EFFECTS OF INHIBITION OF DNA METHYLTRANSFERASE BY 5-AZACYTIDINE ON MANF AND CDNF EXPRESSION

M.Sc. Thesis

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TITLE: Effects of Inhibition of DNA Methyltransferase by 5-Azacytidine on MANF and CDNF Expression

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LAY ABSTRACT

Neurotrophic factors (NTFs) are small proteins that play a role in the survival and maintenance of cells within the nervous system. A new family of NTF proteins was recently identified: mesencephalic astrocyte-derived neurotrophic factor (MANF) and cerebral dopamine neurotrophic factor (CDNF). These proteins play a protective role on dopamine neurons, which are involved in neurodegenerative disorders such as Parkinson's disease. Epigenetics is the modification of gene activity without changing the DNA sequence. Using an epigenetic drug like 5-azacytidine (AZA), which can remove chemical (methyl) groups from DNA, the involvement of DNA demethylation was investigated in the modulation of MANF and CDNF. Treatment with AZA resulted in the increase of CDNF and decrease of MANF expression. The changes seen with CDNF and MANF mRNA expression following treatment indicates that DNA demethylation may play a role in the regulation of these NTFs. These results may provide novel therapeutic approaches for neurodegenerative diseases.

ABSTRACT

The role of neurotrophic factors (NTFs) in neuronal development, differentiation, neuroprotection and maintenance is well documented. The novel family of mesencephalic astrocyte-derived neurotrophic factor (MANF) and cerebral dopamine neurotrophic factor (CDNF) have been found to protect dopaminergic neurons, providing a potential therapeutic avenue for neurodegenerative diseases. Our group has previously shown an induction of NTFs including MANF and CDNF, following treatment with valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, in both cultured cells and rat brain. Furthermore, increased histone H3 acetylation was observed, indicating that epigenetic mechanisms may play a role in the modulation of MANF and CDNF. The interaction between HDAC inhibitors and DNA demethylation prompted us to investigate if DNA demethylation plays a role in the regulation of these NTFs. Treatment with 5-azacytidine (AZA; 1 – 25 μM), a potent DNA demethylating agent, for 24 hours, resulted in a significant increase in CDNF mRNA expression in rat C6 glioma cells. In the same time period, treatment with AZA resulted in a significant decrease in MANF mRNA expression. Furthermore, AZA decreased DNA methyltransferase 1 (DNMT1) mRNA and protein levels, suggesting a decrease in DNMT1 activity. Consistent with these findings, global DNA methylation was decreased following treatment with AZA. In view of the foregoing, the significant changes seen with CDNF and MANF mRNA expression following treatment with AZA, indicate that DNA demethylation may play a role in the regulation of these NTFs. These results may provide novel therapeutic approaches for neurological or related disorders and help to elucidate the mechanisms underlying the regulation of NTFs.

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LIST OF ABBREVIATIONS & SYMBOLS

ΔΔCt	Delta-delta cycle threshold
5-mC	5-methylcytosine
5-hmC	5-hydroxymethylcytosine
6-OHDA	6-hydroxydopamine
Aβ	Amyloid-beta
AD	Alzheimer's disease
ADP	Adenosine diphosphate
AKT	Protein kinase B
ALS	Amyotrophic lateral sclerosis
ANOVA	One-way analysis of variance
AP-1	Activator protein-1
APP/PS1	APP/PS1 are double transgenic mice expressing a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and a mutant human presenilin 1 (PS1-dE9), both directed to CNS neurons (Jackson Labs)
Armet	Arginine rich, mutated in early-stage tumors
ARTN	Artemin
ATF	Activating transcription factor
ATF-6	Activating transcription factor 6
ATP	Adenosine triphosphate
AZA	5-azacytidine
BDNF	Brain derived neurotrophic factor
BER	Base excision repair
Bip	Binding-immunoglobulin protein
C^5	Five-carbon
C^6	Six-carbon
Ca^{2+}	calcium
CDKN2B	Cyclin-dependent kinase 2 inhibitor B
cDNA	Complementary deoxyribonucleic acid
CHOP	C/EBP homologous protein
CLC	Cardiotrophin-like cytokine
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CNTF-R	Cilary neurotrophic factor – receptor
COX-2	Cyclooxygenase-2
CREB	cAMP response element-binding protein
CT-1	Cardiotrophin-1
DAC	5-aza-2'-deoxycytidine
DAergic	Dopaminergic
DM	Diabetes mellitus
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase

ER	Endoplasmic reticulum
ERAD	ER associated degradation
ERK-1/2	Extracellular regulated kinases-1/2
ERSE	ER stress response element
FSL	Flinders Sensitive Line
GABAergic	Gamma-aminobutyric acid-ergic
GDNF	Glial cell line-derived neurotrophic factor
GRFα	GDNF-family receptor-α
GRP78	Glucose regulated protein 78
HAT	Histone acetyltransferase
hCNTs	Human concentrative nucleoside transporters
HDAC	Histone deacetylase
hENTs	Human equilibrative nucleoside transporters
HO-1	Hemoxygenase 1
Hsp90	Heat shock protein 90
ICM	Inner cell mass
IL-1	Interleukin-1
IL-6, IL-11, IL-27, IL-37	Interleukins -6, -11, -27, -37
IRE1	Inositol requiring kinase 1
Jak/STAT	Janus kinases/signal transducer and activator of transcription proteins
JNK	c-Jun N-terminal kinase
KDEL-R	Endoplasmic reticulum protein retention receptor
LDLR	Low-density lipoprotein receptor
L-DOPA	Levodopa
LIF	Leukemia inhibitory factor
LIF-R	Leukemia inhibitory factor – receptor
LPS	Lipopolysaccharide
LTP	Long-term potentiation
MANF	Mesencephalic astrocyte-derived neurotrophic factor
MAPK	Mitogen-activated protein kinase
MeCP2	Methyl-CpG binding protein 2
MPP^+	1-methyl-4-phenylpyridinium
mRNA	Messenger Ribonucleic acid
MTT	[3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide]
NF-Y	Nuclear transcription factor-Y
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B
NGF	Nerve growth factor
NMDA	*N*-methyl-D-aspartic acid
NMR	Nuclear magnetic resonance
Nrf2	Nuclear factor erythroid 2-related factor
NRTN	Neurturin
NT-3	Neurotrophin-3
NT-4	Neurotrohpin-4
NTC	No template control
NTFs	Neurotrophic factors
OD	Optical density
$p75^{NTR}$	Neurotrophin receptor p75

PBS	Phosphate buffered saline
PD	Parkinson's disease
p-eIF2α	phosphorylation of eukaryotic translation initiation factor 2 subunit α
PERK	Protein kinase-like ER kinase
PI3K	Phosphoinositide 3-kinase
p-JNK	phospho - c-Jun N-terminal kinase
PKC	Protein kinase C
PSD95	Postsynaptic density protein 95
PSPN	Persephin
RET	REarranged during Transfection
RNA	Ribonucleic acid
RT-PCR	Reverse transcription – polymerase chain reaction
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SAP	SAF-A/B, Acinus and PIAS
SAPLIPs	Saposin-like proteins
SDS	Sodium dodecyl sulfate
SLC28	Solute carrier family 28
SLC29	Solute carrier family 29
SNpc	Substantia nigra pars compacta
TBP	TATA-box binding protein
TBS-T	Tris-Buffered Saline and Tween 20
TDG	Thymine-DNA-glycosylase
TET	Ten-eleven translocation
TGFβ	Transforming growth factor-β
TH	Tyrosine hydroxylase
TNF-α	Tumor necrosis factor-α
Trk	Tyrosine receptor kinase
TSA	Trichostatin A
UPR	Unfolded protein response
VMCL1	Ventral mesencephalic cell line 1
VPA	Valproic acid
XBP1	X-box binding protein 1

DECLARATION OF ACADEMIC ACHIEVEMENT

My research supervisor, Dr. Lennard Niles, designed all experiments for this project, including the primers used. I was responsible for drug preparation and preparing samples for treatments. I performed all RT-PCR and RT-qPCR, as well as ELISA experiments. With assistance from Emily Hartung and Mahnoor Shah, immunoblotting experiments were performed. Statistical analyses were performed with the assistance of Dr. Niles, with Emily Hartung assisting with some analyses.

CHAPTER 1 – INTRODUCTION

1.1 Neurotrophic Factors

Neurotrophic factors (NTFs) are small, secreted endogenous proteins that support survival and differentiation, especially during early development, as well as the maintenance of synaptic plasticity in the neuronal population (Castrén, 2013; Hefti et al., 1989). They are critical in mediating information to a neuron about the health and status of its contact or neighboring cell (Castrén, 2013). In order for molecules to be defined as neurotrophic factors, they must meet the current criteria: 1) support the survival of a specific developing neuronal population, 2) be synthesized and present in a biologically active form in target neuronal cells that require it for survival, 3) be released by the target neuronal cells in low quantities so that growing axons need to compete for access, thereby selecting the best innervation to the target cell (Castrén, 2013). Beginning with the first discovery of nerve growth factor in the early 1950s, more NTFs have been added and classified into families based on similar molecular characteristics and biological activities (Castrén, 2013). Classically, NTFs have been characterized into three large families: neurotrophins, neurokines and the GDNF family (Castrén, 2013). In recent years, a novel family has been discovered that displays distinct features compared to that of other NTFs. The founding member of the group, mesencephalic astrocyte-derived neurotrophic factor was discovered in 2003 (Petrova et al., 2003), while its homolog, cerebral dopamine neurotrophic factor was discovered in 2007 (Lindholm et al., 2007). Together they form a novel conserved family of neurotrophic factors.

1.1.1 Classical Families

Neurotrophins

The most highly documented and studied family amongst all NTF families is the neurotrophin family. Its most popular members are nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF). Other members included are neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). Collectively, they are thought to be derived from a common ancestral gene with similar structure and sequence (Huang & Reichardt, 2001). Neurotrophins appear to be found only within vertebrates, indicating their importance in higher level processing and less so with early development (Castrén, 2013; Chao, 2003). The synthesis of neurotrophins is derived from their precursors or proneurotrophins, which are about 30-35 kDa in size (Chao, 2003). The proteolytical cleavage of proneurotrophins give rise to the mature proteins that are about 12-13 kDa in size (Chao, 2003).

A common characteristic amongst the majority of the NTFs is their ability to bind to and activate members of the receptor tyrosine kinase family (Trk). BDNF and NT-4 typically bind to TrkB, while NGF binds to TrkA, and NT-3 binds to TrkC and could also bind to other Trk receptors with a lower affinity (Castrén, 2013). In addition to Trk receptors, all neurotrophins could also bind with a lower affinity to neurotrophin receptor p75 ($p75^{NTR}$) (**Figure 1**; Chao, 2003). Interestingly, its been found that when neurotrophins bind to $p75^{NTR}$, this typically leads to activation of cell death pathways. Proneurotrophins promote apoptosis, while mature neurotrophins promote cell survival. This balancing action could be attributed to neurotrophins', such as BDNF's, role in synaptic plasticity and long-term potentiation (LTP) (Chao, 2003).

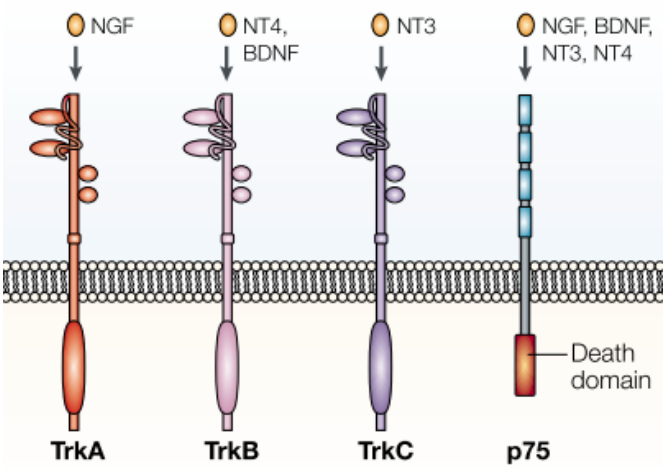

Figure 1. Depiction of neurotrophins and their associated receptors. Each neurotrophin binds selectively to its paired receptor. For example, NT-4 and BDNF selectively bind to TrkB receptor, while NGF binds to TrkA. All neurotrophins bind with a lower affinity to neurotrophin receptor p75 ($p75^{NTR}$), that when activated could result in neuronal apoptosis. Adapted from Chao (2003).

Neurokines

The neuropoietic cytokine family, or neurokines, include ciliary neurotrophic factor (CNTF), interleukins IL-6, IL-11, IL-27, IL-37, leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), and neuropoietin (Halvorsen & Kaur, 2006; Nathanson, 2012). Following the discovery of NGF, CNTF was isolated from the embryonic chick eye where it was found to increase the survival of ciliary neurons, making it the second NTF to be discovered (Halvorsen & Kaur, 2006; Nathanson, 2012). The action of neurokines vary depending on the developmental stage and the cell type (Halvorsen & Kaur, 2006). CNTF, for example, has been linked to promoting the survival of parasympathetic neurons and ciliary ganglion neurons (Halvorsen & Kaur, 2006; Hefti et al., 1989). All neurokines bind to a complex of receptor subunits composed of CNTF receptor (CNTF-R) α-subunit and transmembrane proteins, gp130 and LIF receptor (LIF-R) (Halvorsen & Kaur, 2006). LIF does not require the CNTF-R α-subunit in order to bind to the receptor complex, but it does compete with CNTF for binding (Halvorsen & Kaur,

2006). Signaling activities of neurokines are mediated by the Jak/STAT pathway, which could activate multiple signaling pathways depending on the cell type and tissue (Nathanson, 2012). Neurokines are critical proteins that are present in different types of cells and tissues with varying levels of expression and exerting various actions.

GDNF Family

The GDNF family is named after its founding member, glial cell line-derived neurotrophic factor (GDNF), and is a distant member of the transforming growth factor-β (TGFβ) superfamily (Airaksinen & Saarma, 2002). Other members include neurturin (NRTN), artemin (ARTN), and persephin (PSPN). GDNF was first purified from a rat glioma cell-line supernatant, followed by the purification and identification of other members in this family (Airaksinen & Saarma, 2002). All members of the GDNF family signal through RET (REarranged during Transfection) Trk receptors (Airaksinen & Saarma, 2002; Sidorova & Saarma, 2016). However, in order to activate RET, ligands need to bind to GDNF-family receptor-α (GFRα) receptors, which forms a receptor complex with RET (Airaksinen & Saarma, 2002; Sidorova & Saarma, 2016).

Various tissues synthesize and secrete these neurotrophic factors, which have distinct functions on many neuronal populations. GDNF and NRTN support dopaminergic neurons, cortical, basal forebrain cholinergic and motorneurons in the CNS (Sidorova & Saarma, 2016). In the periphery, they support enteric, sensory, parasympathetic and sympathetic neurons (Sidorova & Saarma, 2016). In non-neuronal tissues, GDNF functions to control kidney development and spermatogenesis (Airaksinen & Saarma, 2002; Sidorova & Saarma, 2016). Their diverse functions in neuronal (and non-neuronal) tissues makes them an ideal therapeutic candidate for diseases targeting areas, such as Parkinson's and addiction, where the dopaminergic pathway is known to be involved.

1.2 MANF/CDNF

Mesencephalic astrocyte-derived neurotrophic factor (MANF; also known as arginine rich, mutated in early-stage tumors or Armet) was first identified from the culture medium of a rat ventral mesencephalic cell line 1 (VMCL1) (Lindholm & Saarma, 2010; Petrova et al., 2003). It has been shown to be selective for dopaminergic neurons and not for GABAergic or serotonergic neurons that are all present in VMCL1 (Petrova et al., 2003). Cerebral dopamine neurotrophic factor (CDNF) was first identified using bioinformatic techniques, and was later shown to have protective effects on dopamine neurons (Lindholm et al., 2007). MANF and CDNF seem to selectively act upon dopaminergic neurons compared to other pathways, making them potential candidates for disorders/diseases affecting this pathway.

1.2.1 Structural Characteristics & Distribution

MANF and CDNF are small soluble proteins with a molecular weight of around 18 kDa (Lindahl et al., 2017; Petrova et al., 2003). CDNF is a paralog of MANF, sharing 59% amino acid identity with human MANF (Lindahl et al., 2017). Human MANF is 179 amino acids long and contains a signal peptide of 21 amino acids, where cleavage of this results in a 158 amino acid mature protein **(Figure 2A)** (Lindahl et al., 2017; Petrova et al., 2003). Human CDNF also contains a signal peptide that is 26 amino acids long and when cleaved results in a 161 amino acid mature protein from its primary sequence of 187 amino acids **(Figure 2A)** (Lindahl et al., 2017; Lindholm et al., 2007). The signal peptide functions to direct MANF/CDNF proteins to the endoplasmic reticulum during protein synthesis, and when cleaved, allows MANF/CDNF to be secreted (Lindahl et al., 2017). MANF and CDNF have dual functions: they could act to mitigate endoplasmic reticulum stress within the cell, and/or as a trophic factor in the extracellular space. In addition, MANF and CDNF do not contain a pro-sequence for enzymatic activation which is a

common characteristic amongst other NTFs (Lindahl et al., 2017). Another distinct feature compared to other NTFs, is their conserved spacing of eight cysteine residues which form four disulphide bridges (Lindahl et al., 2017). Crystal structure of MANF revealed a two-domain protein, where the N-terminal is similar to saposin-like proteins (SAPLIPs) which are known for their ability to interact with lipids and membranes (Parkash et al., 2009). While, NMR spectroscopy revealed the C-terminal to be homologous to SAF-A/B, Acinus and PIAS (SAP) protein superfamily (**Figure 2B**; Hellman et al., 2010). The two domains appear to carry out distinct functions that are partly due to their connection with a flexible linker that provides the freedom to orient the domains into different positions (Parkash et al., 2009).

