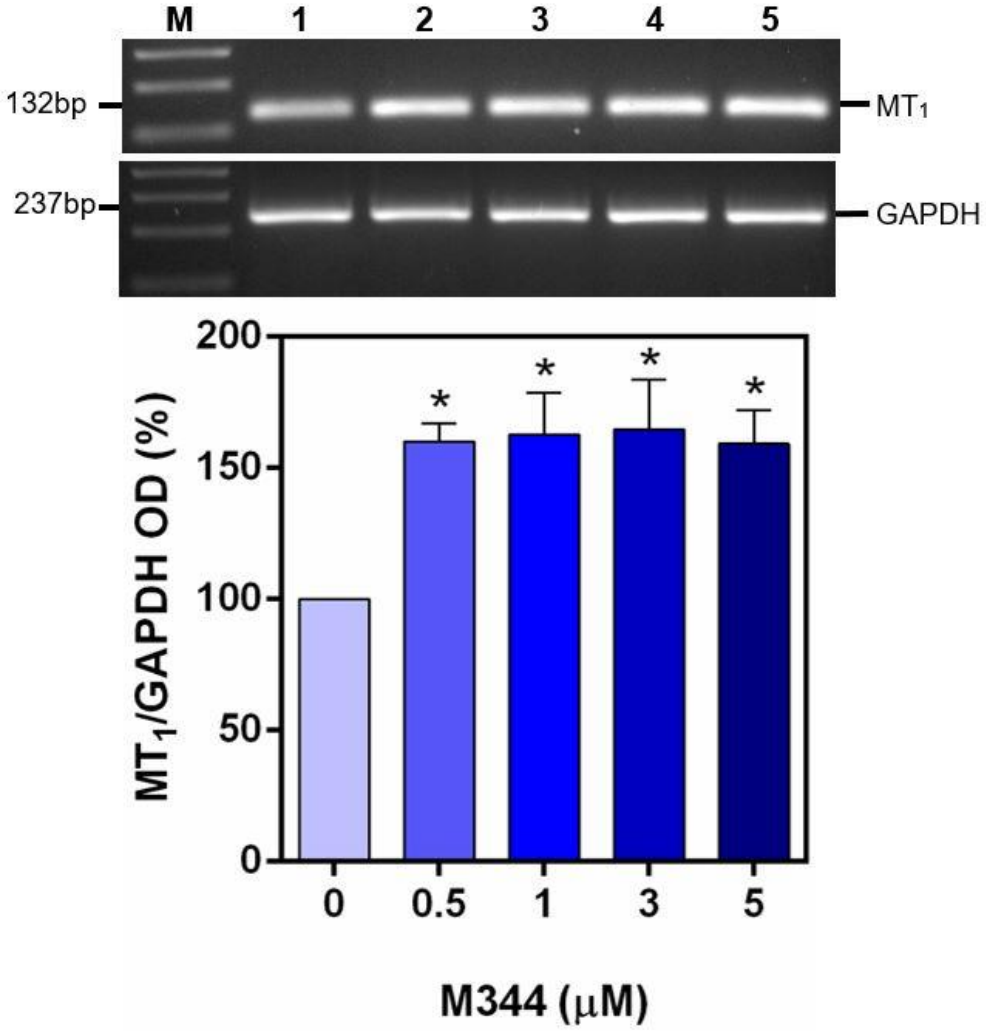

Figure 12. Concentration-dependent induction of MT_1 mRNA expression by HDAC inhibitor M344 treatment for 24 hours in C6 cells. Lanes 1-5: Control (0.04% DMSO), 0.5, 1, 3, 5 μM M344. Histograms represent the means ±S.E.M. (n=3) for percentage (%) values of MT_1/GAPDH optical density (OD) ratios. *P<0.001 versus control.

3.2 DNMT inhibition via Azacytidine induces MT_1 mRNA upregulation

RT-PCR:

Treatment of C6 cells with AZA for 24h, revealed an upregulation of MT_1 expression. One-way ANOVA of the OD values obtained from RT-PCR data on relative expression of MT_1 mRNA levels versus GAPDH showed a significant treatment effect ($F_{(5,17)} = 5.12, P<0.004$) with induction of MT_1 expression by AZA, as described in **Figure 13 A**. Similarly, treatment with AZA for 48h produced a significant treatment effect ($F_{(5,15)} = 2.77, P<0.05$) with an increase in MT_1 mRNA expression versus control (DMSO 0.05%) (**Figure 13 B**). To note, some cytotoxicity was observed at 20 and 25 µM AZA, classified by a reduction in confluence at 24 and 48 hours. However, non-toxic concentrations ranging from 1 – 10 µM, induced MT_1 mRNA expression, indicating that this effect was not due to the cytotoxic effects of AZA.

Real-Time PCR (RT-qPCR):

Furthermore, RT-qPCR data indicate a multifold increase in MT_1 mRNA levels, following treatment of C6 cells with AZA, confirming earlier findings obtained by standard PCR (**Figure 14**). ANOVA after ΔΔCT calculations showed a significant treatment effect ($F_{(5, 11)} = 17.26, P<0.0001$). Post hoc analysis using a Newman-Keuls test showed that the relative levels of MT_1 mRNA were significantly higher in cells treated with 10 µM ($P<0.05$), 20 µM ($P<0.01$), and 25 µM AZA ($P<0.0001$), as compared with controls treated with vehicle (DMSO 0.05%). Sanger sequencing with the MT_1-82 primer confirmed specificity for RT-qPCR amplification of MT_1.

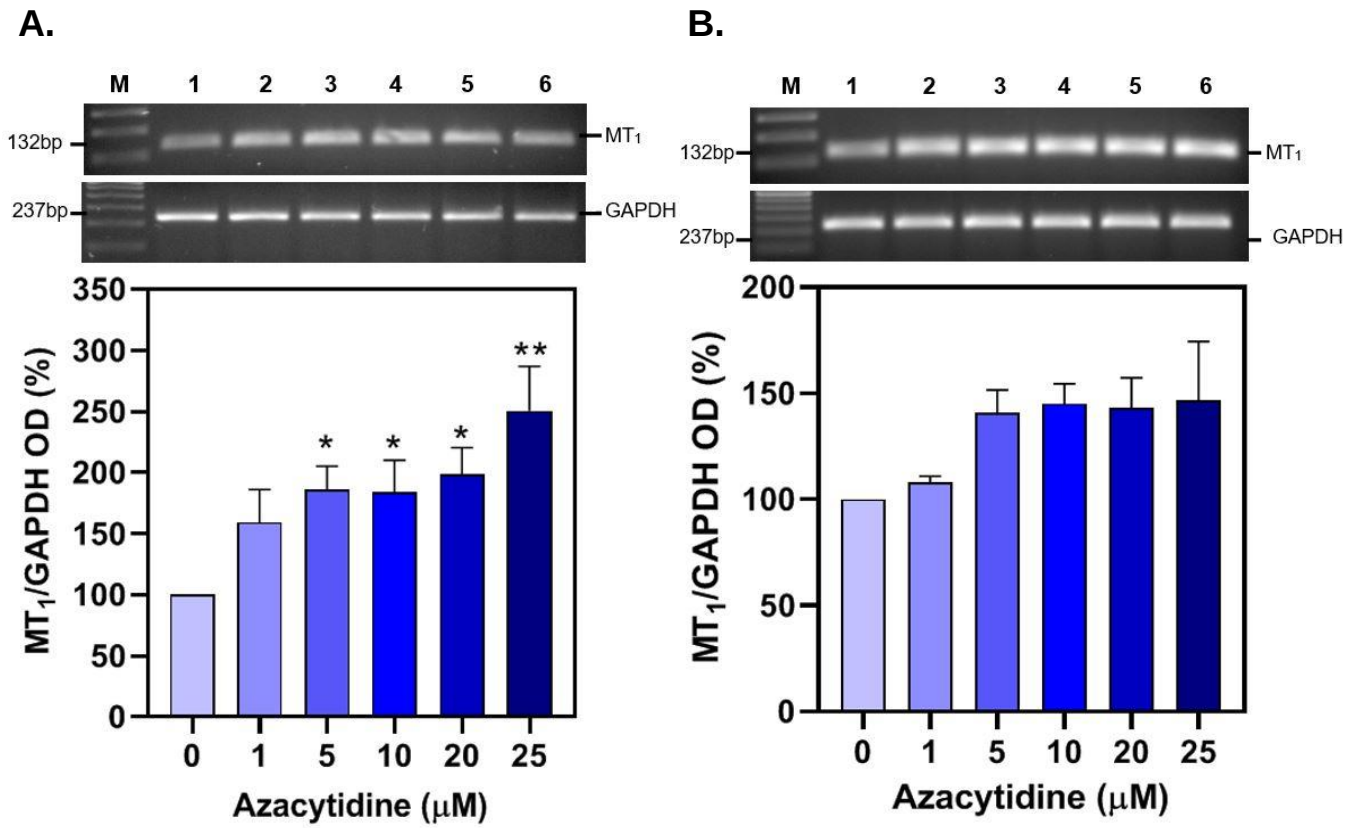

Figure 13. Concentration-dependent induction of MT_1 mRNA expression by AZA for 24 **(A)** or 48 h ours **(B)** in C6 cells. Lanes 1 - 6: Control (0.05% DMSO), 1, 5, 10, 20, 25 μM AZA. **(A)** Histograms represent the means ±S.E.M. (n=4) for percentage (%) values of MT_1/GAPDH optical density (OD) ratios. *P<0.05 versus control, and **P<0.01 versus control. **(B)** Histograms represent the means ±S.E.M. (n=4) for percentage (%) values of MT_1/GAPDH optical density (OD) ratios. ANOVA indicated a significant treatment effect (P<0.05).