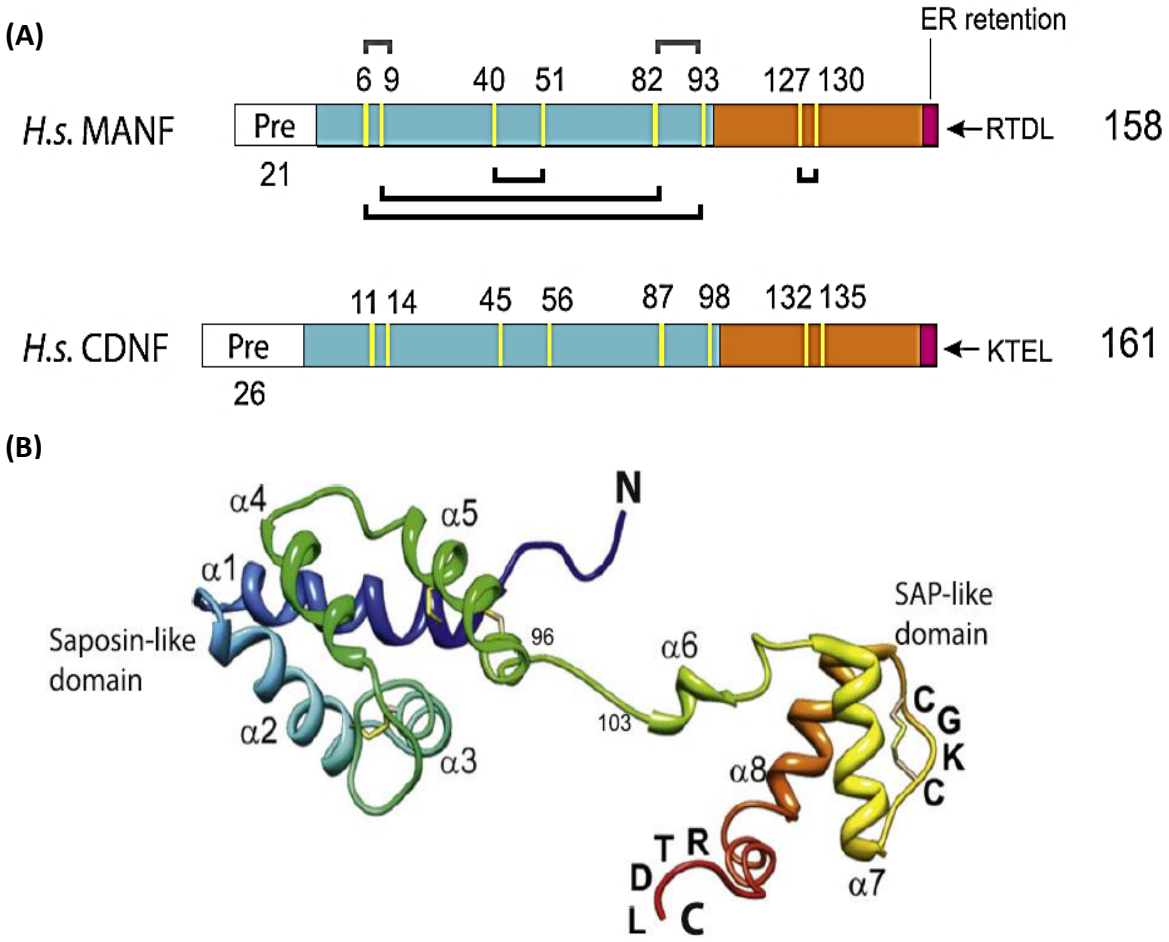

Figure 2. (A) The primary structure of human MANF and human CDNF. **(B)** NMR structure of MANF. (A) The SAPLIPs domain (N-terminal) of MANF and CDNF are depicted in blue and the SAP domain (C-termina) is depicted in orange. (B) NMR structure of MANF shows the N-terminal as indicated, consisting of the SAPLIP domain, the linker region between residues 96 – 103, and the C-terminal containing the SAP domain, ER retention signal (RTDL) and the CXXC motif. Adapted from (Lindahl et al., 2017).

MANF and CDNF are widely expressed in the central nervous system (CNS) and in non-neuronal tissues. In rat models, MANF expression was generally found to be the highest during early postnatal days, followed by a steady decline into adulthood (Wang et al., 2014). This suggests that MANF may play a role in the development of the CNS. In the brain, MANF expression was detected in cerebellar Purkinje cells, the cerebral cortex and hippocampus, where it was generally localized in neurons (Lindholm et al., 2008). High levels of MANF were also detected in secretory tissues such as the salivary glands, spleen, pancreas, liver and testis (Lindahl et al., 2017; Lindholm et al., 2008). CDNF expression, similar to MANF, was found to be primarily within neurons in the CNS (Lindahl et al., 2017). In mouse and human tissues, CDNF transcripts were detected in the brain and non-neuronal tissues (Lindholm et al., 2007). High levels of CDNF were found in the corpus collosum and optic nerve, as well as in the substantia nigra and striatum. At postnatal day 10, CDNF levels were found to be intense in the hippocampus and thalamus in mice. Furthermore, CDNF protein was detected in the Purkinje cells of the cerebellum and in the locus coeruleus, which is located within the brain stem. CDNF protein was generally observed in the cell somas which is consistent with its role as a secretory protein. In non-neuronal tissues, CDNF expression was high in the heart, skeletal muscle, salivary glands and testis in adult mice (Lindholm et al., 2007). CDNF's expression pattern is similar to MANF's throughout the CNS and periphery, however, they differ in intensity, where CDNF levels are generally lower compared to MANF (Lindahl et al., 2017; Lindholm et al., 2007).

1.2.2 ER Stress and MANF/CDNF Regulation

The endoplasmic reticulum (ER) is an organelle found in all eukaryotic cells, that is largely responsible for proper folding of membrane and secreted proteins (Lin et al., 2008). It is also the primary storage of calcium (Ca^{2+}) in the cell, where high levels of Ca^{2+} are required for protein folding reactions and molecular chaperone functions (Malhotra & Kaufman, 2007). Misfolded or partially folded proteins are either retained in the ER with molecular chaperones or directed towards degradation through an ER associated degradation (ERAD) pathway (Malhotra & Kaufman, 2007). Stresses such as the accumulation of misfolded proteins in the ER or increased secretory load can result in the saturation of the ER capacity to fold proteins, commonly referred to as ER stress (Lin et al., 2008). The accumulation of misfolded proteins in the ER leads to the activation of an adaptive signaling cascade known as the unfolded protein response (UPR), which mediates the restoration of ER homeostasis (Malhotra & Kaufman, 2007). If UPR is unable to restore proper balance in the ER, chronic activation of this response leads to apoptosis or cell death (Malhotra & Kaufman, 2007).

There are three ER-localized transmembrane regulators of UPR: protein kinases *inositol requiring kinase 1* (IRE1), double stranded RNA-activated *protein kinase-like ER kinase* (PERK), and the transcription factor *activating transcription factor 6* (ATF-6) (Malhotra & Kaufman, 2007). ATF-6α (an allele of ATF-6) works as a transcription factor, after cleavage into its active form, to induce expression of ER-stress related genes such as chaperones that function as a quality control in the ER (Lin et al., 2008). MANF has been found to be a downstream target of ATF-6α and X-box binding protein 1 (XBP1), a transcription factor activated by IRE1, where it requires either transcription factor for its expression (Lee et al., 2003). ATF-6α recognizes the ER stress response element (ERSE), typically found in the promoter regions of chaperone genes such as

glucose regulated protein 78 (GRP78), or in the presence of nuclear transcription factor-Y (NF-Y) (Mizobuchi et al., 2007). Within the MANF promoter region, ERSE-I and ERSE-II sequences, were identified in mediating MANF mRNA expression under ER stress (Mizobuchi et al., 2007). Compared to other transcription factor, XBP1, ATF-6α was found to have the most potent effect in activating ERSE-II in the promoter region of MANF (Oh-Hashi et al., 2013). In contrast, XBP1 preferentially binds to ERSE-I, regulating the transcription of MANF (Wang et al., 2018b). Moreover, it was found that MANF interacts with XBP1 at the promoter region to further regulate its own transcription (Wang et al., 2018b). The ability of XBP1 and ATF-6α in activating MANF provides insight into its regulation and expression under ER stress.

The functional domains of MANF and CDNF are important in regulating their trafficking and secretion. At the very end of the C-terminal of MANF and CDNF is the highly conserved RTDL (Arginine-Threonine-Aspartic acid-Leucine) and KTEL (Lysine-Threonine-Glutamic acid-Leucine) sequence, respectively, which resembles the standard ER-retention signal KDEL sequence (Lindahl et al., 2017). The human genome contains 3 KDEL receptor isoforms (KDEL1, KDEL2, and KDEL3) located in the Golgi, that generally function by binding to proteins that contain an ER retention signal and promoting their trafficking back to the ER (Henderson et al., 2013). Experiments involving the removal of the RTDL sequence from the C-terminal of MANF resulted in an increase in secretion of MANF via the secretory pathway (Henderson et al., 2013). In another study, authors have found that replacing the RTDL sequence with the KDEL sequence resulted in an increase of the intracellular amount of MANF while secretion decreased (Oh-Hashi et al., 2012). This suggests that the RTDL sequence has a relatively weak affinity for the KDEL receptor, which may be useful in balancing the retention and secretion of MANF (Glembotski et al., 2012; Henderson et al., 2013). In addition to the KDEL receptor (KDEL-R), GRP78 provides

another retention mechanism that regulates MANF's localization to the ER. In conditions that dysregulate ER calcium levels, such as myocardial infarctions or chemical compounds like thapsigargin, the strength of retention is reduced and MANF is secreted into the extracellular space (Glembotski et al., 2012). Once secreted, MANF functions to protect cells from apoptosis in an autocrine or paracrine manner, thereby promoting their survival. However, in normal conditions, MANF was primarily retained in the ER rather than secreted. Thus, the retention mechanism mediated by GRP78 is calcium dependent, while the mechanism of KDEL-R is calcium independent (Glembotski et al., 2012). Furthermore, CDNF secretion is regulated by KDEL-R and GRP78 in similar manner to MANF (Norisada et al., 2016).

The C-terminal domain of MANF and CDNF are important in regulating their trafficking and secretion. The structure of MANF and CDNF are dominated by α-helices; there are 3 α-helices in the C-terminal, where α-7 helix (149-154 amino acids) is important in regulating the secretion of CDNF (Liu et al., 2015b). Disruption of this helix resulted in an increase in secretion in both constitutive and regulated manner (Liu et al., 2015a). The importance of α-7 in regulating secretion could be due to the location of the CXXC motif, which will be discussed later, that is involved in ER-stress. Another study had found similar results, where removal/destruction of this helix impaired the regulated secretion of CDNF by affecting its sorting from the Golgi complex to an appropriate secretory granule, the constitutive secretion was not affected (Sun et al., 2011). In addition to α-7, α-1 was also observed to be critical for the trafficking of CDNF. Using an immunofluorescent microscopy technique, CDNF was found to be distributed within neurosecretory cells and neurons in a punctate manner. Destruction of α-1 resulted in a greater distribution of CDNF within the cell body and a decreased distribution at distal cell processes (Sun et al., 2011). This indicates that α-1 may play a role in the distribution and trafficking of CDNF,

whereby CDNF proteins with intact α-1 may lead to a concentrated distribution within the cell body. Helix α-1 is also critical in the constitutive and regulated secretion of CDNF. Destruction of α-1 led to the blockage of CDNF in the ER, thereby reducing constitutive and regulated secretion (Sun et al., 2011).

The expression of MANF and CDNF are enhanced by pathophysiological stimuli that induce ER stress. Both proteins play a role in attenuating ER stress by promoting cell survival, as will be discussed in the following sections. However, their expression in relation to ER stress is distinct. CDNF mRNA expression is constitutive and was found to be unaffected by ER stress in this particular study (Apostolou et al., 2008). In contrast, MANF's expression was observed to be upregulated in response to ER stress (Apostolou et al., 2008). Thus, MANF could be considered to function in an inducible manner (Apostolou et al., 2008).

1.2.3 Mode of Action

The precise mechanism(s) that MANF and CDNF use to exert their protective effects is unclear. Parkash et al. (2009) proposed that the C-terminal of MANF and CDNF may be involved in cytoprotection and ER stress response, while the N-terminal may be involved with neuroprotective function. The ability of the N-terminal to interact with lipids and membranes makes these proteins susceptible to transverse the lipid membrane into the extracellular space, where they could exert trophic factor effects (Parkash et al., 2009). In the C-terminal of MANF and CDNF, there are two important functional motifs; the conserved CXXC motif located between α7 and α8, and their respective ER retention signal (Mätlik et al., 2015; Parkash et al., 2009). The CXXC motif is important in the survival promoting ability of MANF both intracellularly and extracellularly, where mutation of this motif completely abolished its protective function (Mätlik

et al., 2015). The RTDL motif of MANF was found to be not important in carrying out its extracellular function as a survival promoting factor when applied *in vivo*, but is necessary for its intracellular function as a mediator of ER stress (Mätlik et al., 2015). The CXXC motif is necessary for MANF, and presumably CDNF, to accomplish its neuroprotection and intracellular function.

MANF expression pattern was found to be closely related to the expression of GRP78, a molecular chaperone that plays an important role in folding proteins (Mizobuchi et al., 2007). Under normal conditions, GRP78 retains ATF-6 and other regulators of UPR in the ER, thereby preventing their activation. During ER stress, GRP78 dissociates from ATF-6 , IRE1 and PERK which is then activated and proceeds to initiate ER-stress related genes (Glembotski et al., 2012).The ability of GRP78 to retain MANF in the ER, similar to ATF-6 and the other regulators, suggests that MANF may also be involved in a signaling pathway under ER stress (Glembotski et al., 2012). MANF functioning as a sensor or a signaling molecule in response to ER-stress is in agreement with a recent study where MANF was shown to have a rapid and robust secretion in a ER-stress model (Henderson et al., 2013). On the other hand, CDNF expression under ER-stress is shown to induce an early adaptive UPR. Expressing CDNF in primary rat hippocampal neurons and HEK293-T cells resulted an in early response evidenced by the induction of GRP78 expression, as well as ATF-6 and XBP1 suggesting that it activates the pathways of UPR (Arancibia et al., 2018). Furthermore, CDNF suppressed pro-apoptotic pathways, promoting cellular survival (Arancibia et al., 2018). The function of CDNF under ER-stress provides some insight into why it is an effective regulator of UPR and protective agent in neurodegenerative disease, which will be discussed in the following sections.

The induction of MANF and GRP78 under ER-stress suggests that there is an interaction between these two proteins. GRP78 regulates protein-folding through the binding and removal of

unfolded proteins, which is tightly regulated by the concentration of active GRP78 and the nucleotide bound to it (Yan et al., 2019). When GRP78 is bound to an ATP, an unfolded protein is exchanged for another at a high rate. In contrast, when an ADP is bound to the chaperone, an unfolded protein is stably bounded to GRP78. A longer interaction between the chaperone and the unfolded protein could be useful when the protein requires complex folding. MANF was found to have a role in maintaining protein-folding under ER stress through its interaction with GRP78. MANF's SAP domain was observed to bind to GRP78, modulating chaperone activities by stabilizing GRP78 in an ADP-bound state (Yan et al., 2019), and thereby promoting a longer interaction between unfolded proteins and GRP78. The findings from this study provide insight into a mechanism that MANF may act upon in its role in the UPR pathway.

The cytoprotection of MANF under ER-stress seems to involve nuclear factor erythroid 2-related factor (Nrf2), a transcription factor in the antioxidant response pathway (Zhang et al., 2017). Treatment with MANF led to a significant increase in Nrf-2 expression and its translocation to the nucleus where it induced the anti-oxidant gene, hemoxygenase 1 (HO-1), in a neurotoxin *in vitro* model (Zhang et al., 2017). MANF also was found to increase phosphorylation of AKT in a time and concentration dependent manner (Zhang et al., 2017). Nrf-2 is a downstream target of PI3K/AKT/GSK3β signaling pathway, thus the activation of Nrf-2 by MANF may be mediated by interacting with this pathway (Zhang et al., 2017). The anti-oxidant and protective effect of MANF could partly involve the activation of Nrf-2 in response to ER-stress.

1.3 Neuroprotective Effects of MANF/CDNF

The decline/dysfunction in neurotrophic factor levels may be related to neurodegenerative diseases, such as Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis (ALS) (Lindholm & Saarma, 2009). The ability of NTFs to affect viability and restore lost function in neuronal populations makes them an ideal therapeutic candidate for neurodegenerative diseases. CDNF and MANF have been found to influence dopaminergic neurons, providing a potential therapeutic avenue for neurological disorders that affect this system. Studies have shown that when MANF and/or CDNF are directly injected into the brain, they are able to induce a protective effect in various models, such as Parkinson's or cerebral ischemia. Such results provide evidence of the extracellular action of MANF and CDNF, although an associated receptor has not been identified yet. In addition, due to their selectivity and beneficial effects on dopaminergic neurons, as will be discussed in this section, MANF and CDNF are considered to be ideal therapeutic candidates with relatively similar effects as other NTFs in neurodegenerative models.

1.3.1 Alzheimer's Disease

Alzheimer's disease (AD) is the most common age-related neurodegenerative disease and the most common form of dementia (Finder, 2010; Zhou et al., 2016). The pathological characteristics of AD are the accumulation of amyloid-beta (Aβ) oligomers in the extracellular space and neurofibrillary tangles formed of hyperphosphorylated tau proteins (Finder, 2010). Clinical hallmarks of AD include a progressive impairment in memory, judgement, decision making, and orientation to physical surroundings (Nussbaum & Ellis, 2003). Although there are many theories on the cause of AD, the exact mechanism is still unknown. In addition, there are no effective treatments for this disease currently, thus finding an ideal therapeutic candidate that protects and restores function is greatly needed.

Experiments using APPswe/PS1de9 (APP/PS1) mice modeling AD found that treatment with CDNF protein and gene therapy enhanced long term memory, with no notable effect on Aβ plaque load and neurogenesis (Kemppainen et al., 2015). Interestingly, this effect was seen in the control and AD model mice, suggesting that CDNF may play a role in enhancing long-term memory consolidation in the un-diseased state. The same authors also found weak evidence of CDNF's involvement in synaptic plasticity. APP/PS1 mice receiving CDNF gene transfer showed an increase level of the presynaptic marker, synaptophysin, suggesting a possible involvement of CDNF in synaptic function (Kemppainen et al., 2015). The early stage of AD is characterized by memory deficits which is generally attributed to synaptic dysfunction as a result of Aβ oligomer accumulation in the extracellular space (Zhou et al., 2016). Protein aggregation and accumulation results in the activation of UPR as a result of ER stress. Since CDNF is known to mitigate ER stress, it is reasonable to conclude that it may have beneficial effects in attenuating Aβ cytotoxicity. A recent study had found that treatment with CDNF suppressed Aβ-induced expression of binding-immunoglobulin protein (Bip; also known as GRP78), phosphorylation of eukaryotic translation initiation factor 2 subunit α (p-eIF2α), and phosphorylation of c-Jun N-terminal kinase (p-JNK), proteins that are hallmarks of ER-stress, in primary rat hippocampal cells (Zhou et al., 2016). Furthermore, CDNF was able to alleviate Aβ-induced decrease of synaptic proteins, postsynaptic density protein 95 (PSD95; a post-synaptic protein) and synaptophysin (a presynaptic protein) providing support of its involvement in maintaining synaptic function (Zhou et al., 2016). The ability of CDNF to oppose the negative effects of Aβ through protecting synaptic function and reducing ER stress, suggests a potential therapeutic candidate in treating early stages of AD.

Since CDNF is a paralog of MANF and both are known to mitigate ER stress, it is likely that MANF could also attenuate Aβ-induced ER stress in an AD model. Treatment with Aβ

increased MANF expression in cultured cells, and the overexpression of MANF resulted in protection of cells from Aβ-induced toxicity, whereas a knockdown of MANF furthered Aβ toxicity (Xu et al., 2019). In addition to an upregulation of MANF, exposure to Aβ resulted in an increase in expression of UPR-related proteins, providing further evidence of the involvement of ER stress in AD pathogenesis (Xu et al., 2019). The same authors also found that overexpression of MANF resulted in the attenuation of Aβ-induced ER stress through the suppression of UPR markers such as BiP, ATF6 and C/EBP homologous protein (CHOP) (Xu et al., 2019). The results from this study suggests that ER stress is involved in the pathogenesis of AD which is typically attributed to cell death and neuronal loss. Thus, MANF could prove to be useful in the treatment of AD through its suppression of ER stress. Although treatment with MANF and CDNF in AD models have been shown to have neuroprotective effects, it is worthwhile to investigate the exact mechanism by which they do so.

1.3.2 Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disease after AD (Nussbaum & Ellis, 2003). Clinically, it is commonly marked with a cohort of motor symptoms, referred to as Parkinsonism, which includes bradykinesia, rigidity and tremor (Bartels & Leenders, 2009). A pathological hallmark of PD is the progressive loss of dopaminergic (DAergic) neurons especially in the substantia nigra pars compacta (SNpc) (Nussbaum & Ellis, 2003). The exact pathogenesis of PD is still unknown. Currently, there is no cure with most treatment plans addressing each symptom associated with the disease.

Levodopa (L-DOPA) is currently used to treat the symptoms of PD, but does not address the progressive loss of DAergic neurons associated with PD. In *in vivo* experiments, CDNF was

found to have a neuroprotective and a neurorestorative effect in a 6-hydroxy dopamine (6-OHDA; a hydroxylated analogue of dopamine) rat model of PD. Injection of CDNF or GDNF into the striatum, before infusion of 6-OHDA, resulted in a reduction of amphetamine-induced rotational behaviour at 2 (for both GDNF and CDNF) and 4 weeks (only CDNF) following infusion (Lindholm et al., 2007). In addition, CDNF and GDNF significantly increased the number of tyrosine hydroxylase (TH)-positive cell bodies in the SNpc, as well as the density of TH-positive fibers in the striatum (Lindholm et al., 2007). CDNF protected DAergic neurons from the toxic effects of 6-OHDA, and its effect was comparable to that of GDNF, which is well studied for its effect in PD models. CDNF may be an alternative, or possibly even better, candidate in treating PD.

In addition to its neuroprotective effect, CDNF was also found to restore DAergic neurons by preventing apoptosis of remaining neurons following infusion of 6-OHDA (Lindholm et al., 2007). Post-treatment with CDNF or GDNF into the striatum led to a reduced effect of amphetamine-induced rotational behaviour compared to control, similar to what was seen with pre-treatment of CDNF. Furthermore, the number of TH-positive cells in the lesion side of the SNpc was partially recovered by CDNF (58%) and GDNF (57%) (Lindholm et al., 2007). Thus, CDNF was able to protect remaining neurons from 6-OHDA-induced cell death, which could be useful in treating the progressive loss of neurons in the pathogenesis of PD. The neurorestorative effect of CDNF seen in the afore mentioned study is consistent with a more recent study, where chronic infusion (i.e. 14 days) of CDNF into the striatum resulted in a reduction of amphetamine-induced rotations and a partial protection of TH-positive cell bodies and fibers in the SNpc from degradation (Voutilainen et al., 2011). The authors compared the effect of CDNF, MANF and GDNF in a 6-OHDA rat model of PD, and interestingly, CDNF was the only NTF to have

significant neuroprotective effects in this *in vivo* experiment (Voutilainen et al., 2011). The lack of significant effect with MANF and GDNF could be due to the difference in the injection sites of the toxin (i.e. 6-OHDA) and the NTFs; 6-OHDA was injected into two sites of the left striatum of the rat models, while NTFs were injected in between the two sites (Voutilainen et al., 2011). Kirik et al. (2000) demonstrated that GDNF administered in the same location as the toxin resulted in a significant protective effect. Thus, it is possible that MANF and GDNF were not able to exert their neuroprotective effects in the former study because they were not injected in the same location as the toxin. The neuroprotective effect of CDNF, however, was observed regardless of administered location, suggesting that CDNF may be able to disperse and exert its action compared to other NTFs (Voutilainen et al., 2011). Furthermore, the distribution volume of CDNF, MANF and GDNF during chronic infusion was different; after 3 days of continuous infusion, MANF's distribution volume was larger than that of CDNF, which was larger than GDNF (Voutilainen et al., 2011). The rapid secretion of MANF is consistent with a previous study (Henderson et al., 2013), suggesting that MANF may be easily diffused in the brain compared to other NTFs. While the diffusion of a trophic factor is important, it is not a critical determinant of its therapeutic efficacy, as seen with the effect of CDNF compared to that of MANF and GDNF (Voutilainen et al., 2011).

Lewy bodies are fibrillar aggregates that are primarily composed of a soluble protein called α-synuclein, which is found in the presynaptic nerve terminal (Bartels & Leenders, 2009; Wakabayashi et al., 2013). It is thought that oligomers of α-synuclein induce cytotoxicity in the pathogenesis of PD (Wakabayashi et al., 2013). Thus, the modulation or the attenuation of the negative effects of α-synuclein aggregates may prove to be beneficial in treating PD. Treatment with CDNF resulted in the protection of neuronal cells from the toxicity effect of α-synuclein, by

increasing cell viability as shown by MTT and lactate dehydrogenase assays (Latge et al., 2015). Furthermore, the authors did not observe CDNF binding to α-synuclein, suggesting that CDNF may have attenuated the effect of α-synuclein through a survival signaling cascade (by binding to an unknown transmembrane receptor) or promoting cell survival through its interaction with Bax, a protein that induces apoptosis (Latge et al., 2015). As mentioned previously, CDNF, like MANF, has two functional domains such that it could induce different mechanisms in promoting cell survival.

The neuroprotective effect of CDNF was found to be through its attenuation of apoptosis mechanisms, rather than its inhibition. The neuroprotective and reversal effect of CDNF observed in PD studies was attributed to its modulation of apoptotic factors, Bcl-2/Bax, and activation of caspase-3 (Mei & Niu, 2014). In an *in vitro* experiment using 6-OHDA, pre- or post-treatment with CDNF resulted in increased cell viability in a dose-dependent manner. PC12 cells exposed to 6-OHDA were found to have a decrease in Bcl-2 expression, which is involved in preventing apoptosis, and an increase in Bax expression, a pro-apoptotic factor. However, the decrease in Bcl-2 expression was attenuated by treatment with CDNF, suggesting that CDNF exerts its neuroprotective and reversal effect through apoptosis-signaling (Mei & Niu, 2014).

GDNF is one of the most well-documented NTFs for its therapeutic potential in PD. GDNF was found to have neuroprotective effects on DAergic neurons and preserve motor functions in a rat model of PD (Kirik et al., 2000). The effect of GDNF and CDNF are comparable in PD models, thus a combinatorial treatment of these two factors may be more efficacious than alone. A combinatorial treatment of GDNF and CDNF resulted in an additive effect in restoring DAergic function compared to single treatment of either proteins (Voutilainen et al., 2017). This study also found that GDNF and CDNF may exert their effects, at least partially, through different

mechanisms: GDNF activated ERK-1/2 and AKT pathways but did not affect ER-stress markers, while CDNF activated the PI3K/AKT pathway and attenuated ER-stress as expected, as evidenced by the downregulation of GRP78 and a decrease in the phosphorylation of eIF2α (Voutilainen et al., 2017). The additive effect seen with submaximal doses of GDNF and CDNF, may provide a potential avenue for treatment of PD with possibly fewer side effects.

The combinatorial treatment of MANF and CDNF was also studied in a 6-OHDA rat model of PD (Cordero-Llana et al., 2015). However, instead of intra-striatal or intra-nigral injections, which are commonly utilized in other *in vivo* experiments, gene therapy involving lentiviral vectors was used. The authors found that expression of CDNF and MANF together had a synergistic effect in recovery of behavioural function, as well as a significant increase in TH-positive cell bodies in the SNpc and preservation of TH-positive terminals in the striatum (Cordero-Llana et al., 2015). The synergistic effect seen with MANF and CDNF suggests that they may act through different mechanisms. Interestingly, expression of MANF or CDNF alone did not induce neuroprotective effects as was reported in other studies (Cordero-Llana et al., 2015). However, this could be due to the parameters used in this study making it difficult to directly compare results with previous reports, as well as a more severe lesion was induced by the injection of 6-OHDA into two sites of the cortex striatum (Cordero-Llana et al., 2015), whereas in other studies, 6-OHDA was only injected into one site creating a moderate lesion.

1.4 MANF/CDNF in the Periphery

1.4.1 Anti-Inflammatory Effects

Inflammation could induce ER stress, thereby activating UPR. The three main pathways of UPR have been linked to modulating the induction of pro-inflammatory factors (Chen et al., 2015). MANF was detected in the peripheral white blood cells from patients with inflammatory diseases such as rheumatoid arthritis and systemic lupus erythematosus, suggesting that MANF may play a role in modulating the immune response (Chen et al., 2015). NF-kB and activator protein-1 (AP-1) are both key players in activating pro-inflammatory signaling pathways (Chen et al., 2015; Wang et al., 2018a). NF-κB is a transcription factor that is activated by pro-inflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor α (TNF-α) (Lawrence, 2009). Following activation, NF-κB signaling induces the expression of other inflammatory genes including cytokines and chemokines (Lawrence, 2009). Chen and colleagues (2015) demonstrated that MANF was significantly upregulated in patients with rheumatoid arthritis and systemic lupus erythematosus. The upregulation of MANF resulted in the suppression of NF-κB signaling through its interaction with the DNA binding domain of p65, an NF-κB subunit. The interaction between MANF and p65 was mediated through the C-terminal SAP-like domain of MANF. The findings suggest that MANF may be a novel negative regulator of NF-κB signaling, thereby attenuating inflammatory responses. In the same study, the authors also observed MANF re-localizing to the nuclei following treatment with an inflammatory stimulator and ER-stress inducer. This suggests that under such conditions, MANF may localize to the nuclei in order to exert its protective function. Knockdown of MANF resulted in the enhancement of p65 binding to NF-κB regulated genes (Chen et al., 2015).

AP-1 is another transcription factor that regulates the response to an inflammatory stimulus. It is composed of heterodimers or homodimers of proteins belonging to Fos, Jun, activating transcription factor (ATF) and MAF families (Shaulian & Karin, 2001; Wang et al., 2018a). Similarly to NF-κB, AP-1 could also be activated by pro-inflammatory factors such as TNF-α and IL-1 (Shaulian & Karin, 2001). Three putative binding sites were identified within the promoter region of MANF, though only one of the binding sites were effective in inducing MANF transcription (Wang et al., 2018a). AP-1, composed of subunits c-Jun and c-Fos, was found to upregulate MANF expression under inflammatory conditions through direct binding (Wang et al., 2018a). This finding provides evidence of MANF as a downstream target of AP-1, elucidating its regulation under inflammatory stimuli. The enhancement of MANF via AP-1 and consequently the suppression of NF-κB signaling, demonstrates its involvement in fine-tuning two of the most critical components (i.e. NF-κB and AP-1) of the inflammatory response.

Neuroinflammation is mediated by the production of pro-inflammatory factors including cytokines, chemokines and reactive oxygen species by microglia or astrocytes within the CNS (DiSabato et al., 2016). In rat primary microglial cells, CDNF expression was induced by an inflammatory stimulator, lipopolysaccharide (LPS) (Zhao et al., 2014). Pre-treatment of CDNF attenuated the upregulation of proinflammatory cytokines prostaglandin E2 and interleukin-1β by LPS (Zhao et al., 2014). In addition, CDNF was observed to alleviate LPS-induced inflammatory injuries by protecting cells from cytotoxicity. It is possible that CDNF may exert its anti-inflammatory effects through the modulation of the JNK pathway, as pre-treatment with CDNF significantly reduced the phosphorylation of JNK thus suppressing its signaling pathway (Zhao et al., 2014). Thus, MANF and CDNF seem to play a role in the immune response, which could be in addition to or part of their response to ER stress.

1.4.2 Diabetes Mellitus and Enteric Nervous System

As mentioned previously, high levels of MANF and CDNF mRNA and protein were detected in secretory tissues such as the pancreas and salivary glands, suggesting a possible involvement in organ function (Lindholm et al., 2007, 2008). MANF knockout mice had developed severe diabetes mellitus due to the progressive loss of insulin producing pancreatic β-cells (Lindahl et al., 2014; Voutilainen et al., 2015). Diabetes mellitus (DM) is a group of metabolic disorders that are characterized by hyperglycemia due to impairment in insulin secretion, action or both (Association, 2012). There are two broad categories for DM: Type 1 and Type 2 diabetes. Type 1 diabetes is generally caused by a progressive autoimmune destruction of pancreatic β-cells, while Type 2 is due to a combination of insulin resistance and deficient insulin secretion (Danilova & Lindahl, 2018). It has been theorized that a reduction in β-cell mass could be due to chronic activation of UPR (Danilova & Lindahl, 2018). In MANF deficient mice, activation of UPR genes and proteins was observed in pancreatic islets, suggesting that unresolved ER stress could contribute to the pathogenesis of DM (Lindahl et al., 2014). Furthermore, increased MANF levels were observed in young children recently diagnosed with DM, as MANF plays a key role in attenuating ER stress (Danilova & Lindahl, 2018). In conditional knockout mice, removal of MANF from the pancreas resulted in a similar diabetic phenotype with the global knockout model (Lindahl et al., 2014). Taken all together, chronic UPR activation seems to be involved in DM which consequently results in MANF upregulation. Loss of MANF leads to a severe form of DM, suggesting its role in the survival and proliferation of β-cells.

Interestingly, knockdown of CDNF in mice did not result in the development of severe diabetes, nor was poor survival and severe growth defects observed as was seen in MANF deficient mice (Lindahl et al., 2014, 2020). However, deletion of CDNF did have an effect on the

development of the enteric nervous system. CDNF knockdown mice at 1.5 or 3 months had fewer submucosal neurons than their wildtype counterparts (Lindahl et al., 2020). The degeneration of submucosal neurons was continued into 9 to 12 month mice, suggesting that CDNF plays an important role in the survival of these neurons (Lindahl et al., 2020). Although, MANF and CDNF are structurally similar and share a relatively similar expression pattern, the different biological effects of these proteins indicate that their unique mechanisms of action could be complementary.

Type 1 diabetes is characterized by an autoimmune response that leads to the loss of β-cells. It was recently demonstrated that MANF expression is upregulated in cytokine-stimulated human β-cells (Hakonen et al., 2018). Furthermore, administration of MANF resulted in increased survival of primary and clonal human β-cells from cytokine-induced cell death, which could be attributed to its attenuation of UPR by reducing the expression of ER-stress related genes. MANF was also found to suppress NF-κB signaling pathway by reducing the nuclear localization and phosphorylation of p65, a subunit of the NF-κB complex (Hakonen et al., 2018). These results are consistent with previous reports of MANF's function as a regulator of ER-stress and immune signaling pathways. Treatment with MANF could be useful in promoting the survival of β-cells in Type 1 diabetes.

1.5 Epigenetic Mechanisms

Epigenetics is classically defined as the study of any process that alters gene activity without changing the DNA sequence, leading to modifications that are heritable (Weinhold, 2006). Although each cell has the same genetic information, the diverse levels of gene expression that is found within a variety of cells and tissues differ largely in part due to epigenetic mechanisms (Moore et al., 2013). One such mechanism of altering gene expression is through chromatin modification. Chromatin is a complex of proteins, that includes histones and DNA that are tightly bound to fit into the nucleus (Weinhold, 2006). Generally, chromatin is present in two states: heterochromatin and euchromatin. Heterochromatin exists in the condensed form, with the DNA strands tightly wrapped around the histone cores, preventing gene transcription (Bannister & Kouzarides, 2011; Romanowska & Joshi, 2019). While, euchromatin exists in the decondensed form with the DNA strands loosely wrapped around the histone cores, allowing gene transcription to occur (Romanowska & Joshi, 2019).

Histone Acetylation

A major process that is involved in chromatin modification is histone acetylation, which is controlled by histone acetyltransferase (HAT) enzymes (acts as a co-activator) and histone deacetylase (HDAC) enzymes (acts as a co-repressor) (Vaissière et al., 2008). HAT enzymes catalyze the acetylation of histones, resulting in a "looser" chromatin structure (i.e. euchromatin) thus allowing transcriptional enzymes and proteins access to the DNA strand (Bannister & Kouzarides, 2011). On the other hand, HDAC enzymes act by deacetylating histones, returning the chromatin structure to that of a more tightly bounded form (i.e. heterochromatin) effectively silencing gene transcription (**Figure 3**; Vaissière et al., 2008).

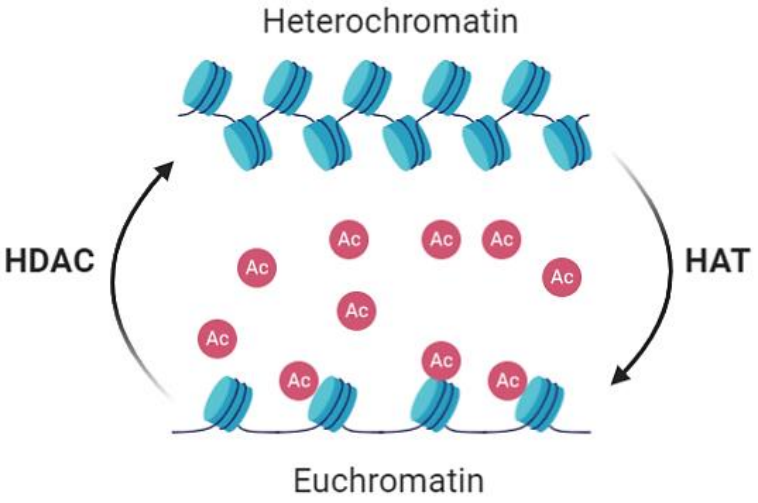

Figure 3. Depiction of histone acetylation. HAT enzymes add acetyl groups to histone tails, which results in a looser chromatin structure (euchromatin). While HDAC removes acetyl groups resulting in a tighter chromatin structure (heterochromatin).