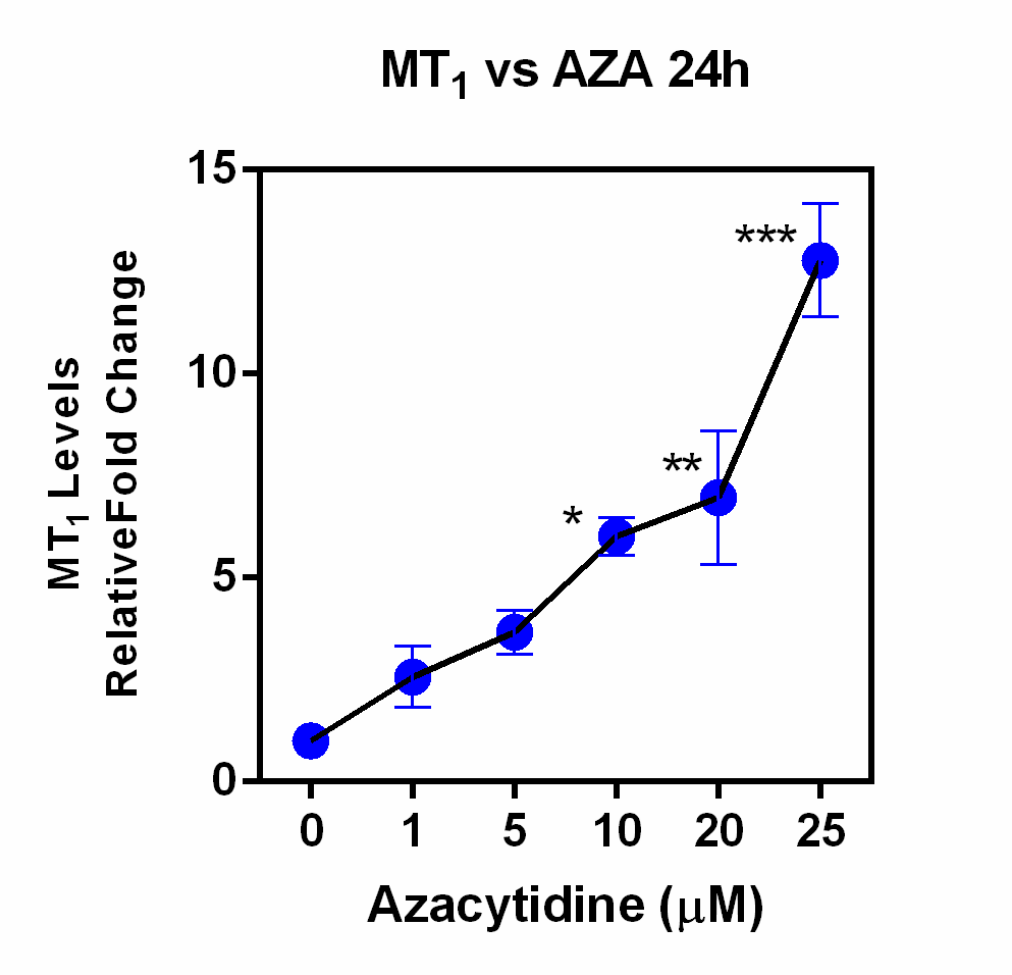

Figure 14. Concentration-dependent induction of MT_1 mRNA expression by AZA for 24 hours in C6 cells. RT-qPCR data shown are the means ±S.E.M. (n=2-3) for values of $2^{-\Delta\Delta Ct}$ MT_1 normalized with 18S rRNA. *P<0.05, **P<0.01, and ***P<0.0001 versus control.

3.3 Treatment with Azacytidine diminishes DNMT1 protein and mRNA expression

Western Blot Analysis:

Results from Western blotting indicate a knockdown of DNMT1 protein in C6 cells after treatment with AZA for 24 or 48 hours, relative to the control protein, β-Actin (**Figure 15 A**). One-way ANOVA on data obtained from OD analyses on Western blot images indicated a significant treatment effect *($F_{(3,10)} = 11.25, P<0.001$)* for 24 hours (**Figure 15 B**) and 48 hours ($F_{(2,3)} = 81.72, P<0.002$) at 10 and 20 µM versus control (0.05% DMSO) (**Figure 15 C**). Newman-Keuls testing indicated that the relative levels of DNMT1 protein were significantly knocked down at 10 and 20 µM at both 24 and 48hr timepoints (*P<0.01*).

RT-qPCR:

Additionally, RT-qPCR experiments assessing DNMT1 mRNA levels, indicated that AZA caused a significant decrease in DNMT1 mRNA expression. One-way ANOVA indicated a significant knockdown of DNMT1 mRNA expression after 24 ($F_{(5,12)} = 11.84, P <0.0003$) and 48 hours ($F_{(5,12)} = 4.729, P <0.05$) (**Figure 16 A**). A Newman-Keuls test indicated that the relative levels of DNMT1 mRNA were significantly reduced in cells treated with 1 µM AZA for 24hr (*P <0.01*), and even more of an effect is seen at 5 µM (*P <0.001*) versus control. This effect was also seen at 48 hrs, as the relative levels of DNMT1 were significantly reduced at concentrations of 20 µM and 25 µM (*P <0.01, P<0.05*) (**Figure 16 B**). Sanger sequencing with the DNMT1 primer confirmed specificity for RT-qPCR amplification of DNMT1.

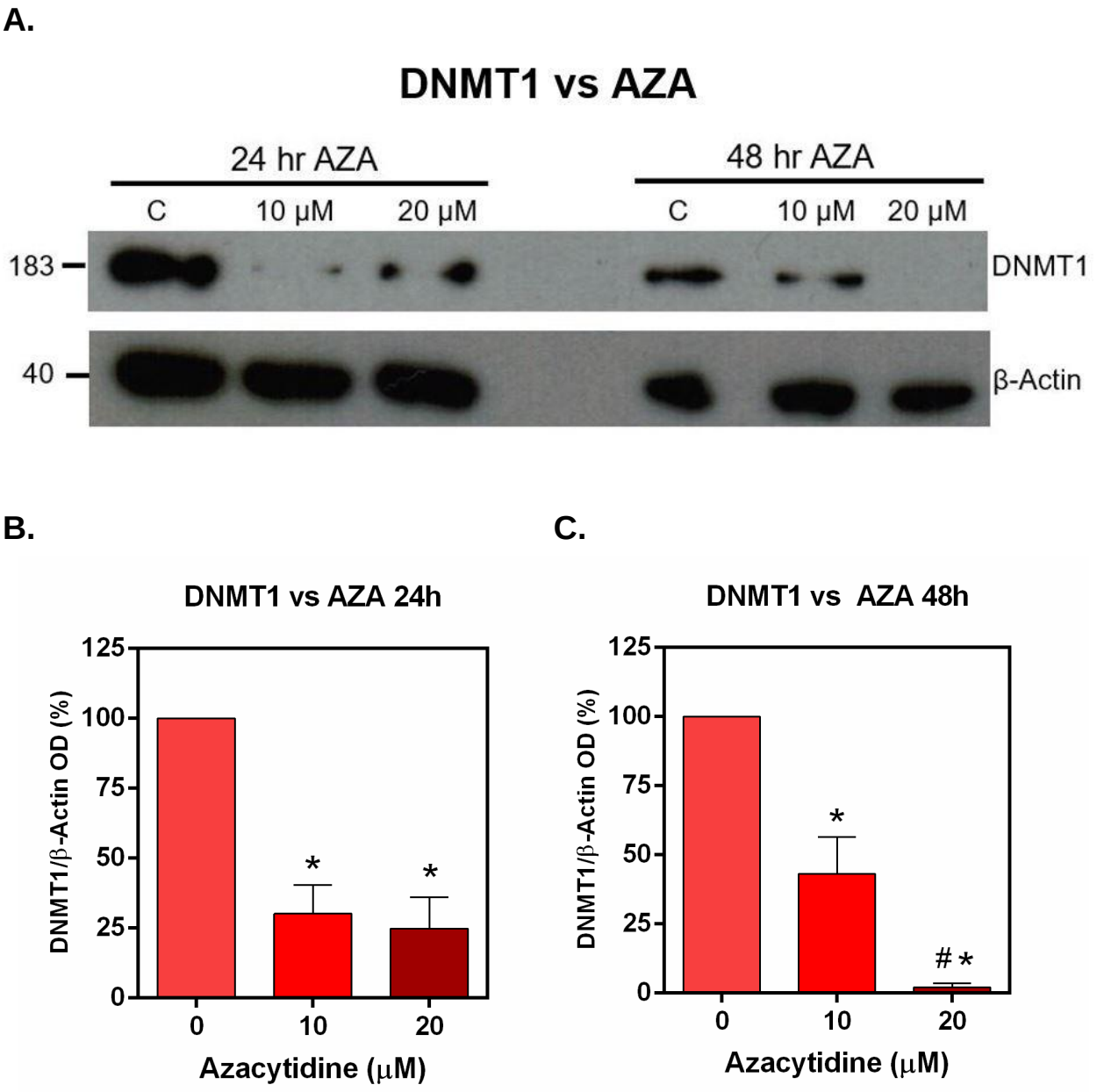

Figure 15. Concentration-dependent knockdown of DNMT1 protein expression by AZA treatment for 24 or 48 hours. in C6 cells. **(A)** Representative immunoblots for DNMT1 protein expression vs AZA for 24 or 48h. **(B)** Histogram represents the means ±S.E.M. (n=3) for percentage (%) values of DNMT1/β-Actin optical density (OD) ratios (24h). *P<0.01versus control. **(C)** Histogram represents the means ±S.E.M. (n=2) for percentage (%) values of DNMT1/β-Actin optical density (OD) ratios (48h). *P<0.01, versus control and #P<0.05 versus 10 µM AZA.