DNA Methylation

DNA methylation is another major and extensively studied epigenetic mechanism, which is often used as a marker for silenced genes. This process is typically carried out by the covalent addition of a methyl group to the five-carbon (C^5) position of cytosine bases in CpG dinucleotides found along the DNA strand (Vaissière et al., 2008). Concentrated regions of CpG dinucleotides are known as CpG islands, which are mostly found within the promoter regions of genes (Gräff et al., 2011). Methylation of the cytosine bases is carried out by DNA methyltransferase (DNMT) enzymes, which are divided into two types: maintenance DNMT and *de novo* DNMTs. Maintenance DNMT or the isoform, DNMT1, preserves the methylation pattern between cell generations. Whereas *de novo* DNMTs, isoforms DNMT3a and DNMT3b, establish new methylation patterns that could be inherited in the daughter strand (Vaissière et al., 2008).

DNA Demethylation

DNA methylation was traditionally thought of as a permanent form of silencing transcriptional activity; however, recent evidence indicates that DNA methylation is a complex dynamic process that could be reversible (Gräff et al., 2011). DNA demethylation is thought to occur via two processes: active or passive demethylation. Passive demethylation involves the downregulation of DNMT1 in the new daughter cell, restoring cytosine bases to their original form (Santiago et al., 2014). On the other hand, active demethylation involves the removal of the methyl group from the cytosine base through enzymatic reactions which could occur in dividing or non-dividing cells (such as neurons) (Rasmussen & Helin, 2016). The discovery of the ten-eleven translocation (TET) enzymes provide a mechanism for active demethylation (Rasmussen & Helin, 2016). The TET enzymes (isoforms: TET1, TET2, & TET3) mediate DNA demethylation by catalyzing the conversion of 5mC (5-methylcytosine) to 5hmC (5-hydroxymethylcytosine), an intermediate product in the TET-mediated demethylation process (Santiago et al., 2014). Subsequent steps result in the formation of 5-carboxylcytosine, removal of this oxidized cytosine base by thymine-DNA-glycosylase (TDG) and restoration via the base excision repair (BER) pathway returning the cytosine base to its unmodified form (Rasmussen & Helin, 2016).

TET Enzymes

The TET family are found in various organs and tissues, where they play important roles in development, cell differentiation and stem cell function. TET-1, through its demethylating function, has been implicated in regulating transcription factor, NANOG, which plays a role in maintaining the pluripotent state of ESC cells (Santiago et al., 2014). Moreover, the level of TET expression varies depending on the stage of development. For example, TET-3 expression is the highest in oocytes and zygotes followed by a steady decline until cells form an inner cell mass

(ICM) in blastocysts where TET-1 and TET-2 levels start to increase in ICM and undifferentiated embryonic stem cells (ESC) (Rasmussen & Helin, 2016). During differentiation, TET-1 levels follow a steady decline while TET-2 and TET-3 expression is consistent or steadily increases till the three germ layers (Rasmussen & Helin, 2016). The differential levels of TET enzymes during embryogenesis suggests that they each may play a unique role during different stages.

DNA Methylation & Gene Transcription

The inhibition of gene transcription through DNA methylation is thought to happen through either direct or indirect repression. Direct repression involves the blockage of transcription factor access to promoter regions due to methylated CpG sites (Bird, 2002). In contrast, indirect repression involves the recruitment of proteins, such as methyl-CpG binding protein 2 (MeCP2) that bind to methylated DNA via the methyl-CpG-binding domain (Bird, 2002; Vaissière et al., 2008). Binding of MeCP2 to methylated DNA results in the recruitment of HDACs, effectively inhibiting transcriptional activity and resulting in a deacetylated repressive chromatin structure (Bird, 2002; Vaissière et al., 2008). Thus, it has been suggested that there is an interplay or crosstalk between histone deacetylation and DNA methylation resulting in effective gene transcription repression.

1.6 Epigenetic Drugs: DNMT Inhibitors

1.6.1 Azanucleosides

5-azacytidine (AZA) and 5-aza-2'-deoxycytdine (DAC) are cytosine analogs that are widely used in cancer therapies (**Figure** 4; Stresemann & Lyko, 2008). AZA and DAC are effective in inhibiting DNMT function by incorporating into DNA and/or RNA, leading to the loss of methylation markers (Christman, 2002). Due to its ribonucleoside structure, AZA is a less potent DNA methylation inhibitor compared to DAC, and more likely to be incorporated into RNA, affecting protein synthesis (Stresemann & Lyko, 2008). Once incorporated into the DNA, AZA and DAC have similar mechanisms of action. Generally, AZA and DAC are metabolized into the active nucleotide, 5-aza-2'-deoxycytidine-5'-triphosphtate, where they are then incorporated into the DNA strand **(Figure 5)**. DNMTs recognize azacytosine-guanine dinucleotides as a natural substrate and initiate the addition of methyl groups. This leads to the formation of a covalent bond with DNMT enzymes and six-carbon (C^6) atom of the cytosine ring. In normal circumstances, the bond is resolved by beta-elimination through the 5-carbon (C^5) atom. However, azacytosines contain a nitrogen in place of C^5, resulting in effectively trapping DNMT enzymes through the covalent bond. The trapping of DNMT enzymes results in the blockage of DNA functionality, thereby triggering DNA damage signaling leading to the degradation of the enzymes (**Figure 6**; Stresemann & Lyko, 2008). As a consequence, cellular DNMT enzymes are depleted and methylation marks are lost during replication, resulting in passive DNA demethylation. In addition to passive DNA demethylation, AZA was also been found to play a role in active DNA demethylation through the induction of TET enzymes, as will be discussed in later sections (Sajadian et al., 2015).

The effectiveness of azanucleosides depends on the expression of nucleoside transporters (Damaraju et al., 2012; Stresemann and Lyko, 2008). Cellular uptake of nucleoside and their analogs are typically mediated by two families of nucleoside transporters: human equilibrative nucleoside transporters (hENTs) or the SLC29 family and the human concentrative nucleoside transporters (hCNTs) or the SLC28 family (Damaraju et al., 2012). Thus, the presence and concentration of nucleoside transporters on the cell membrane are crucial components of AZA and DAC's function.

In addition to its effect on DNMT1, AZA has been noted to induce demethylating changes through the activation of signaling pathways. Cyclooxygenase-2 (COX-2) is an inducible enzyme that plays a role in the inflammatory pathway, and is linked with the production of pro-inflammatory factor, prostaglandin E2 (PGE_2) (Yu & Kim, 2015). Treatment with AZA induced COX-2 expression in human fibrosarcoma HT-1080 cells through the process of DNA demethylation, as a DNA methylation agent was observed to antagonize AZA's effect on COX-2 expression (Yu & Kim, 2015). Interestingly, ERK-1/2 and AKT was phosphorylated following treatment with 10 µM of AZA, and this was evident after 1 hour of treatment (Yu & Kim, 2015). Pre-treatment with inhibitors of PI3K/AKT and ERK-1/2 inhibited AZA-induced COX-2 expression, suggesting that PI3K/AKT and ERK-1/2 pathways play a role in the induction of COX-2 expression by AZA (Yu & Kim, 2015). Another study had also found that treatment with AZA (10 µM) activated ERK-1/2, which was associated with the upregulation of low-density lipoprotein receptor (LDLR) mRNA and protein expression in human hepatic cell lines (Mnasri et al., 2018). The findings from these studies suggest that AZA may be able to activate PI3K/AKT and ERK-1/2 pathways, which may induce changes in addition to epigenetic modification.

Figure 4. Azanucleoside structures of azacytidine and decitabine compared to cytidine. (Modified from Gnyszka et al., 2013).

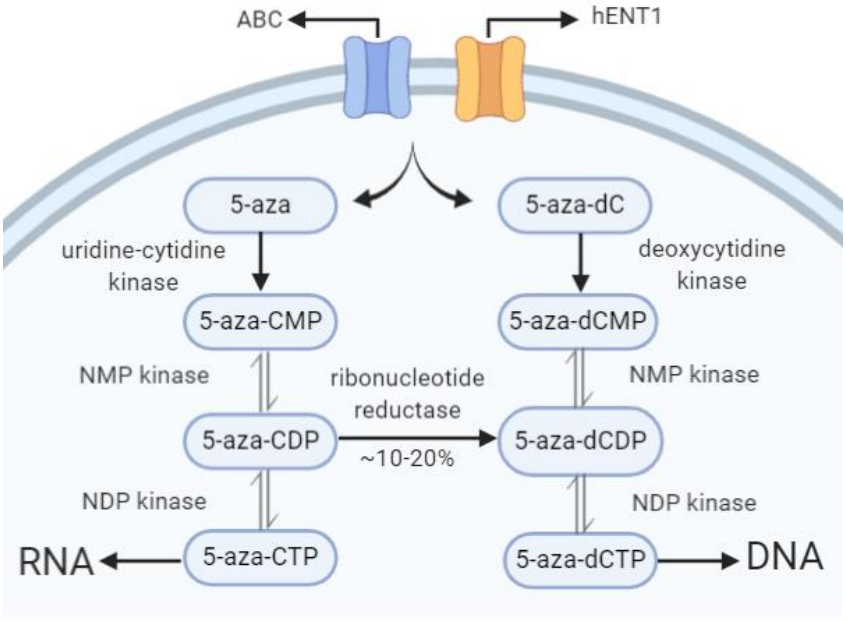

Figure 5. Depiction of the different metabolic pathways that 5-azacytidine (5-aza) and 5-2'-deoxycytidine (5-aza-dC) use once absorbed into the cell. Possible transporter candidates, such as human equilibrative nucleoside transporter 1 (hENT1) or the ATP-dependent exporters (the ABC family), mediate the movement of 5-aza or 5-aza-dC into the cell. Ribonucleotide reductase is the rate-limiting enzyme for AZA to be converted to *5-aza-deoxyribonucleotides*. Both 5-aza and 5-aza-dC are converted into the active nucleotide, *5-aza-2'-deoxycytidine-5'-triphosphtate* (5-aza-dCTP), followed by incorporation into the DNA strand. (Modified from Stresemann and Lyko, 2008).

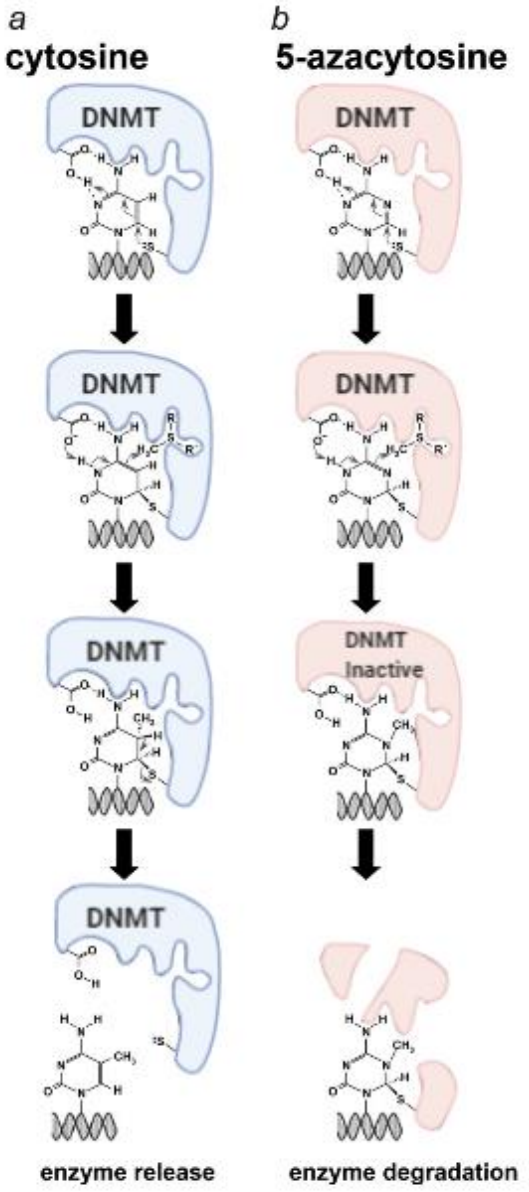

Figure 6. Interaction between DNMT1 enzyme and 5-azacytidine. (A) DNMT1 catalyzing the addition of a methyl group to the 5-carbon of the cytosine base. Addition of the methyl group leads to repression of gene transcription. (B) 5-azacytidine is incorporated into the DNA strand, and a covalent bond is formed with the DNMT1 enzyme. This results in the trapping of the enzyme and triggering DNA damage signaling, which leads to the degradation of DNMT1 enzymes. (Modified from Stresemann and Lyko, 2008).

1.7 HDAC Inhibitors and DNA Demethylation

The interaction between histone deacetylation and DNA methylation, suggests that HDACs may exert epigenetic changes through processes separate from the modification of histone proteins. HDAC inhibitors such as valproic acid (VPA), have been implicated in inducing DNA demethylation (Detich et al., 2003). Treatment with VPA resulted in a dose-dependent demethylation of ectopically methylated CMV-GFP plasmid transfected into human embryonal kidney 293 cells (Detich et al., 2003). Furthermore, different classes of HDAC inhibitors, such as sodium butyrate or suberoylanilide hydroxamic acid, reversed the methylation status of tumor suppressor genes (Sarkar et al., 2011). These results are consistent with another report that showed HDAC inhibitor, trichostatin A (TSA) inducing global DNA hypomethylation and promoter specific demethylation (Arzenani et al., 2011). In addition, DNMT1 protein levels were found to decrease following TSA treatment in human hepatocellular carcinoma Hep3B cells (Arzenani et al., 2011). This finding suggests that the demethylation effect of HDAC inhibitors may involve the modulation of DNMT1 levels. In support of this, it has been recently observed that treatment with HDAC inhibitor LBH589, induced proteasomal degradation of DNMT1 in human breast cancer cells (Zhou et al., 2008). DNMT1 associates with molecular chaperone heat shock protein 90 (Hsp90), which functions to provide stability and transcriptional regulation of DNMTs (Nagaraju et al., 2017). Treatment with LBH589 resulted in the acetylation of Hsp90, which led to its dissociation from DNMT1 and the subsequent ubiquitin-degradation of DNMT1 (Zhou et al., 2008). The findings from these studies provide evidence that HDAC inhibitors can contribute to DNA demethylation, leading to the transcriptional activation of previously suppressed genes. Furthermore, the ability of different classes of HDAC inhibitors in inducing DNA demethylation suggests that it is a universal effect of HDAC inhibitors.

The action of HDAC inhibitors in inducing DNA demethylation could be through a process different from that of DNMT inhibitors, as combinatorial treatments with both of these inhibitors caused a synergistic reactivation of silenced genes (Primeau et al., 2003; Yang et al., 2005). A combination treatment of 5-aza-2'-deoxycytidine (DAC - a DNMT inhibitor) and depsipeptide (a HDAC inhibitor) in human breast carcinoma cell lines resulted in a prominent increase of maspin, a tumor suppressor gene, compared to effects seen with either drug alone (Primeau et al., 2003)(Primeau et al., 2003). Similar findings were seen in leukemic cell lines HL-60 and MOLT4, where combination treatment of VPA and 5-AZA-CdR resulted in a greater induction of previously silenced genes compared to treatment with either drug (Yang et al., 2005). The link between DNA demethylation and histone acetylation in gene activation could provide a viable target in regulating gene expression as a form of treatment for various diseases. It is possible that modulation of both DNA methylation and histone acetylation may play a role in the induction of NTFs.

1.8 Epigenetic Modulation of Neurotrophic Factors – Previous Research

Recent reports demonstrate the modulation of NTF expression through epigenetic changes, which could prove to have beneficial effects in the treatment of neurodegenerative or neurological disorders. HDAC inhibitor, VPA, is a branched short-chain fatty acid that is widely used in treatments of epilepsy and bipolar disorder due to its anti-convulsive and mood-stabilizing effects (Löscher, 1999; Phiel et al., 2001). Although it has been widely studied in different context, its exact mechanism of action is still unclear. It is thought to have multiple kinase targets, and it could also enhance GABAergic function, while attenuating excitatory neurotransmitter (e.g. NMDA) release (Löscher, 1999; Monti et al., 2010). Furthermore, VPA has also been shown to downregulate expression of PKC and modulate CREB, leading to various changes intracellularly (Phiel et al., 2001). VPA has also been reported to induce NTF expression in *in vitro* and *in vivo*

models. Treatment with VPA significantly increased GDNF and BDNF mRNA expression in rat C6 glioma cells (Castro et al., 2005). Moreover, chronic treatment with VPA elevated the expression of CDNF, MANF, GDNF and PSPN in rat hippocampus (Niles et al., 2012). A significant increase in CDNF, MANF and BDNF levels were also observed in the striatum (Niles et al., 2012). The findings from these studies provide support for NTFs being a potential target of VPA, providing an explanation for this drug's neuroprotective effect.

The implication of NTFs being a target of VPA provides a possible avenue to explore in the context of neurodegenerative and/or neurological disorders. GDNF has been extensively studied for its protective effects in PD models, recently, it has been recently demonstrated that it could be induced in response to treatment with HDAC inhibitors. Treatment with VPA, sodium butyrate and TSA induced the expression of GDNF and BDNF in primary rat cortical astrocytes (Wu et al., 2008). The upregulation of GDNF was associated with increased acetylation at its promoter region following treatment with all three HDAC inhibitors (Wu et al., 2008). The authors also found that treatment with VPA, sodium butyrate and TSA had protective effects on dopamine neurons in the presence of neurotoxin, 1-methyl-4-phenylpyridinium (MPP^+) in neuron glia culture, suggesting that histone acetylation may a role in neuroprotection (Wu et al., 2008). The observed effects in this study provide evidence of NTFs being targets of HDAC inhibitors, as structurally diverse inhibitors were able to induce their expression. Furthermore, histone acetylation was shown to promote the survival of neuronal cells against a neurotoxin, which may be associated with the elevation of NTFs.

The transcriptional regulation of NTFs may be associated with epigenetic mechanisms, as seen in reports where histone acetylation induced their expression. DNA demethylation has been implicated in the modulation of GDNF and BDNF expression as well. In human U251 glioma

cells, 5-azacyditine (5 µM) was shown to significantly decrease methylation levels in the promoter region of GDNF, which was associated with an increase in GDNF mRNA expression at similar doses (Yu et al., 2013). GDNF transcription may be modulated by epigenetic mechanisms, as both HDAC and DNMT inhibitors were able to increase its expression. HDAC inhibitor, sodium butyrate was found to have a demethylating effect *in vivo* as evidenced by an increase in 5hmC levels at the BDNF promoter 4 region, which was associated with an upregulation of BDNF mRNA levels (Wei et al., 2015). Thus, the modulation of NTFs by epi-drugs, both HDAC and DNMT inhibitors, suggests that an epigenetic mechanism may underlie their transcriptional regulation. The ability to modulate the expression of NTFs has implications for the possible treatment of neurodegenerative and/or neurological disorders.