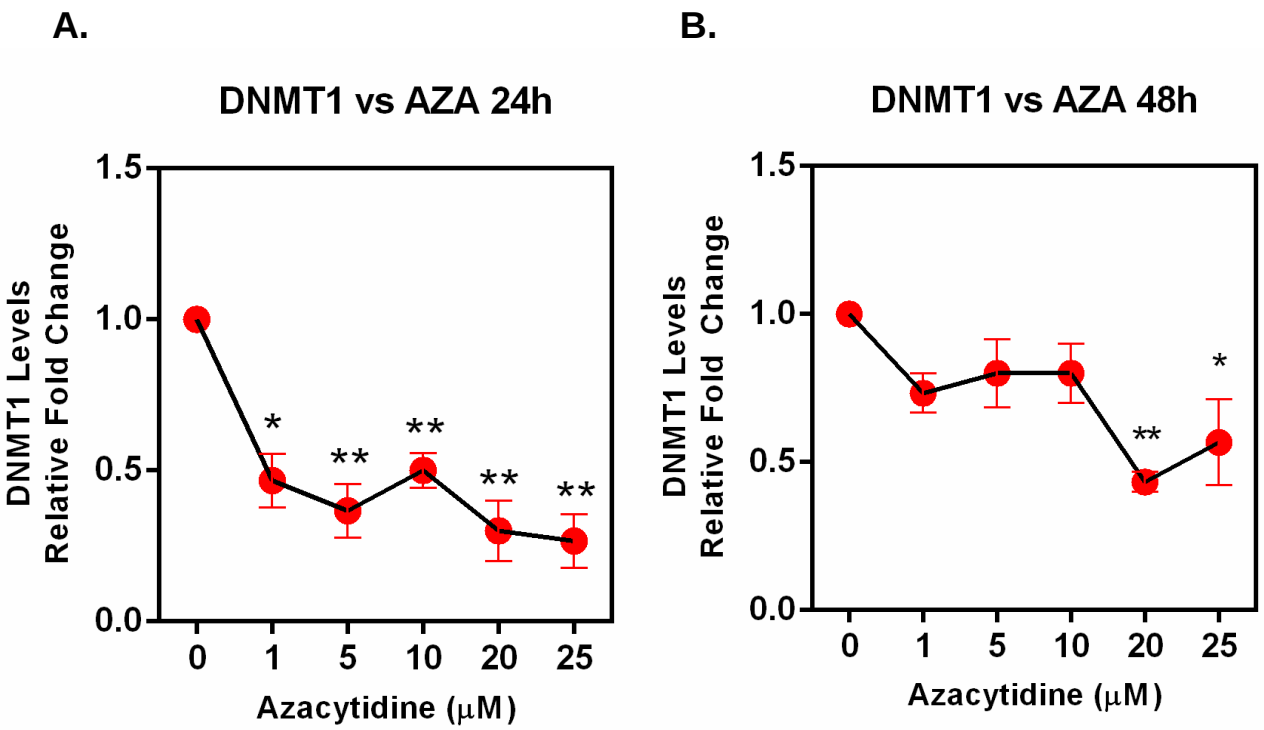

Figure 16. Concentration-dependent knockdown of DNMT1 mRNA expression after treatment with AZA for 24 **(A)** or 48 hours **(B)**. RT-qPCR data shown are the means ±S.E.M. (A: n=3, B: n=3) for values of $2^{-\Delta\Delta Ct}$ DNMT1 normalized with 18S RNA. **(A)** *P<0.01, **P<0.001 versus control and **(B)** *P <0.05, **P<0.01 versus control.

3.4 Effect of the combination of VPA and AZA on MT_1 expression

Experiments using the combination of AZA at concentrations ranging from 1 – 10 µM, and VPA at a concentration of either 1 or 3 mM caused induction of MT_1 mRNA, as observed using RT-qPCR. Treatment with VPA alone caused about a 10-fold induction of MT_1 (*P<0.01* versus control), and treatment with 5 µM AZA induced MT_1 approximately 2-fold versus control (**Figure 17 A**). Treatment with the combination of VPA and AZA, at concentrations of 3 mM and 1 µM, respectively, generated about an 11-fold induction of MT_1, which was not significantly different from the effect of just VPA (3 mM). Interestingly, as the concentration of AZA was increased to 5 µM in combination with 3mM VPA, a trend toward an additive or greater increase in MT_1 expression was observed (**Figure 17 A**). ANOVA after ΔΔCT calculations revealed a significant treatment effect ($F_{(12, 26)} = 10.11$, *P<0.0001*). Post-hoc testing revealed that at with this combination, there was a significant upregulation of MT_1 versus control (*P<0.001*). However, the high variance precluded detection of a significant difference versus VPA (3 mM) alone.

In view of the above variance and the apparent skewness of data from combination experiments, a log transformation was used to obtain a more normal distribution of data, which would improve the validity of statistical analysis. A Log2 transformation followed by ANOVA revealed a significant treatment effect ($F_{(12, 26)} = 20.52$, *P<0.0001*). Newman-Keuls testing of the data indicated that cells treated with 1 mM and 3 mM VPA alone had a significant effect on MT_1 levels (*P<0.05*, *P<0.0001* respectively) (**Figure 17 B**). In addition, the combination of VPA 1 mM and AZA 1 µM, and VPA 1 mM and AZA 5 µM increased MT_1 expression 2-fold (*P<0.01*). The 3 mM VPA concentration plus 1, 5, or 10 µM AZA showed a powerful induction of MT_1 (*P<0.0001*), although this effect was not

different from that of VPA 3 mM alone (**Figure 17 B**). Furthermore, the combinations of VPA 1 mM and AZA 1 and 10 µM do not show significant induction of MT_1 versus VPA 1 mM alone, although these combinations show significant induction versus AZA 1 µM (*P<0.05*). This suggests that VPA has a greater effect than that of AZA alone or in combination.

A.

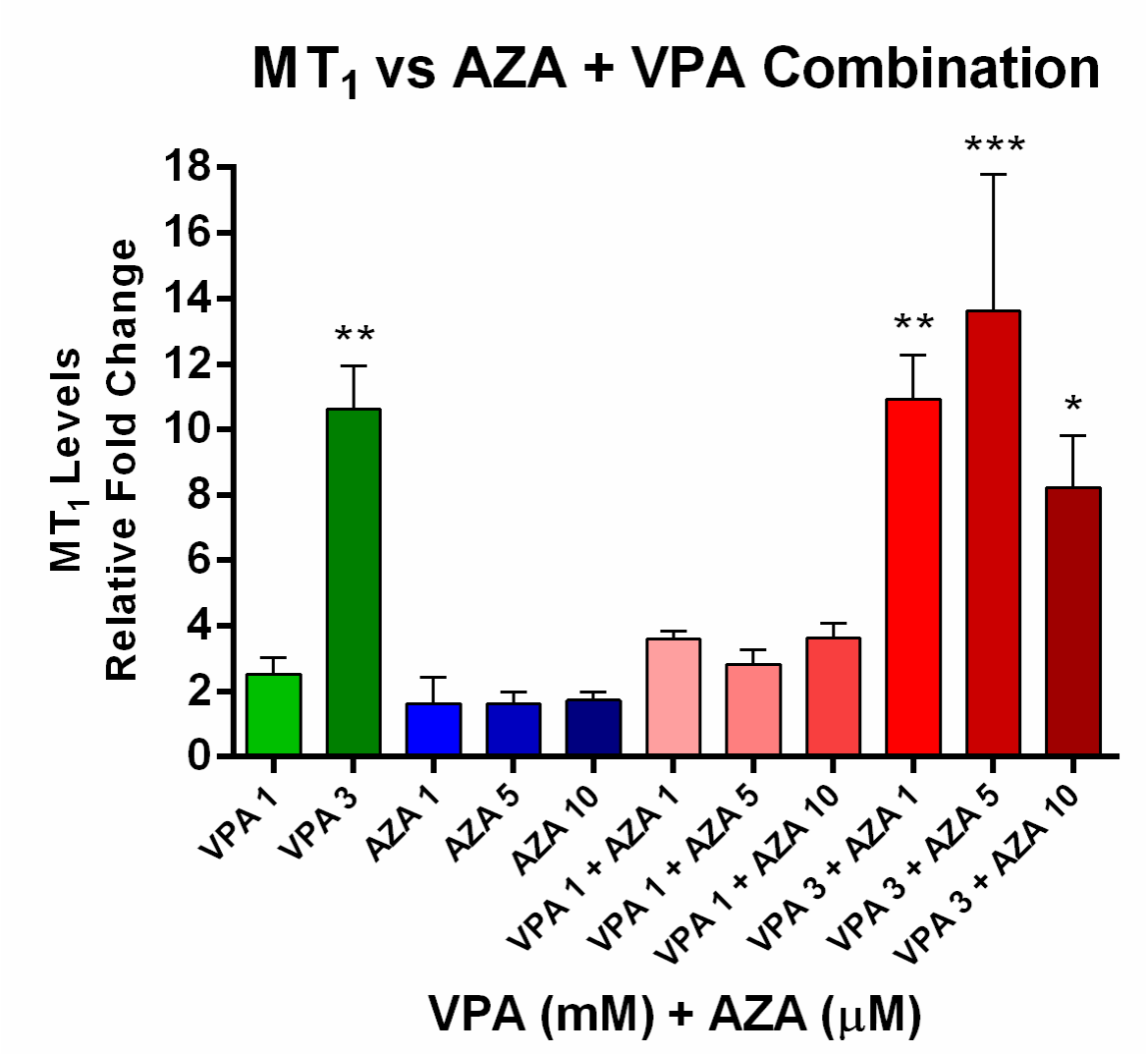

Figure 17. See full legend on next page.