1.9 Rationale, Hypothesis & Specific Aims

Rationale

The importance of NTFs in the development, maintenance and survival of the nervous system is well documented. MANF and CDNF, although a novel family, have already been shown to have protective effects in neurodegenerative models such as PD and AD, and other diseases. Though they are known to be localized within the ER and play an important role in mitigating ER stress, the understanding of their regulation and mode of action is still limited. Recent findings have indicated that MANF and CDNF expression could be regulated by epigenetic mechanisms. VPA, an HDAC inhibitor, was shown to induce MANF and CDNF expression in neural stem cells and in rat brain (Almutawaa et al., 2014; Niles et al., 2012). Thus, it is possible that the modulation of these proteins could be mediated by epigenetic mechanisms. As discussed previously, DNA methylation could involve HDAC enzymes in gene repression. Therefore, it may be that DNA methylation could also play a role in the transcriptional regulation of MANF and CDNF **(Figure**

7). Modulating the regulation of such proteins with the use of epigenetic drugs, may provide a therapeutic avenue that could be used in disease models like PD.

Hypothesis

Inhibition of DNA methylation will alter neurotrophic factor gene expression.

Specific Aims

1. Examine the effects of DNMT inhibition by AZA on the mRNA or protein expression of DNMT1 and TET enzyme isoforms.
2. Examine the effects of AZA on global DNA methylation and/or the methylation status of selected NTF promoters.
3. Examine the expression of CDNF and MANF after treatment with AZA, in a time- and concentration-dependent manner.
4. Examine the combinatorial effects of AZA/other DNMT inhibitors plus VPA or other HDAC inhibitors, on the expression of the above NTFs.

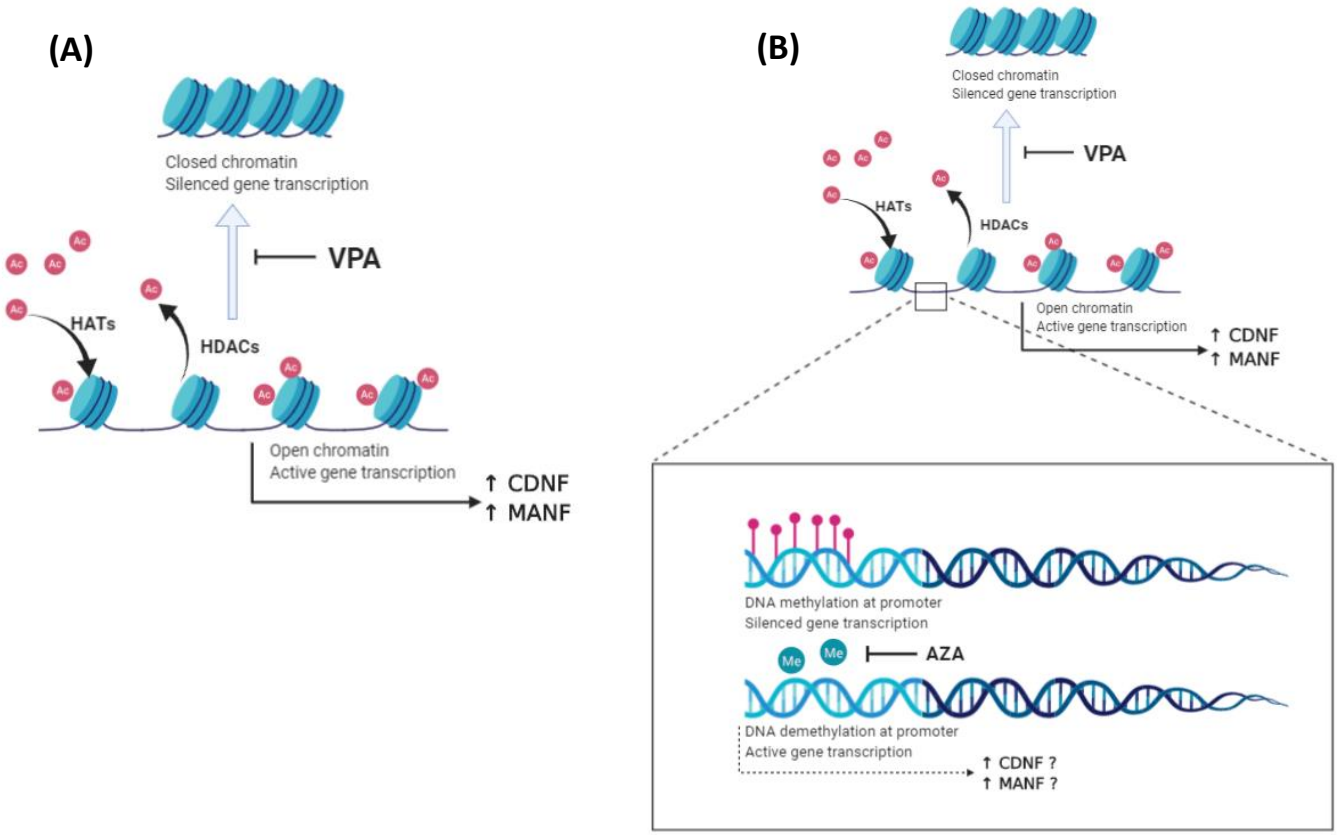

Figure 7. Overview of thesis rationale. **(A)** Previous studies have shown that VPA could induce CDNF and MANF expression by the inhibition of HDAC enzymes. **(B)** In light of the interaction between HDAC enzymes and DNA methylation, we sought to investigate the effects of DNMT inhibitors on the expression of CDNF and MANF.

CHAPTER 2 – MATERIALS & METHODS

2.1 Cell Culture and Treatments

The rat C6 glioma cell line was used to investigate the above hypothesis. Cells were grown on 10-cm Corning culture dishes (Fisher Scientific Ltd., Nepean, ON, CA) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), fungizone and penicillin/streptomycin, as reported previously (Kim et al., 2008). Cells from passages 15-25 were seeded at a density of $10^4/cm^2$ and maintained at 37°C in a humidified incubator with 5% CO_2/air.

Different culture environments were tested to select an optimal culture condition for drug treatments **(Table 1; Figure 8)**. Based on the results **(Figure 8)**, condition #1 was chosen as the culture condition for this study since basal expression of tested trophic factors were not upregulated compared to other conditions.

5-azacytidine (AZA) was obtained from Sigma Aldrich (Oakville, ON, CA). Before every drug treatment, a fresh 100 mM stock of 5-azacytidine was prepared in 100% DMSO, then working stocks of 50 mM and 10 mM were prepared in 100% DMSO. Using condition #1 parameters, cells were treated with AZA at concentrations of 1, 5, 10, 20 and 25 µM for 24 hours, at a confluency of about 60-70%. AZA vehicle (0.05 % DMSO) controls were included for each experiment.

Table 1. Different culture conditions that were tested in selecting the most optimal condition.

Condition #	Environment
1	Maintain in 10 % FBS and treat when confluency is at 60-70%
2	Change to 1% FBS at 40% confluency for 24 hours then treat at 70% confluency
3	Change to 1% FBS at 20% confluency then treat at 70% confluency
4	Seed in 1% FBS then treat at 70% confluency

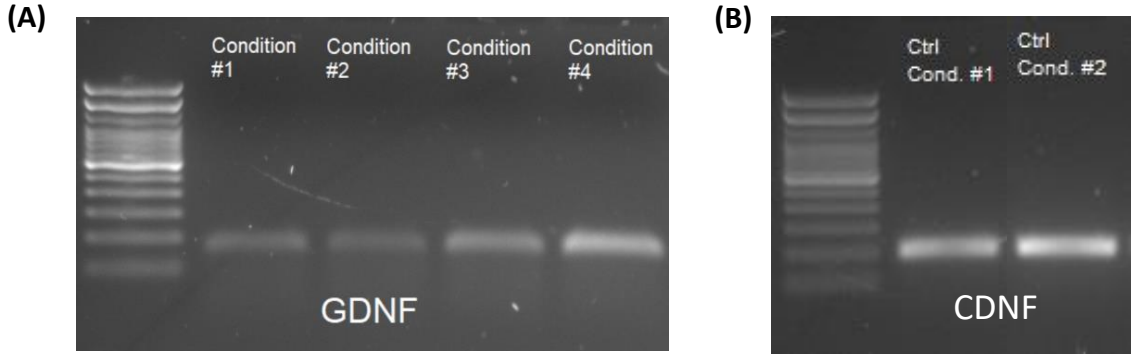

Figure 8. Comparing different culture conditions on the basal expression of trophic factors following treatment. (A) Four different types of culture conditions were tested to assess basal levels of trophic factors. Condition #1 and Condition #2 seemed to be the best contenders in not upregulating basal levels of the tested trophic factor, GDNF. (B) Comparing condition #1 to condition #2 on the basal expression of CDNF. Condition #1 had the least effect on the basal expression of CDNF, thus, it was selected as the culture condition for this study.

2.2 Cell Counting/Trypan Blue Assay

In order to assess viability, the Trypan Blue assay was used. Briefly, cells were brought into suspension using 1 mL of Trypsin, then re-suspended in 3-4 mL of media. 10 µL of cell suspension was mixed with 90 µL of Trypan Blue for a final dilution of 1:10. After vortexing for a few seconds, 10 µL of the cell suspension with the Trypan Blue was loaded onto a prepared hemocytometer where a light microscope was used to count cells. Dead cells were identified as cells with a blue stain and a disrupted membrane, while alive cells were identified as cells that did not have a blue stain, were bright and had an intact membrane. To calculate the viability percentage, the number of viable cells (or alive cells) was divided by the total number of cells (number of alive + number of dead cells), multiplied by 100.

2.3 RNA Extraction and cDNA Synthesis

Following drug treatment, total RNA was isolated using 1mL TRIzol as per instructions of the supplier (Invitrogen Canada Inc., Burlington, ON, CA). Briefly, following the addition of TRIZol, cells were homogenized using a 21G needle and 200 µL of chloroform was added in order to separate total RNA from the rest of the solution. Following separation, total RNA was precipitated using 100% isopropanol, where samples were incubated at room temperature for 10 mins then spun at 13 000 rpm in 4°C. The pellet was resuspended and washed with 1 mL of 75% ethanol at least twice. The pellet was resuspended in 20-25 µL of RNase-free water and incubated at 55 – 60°C for 15 mins. RNA concentration was determined using the NanoDrop One spectrophotometer and purity was assessed using the $A_{260/280}$ and $A_{260/230}$ ratios (ThermoFisher Scientific).

DNase treatments were done in order to ensure purity of RNA samples and remove any traces of DNA residuals. Master mixes of DNase I and DNase I Reaction Buffer reagents (ThermoFisher Scientific) were prepared with 25 µg of RNA sample. For RT-PCR assays, cDNA was synthesized from ~2µg DNase-treated RNA using the Omniscript Reverse Transcriptase Kit (Qiagen Inc. Canada, Mississauga, ON, CA) and Oligo(dT) primers as done previously (Castro et al., 2005). Whereas for RT-qPCR assays, cDNA was synthesized from ~1ug of DNase-treated RNA, using the SensiFAST cDNA Kit (FroggaBio, Toronto, ON, CA). The thermal cycler was set up using the following protocol: 25°C for 10 min, then 42°C for 15 min, followed by an inactivation step of 85°C for 5 min and held at 4°C. cDNA product was stored at -20°C until use.

2.4 RT-PCR

In order to save time while optimizing RT-qPCR, initial assessments of basal target gene expression under different cell culture conditions, were performed using a standard RT-PCR protocol. Generally, RT products were amplified using the HotStarTaq Master Mix Kit (Qiagen Inc. Canada, Mississauga, ON, CA). The thermal cycler was programmed with an initial heat activation of the HotStarTaq DNA polymerase at 95°C for 15 min. Following this, samples were amplified at 94°C for 30 sec, about 55-59°C for 30 sec, 72°C for 1 min, with a final extension period at 72°C for 10 min. As previously reported, negative template controls containing RNA without reverse transcription or cDNA were processed to confirm RT-PCR specificity and the absence of DNA contamination (Castro et al., 2005). Furthermore, housekeeping genes such as 18S ribosomal RNA or TATA-box binding protein (TBP), were amplified as an internal control. Agarose (2%) gels stained with SYBR Safe DNA Gel Stain (Invitrogen Canada Inc., Burlington, ON, CA) were used to separate amplified cDNA bands. These gels were scanned using an AlphaImager 2200 system (Alpha Innotech Corp.).

2.5 RT-qPCR

For each set of primers used, temperature gradient tests were performed to determine optimal melting and annealing temperatures, while efficiency tests were completed to determine amplification efficiency and primer validation. RT products were amplified using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Ltd, Mississauga, ON, CA). Before each assay, master mixes were prepared containing SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories Ltd, Mississauga, ON, CA), a final primer concentration of 500 nM and 100 ng of cDNA template. Assays were usually done in duplicates in a final volume of 20 µL of the master mix. Generally, the following protocol was used: initial heat activation at 95°C

for 30 sec, followed by denaturation at 95°C for 15 secs and annealing/extension at 60°C for 30 sec for 40 cycles. Then 95°C for 10 secs, ending with a melt curve of 65°C for 5 secs to 95°C for 5 secs. Internal controls, such as 18S ribosomal RNA or TATA-box binding protein (TBP), were used in each experiment **(Table 2)**. No template controls (NTCs - without the cDNA template), were also included, along with melt curve analysis, to confirm specificity and absence of contamination (Niles et al., 2012).

Table 2. Primer sequences used in RT-qPCR

Gene	Primers (5' -> 3')	Nucleotides	Size (bp)
MANF	GGCGACTGCGAAGTTTGTAT CGATTTTCTTTGCCTCTTGC	133 – 152 269 – 250	137
CDNF	AAAGAAAACCGCCTGTGCTA TCATTTTCCACAGGTCCACA	289 – 308 487 – 468	199
TET-1	ACATTGCTGGAGACTGTCGA TTCTGTCACGGCCATCTTCT	5406 – 5425 5567 – 5548	162
DNMT1	CCTGGAGAACGAACACTCT CATGGTCTCACTGTCCGACT	113 – 132 275 – 256	163
18S RNA	CGTTCTTAGTTGGTGGAGCG AACGCCACTTGTCCCTCTAA	1341 – 1360 1468 – 1449	127
TBP	CTCAGTTACAGGTGGCAGCA CTCAGTGCAGAGGAGGGAAC	1264 – 1283 1343 – 1324	80

2.6 Global DNA Methylation Analysis

Total genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen Inc. Canada, Mississauga, ON, CA) according to manufacturer's instruction. Briefly, cells were collected and spun at 300 x *g*, followed by resuspension in 200 µL of PBS. Proteinase K was added to the samples, where they were then lysed using the provided lysis buffer (Buffer AL) and incubated at 56°C for 10 mins. Before transfer into the provided spin columns, 200 µL of ethanol (96-100%) was added to each sample. Samples were spun at 8 000 rpm for 1 min and flow-through was discarded. They were subsequently washed with the provided wash buffers; Buffer AW1 was added and samples were spun at 8 000 rpm for 1 min, then Buffer AW2 was added, where samples were spun at 14 000 rpm for 3 mins. DNA was eluted using the provided elution buffer (Buffer AE) which was added to the center of the spin columns, followed by an incubation period of 1 min at room temperature and then spun at 8 000 rpm for 1 min. This step was done twice in order to increase DNA yield. DNA concentration was determined using a NanoDrop One, and quality was assessed using the $A_{260/230}$ and $A_{260/280}$ ratios. DNA samples were stored at -80°C for use the next day.

Following extraction, the DNA Colorimetric Quantification Kit (Abcam, Cambridge, UK) was used to assess global methylation status or 5-methylcytosine (5-mC) content. To summarize, binding solution was added to each well, followed by the addition of the provided negative and positive control, and 200 ng of gDNA. The plate was then incubated at 37°C for 90 mins, then washed and incubated with the capture antibody at room temperature for 60 mins. The plate was washed again and incubated with the detection antibody at room temperature for 30 mins, followed by a wash and incubation of the enhancer antibody at room temperature for 30 mins. After

incubation, the plate underwent a final wash and the developer solution was added. The stop solution was shortly added, and absorbance was read at 450 nm.

Relative global DNA methylation levels were determined by calculating the percentage of 5-mC in a sample. The following formula was used:

$$\text{5-mC\%} = \frac{(Sample\ OD - Negative\ Control\ OD) \div S}{(Positive\ Control\ OD - Negative\ Control\ OD)\ x\ 2 \div P}\ x\ 100\%$$

Where S is the amount of sample DNA in ng, P is the amount of positive control in ng (this was 5 ng in all experiments) and 2 is a factor that normalizes 5-mC in the positive control to 100%.

2.7 Nuclear Protein Preparation

In order to assess the status of DNMT1 protein expression following treatment with AZA, western blots were performed. Nuclear protein fractions were extracted from treated cells and purified using the following technique. Cells were harvested in lysis buffer containing 0.1% NP-40, then spun at 3 000 rpm for 10 min. Once the supernatant containing the cytosolic fraction was removed, the nuclear pellets were resuspended in a buffer containing 4.6M NaCl which aids in forcing the DNA extract into solution. The samples were homogenized using a glass homogenizer, then by a 21G needle. Following homogenization, samples were incubated on ice for 30 mins – 1 hour, and finally, spun at 14 000 rpm for 35 mins.

2.8 Protein Quantification

The purified nuclear protein fractions were then quantified using a DC Assay protocol (Bio-Rad Laboratories Ltd, Mississauga, ON, CA). A standard curve using bovine serum albumin (BSA) standards was created, with concentrations ranging from 0.2 mg/ml to 1.4 mg/ml. This was used to determine protein concentration for each assay. Using a spectrophotometer, absorbances of standards and sample protein were read at 750nm. Absorbances of the standards were plotted versus their known protein concentration. The equation of the line generated from the standard curve was used to calculate sample protein concentration.

2.9 Western Blot

Nuclear extract (15ug) prepared in 2X SDS solution were loaded onto a 10% Acrylamide gel, that was set to run for 30 mins at 60 volts then for 2 hours (or until the ladder was well separated) at 100 volts. A PVDF membrane was used for transfer, which was set up at 25 volts for 20 hours or overnight in 4°C. Following transfer, the membranes were blocked in a Tris-Buffer Saline and Tween (TBS-T) with 5% milk blocking solution for 1 hour at room temperature. Membranes were then incubated with DNMT1 (1: 500, Cell Signaling) or β-Actin (1: 10,000, Sigma-Aldrich) primary antibodies for 48 hours in 4°C. After incubation with primary antibodies, membranes underwent a few washes in TBS-T solution, and were incubated with secondary antibodies for 1 hour at room temperature. For DNMT1 blots, anti-rabbit IgG-HRP secondary antibody (Santa Cruz Biotechnology) diluted 1: 5,000, was used, while for β-actin, anti-mouse IgG-HRP secondary antibody (Sigma-Aldrich) diluted 1: 20,000, was used. Following incubation, enhanced chemiluminescence (ECL) reagents were prepared and added to the blots for visualization by film autoradiography, as reported previously (Castro et al., 2005; Niles et al., 2012).

2.10 Data and Statistical Analysis

The delta-delta cycle threshold (ΔΔCt) method was used to analyze relative fold change for each target gene, following treatment with AZA or vehicle. Once the cycle threshold (Ct) values were obtained, the ΔCt was determined by calculating the difference between the mean Ct of the target gene and the mean Ct of the housekeeping gene ($\Delta Ct = Ct_{target\ avg.} - Ct_{housekeeping\ avg.}$). To calculate ΔΔCt, the control ΔCt was subtracted from the sample ΔCt ($\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{control}$). To determine the relative fold change for each target gene, the equation $2^{(-\Delta\Delta Ct)}$ was used (Niles et al., 2012).

Optical density (OD) normalization was done following visualization of western blots. This included digitally scanning films generated from the film autoradiography onto the AlphaImager 2200 system (Alpha Innotech Corporation). OD ratios of DNMT1 bands over the β-actin bands were taken, and subsequently used in data normalization and analysis (Castro et al., 2005).

Once the ΔΔCt analysis was completed for RT-qPCR and OD normalization for western blots, one-way analysis of variance (ANOVA) was used to determine whether there were significant treatment effects. The p value was < 0.05 for all tests when determining the level of significance. In addition, a post-hoc analysis (Newman-Keuls) was used to determine significant differences between drug doses and controls, as reported previously (Almutawaa et al., 2014; Castro et al., 2005; Niles et al., 2012).

CHAPTER 3 – RESULTS

3.1 5-Azacytidine diminished DNMT1 mRNA expression

A concentration-dependent decrease in DNMT1 mRNA expression was observed following treatment with AZA for 24 or 48 hours. One-way ANOVA revealed a significant treatment effect of $F_{(5, 12)} = 11.84$, $p < 0.0003$ for 24 hours **(Figure 9A)**, and $F_{(5, 12)} = 4.729$, $p < 0.05$ for 48 hours **(Figure 9B)**. Furthermore, Newman-Keuls testing indicated a significant decrease in DNMT1 mRNA levels at 1 µM AZA ($p < 0.01$) and a more pronounced effect at higher (5 – 25 µM) doses ($p < 0.001$) compared to control at 24 hours. A similar suppression of DNMT1 mRNA expression was seen at the 20 µM ($p < 0.01$) and 25 µM ($p < 0.05$) doses, following treatment with AZA for 48 hours.

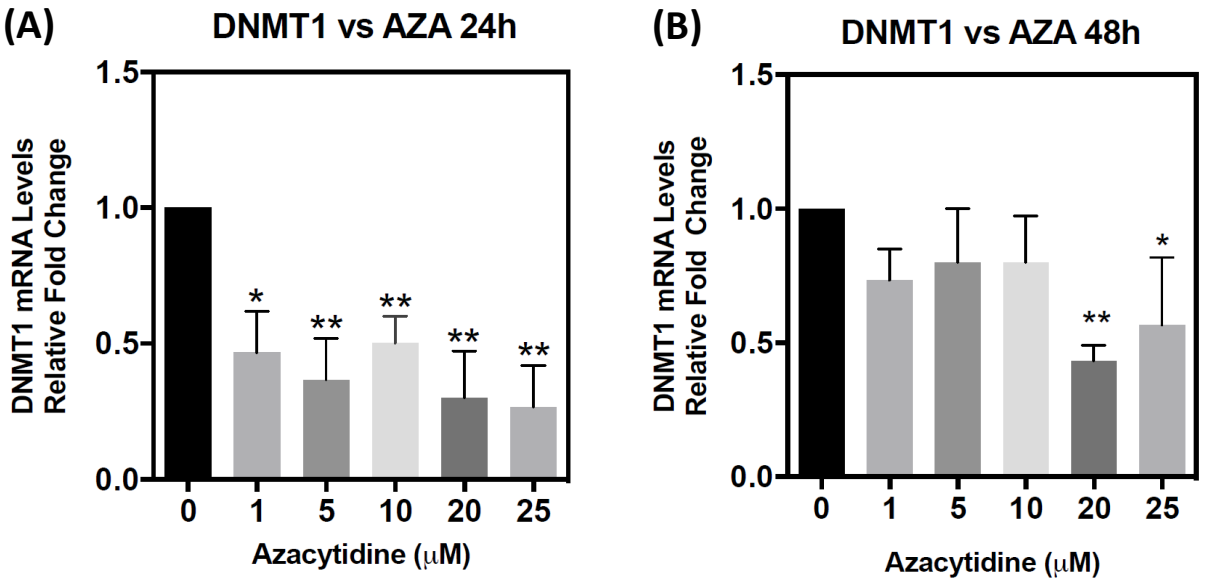

Figure 9. Decrease in DNMT1 mRNA expression after AZA treatment for 24 (A) or 48 hours (B). (A) A significant reduction in DNMT1 mRNA was seen at the 1 µM dose, and a more marked decrease at the higher doses at 5 – 25 µM. (B) A significant decline was seen at 48 hours at the 20 and 25 µM doses. Data shown are means ±S.E.M. (n=3), normalized to 18S. (A) *$p<0.01$, **$p<0.001$ vs control, and (B) *$p<0.05$, **$p<0.01$ vs control.

3.2 5-Azacytidine suppressed DNMT1 protein expression

Protein analysis was also done to corroborate mRNA findings. A suppression of DNMT1 protein was observed after treatment with AZA for 24 or 48 hours **(Figure 10A)**. One-way ANOVA revealed a significant treatment effect of $F_{(3, 10)} = 11.25, p < 0.001$ for 24 hours, and $F_{(2, 3)} = 81.72, p < 0.002$ for 48 hours. Post-hoc analysis (Newman-Keuls) indicated a significant decrease in DNMT1 protein levels at the 10 µM and 20 µM doses of AZA after 24 and 48 hours ($p < 0.01$) **(Figure 10B & C)**.

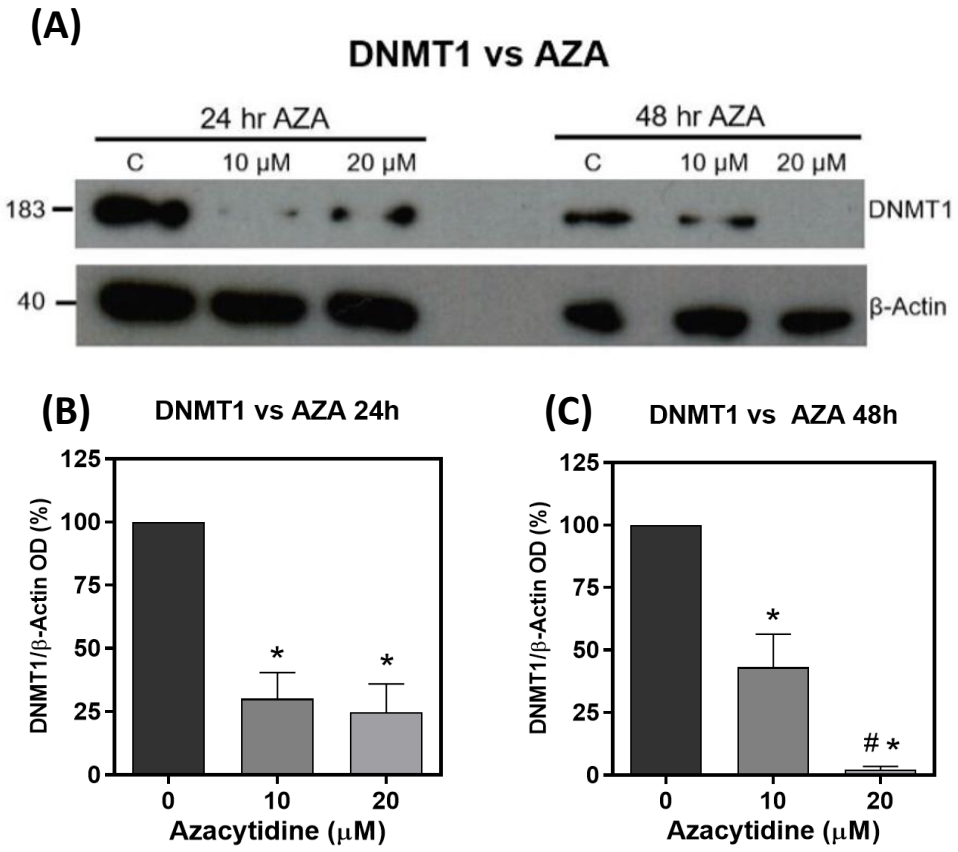

Figure 10. Suppression of DNMT1 protein levels following treatment with AZA for 24 or 48 hours. Immunoblot (A) and histogram (B, C) of DNMT1 protein expression after treatment with AZA for 24 or 48 hours. (B, C) Data shown represent the means ±S.E.M. (n=2-3) in percentage (%) values for DNMT1/β-actin optical density (OD) ratios. *$p<0.01$ vs control; #$p<0.05$ vs 10 µM AZA.

3.3 5-Azacytidine decreased global DNA methylation

To assess the effect of AZA on the global DNA methylation status in C6 cells, an ELISA assay (DNA Colorimetric Quantification Kit, Abcam, Cambridge, UK) was performed. Preliminary findings from a single experiment conducted in duplicate or triplicate, revealed a decrease in 5-methylcytosine (a marker for DNA methylation) content of about 17 % at 5 µM AZA to greater than 90% at10 µM or 20 µM AZA **(Figure 11)**.

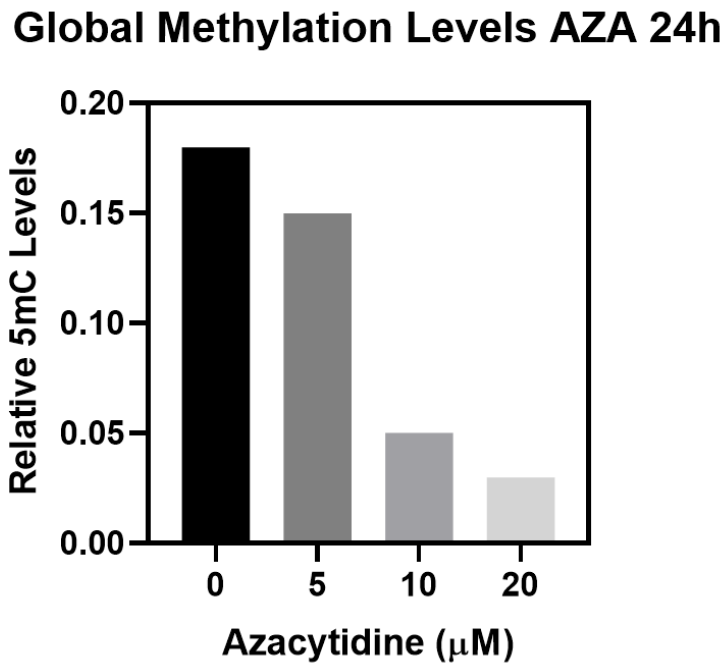

Figure 11. Effect of AZA on 5-methylcytosine (5-mC) content following treatment for 24 hours. Data shown are the means of 2-3 replications from one assay, which show a decrease in 5-methylcytosine (5-mC) levels in C6 cells treated with AZA.

3.4 Effect of 5-Azacytidine on TET-1 mRNA expression

An increase in mRNA expression of TET-1 was observed at the 1 µM dose after treatment with AZA for 24 hours, followed by a decrease in expression with the higher doses (i.e. 5 – 25 µM) **(Figure 12)**. A significant treatment effect of $F_{(5, 12)} = 22.62$, $p < 0.0001$ was revealed by one-way ANOVA. Post-hoc analysis using Newman Keuls indicated a significant increase at the 1 µM dose ($p < 0.001$) as compared to control. Whereas, at higher concentrations a significant decrease was observed at 10 – 25 µM ($p < 0.05$) compared to control.

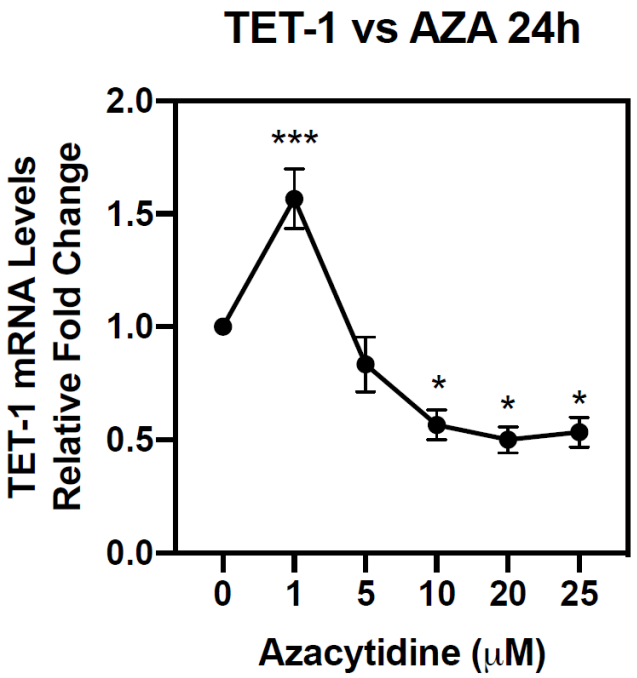

Figure 12. Effect of AZA on TET-1 mRNA expression, following treatment for 24 hours. Data shown are means ±S.E.M. (n=3), normalized to TBP. Increase of TET-1 expression at the 1 µM dose was observed, followed by a decrease at the higher doses: 10, 20 and 25 µM. *$p<0.05$ and ***$p<0.001$ vs control.

3.5 Effect of 5-Azacytidine on CDNF and MANF mRNA expression

A concentration-dependent increase of CDNF mRNA expression was observed after treatment with AZA (1 µM to 25 µM) **(Figure 13A)**. One-way ANOVA revealed a significant treatment effect of $F_{(5, 12)} = 8.256$, $p < 0.0014$. Furthermore, a post-hoc analysis using a Newman-Keuls test showed a significant increase in CDNF expression at the 20 µM ($p < 0.01$) and 25 µM ($p < 0.01$) doses compared to control.

In contrast, a decrease in MANF expression, in response to AZA treatment, was observed during the same time period **(Figure 13B)**. Following ΔΔCt analysis, one-way ANOVA revealed a significant treatment effect of $F_{(5, 12)} = 12.62$, $p < 0.0002$, while a Newman-Keuls test revealed a significant decreases at 1 µM and 5 µM ($p < 0.05$), and at 10 – 25 µM ($p < 0.001$), compared to control (DMSO 0.05%).

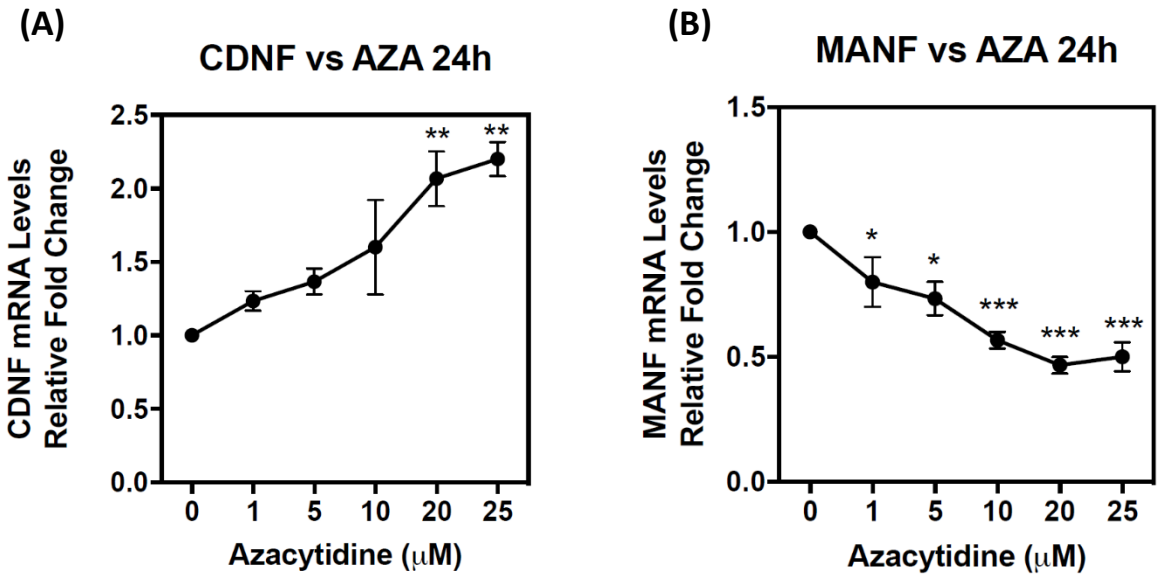

Figure 13. Effect of AZA on CDNF and MANF mRNA expression following treatment for 24 hours. Data shown are means ±S.E.M. (n=3), normalized to TBP. A concentration-dependent increase in CDNF expression (A) and decrease in MANF expression (B) was observed. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ vs control.

CHAPTER 4 – DISCUSSION

4.1 Effect of 5-Azacytidine on Methylation Status

Although the methylation status of the promoter regions for CDNF and MANF were not analyzed, it is reasonable to infer that DNA demethylation had occurred as AZA has been shown to induce this epigenetic change in other cell lines. Analysis of methylation levels at 27 000 CpG dinucleotides in human HCT116 colon carcinoma cells and HL-60 myeloid leukemia cells revealed a 50% decrease in methylation levels following treatment with AZA (1 µM) for 24 hours and a 60% decrease following treatment with DAC (1 µM) (Hagemann et al., 2011). The authors also found that AZA and DAC efficiently demethylated CpG dinucleotides found within non-CpG islands compared to CpG islands, providing insight into the demethylating effect of AZA and DAC (Hagemann et al., 2011). In the context of regulating a specific gene, AZA (0.5 and 1 µM) and DAC (0.5 and 1 µM) were observed to induce hypomethylation of the previously silenced tumor suppressor gene, CDKN2B, in a leukemia cell line (SKM-1) derived from a patient with myelodysplastic syndromes (MDS) (Kimura et al., 2012). AZA and its analog, DAC, are potent DNA demethylating agents that have beneficial effects in treating cancer such as MDS.