B.

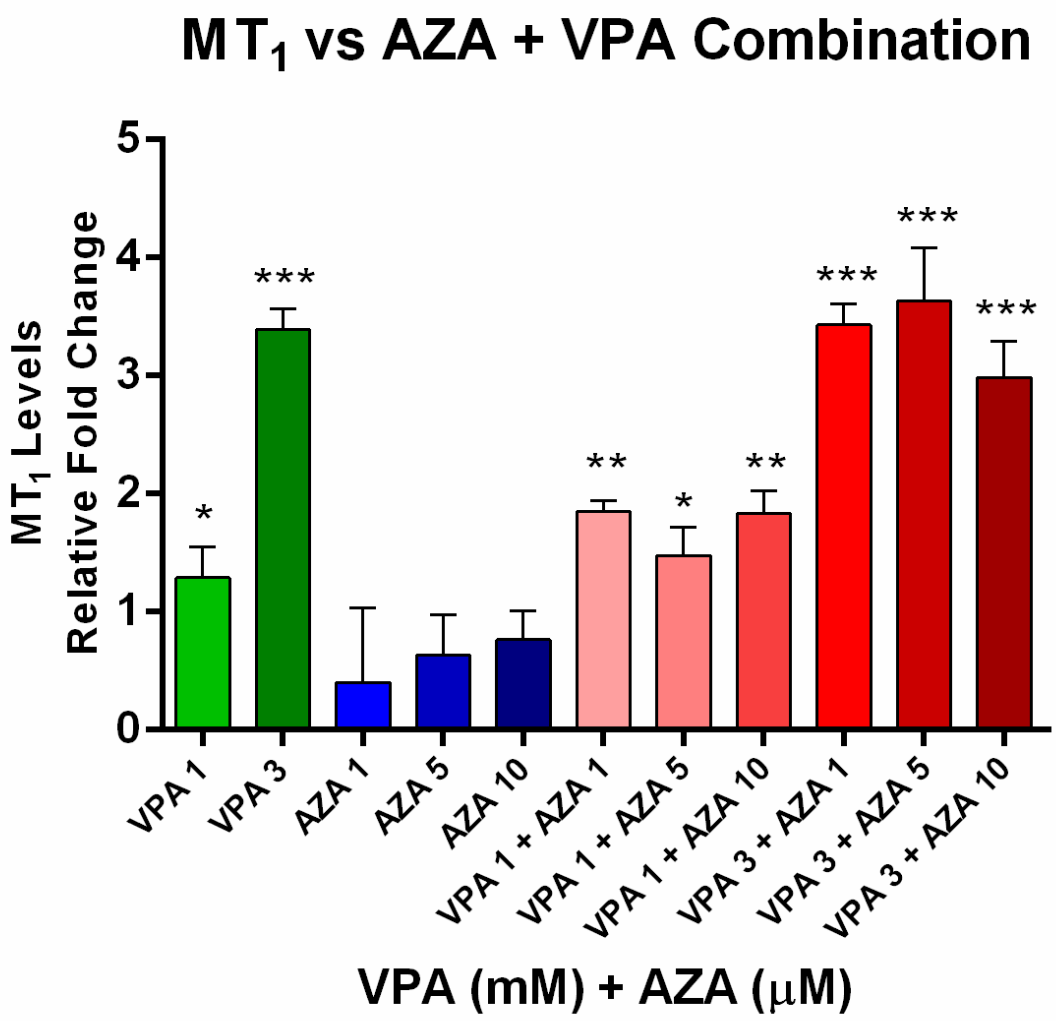

Figure 17. Enhanced induction of MT_1 mRNA expression by combinatorial treatment with AZA and VPA for 24 hours in C6 cells. RT-qPCR data shown represents the means (n=3) for values of $2^{-\Delta\Delta Ct}$ MT_1 normalized with 18S rRNA or TBP. Fold changes in MT_1 relative to vehicle controls for VPA (DMEM) and AZA (DMSO 0.05%), are shown. **A.** ANOVA indicated a significant treatment effect (*P<0.0001*). *P <0.05, **P <0.01, ***P<0.0001 versus control. **B.** Data represents the Log2 of the relative fold change values shown in A. ANOVA indicated significant treatment effect (*P<0.0001*). *P <0.05, **P <0.01, ***P<0.0001 versus control.

CHAPTER 4: DISCUSSION

4.1 HDAC Inhibition via M344 Induces MT_1 mRNA upregulation

Initial experiments, to assess the ability of HDACi to upregulate the MT_1 receptor, were completed to ensure that HDACi was effective in the rat C6 cells currently in use. Treatment with the HDACi M344 at concentrations ranging from 0.5 – 5 µM for 24 hours showed a concentration-dependent increase of MT_1 mRNA levels, and the lowest dose of 0.5 µM induced a significant treatment effect, which is likely due to the potent HDAC inhibitory effect leading to hyperacetylation of histones, causing open chromatin conformation near the MT_1 gene. This effect on MT_1 seemed to remain stable at around 1.5-fold induction of MT_1 versus the control gene, GAPDH, as concentration was increased up to 5 µM (**Figure 12**). Future studies assessing the acetylation levels at the MT_1 promoter region could confirm HDAC inhibition, similar to what was done previously to confirm promoter-associated histone acetylation levels after treatment of C6 cells with VPA (Bahna & Niles, 2017).

Additional dose-response and time-course experiments would reveal the optimal concentration and treatment length for M344 in C6 cells, which would be useful to identify for pursuing use of M344 in combination experiments with other epi-drugs. Furthermore, identifying the lowest dose that causes the observed 150% induction of MT_1 mRNA (relative to control) would be beneficial as M344 has a cytotoxic effect in C6 cells; identifying this dose would allow for avoidance of off-target effects that may affect gene transcription.

4.2 DNMT inhibition via Azacytidine induces MT_1 mRNA upregulation

This study shows for the first time that treatment of rat C6 cells with varying concentrations of the DNA demethylating drug, AZA, upregulates melatonin MT_1 receptor expression. Initial studies were conducted with standard PCR due to the need for identifying successful parameters for detecting the expression of this low-basal level gene, which involved troubleshooting of the RNA extraction, and the use of a nested PCR strategy. These initial studies were able to detect a significant increase in MT_1 mRNA expression after 24 hours of AZA treatment, as a 2.5-fold increase in the receptor expression was observed at a concentration of 25 µM, as seen in **Figure 13 A**. Similar results were seen at 48 hours, and there was a significant treatment effect, although due to large variances between groups, no inter-group significance was obtained. This can be attributed to issues with the stability of the AZA, as it was later determined that fresh preparations of the drug should be used, which was not done for every replication. In addition, other researchers have added fresh AZA (or DAC) every 24 hours for the course of the treatment, so perhaps this would improve the effect of AZA over 48 hours. Importantly, subsequent real-time (RT-qPCR) studies confirmed the AZA-induced MT_1 upregulation observed with nested PCR. Consistent with the significantly greater sensitivity of qPCR, a multifold (up to 14-fold) upregulation of the MT_1 receptor was observed, following AZA treatment for 24 hours (**Figure 14**).

These results are consistent with several lines of evidence that show the ability of AZA (or DAC) to induce gene expression. For instance, AZA at concentrations ranging from 5 – 20 µM can induce cyclooxygenase-2 (COX-2) expression in human fibrosarcoma cells after 24 hours (Yu & Kim, 2015). Although the methylation status of the MT_1

promoter region, in the presence or absence of AZA, was not examined, it is reasonable to conclude that demethylation of this promoter was involved in the observed induction of MT_1, due to the known potent demethylating effects of AZA (Stresemann & Lyko, 2008).

This view is supported by several studies, which have shown that knockdown of DNMT by AZA/DAC is linked to demethylation of target gene promoters, and subsequent changes in gene expression (Lemarie et al., 2005; Yang, 2005). As previously discussed, AZA is commonly used as a chemotherapy drug, and is FDA approved. Aberrant patterns of methylation are present in some cancers, these can manifest as DNA hypermethylation with consequent silencing of tumor suppressor genes (Seelan, 2018). In general, AZA/DAC is thought to facilitate promoter demethylation and therefore reactivate silenced tumor suppressor genes. This phenomenon is observed at the promoters of several genes related to cancers such as breast cancer, colorectal cancer, and neuroendocrine tumors (NETs) (Sayar, 2015; Rawluszko-Wieczorek, 2015; Kim et al., 2015). Of particular interest, is the finding that treatment of various human NET cell lines with DAC (10 µM) for four days resulted in significant upregulation of neurotensin receptor 1 (NTSR1) mRNA and protein levels. The effect on NTSR1 mRNA ranged from 5- to as high as 57-fold induction of expression, depending on the cell line treated (Kim et al., 2015). Neurotensin is a neurotransmitter found in the CNS, moreover NTSR1 is a GPCR, also broadly distributed in the CNS, including the suprachiasmatic nucleus (Boules, 2013). These results lend to the idea that demethylation is an important regulator of GPCR expression in the CNS and corroborates the present findings with AZA induction of MT_1 mRNA expression. Furthermore, it can be expected that similar findings at the protein level could be obtained, however the lack of reliable antibodies for the rat melatonin MT_1

receptor is a hinderance. Future experiments could be conducted to assess the ability of AZA to induce MT_1 expression, mRNA and protein, in human cell lines such as neuroblastoma (SH-5YSY), or breast cancer (MCF7). It should be noted that, as seen with the NTSR1 results, the effect of demethylation varies quite dramatically by cell line or tissue type.

4.3 Treatment with Azacytidine diminishes DNMT1 protein and mRNA expression

In this study the ability of the demethylating agent, AZA, to inhibit, or downregulate DNMT1 was assessed. It was found that treatment with AZA at concentrations of 10 or 20 µM for 24 or 48 hours caused significant downregulation of DNMT1 protein levels in C6 cells. As shown in **Figure 15 B** and **C**, the 24-hour time point indicated a 75% knockdown of DNMT1 protein, where increasing the treatment time to 48 hours caused almost complete knockdown of the protein at the 20 µM concentration of AZA. This reflects the mechanism of action for AZA, as the DNMT protein becomes trapped, triggering its degradation (Stresemann & Lyko, 2008). Furthermore, the observed decrease in DNMT1 protein levels could also be due to the inhibition of DNMT1 transcription, as this phenomenon was seen at the mRNA level in the present study. RT-qPCR experiments showed a concentration-dependent knockdown of DNMT1 mRNA levels with as low as 1 µM AZA for 24 hours inducing an approximately 50% knockdown relative to control. This corroborates previous research that indicates DAC exposure (5 or 20 µM) to a mouse hippocampus derived cell line results in decreased DNMT1 mRNA, protein, and activity levels (Yang et al., 2017). Consequently, it can also be predicted that if activity assays were conducted to assess DNMT1 activity before and after AZA treatment in C6 cells, the activity would be significantly decreased. However, there is

some conflicting evidence concerning the effect of AZA on DNMT1. For example, Groshal et al. (2005) found that DNMT1 depletion is a post-translational event, as they observed no change in DNMT1 mRNA levels after treatment with DAC for 24 hours in HeLa cells. One explanation for the observed conflicts could be due to differences in cell line, treatment protocol, and analyses of RT-qPCR data.

In the present study, the inhibition of both DNMT1 mRNA and protein expression by similar concentrations of AZA supports our hypothesis that DNA demethylation plays a role in the regulation of the MT_1 receptor, consistent with the well-known effects of this epigenetic mechanism on gene transcription. While this study focused on induction of passive demethylation, via inhibition of DNMT1 by AZA, future experiments could examine mechanisms of active demethylation, such as the effects of AZA on TET proteins. Previous research has indicated that AZA is able to trigger TET2 or TET3-dependent demethylation *in vitro*, via upregulation of these TET proteins (Sajadian et al., 2015). Such studies assessing TET and MT_1 levels would elucidate the possible interplay of passive and active demethylation in the upregulation of the MT_1 receptor by AZA.

4.4 Effect of the combination of VPA and AZA on MT_1 expression

As discussed previously, HDAC inhibition by VPA or other drugs, upregulates melatonin receptor expression in cultured cells or in the rat brain. Given that these epi-drugs can also modify DNA methylation, it was of interest to determine whether combinatorial treatment with VPA plus AZA, results in enhanced induction of MT_1 expression, in order to better understand the mechanisms involved. VPA by itself performed similarly to what was formerly observed, as it caused a dramatic upregulation of MT_1 at the 3 mM concentration (Kim et al., 2008; Niles et al., 2012; Bahna et al., 2014).

The combination of VPA and AZA at concentrations of 3 mM and 5 µM respectively, induced an upregulation of MT_1 mRNA which suggested additivity, but this trend was not seen with transformed data. Interestingly, log transformation of data revealed additive effects when a lower dose of VPA and AZA were combined.

In order to obtain a better understanding of the impact of the combination treatment on MT_1 mRNA expression, replications and tests at different time points will be required. However, the present results are very promising and suggest a possible additive or synergistic interaction of HDAC inhibition by VPA and DNA demethylation by AZA, resulting in enhanced induction of MT_1 mRNA expression. This phenomenon has been observed previously in the context of cancer. As mentioned previously, Yang et al. (2005) observed synergistic induction of previously silenced genes (p57KIP2 and p21CIP1) in a leukemic cell line after treatment with a combination of DAC and VPA. Additionally, similar results were found when assessing the combination of DAC and phenylbutyrate in a murine leukemia cell line, where synergistic activation of the tumor suppressor gene p15CDN2B was observed after treatment for three days (Lemarie, 2004).

It is not clear whether HDACi-induced demethylation is essential for MT_1 upregulation, or if both mechanisms (HDACi and DNA demethylation) are required for the most powerful induction of MT_1. Currently, it appears that both mechanisms are involved in MT_1 induction; however, a more targeted approach may be beneficial in teasing out which of the two mechanisms is the driver of this process. If it were possible to identify an HDACi that had no DNA demethylating ability, for comparison against VPA, it could be used to determine if the DNA demethylating ability is necessary for the induction of MT_1 expression. Alternatively, use of a non-drug technique to knockdown HDACs, such

as shRNA, could be done to assess the DNA demethylating abilities of HDAC inhibitor drugs on MT_1 mRNA expression.

4.5 Future Directions

In addition to what was discussed above, it can be predicted that comparable results after treatment with AZA would be found for the MT_2 receptor, as Niles et al. (2012) determined that VPA caused significant induction of MT_2 in the rat hippocampus. However, MT_2 has low basal expression in C6 cells, as previously identified by Castro et al. (2005), and troubleshooting and use of the nested PCR strategy would likely have to be conducted similar to that of MT_1. It would be interesting to assess the effect of AZA on MT_2 because, the formation of MT_1/MT_2 heterodimers is important to the maintenance of the effects of melatonin and downstream signaling (Baba, 2013). Furthermore, many of the aforementioned melatonergic agonists affect the MT_2 receptor as well, such as PD-6735 and Ramelteon. MT_2 is also involved in age-related differences in expression that is observed for MT_1, therefore understanding the epigenetic regulation of this receptor, and how it interacts with MT_1 is equally important to identify (Sanchez-Hidalgo, 2009).

Taking the present findings that HDACi and DNA demethylation generate significant increases in levels of MT_1, future studies could assess how increasing MT_1 levels affects downstream melatonin signaling, or the effects of melatonergic agonists. For example, examining if activating MT_1 after treatment with AZA or an HDACi causes amplified inhibition of the cAMP-PKA-pCREB pathway, or if the presence of more MT_1 receptors causes enhanced activation of downstream genes such as GDNF (or other neurotrophic factors). Furthermore, the upregulation antioxidant genes downstream of the MAPK-ERK pathway, which is upregulated by melatonin signaling, such as activation

of NRF-2-ARE, could be targeted after triggering upregulation of MT_1 (Wang et al., 2012; Shin et al., 2015; Pan & Niles, 2015). Future studies such as this would be beneficial in identifying direct therapeutic benefits to epigenetic modulation of the melatonin receptors.

CHAPTER 5: CONCLUSIONS

5.1 Epigenetic regulation of the melatonin receptor

This thesis indicates for the first time that DNA demethylation, induced by AZA, stimulates melatonin receptor expression. These results suggest that DNA demethylation, induced by HDACi, could be part of the mechanism behind previous findings that treatment with VPA, or other HDACi, induce MT_1 mRNA and/or protein expression. Studies have shown a link between promoter demethylation following treatment with HDAC inhibitors and gene expression. For example, the HDAC inhibitors sodium butyrate (1 mM) and suberoylanilide hydroxamic acid (SAHA, 10 μM) can activate the tumor suppressor genes, p21 and RARB2 in LnCAP cells, by decreasing the methylation of their promoter regions (Sarkar et al., 2011). Additionally, Zhou et al. (2008) found that two different HDAC inhibitors, LBH589 and TSA, caused significant degradation of DNMT1 protein levels. These HDACi, when in combination with AZA (2.5 μM) induced a greater inhibition of DNMT1 than either drug alone in a breast cancer cell line.

Therefore, it appears that the observation of HDACi causing upregulation of the MT_1 receptor could involve the demethylating ability of HDACi, as supported by the present findings using AZA individually, and in combination with VPA. Future experiments could be conducted to confirm a change in the methylation status of the MT_1 promoter region after treatment with AZA and/or HDAC inhibitors, using appropriate methods such as methylation-specific PCR. This would further show that AZA-induced demethylation and HDAC inhibition have a direct effect on the MT_1 gene, and that these mechanisms drive the upregulation of the MT_1 receptor. Additionally, translating these

findings to other systems, such as human neuronal cell lines, and *in vivo* models of neurodegenerative diseases would provide more support of how these epigenetic mechanisms function in a human system. However, the evidence presented in this thesis supports the notion that the MT_1 receptor expression can be modulated by epigenetic mechanisms, including histone acetylation and DNA demethylation, which provide an interesting avenue for treatment of diseases involving this receptor, or other GPCRs.