In accordance with above, there were significant decreases in DNMT1 mRNA and protein expression following treatment with AZA for 24 hours **(Figures 9 & 10)**. Treatment with 10 or 20 µM AZA diminished DNMT1 protein expression **(Figure 10)**, which is in agreement with a previous study that showed treatment with AZA resulted in depletion of DNMT1 protein expression in human AML lines (KG-1a and THP-1) (Hollenbach et al., 2010). This result is also in keeping with the occurrence of DNMT1 degradation as a result of AZA incorporation into the DNA strand, which leads to the loss of the methylated DNA marker, 5-mC (Christman, 2002). In addition, DNMT1 mRNA expression was significantly decreased with 1 µM of AZA, and a more

pronounced effect was observed with the higher doses (5 – 25 µM), following a 24 hour treatment period **(Figure 9A)**. With the reduction of DNMT1 mRNA and protein levels following treatment with AZA, it could be expected that DNMT1 activity levels would also decrease. An examination of DNMT1 activity will help to clarify this possibility. Lastly, a concentration-dependent decrease in global methylation levels following treatment was observed **(Figure 11)**. These findings indicate that DNA demethylation had occurred following treatment with AZA, which may play a role in the changes in CDNF and MANF mRNA levels observed in this study.

In addition to their ability to induce passive demethylation, AZA and DAC have also been implicated in active DNA demethylation through the induction of TET enzymes. As discussed previously, TET enzymes are involved in the active demethylation of DNA (replication-independent) by oxidizing the conversion of 5-mC to 5-hmC (Rasmussen & Helin, 2016; Santiago et al., 2014). A previous report demonstrated that AZA induced expression of TET-2 and TET-3 in hepatocellular carcinoma cells which resulted in the enhanced levels of 5-hmC (Sajadian et al., 2015). Furthermore, TET-2 knockdown prevented the induction of 5-hmC by AZA, suggesting that AZA promotes active demethylation through TET-2 compared to the other TET enzymes (Sajadian et al., 2015). The authors found little to no detection of TET-1 expression in the cell lines used (Sajadian et al., 2015). The induction of TET-2 and TET-3 by AZA was also seen in adipose-derived mesenchymal stem cell, where 48 hours of treatment with AZA (1 µM) resulted in an increase of TET-2 and TET-3 mRNA expression (Kornicka et al., 2017).

In the current study, a significant increase in TET-1 mRNA expression was seen at 1 µM AZA after a 24 hour treatment in C6 cells, with significant decreases at higher drug doses **(Figure 12)**. The pattern of TET-1 expression following treatment with AZA could be due to time or concentration dependent factors, which would not be revealed in a 24 hour experiment.

In order to better understand the dynamic response to AZA, a detailed time course study that includes periods ranging from hours to days, and the inclusion of doses lower than 1 µM AZA would be useful in correlating changes in DNA methylation with the expression of TET1 or other isoforms. In other studies, treatment with DAC for 5 days was seen to induce global hypomethylation at lower concentrations, with the greatest decrease in methylation levels seen at 0.3 µM, which was followed by a steady increase of methylation levels with a return to baseline at 20 µM (Kopp et al., 2013). This U-shaped pattern was associated with DAC's ability to inhibit NK lysis function, where DAC had the greatest effect at intermediate concentrations with low and high concentrations having little effect on lysis activity (Kopp et al., 2013). As such, it is possible that AZA may have had a biphasic effect on TET-1 mRNA expression, with an increase or decrease at lower or higher concentrations, respectively. An examination of mRNA and protein expression of the three TET isoforms, under the above-mentioned conditions, will help to elucidate AZA's effect on these enzymes.

4.2 Effect of 5-Azacytidine on MANF and CDNF mRNA expression

Epi-drugs which can modify gene expression, were previously shown to modulate the expression of neurotrophic factors (NTFs). Treatment with VPA, an HDAC inhibitor, upregulated CDNF mRNA and protein expression in mouse C17.2 neural stem cells, as well as MANF and GDNF expression (Almutawaa et al., 2014). The authors also observed an increase in H3 acetylation following VPA treatment, suggesting that this epigenetic mechanism may be involved in the modulation of these NTFs (Almutawaa et al., 2014). Moreover, *in vivo* studies revealed that chronic treatment with VPA increased the expression of NTFs: CDNF, MANF, GDNF and PSPN in rat hippocampus, as well as an increase of CDNF, MANF and BDNF expression in the striatum (Niles et al., 2012). The observed increase of NTFs following treatment with VPA supports the

involvement of histone acetylation in the regulation of their expression. In addition, and of particular relevance to the present study, there is evidence that VPA and other HDAC inhibitors can also alter DNA methylation (Arzenani et al., 2011; Detich et al., 2003; Sarkar et al., 2011), which suggests that this other epigenetic mechanism is involved in the induction of CDNF and MANF by VPA.

In accordance with the above view, the DNMT inhibitor, 5-aza-2'-deoxycytidine (DAC), which decreases DNA methylation, was found to induce NTF expression. An increase in BDNF mRNA expression was observed after treatment with DAC in mouse neuroblastoma Neuro-2a cells, which was associated with demethylation of CpG sites in the promoter region of BDNF by DAC (Ishimaru et al., 2010). Neuro-2a cells were also treated with TSA (100 – 1 000 nM) for 24 hour, where an increase in acetylation of H3 and H4 was observed at the promoter region of BDNF, which was associated with an increase in BDNF mRNA levels (Ishimaru et al., 2010). These results suggest that histone acetylation and DNA demethylation play a role in the transcriptional regulation of BDNF. Similarly, an *in vivo* study showed that systemic administration of DAC or AZA suppressed global DNA methylation and increased BDNF levels in the rat hippocampus (Sales et al., 2011). These results provide support for the role of the two major epigenetic mechanisms, DNA methylation and histone acetylation, in modulating the expression of NTFs. Given the interplay between histone acetylation and DNA methylation in modifying gene transcription, the effect of DNA demethylation on the expression of CDNF and MANF was studied. Treatment with the DNMT inhibitor, AZA, for 24 hours resulted in a concentration-dependent increase of CDNF mRNA expression in rat C6 glioma cells **(Figure 13A)**. In contrast, MANF mRNA expression was downregulated during the same time period in a concentration-dependent manner **(Figure 13B)**.

The biological effects of AZA in addition to its demethylating ability are quite broad. It is well established that AZA is able to inhibit cell growth, induce epigenetic modifications and activate previously silenced gene transcription (Christman, 2002; Hollenbach et al., 2010). AZA has also been linked to mitigation of oxidative stress in a model of aged-related degeneration of mesenchymal stem cells. Treatment with AZA in adipose-derived mesenchymal stem cells resulted in a decrease of nitric oxide and reactive oxygen species (ROS) levels and attenuation of oxidative stress, which has been known to activate ER stress (Kornicka et al., 2017).

Emerging evidence suggests that AZA may have an effect on the modulation of ER homeostasis. ER stress is a result of any stimuli that disrupts the homeostasis of the ER, which consequently leads to the activation of a series of signaling cascades that are collectively known as the unfolded protein response (UPR). Briefly, there are three major transmembrane regulators of UPR: inositol-requiring protein 1α (IRE1α), protein kinase RNA-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF-6) (Hetz, 2012; Xu et al., 2005). All three sensors work to restore homeostasis of the ER by activating various signaling cascades that function to restore proper protein folding capacity (Xu et al., 2005). Under unstressed conditions, major ER chaperone, glucose regulated protein 78 (GRP78), binds to all three sensors inhibiting their activation, whereas in stressed conditions, GRP78 preferentially binds to misfolded proteins, releasing its hold from IRE1α, PERK and ATF-6 and allowing their activation (Hetz, 2012). AZA was observed to activate IRE1α in human hepatoma cell line Huh-7, independent of ER-stress (Mnasri et al., 2018). Treatment with AZA did not result in the increase of GRP78 nor in the splicing of XBP1, which are major players in the UPR and involved in MANF expression (Mnasri et al., 2018; Pakarinen et al., 2020). However, another study had found that treatment with 0.5 μM of DAC for up to 6 days resulted in a decrease of IRE1α and CHOP expression in macrophages

(Cao et al., 2014). Pre-treatment of macrophages with 0.5 μM of DAC for 2 days, followed by exposure to stearate (an inducer of ER stress) for 1 day, resulted in a decrease in the phosphorylation and total protein expression of IRE1α (Cao et al., 2014). The findings from this study suggests that DAC may have an inhibitory effect on ER stress. DAC's analog, AZA, was also able to attenuate ER stress when administered in combination with resveratrol in adipose stem cells, evidenced by the decrease in ER-stress markers CHOP and PERK (Marycz et al., 2019). These studies provide support for the involvement of AZA in the mitigation of ER stress.

Moreover, a recent report demonstrated that AZA may have a role in inactivating ER stress through the modulation of microRNAs (miRNA). miRNAs are small non-coding RNAs that can modulate gene activity by affecting post-transcription gene expression, and are found to play an important role in the UPR (Li et al., 2020; Maurel & Chevet, 2013). A number of miRNAs are induced upon ER stress and are thought to be regulators of the UPR pathway including IRE1α, GRP78 and PERK (Maurel & Chevet, 2013). miR-199a-5p, a regulator of the transforming growth factor-β1 (TGF-β1) pathway, has been linked to the modulation of GRP78, ATF-6α and XBP1 expression (Li et al., 2020). Under UPR activation, miR-199a-5p has been found to be hypermethylated which results in the silencing of its transcription. Treatment with AZA (5 μg/mL) for 16 hours restored the expression of previously silenced miR-199a-5p in subepithelial myofibroblasts isolated from patients with Crohn's disease (Li et al., 2020). Restored expression of miR-199a-5p was also associated with a decrease in TGF-β1 expression, which is induced upon ER stress (Li et al., 2020). A decrease in protein levels of GRP78, and transcription factors: XBP1 and ATF-6α, following transfection with miR-199a-5p was observed, suggesting that miR-199a-5p inhibited the ER stress response. Furthermore, knockdown of DNMT1 decreased expression of GRP78, after its increase by tunicamycin-induced ER stress, which points to the possible

involvement of an epigenetic regulatory mechanism in ER stress (Li et al., 2020). As discussed earlier, MANF is induced by ER stress, which appears to be reduced by AZA. Therefore, it is likely that the downregulation of MANF mRNA expression observed in C6 cells is due to the inhibition of endogenous ER stress by AZA. An examination of ER stress markers such as GRP78, ATF-6 or XBP1, will help to confirm the suggested decrease of ER stress, following treatment with AZA in C6 cells.

When assessing the implications of the changes seen with CDNF and MANF levels after AZA treatment, it is important to consider the possible involvement of signaling pathways, PI3K/AKT and MAPK/ERK-1/2, in their modulation as well. Signaling pathways, PI3K/AKT and MAPK/ERK-1/2, are highly complex and mediate various cellular functions including gene transcription, protein synthesis, cellular survival and growth. Moreover, there are recent reports suggesting that these two pathways may be involved with ER stress. In primary glial cells, activation of AKT was observed following treatment with ER stress inducers, tunicamycin and thapsigargin (Hosoi et al., 2007). Interestingly, the authors observed an upregulation of activated AKT during short term exposure to ER stress (i.e. up to 2 hours), while a downregulation of activated AKT was seen during long term exposure to ER stress (i.e. over 12 hours) (Hosoi et al., 2007). The inactivation of AKT due to prolonged ER stress was found to be associated with an upregulation of ERK in another study, suggesting that there is a cross-talk between these two pathways under ER stress (Dai et al., 2009). As AKT activation decreased, there was a gradual increase in ERK activation suggesting that MAPK/ERK may play a long term role in cellular survival, while PI3K/AKT may be part of a rapid response to ER stress (Dai et al., 2009). In the context of AZA and these two signaling pathways, recent reports have shown that AZA induces phosphorylation of both AKT and ERK-1/2 in different cell lines (Mnasri et al., 2018; Yu & Kim,

2016). In human fibrosarcoma HT1080 cells, AZA's effect on inducing gene expression was diminished by exposure to AKT and ERK-1/2 inhibitors, suggesting that AZA may induce gene expression partially through the activation of these pathways (Yu & Kim, 2016). Some of the downstream targets of these pathways are the activation of transcription factors that may have an indirect or direct effect on modulating CDNF and MANF expression. In addition, ERK-2 was recently found to directly bind to DNA, modulating gene transcription activity (Hu et al., 2009). Thus, ERK-2 may have played a role in modulating CDNF and MANF expression in C6 cells following treatment with AZA either through its direct interaction with the promoter regions or through the activation of MAPK/ERK-1/2 pathway. As CDNF and MANF are both localized in the ER and play a role in ER stress, it is possible that treatment with AZA for 24 hours in C6 cells may have resulted in the activation of PI3K/AKT and/or MAPK/ERK-1/2 signaling pathways, and consequently, the modulation of CDNF/MANF levels. In order to examine the possible involvement of PI3K/AKT and MAPK/ERK-1/2 pathways in modulating CDNF and MANF levels, their activation and downstream targets could be assessed following AZA treatment with or without selective kinase inhibitors.

CDNF mRNA expression showed a clear upward trend at all AZA concentrations examined, with significant increases observed at the higher concentrations (20 and 25 µM), following a 24 hour treatment **(Figure 13A)**. In addition to its DNA demethylating effect, AZA is also cytotoxic at higher concentrations and has been demonstrated to exert antiproliferative effects in various cancer models including human myelodysplastic syndrome (Hollenbach et al., 2010; Kimura et al., 2012). In human acute myeloid leukemia (AML) cells, AZA was shown to have a significant effect on reducing cell viability with concentrations greater than 1 µM after 72 hours (Hollenbach et al., 2010). Preliminary results from hemocytometer/trypan blue cell counting

revealed a loss in cell viability of about 30-35% in C6 cells after treatment with 20 or 25 µM AZA, but this was not seen at lower drug concentrations. Thus, it is important to consider if the upregulation seen with CDNF at higher concentrations (20 and 25 µM) is a result of AZA's cytotoxic effect. It should be noted that both NTFs have been shown to protect cells from toxin - induced apoptosis and to increase cell viability (Huang et al., 2016; Lindholm et al., 2007). MANF suppressed 6-OHDA-induced apoptosis in SH-SY5Y cells and inhibited the increase of cleaved caspase-3, which plays a role in the apoptotic pathway (Huang et al., 2016). Moreover, in PC12 cells, treatment with CDNF attenuated the decrease in Bcl-2, as well as the increase of Bax and caspase-3 expression caused by 6-OHDA (Mei & Niu, 2014). Results from these studies suggest that CDNF and MANF promote cell survival and exert protective effects against drug-induced toxicity.

Furthermore, it was previously reported that AZA was unable to induce DNA fragmentation (a marker of AZA's cytotoxicity) and morphological changes that are associated with apoptosis in rat C6 glioma cells (Nakayama, 1999). However, it should be noted that the specifics of the study, including concentrations used, were not clearly stated. In a different study, AZA (3 µM) was also shown to inhibit programmed cell death induced by1,25-dihydroxyvitamin D3 in rat C6.9 glioma cells, which are isolated from the C6 glioma cell line (Canova et al., 1998). In addition, DNA fragmentation was not observed in cells exposed to 1,25-dihydroxyvitamin D3 then treated with AZA (0.1 – 5 µM) or just AZA alone (Canova et al., 1998). The authors did not examine the demethylating effect of AZA in this model, but it was suggested that the protective effect of AZA against apoptosis could be due to the demethylation of previously silenced genes, including the induction of anti-apoptotic family Bcl and any other genes involved in the suppression of programmed cell death (Canova et al., 1998). While AZA has been documented to

have cytotoxic effects in cancer cell lines such as AML cells (Hollenbach et al., 2010), it could be possible that some cell lines are resistant. In multiple myeloma cells, AZA (1 – 5 μM) was shown to have significant cytotoxic effects after 72 hours, as evidenced by a pronounced decrease in cell viability, however it had no effect on the cell viability of peripheral blood mononuclear cells or patient- derived bone marrow stromal cells (Kiziltepe et al., 2007). The findings from the previous studies suggest that AZA may have selective cytotoxic effects against some cell lines, while not affecting others. Thus, the toxicity of AZA may be considered to be cell-type dependent, as well as dependent on treatment period. Nonetheless, in the absence of more detailed studies of cell viability and related parameters, the involvement of a cytotoxic effect in the observed increase of CDNF cannot be ruled out. Detailed assays of cell viability using MTT or WST-1 protocols could be done following treatment with AZA. To assess AZA's effect on apoptosis in C6 cells, expressions of proapoptotic factors such as Bax or caspase-3, could be analyzed. In assessing the implications of these findings, it is important to determine whether protein levels, which provide an indication of possible alterations in the functional activities of CDNF and MANF, reflect the observed changes in the mRNA levels of these NTFs. The examination of the effects of AZA on the methylation status of the promoter regions of both MANF and CDNF will also help to elucidate the mechanisms involved in the modulation of these NTFs by AZA.

4.3 Future Directions

In this study we had intended to investigate the aims outlined in the previous section (i.e. Chapter 1, section 1.9) including the examination of HDAC and DNMT inhibitors (including combinatorial treatments) on the expression of CDNF and MANF in a time-dependent manner, as well as looking at their effects on DNMT1 and TET enzymes. However, we were only able to complete the analysis of DNMT inhibitors on CDNF/MANF, TET-1 and DNMT1 levels in a 24

hour period before our lab was shut down in response to the COVID-19 pandemic. As a result, many of our planned experiments that would have completed the outlined aims were put on a halt. As such, to complete these aims, future experiments could involve treatment at various time periods to assess the time-dependent effects of AZA on these targets. Protein analysis and ELISA can be completed to provide results of protein levels and/or enzyme activity after treatment. In addition, a combinatorial treatment of HDAC and DNMT inhibitors will aid in exploring the possible synergistic effect of these epi-drugs on the modulation of NTFs. Other classes of HDAC inhibitors, such as valproate or TSA, and AZA analog, DAC, may be used to assess if the effects seen with VPA and/or AZA is attributed to the drug itself or to their epigenetic activity. This study design could also be replicated in other cell types such as neuronal cell lines (e.g. SH-SY5Y) to investigate the possible implications for neuronal function.

Depending on the results from the above *in vitro* experiments, the application of epi-drugs, such as the combination of HDAC and DNMT inhibitors, in a neurodegenerative model such as 6-OHDA rat model of PD could be done to assess the potential therapeutic effect of this strategy. The examination of other NTFs including BDNF and GDNF following treatment with epi-drugs may help to elucidate if epigenetic mechanisms are involved in the transcriptional regulation of other NTF family members. The modulation of other NTFs including CDNF and MANF, could have various therapeutic implications in neurological or neurodegenerative diseases, especially in cases where levels of NTFs are decreased.