5.2 Application of research to medicine

Understanding the interplay between these two mechanisms will generate knowledge about the epigenetic regulation of MT_1, and how these mechanisms can be exploited for potential therapeutic avenues aimed at diseases involving melatonin signaling and its receptor. As discussed previously, the expression of MT_1 and MT_2 receptors decreases with age and is significantly lower in patients with neurodegenerative disorders such as AD and PD (Brunner, 2006; Aldi, 2010). Furthermore, several lines of evidence indicate that signaling of melatonin via its receptors has neuroprotective effects. For instance, inhibition of mitochondrial dysfunction in AD, and α-synuclein assembly in PD, both of which are involved with disease progression (Dragicevic, 2011; Ono, 2012). Therefore, inducing melatonin receptor expression could have beneficial protective effects for patients with neurodegenerative disorders. This research also applies to depressive disorders, as patients afflicted have a reduction in levels of BDNF, and melatonin receptor agonism by agomelatine has anti-depressive effects (Martinotti, 2016; Liu, 2016). Upregulation of neurotrophic factors such as GDNF by melatonin has been shown in previous research, so perhaps treatment with melatonin or its agonists would also increase BDNF levels, and this effect would be strengthened by increased

expression of melatonin receptors (Armstrong & Niles, 2002). Melatonin receptors also have a protective role in breast cancer, as overexpression of MT_1 *in vitro* and *in vivo* has significant anti-tumor effects (Yuan, 2002; Collins, 2003). Recent evidence suggests, that melatonin inhibits breast cancer growth via activation of MT_1, and paclitaxel (a cytotoxic chemotherapy drug) resistance can be inhibited by the upregulation of MT_1 induced by the HDAC inhibitor TSA (Xiang, 2019). Therefore, the epigenetic mechanisms explored in this thesis will generate knowledge about the regulation of melatonin receptors, GPCRs, and other genes. Additionally, this project demonstrates how epigenetics can be applied as a promising avenue of treatment that can be explored for disorders involving genetic dysregulation.

REFERENCES

Armstrong, K.J. & Niles, L.P. (2002). Induction of GDNF mRNA expression by melatonin in rat C6 glioma cells. *Neuroreport, 13(4):* 473 – 475.

Baba, K., et al. (2013). Heteromeric MT1/MT2 Melatonin Receptors Modulate Photoreceptor Function. *Science Signaling, 6(296):* 1 – 27.

Bahna S.G., Sathiyapalan A., Foster J.A., Niles L.P. (2014). Regional upregulation of hippocampal melatonin MT receptors by valproic acid: therapeutic implications for Alzheimer's disease. *Neuroscience Letters, 576C*: 84–87.

Bahna, S.G. & Niles, L.P. (2017). Epigenetic induction of melatonin MT1 receptors by valproate: Neurotherapeutic implications. *European Neuropsychopharmacology, 27:* 828 – 832.

Bannister, A.J., Kouzarides, T. (2011). Regulation of chromatin by histone modifications. *Cell Research 21:* 381–395.

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. *Molecular Endocrinology, 10(7):* 892 – 902.

Bassani, T.B., et al. (2014). Neuroprotective and anti-depressant-like effects of melatonin in a rotenone-induced Parkinson's disease model in rats. *Brain Research, 1593:* 95 – 105.

Bayraktar G., Kreutz M.R. (2017). Neuronal DNA methyltransferases: epigenetic mediators between synaptic activity and gene expression? *Neuroscientist.*

Blask, D.E., Dauchy, R.T., Sauer, L.A., et al. (2003). Growth and fatty acid metabolism of human breast cancer (MCF-7) xenografts in nude rats: impact of constant light-induced nocturnal melatonin suppression. *Breast Cancer Research and Treatment, 79:* 313 – 320.

Blask, D.E., Brainard, G.C., Dauchy, R.T., et al. (2005). Melatonin-depleted blood from premenopausal women exposed to light at night stimulates growth of human breast cancer xenografts in nude rats. *Cancer Research, 65:* 11174 – 11184.

Bosetti, F., Brizzi, F., Barogi, S. *et al.* (2002). Cytochrome c oxidase and mitochondrial F1F0-ATPase (ATP synthase) activities in platelets and brain from patients with Alzheimer's disease. *Neurobiology of Aging, 23:* 371 – 376.

Boules, M., Li, Z., Smith, K., Fredrickson, P., Richelson, E. (2013). Diverse roles of neurotensin agonists in the central nervous system. *Frontiers in Endocrinology, 4(36):* 1 – 16.

Brunner, P. et al. (2006). Pineal and cortical melatonin receptors MT1 and MT2 are decreased in Alzheimer's disease. *European Journal of Histochemistry, 50(4):* 311 – 316.

Brydon, L., et al. (1999). Dual Signaling of Human Mel1a Melatonin Receptors via G_{i2}, G_{i3}, and $G_{q/11}$ Proteins. *Molecular Endocrinology, 13(12):* 2025 – 2037.

Burton, G.J. & Jauniaux, E. (2011). Oxidative stress. *Best Practice & Research Clinical Obstetrics & Gynaecology, 25(3):* 287 – 299.

Capitelli, C., Sereniki, A., Santos Lima, M.M., Reksidler, A.B., Tufilk, S., Frazao Vital, M.A.B. (2008). Melatonin attenuates tyrosine hydroxylase loss and hypolocomotion in MPTP-lesioned rats. *European Journal of Pharmacology, 594:* 101 – 108.

Carriere, C.H., Kang, N.H., Niles, L.P. (2016). Chronic low-dose melatonin treatment maintains nigrostriatal integrity in an intrastriatal rotenone model of Parkinson's disease. *Brain Research, 1633:* 115 – 125.

Castro, L.M.R., Galland, M., Niles, L.P. (2005). Novel targets for valproic acid: Up-regulation of melatonin receptors and neurotrophic factors in C6 glioma cells. *Journal of Neurochemistry, 95(5):* 1227 – 1236.

Chaste, P., et al. (2010). Identification of Pathway-Biased and Deleterious Melatonin Receptor Mutants in Autism Spectrum Disorders and in the General Population. *PLoS ONE 5(7):* e11495.

Collins, A., Yuan, L., Kiefer, T.L., Cheng, Q., Lai, L., Hill, S.M. (2003). Overexpression of the MT1 melatonin receptor in MCF-7 human breast cancer cells inhibits mammary tumor formation in nude mice. *Cancer Letters, 189:* 49 – 57.

Conway, S., Drew, J.E., Mowat, E.S., Barrett, P., Delagrange, P., Morgan, P.J. (2000). Chimeric melatonin mt1 and melatonin-related receptors. Identification of domains and residues participating in ligand binding and receptor activation of the melatonin mt1 receptor. *The Journal of Biological Chemistry, 275(20):* 602 – 620.

Davie, J.R. (2003). Inhibition of Histone Deacetylase Activity by Butyrate. *The Journal of Nutrition, 133(7):* 2485S – 2493S.

de Bodinat, C., Guardiola-Lemaitre, B., Mocaer, E., Renard, P., Munoz, C., Millan, M.J. (2010). Agomelatine, the first melatonergic antidepressant: discovery, characterization and development. *Nature Reviews Drug Discovery, 9(8):* 628 – 642.

Detich, N., Bovenzi, V., Szyf, M. (2003). Valproate Induces Replication-independent Active DNA Demethylation. *Journal of Biological Chemistry, 278:* 27586 – 27592.

Dhe-Paganon, S., Syeda, F., Park, L. (2011). DNA methyl transferase 1: regulatory mechanisms and implications in health and disease. *International Journal of Biochemistry and Molecular Biology, 2(1):* 58 – 66.

Dragicevic, N., et al. (2011). Melatonin treatment restores mitochondrial function in Alzheimer's mice: a mitochondrial protective role of melatonin membrane receptor signalling. *Journal of Pineal Research, 51(1):* 75 – 86.

Evans, P.H. (1993). Free radicals in brain metabolism and pathology. *British Medical Bulletin, 49(3):* 577 – 587.

Friedlander, R.M. (2003). Apoptosis and Caspases in Neurodegenerative Diseases. *The New England Journal of Medicine, 348:* 1365 – 1375.

Gooley, J.J. & Saper, C.B. (2017). *Chapter 33 – Anatomy of the Mammalian Circadian System.* In: Kryger, M., Roth, T., Dement, W.C. (eds) Principles and Practice of Sleep Medicine (6th Edition). Elsevier, Philadelphia, PA.

Groshal, K., Datta, J., Majumder, S., Bai, S., Kutay, H., Motiwala, T., Jacob, S.T. (2005). 5-Aza-Deoxycytidine Induces Selective Degradation of DNA Methyltransferase 1 by a Proteasomal Pathway That Requires the KEN Box, Bromo-Adjacent Homology Domain, and Nuclear Localization Signal. *Molecular and Cellular Biology, 25:* 4727 – 4741.

Hamdi, A. (1998). Melatonin administration increases the affinity of d2 dopamine receptors in the rat striatum. *Life Sciences, 63:* 2115 – 2120.

Heerboth, S., Lapinska, K., Snyder, N., Leary, M., Rollinson, S., Sarkar, S. Use of Epigenetic Drugs in Disease: An Overview. *Genetics & Epigenetics, 6: 9 – 19.*

Jablonska, K. et al. (2013). Expression of melatonin receptor MT1 in cells of human invasive ductal breast carcinoma. *Journal of Pineal Research, 54:* 334 – 345.

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. *European Journal of Pharmacology, 560:* 17 – 22.

Jeltsch, J. & Jurkowska, R.Z. (2016). Allosteric control of mammalian DNA methyltransferases – a new regulatory paradigm. *Nucleic Acids Research, 44(18):* 8556 – 8575.

Jockers, R., Maurice, P., Boutin, J.A., Delagrange, P. (2008). Melatonin receptors, heterodimerization, signal transduction and binding sites: what's new? *British Journal of Pharmacology, 154:* 1182 – 1195.

Jung, M., Brosch, G., Kolle, D., Scherf, H., Gerhauser, C., Loidl, P. (1999). Amide Analogues of Trichostatin A as Inhibitors of Histone Deacetylase and Inducers of Terminal Cell Differentiation. *Journal of Medicinal Chemistry, 42:* 4669 – 4679.

Kamal, M., et al. (2015). Convergence of Melatonin and Serotonin (5-HT) Signaling at MT2/5-HT2C Receptor Heteromers. *The Journal of Biological Chemistry, 290(18):* 11537 – 11546.

Kato, K. et al. (2005). Neurochemical properties of ramelteon (TAK-375), a selective MT1/MT2 receptor agonist. *Neuropharmacology, 48(2):* 301 – 310.

Kim, B., Castro, L.M.R., Jawed, S., Niles, L.P. (2008). Clinically relevant concentrations of valproic acid modulate melatonin MT1 receptor, HDAC and MeCP2 mRNA expression in C6 glioma cells. *European Journal of Pharmacology, 589:* 45 – 48.

Kim, H., & Bae, S. (2011). Histone deacetylase inhibitors: molecular mechanisms of action and clinical trials as anti-cancer drugs. *American Journal of Translational Research, 3(2):* 166 – 179.

Kim, J. et al. (2015). Differential expression and tumorigenic function of neurotensin receptor 1 in neuroendocrine tumor cells. *Oncotarget, 6(29):* 26960 – 26970.

Klein, D.C., et al. (1997). The Melatonin Rhythm-generating Enzyme: Molecular Regulation of Serotonin *N*-acetyltransferase in the Pineal Gland. *Recent Progress in Hormone Research, 52:* 307 – 358.

Kon, P.O. (2008). Melatonin attenuates the cerebral ischemic injury via the MEK/ERK/p90RSK/bad signaling cascade. *The Journal of Veterinary Medical Science, 70(11):* 1219 – 1223.

Korkmaz, A., Topal, T., Tan, D. (2009). Role of melatonin in metabolic regulation. *Reviews in Endocrine and Metabolic Disorders, 10:* 261 – 270.

Kusaczuk, M., Kretowski, R., Bartoszewicz, M., Cechowska-Pasko, M. (2016). Phenylbutyrate – a pan-HDAC inhibitor – suppresses proliferation of glioblastoma LN-229 cell line. *Tumor Biology, 37(1):* 931 – 942.

Lacoste, B., et al. (2015). Anatomical and cellular localization of melatonin MT1 and MT2 receptors in the adult rat brain. *Journal of Pineal Research, 58*: 397 – 417.

Lahiri, D.K., Ge, Y.W., Sharman, E.H., Bondy S.C. (2004). Age-related changes in serum melatonin in mice: higher levels of combined melatonin and 6-hydroxymelatonin sulfate in the cerebral cortex than serum, heart, liver and kidney tissues. *Journal of Pineal Research, 36(4):* 217 – 223.

Landolt, H.P., Wehrle, R. (2009). Antagonism of serotonergic 5-HT2A/2C receptors: mutual improvement of sleep, cognition and mood*? European Journal of* Neuroscience, 29: 1795 – 809.

Lauffer, B.E.L. et al. (2013). Histone Deacetylase (HDAC) Inhibitor Kinetic Rate Constants Correlate with Cellular Histone Acetylation but Not Transcription and Cell Viability. *The Journal of Biological Chemistry, 288(37):* 26926 – 26943.

Lemarie, M., Momparler, L.F., Farinha, N.J., Bernstein, M., Momparler, R.L. (2004). Enhancement of Antineoplastic Action of 5-Aza-2'-deoxycytidine by Phenylbutyrate on L1210 Leukemic Cells. *Leukemia & Lymphoma, 45(1):* 147 – 154.

Lemaire, M., Momparler, L.F., Bernstein, M.L., Marquez, V.E., Momparler, R.L. (2005). Enhancement of antineoplastic action of 5-aza-2'-deoxycytidine by zebularine on L1210 leukemia. *Anti-Cancer Drugs, 16(3):* 301 – 308.

Li, X. & Chen, B.D. (2009). Histone Deacetylase Inhibitor M344 Inhibits Cell Proliferation and Induces Apoptosis in Human THP-1 Leukemia Cells. *American Journal of Biomedical Sciences, 1(4):* 352 – 363.

Lin, L., Huang, Q., Yang, S., Chu, J., Wang, J., Tian, Q. (2013). Melatonin in Alzheimer's Disease. *International Journal of Molecular Sciences, 14:* 14575 – 14593.

Liu, J., Clough, S.J., Hutchinson, A.J., Adamah-Biassi, E.B., Popovska-Gorevski, M., Dubocovich, M.L. (2016). MT1 and MT1 Melatonin Receptors: A Therapeutic Perspective. *The Annual Review of Pharmacology and Toxicology, 56:* 361 – 383.

Long, H.K., Blackledge, N.P., Klose, R.J. (2013). ZF-CxxC domain-containing proteins, CpG islands and the chromatin connection. *Biochemical Society Transactions, 41(Pt 3):* 727 – 740.

Martinotti, G., et al. (2016). Agomelatine Increases BDNF Serum Levels in Depressed Patients in Correlation with the Improvement of Depressive Symptoms. *International Journal of Neuropsychopharmacology, 19(5):* 1 – 6.

Mas, A., Guillamon, J.M., Torija, M.J., Beltran, G., Cerezo, A.B., Troncoso, A.M., Garcia-Parilla, M.C. (2014). Bioactive Compounds Derived from the Yeast Metabolism of Aromatic Amino Acids during Alcohol Fermentation. *Journal of Biomedicine and Biotechnology:* 1 – 7.

Melamet, P., Yosefzon, Y., David, C., Tsukerman, A., Pneuli, L. (2018). Tet Enzymes, Variants, and Differential Effects on Function. *Frontiers in Cell Development and Developmental Biology, 6(22).*

Mellen, M., Ayata, P., Dewell, S., Kriaucionis, S., Heintz, N. (2012). MeCP2 Binds to 5hmC Enriched within Active Genes and Accessible Chromatin in the Nervous System. *Cell, 151(7):* 1417 – 1430.

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. *The World Journal of Biological Psychiatry, 11:* 148 – 153.

Monti, B., Polazzi, E., Contestabile, A. (2009). Biochemical, Molecular and Epigenetic Mechanisms of Valproic Acid Neuroprotection. *Current Molecular Pharmacology, 2:* 1 – 16.

Nebbioso, A., Carafa, V., Beneditti, R., Altucci, L. (2012). Trials with 'epigenetic' drugs: An update. *Molecular Oncology, 6:* 657 – 682.

Ng, K.Y., Leong, M.K., Liang, H., Paxinos, G. (2017). Melatonin receptors: distribution in mammalian brain and their respective putative functions. *Brain Structure and Function, 222:* 2921 – 2939.

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. *International Journal of Neuropsychopharmacy, 15:* 1343 – 1350.

Ono, K., Mochizuki, H., Ikeda, T., Nihira, T., Takasaki, J., Teplow, D.B., Yamada, M. (2012). Effect of melatonin on α-synuclein self-assembly and cytotoxicity. *Neurobiology of Aging, 33:* 2172 – 2185.

Oprea-Ilies, G. et al. (2013). Expression of melatonin receptors in triple negative breast cancer (TNBC) in African American and Caucasian women: relation to survival. *Breast Cancer Research and Treatment, 137:* 677 – 687.

Pala, D., Lodola, A., Bedini, A., Spadoni, G., Rivara, S. (2013). Homology Models of Melatonin Receptors: Challenges and Recent Advances. *International Journal of Molecular Sciences, 14:* 8093 – 8121.

Pan, Y. & Niles, L.P. (2015). Epigenetic mechanisms of melatonin action in human SH-SY5Y neuroblastoma cells. *Molecular and Cellular Endocrinology, 402:* 57 – 63.

Pang, S.F., Tsang, C.W., Hong, G.X., Yip, P.C., Tang, P.L., Brown, G.M. (1990). Fluctuation of blood melatonin concentrations with age: result of changes in pineal melatonin secretion, body growth, and aging. *Journal of Pineal Research, 8(2):* 179 – 192.

Pastor-Anglada, M., Molina-Arcas, M., Casado, F.J., Bellosillo, B., Colomer, D., Gil, J. (2004). Nucleoside transporters in chronic lymphocytic leukaemia. *Leukemia, 18:* 385 – 393.

Pevet, P. (2002). Melatonin. *Dialogues in Clinical Neuroscience, 4(1):* 57 – 72.

Phaniendra, A., Jestadi, D.B., Periyasamy, L. (2015). Free Radicals: Properties, Sources, Targets and Their Implication in Various Diseases. *Indian Journal of Clinical Biochemistry, 30(1):* 11 – 26.

Ravichandran, M., Jurkowska, R.Z., Jurkowski, T.P. (2017). Target specificity of mammalian DNA methylation and demethylation machinery. *Organic & Biomolecular Chemistry, 16(9).*

Rawłuszko-Wieczoreka, A.A., Horstb, N., Horbackab, K., Banduraa, A.S., Swiderskaa, M., Krokowiczb, P., Jagodzinski, P.P. (2015). Effect of DNA methylation profile on *OATP3A1* and *OATP4A1* transcript levels in colorectal cancer. *Biomedicine & Pharmacotherapy, 74:* 233 – 242.

Redman, J.R. & Francis, A.J.P. (1998). Entrainment of Rat Circadian Rhythms by the Melatonin Agonist S-20098 Requires Intact Suprachiasmatic Nuclei But Not the Pineal. *Journal of Biological Rhythms, 13(1):* 39 – 51.

Reiter, R.J., Tan, D., Burkhardt, S. (2002). Reactive oxygen and nitrogen species and cellular and organismal decline: amelioration with melatonin. *Mechanisms of Ageing and Development, 132:* 1007 – 1019.

Sajadian, S.O., et al. (2015). Induction of active demethylation and 5hmC formation by 5-azacytidine is TET2 dependent and suggests new treatment strategies against hepatocellular carcinoma. *Clinical Epigenetics, 7(1):* 98.

Sanchez-Hidalgo, M., Montavez, J.M.G., del Pilar Carrascosa-Salmoral, M., del Carmen Naranjo Gutierrez, M., Lardone, P.J., Alarcon De La Lastra Romero, C. (2009). Decreased MT1 and MT2 melatonin receptor expression in extrapineal tissues of the rat during physiological aging. *Journal of Pineal Research, 46(1):* 29 – 35.

Sarkar, S. Abujamra, A.L., Loew, J.E., Forman, L.W., Perrine, S.P., Faller, D.V. (2011). Histone Deacetylase Inhibitors Reverse CpG Methylation by Regulating DNMT1 through ERK Signaling. *Anticancer Research, 31:* 2723 – 2732.

Sayar, N., Karahan, G., Konu, O., Bozkurt, B., Bozdogan, O., Yulug, I.G. (2015). Transgelin gene is frequently downregulated by promoter DNA hypermethylation in breast cancer. *Clinical Epigenetics, 7(104):* 1 – 16.

Schomerus, C. & Korf, H.W. (2005). Mechanisms regulating melatonin synthesis in the mammalian pineal organ. *Annals of the New York Academy of Sciences, 1057:* 372 – 383.

Seelan, R.S., Mukhopadhyay, P., Pisano, M.M., Greene, R.M. (2018). Effects of 5-Aza-2'-deoxycytidine (decitabine) on gene expression. *Drug Metabolism Reviews, 50(2):* 193 – 207.

Sharma, R., Ottenhof, T., Rzeczkowska, P., Niles, L.P. (2008). Epigenetic targets for melatonin: induction of histone H3 hyperacetylation and gene expression in C17.2 neural stem cells. *Journal of Pineal Research, 45:* 277- 284.

Shankar, S. & Srivastava, R.K. (2008). *Histone Deacetylase Inhibitors: Mechanisms and Clinical Significance in Cancer.* In: Khosravi-Far, R., & White, E. (eds) Programmed Cell Death in Cancer Progression and Therapy. Springer.

Shieh, K.R., Chu, Y.S., Pan, J.T. (1997). Circadian change of dopaminergic neuron activity: Effects of constant light and melatonin. *NeuroReport, 8:* 2283 – 2287.

Shin, E., et al. (2015). Melatonin Attenuates Memory Impairment Induced by *Klotho* Gene Deficiency Via Interactive Signaling Between MT2 Receptor, ERK, and Nrf2-Related Antioxidant Potential. *International Journal of Neuropsychopharmacology, 18(6):* 1 – 14.

Stephan, L. & Momparler, R.L. (2015). Combination chemotherapy of cancer using the inhibitor of DNA methylation 5-aza-2'-deoxycytidine (decitabine). *Journal of Cancer Research & Therapy, 3(5):* 56 – 65.

Stevens, R.G., Brainard, G.C., Blask, D.E., Lockley, S.W., Motta, M.E. (2014). Breast Cancer and Circadian Disruption from Electric Lighting in the Modern World. *CA: A Cancer Journal for Clinicians, 64(3):* 207 – 218.

Stresemann & Lyko (2008). Modes of action of the DNA methyltransferase inhibitors azacytidine and decitabine. *International Journal of Cancer, 132(1).*

Suetake I., Watanebe M., Takeshita K., Takahashi S., Carlton P. (2017) *The Molecular Basis of DNA Methylation.* In: Kaneda A., Tsukada Y. (eds) DNA and Histone Methylation as Cancer Targets. Cancer Drug Discovery and Development. Humana Press, Cham.

Tan, D., et al. (2002). Chemical and Physical Properties and Potential Mechanisms: Melatonin as a Broad Spectrum Antioxidant and Free Radical Scavenger. *Current Topics in Medicinal Chemistry, 2:* 181 – 197.

Tan, H.H. & Porter, A.G. (2009). $P21^{WAF1}$ negatively regulates DNMT1 expression in mammalian cells. *Biochemical and Biophysical Research Communications, 382:* 171-176.

Tosini, G., Owino, S., Guillaume, J., Jockers, R. (2014). Understanding melatonin receptorpharmacology: Latest insights from mouse models, and their relevance to human disease. *BioEssays, 36(8):* 778 – 787.

Truong, T. et al. (2014). Breast cancer risk, nightwork, and circadian clock gene polymorphisms. *Endocrine-Related Cancer, 21:* 629 – 638.

Ulrey, C.L., Liu, L., Andrews, L.G., Tollefsbol, T.O. (2005). The impact of metabolism on DNA methylation. *Human Molecular Genetics, 14:* R139 – R147.

von Gall, C., Stehle, J.H., Weaver, D.R. (2002). Mammalian melatonin receptors: molecular biology and signal transduction. *Cell and Tissue Research, 309:* 151 – 162.

Wang, X. et al. (2008). Inhibitors of Cytochrome *c* Release with Therapeutic Potential for Huntington's Disease. *The Journal of Neuroscience, 28(38):* 9473 – 9485.

Wang, X. & Michaelis, E.K. (2010). Selective Neuronal Vulnerability to Oxidative Stress in the Brain. *Frontiers in Aging Neuroscience, 2(1):* 1 – 13.

Wang, Z. et al. (2012). Melatonin activates the Nrf2-ARE pathway when it protects against early brain injury in a subarachnoid hemorrhage model. *Journal of Pineal Research, 53(2):* 1 – 9.

Xiang, S., Dauchy, R.T., Hoffman, A.E., Pointer, D., Frasch, T., Blask, D.E., Hill, S.M. (2019). Epigenetic inhibition of the tumor suppressor ARHI by light at night-induced circadian melatonin disruption mediates STAT3-driven paclitaxel resistance in breast cancer. *Journal of Pineal Research, e12586:* 1 – 15.

Yang, H., Hoshino, K., Sanchez-Gonzalez, B., Kantarjian, H., Garcia-Manero, G. (2005). Antileukemia activity of the combination of 5-aza-2′-deoxycytidine with valproic acid. *Leukemia Research, 29(7):* 739 – 748.

Yin, X., Xu, Y. (2016). Structure and Function of TET Enzymes. In: Jeltsch A., Jurkowska R. (eds) DNA Methyltransferases - Role and Function. Advances in Experimental Medicine and Biology, vol 945. Springer, Cham.

Yoshida, T., et al. (2012). Decreased serum levels of mature brain-derived neurotrophic factor (BDNF), but not its precursor proBDNF, in patients with major depressive disorder. *PLoS One, 7*: e42676.

Yu, S. & Kim, S. (2015). DNA-hypomethylating agent, 5'-azacytidine, induces cyclooxygenase-2 expression via the PI3-kinase/Akt and extracellular signal-regulated kinase-1/2 pathways in human HT1080 fibrosarcoma cells. *International Journal of Oncology, 47:* 1469 – 1475.

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. *Molecular and Cellular Endocrinology, 192:* 147 – 156.

Zhang, Y., et al. (2013). Melatonin inhibits the caspase-1/cytochrome c/caspase-3 cell death pathway, inhibits MT1 receptor loss and delays disease progressing in a mouse model of amyotrophic lateral sclerosis. *Neurobiology Disease, 55:* 26 – 35.

Zhou, Q., et al. (2008). Inhibition of Histone Deacetylases Promotes Ubiquitin-Dependent Proteasomal Degradation of DNA Methyltransferase 1 in Human Breast Cancer Cells. *Molecular Cancer Research, 6(5):* 873 – 883.

Zlotos, D.P., Jockers, R., Cecon, E., Rivara, S., Witt-Enderby, P.A. (2014). MT_1 and MT_2 Melatonin Receptors: Ligands, Models, Oligomers, and Therapeutic Potential. *Journal of Medicinal Chemistry, 57:* 3161 – 3185.