CHAPTER 5 – CONCLUSION

Emerging reports have implicated histone acetylation and DNA demethylation in the modulation of NTFs including BDNF, GDNF and the novel family, CDNF and MANF (Almutawaa et al., 2014; Ishimaru et al., 2010; Niles et al., 2012; Sales et al., 2011). In this study, we had observed an upregulation of CDNF mRNA expression following a 24 hour treatment with demethylating agent, AZA. During the same time period, we had also observed a decrease of MANF mRNA expression, which could be due to the inhibition of ER stress activation by AZA, as MANF expression is greatly enhanced by its activation (Apostolou et al., 2008). The changes seen with CDNF and MANF mRNA levels were associated with the suppression of DNMT1 mRNA and protein expression after a 24 hour AZA treatment. As AZA is known to activate the expression of previously silenced genes, it is possible that with the diminished levels of DNMT1 and consequently increased DNA hypomethylation, CDNF levels were induced. Whereas a decrease in DNMT1 levels may had an effect on decreasing GRP78 levels (Li et al., 2020), which could have led to the observed decrease of MANF expression, as MANF expression closely resembles that of GRP78 (Mizobuchi et al., 2007). Analysis of the protein levels, ER stress markers, time-dependent factors and other experiments need to be done in order to provide a clearer picture of AZA's demethylating effect on CDNF and MANF expression. Moreover, a significant increase in TET-1 mRNA expression at the 1 µM dose was also observed, which was followed by a decrease with higher doses. The biphasic response of TET-1 could be due to various factors including time or concentration-dependent. In order to make a conclusive statement of AZA's effect on TET-1, various time points and concentrations need to be assessed, as well as an examination of 5-hmC and protein levels following treatment. Investigating the process of DNA demethylation and histone acetylation on CDNF and MANF regulation, will help to further our

understanding of the role that epigenetic mechanisms play in modulating NTF expression. Furthermore, elucidating the regulation of CDNF and MANF expression could provide possible avenues in treatment of neurodegenerative diseases.

NTFs play an important role in the maintenance and survival of the nervous system. They have broad actions in various tissues and have been proved to have therapeutic effects in neurodegenerative or neurological disorders. BDNF, for example, is a well-established NTF that plays a critical role in the nervous system having effects on synaptic activity, memory formation and cognitive function. Decreased levels of BDNF were observed in patients with bi-polar disorders, schizophrenia and major depressive disorder, which was typically associated with increase methylation levels at the promoter region (Ikegame et al., 2013). Treatment with HDAC inhibitor, sodium butyrate, resulted in an anti-depressant effect in Flinders Sensitive Line (FSL) rat models, which are an established genetic model to investigate depression or anti-depression effects *in vivo* (Wei et al., 2015). The authors also found that sodium butyrate increased TET-1 levels, which was associated with an increase of 5-hmC at BDNF promoter regions, that consequently lead to an increase of BDNF expression (Wei et al., 2015). The results from this study implicates HDAC inhibitors to have a DNA demethylating effect, as well as providing support for epigenetic modulation of NTF in treating a psychiatric disorder. Thus, the investigation of histone acetylation and DNA demethylation in modulating NTF expression could have positive implications in the treatment of diseases that affect their pathway.

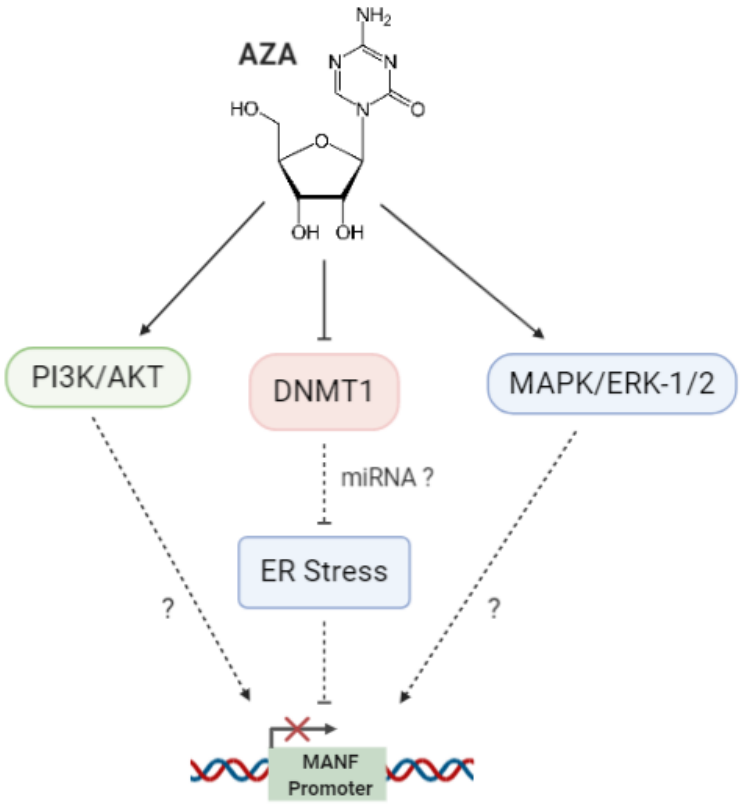

Figure 14. Hypothetical mode of action of AZA on MANF mRNA expression in rat C6 glioma cells. AZA downregulated MANF expression after a 24 hour treatment period in C6 cells. This could be due to the inhibition of ER stress activation, possibly through the involvement of miRNAs, as they have been implicated to be regulators of the UPR pathway (Maurel & Chevet, 2013). AZA has been shown to hypomethylate miRNA in subepithelial fibroblasts which resulted in the attenuation of ER stress (Li et al., 2020). In addition, AZA has been linked to activating PI3K/AKT and ERK-1/2 pathways, suggesting that it modulates gene activity through these pathways (Mnasri et al., 2018; Yu & Kim, 2015; Yu & Kim 2016) which could account for

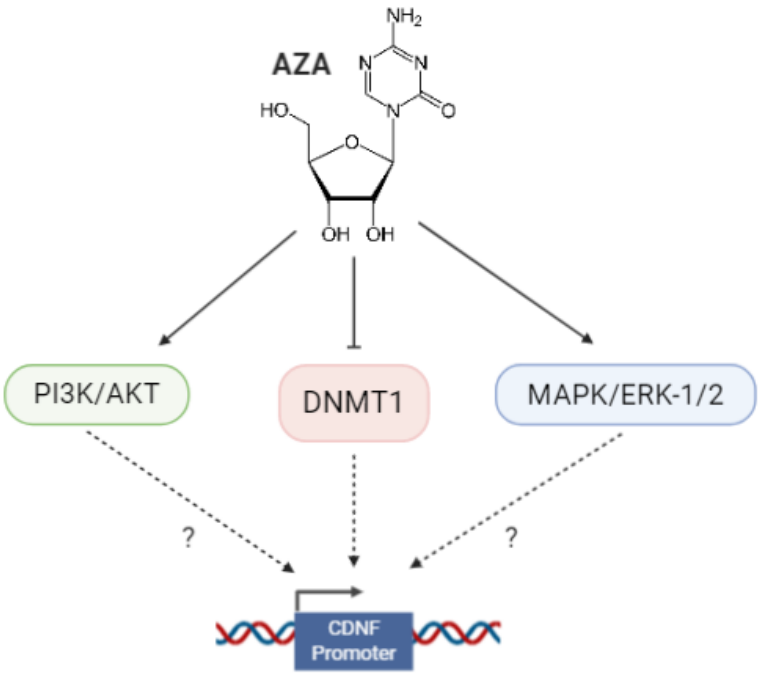

Figure 15. Hypothetical mode of action of AZA on CDNF mRNA expression in rat C6 glioma cells. The downregulation of DNMT1 was associated with the upregulation of CDNF in C6 cells, which may be due to hypomethylation of CDNF's promoter region. As part of its demethylation effect, AZA has been implicated in activating the PI3K/AKT and the MAPK/ERK-1/2 pathway, through increasing the phosphorylation of AKT and ERK-1/2 in human fibrosarcoma HT-1080 cells (Yu & Kim, 2015). Thus, AZA may have also induced CDNF expression partially through the modulation of AKT and/or ERK-1/2.

REFERENCES

Airaksinen, M. S., & Saarma, M. (2002). The GDNF family: Signalling, biological functions and therapeutic value. In *Nature Reviews Neuroscience* (Vol. 3, Issue 5, pp. 383–394). Nature Publishing Group. https://doi.org/10.1038/nrn812

Almutawaa, W., Kang, N. H., Pan, Y., & Niles, L. P. (2014). Induction of Neurotrophic and Differentiation Factors in Neural Stem Cells by Valproic Acid. *Basic & Clinical Pharmacology & Toxicology*, *115*(2), 216–221. https://doi.org/10.1111/bcpt.12201

Apostolou, A., Shen, Y., Liang, Y., Luo, J., & Fang, S. (2008). Armet, a UPR-upregulated protein, inhibits cell proliferation and ER stress-induced cell death. *Experimental Cell Research*, *314*(13), 2454–2467. https://doi.org/10.1016/j.yexcr.2008.05.001

Arancibia, D., Zamorano, P., & Andrés, M. E. (2018). CDNF induces the adaptive unfolded protein response and attenuates endoplasmic reticulum stress-induced cell death. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1865*(11), 1579–1589. https://doi.org/10.1016/j.bbamcr.2018.08.012

Arzenani, M. K., Zade, A. E., Ming, Y., Vijverberg, S. J. H., Zhang, Z., Khan, Z., Sadique, S., Kallenbach, L., Hu, L., Vukojevic, V., & Ekstrom, T. J. (2011). Genomic DNA Hypomethylation by Histone Deacetylase Inhibition Implicates DNMT1 Nuclear Dynamics. *Molecular and Cellular Biology*, *31*(19), 4119–4128. https://doi.org/10.1128/mcb.01304-10

Bannister, A. J., & Kouzarides, T. (2011). Regulation of chromatin by histone modifications. *Cell Research* (Vol. 21, Issue 3, pp. 381–395). https://doi.org/10.1038/cr.2011.22

Bartels, A. L., & Leenders, K. L. (2009). Parkinson's disease: The syndrome, the pathogenesis and pathophysiology. *Cortex*, *45*(8), 915–921. https://doi.org/10.1016/j.cortex.2008.11.010

Bird, A. (2002). DNA methylation patterns and epigenetic memory. *Genes and Development* (Vol. 16, Issue 1, pp. 6–21). https://doi.org/10.1101/gad.947102

Canova, C., Chevalier, G., Remy, S., Brachet, P., & Wion, D. (1998). Epigenetic control of programmed cell death: inhibition by 5-azacytidine of 1,25-dihydroxyvitamin D3-induced programmed cell death in C6.9 glioma cells. *Mechanisms of Ageing and Development*,

101(1–2), 153–166. https://doi.org/10.1016/s0047-6374(97)00172-3

Cao, Q., Wang, X., Jia, L., Mondal, A. K., Diallo, A., Hawkins, G. A., Das, S. K., Parks, J. S., Yu, L., Shi, H., Shi, H., & Xue, B. (2014). Inhibiting DNA methylation by 5-Aza-2'-deoxycytidine ameliorates atherosclerosis through suppressing macrophage inflammation. *Endocrinology*, *155*(12), 4925–4938. https://doi.org/10.1210/en.2014-1595

Castrén, E. (2013). Trophic factors: Neurotrophic factors. In *Neuroscience in the 21st Century: From Basic to Clinical* (pp. 1555–1589). https://doi.org/10.1007/978-1-4614-1997-6_55

Castro, L. M. R., Gallant, M., & Niles, L. P. (2005). Novel targets for valproic acid: up-regulation of melatonin receptors and neurotrophic factors in C6 glioma cells. *Journal of Neurochemistry*, *95*(5), 1227–1236. https://doi.org/10.1111/j.1471-4159.2005.03457.x

Chao, M. V. (2003). Neurotrophins and their receptors: A convergence point for many signalling pathways. *Nature Reviews Neuroscience*, *4*(4), 299–309. https://doi.org/10.1038/nrn1078

Chen, L., Feng, L., Wang, X., Du, J., Chen, Y., Yang, W., Zhou, C., Cheng, L., Shen, Y., Fang, S., Li, J., & Shen, Y. (2015). Mesencephalic Astrocyte-Derived Neurotrophic Factor Is Involved in Inflammation by Negatively Regulating the NF-κB Pathway. *Scientific Reports*, *5*(1), 1–13. https://doi.org/10.1038/srep08133

Christman J. K. (2002). 5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene*, *21*(35), 5483–5495. https://doi.org/10.1038/sj.onc.1205699

Cordero-Llana, Ó., Houghton, B. C., Rinaldi, F., Taylor, H., Yáñez-Muñoz, R. J., Uney, J. B., Wong, L. F., & Caldwell, M. A. (2015). Enhanced efficacy of the CDNF/MANF family by combined intranigral overexpression in the 6-OHDA rat model of parkinson's disease. *Molecular Therapy*, *23*(2), 244–254. https://doi.org/10.1038/mt.2014.206

Dai, R., Chen, R., & Li, H. (2009). Cross-talk between PI3K/Akt and MEK/ERK pathways mediates endoplasmic reticulum stress-induced cell cycle progression and cell death in human hepatocellular carcinoma cells. *International Journal of Oncology*, *34*(6), 1749–1757. https://doi.org/10.3892/ijo_00000306

Damaraju, V. L., Mowles, D., Yao, S., Ng, A., Young, J. D., Cass, C. E., & Tong, Z. (2012). Role of Human Nucleoside Transporters in the Uptake and Cytotoxicity of Azacitidine and Decitabine. *Nucleosides, Nucleotides and Nucleic Acids*, *31*(3), 236–255. https://doi.org/10.1080/15257770.2011.652330

Danilova, T., & Lindahl, M. (2018). Emerging Roles for Mesencephalic Astrocyte-Derived Neurotrophic Factor (MANF) in Pancreatic Beta Cells and Diabetes. *Frontiers in Physiology*, *9*(OCT), 1457. https://doi.org/10.3389/fphys.2018.01457

Detich, N., Bovenzi, V., & Szyf, M. (2003). Valproate induces replication-independent active DNA demethylation. *The Journal of biological chemistry*, *278*(30), 27586–27592. https://doi.org/10.1074/jbc.M303740200

DiSabato, D. J., Quan, N., & Godbout, J. P. (2016). Neuroinflammation: the devil is in the details. *Journal of Neurochemistry*, *139*(Suppl 2), 136–153. https://doi.org/10.1111/jnc.13607

Finder, V. H. (2010). Alzheimer's Disease: A General Introduction and Pathomechanism. *Journal of Alzheimer's Disease*, *22*, 5–19. https://doi.org/10.3233/JAD-2010-100975

Glembotski, C. C., Thuerauf, D. J., Huang, C., Vekich, J. A., Gottlieb, R. A., & Doroudgar, S. (2012). Mesencephalic astrocyte-derived neurotrophic factor protects the heart from ischemic damage and is selectively secreted upon sarco/endoplasmic reticulum calcium depletion. *The Journal of Biological Chemistry*, *287*(31), 25893–25904. https://doi.org/10.1074/jbc.M112.356345

Gräff, J., Kim, D., Dobbin, M. M., & Li-Huei, T. (2011). Epigenetic Regulation of Gene Expression in Physiological and Pathological Brain Processes. *Physiological Reviews*, *91*(2), 603–649. https://doi.org/10.1152/physrev.00012.2010

Hagemann, S., Heil, O., Lyko, F., & Brueckner, B. (2011). Azacytidine and decitabine induce gene-specific and non-random DNA demethylation in human cancer cell lines. *PLoS ONE*, *6*(3). https://doi.org/10.1371/journal.pone.0017388

Hakonen, E., Chandra, V., Fogarty, C. L., Yu, N. Y. L., Ustinov, J., Katayama, S., Galli, E., Danilova, T., Lindholm, P., Vartiainen, A., Einarsdottir, E., Krjutškov, K., Kere, J., Saarma,

M., Lindahl, M., & Otonkoski, T. (2018). MANF protects human pancreatic beta cells against stress-induced cell death. *Diabetologia*, *61*(10), 2202–2214. https://doi.org/10.1007/s00125-018-4687-y

Halvorsen, S. W., & Kaur, N. (2006). CNTF and Related Neurokines. *Handbook of Neurochemistry and Molecular Neurobiology* (pp. 43–68). https://doi.org/10.1007/978-0-387-30381-9_3

Hefti, F., Hartikka, J., & Knusel, B. (1989). Function of neurotrophic factors in the adult and aging brain and their possible use in the treatment of neurodegenerative diseases. *Neurobiology of Aging*, *10*(5), 515–533. https://doi.org/10.1016/0197-4580(89)90118-8

Hellman, M., Arumäe, U., Yu, L. Y., Lindholm, P., Peränen, J., Saarma, M., & Permi, P. (2011). Mesencephalic astrocyte-derived neurotrophic factor (MANF) has a unique mechanism to rescue apoptotic neurons. *The Journal of biological chemistry*, *286*(4), 2675–2680. https://doi.org/10.1074/jbc.M110.146738

Henderson, M. J., Richie, C. T., Airavaara, M., Wang, Y., & Harvey, B. K. (2013). Mesencephalic astrocyte-derived neurotrophic factor (MANF) secretion and cell surface binding are modulated by KDEL receptors. *Journal of Biological Chemistry*, *288*(6), 4209–4225. https://doi.org/10.1074/jbc.M112.400648

Hetz, C. (2012). The unfolded protein response: Controlling cell fate decisions under ER stress and beyond. *Nature Reviews Molecular Cell Biology* (Vol. 13, Issue 2, pp. 89–102). https://doi.org/10.1038/nrm3270

Hollenbach, P. W., Nguyen, A. N., Brady, H., Williams, M., Ning, Y., Richard, N., Krushel, L., Aukerman, S. L., Heise, C., & MacBeth, K. J. (2010). A comparison of azacitidine and decitabine activities in acute myeloid leukemia cell lines. *PLoS ONE*, *5*(2). https://doi.org/10.1371/journal.pone.0009001

Hosoi, T., Hyoda, K., Okuma, Y., Nomura, Y., & Ozawa, K. (2007). Akt up- and down-regulation in response to endoplasmic reticulum stress. *Brain Research*, *1152*(1), 27–31. https://doi.org/10.1016/j.brainres.2007.03.052

Hu, S., Xie, Z., Onishi, A., Yu, X., Jiang, L., Lin, J., Rho, H. sool, Woodard, C., Wang, H.,

Jeong, J. S., Long, S., He, X., Wade, H., Blackshaw, S., Qian, J., & Zhu, H. (2009). Profiling the Human Protein-DNA Interactome Reveals ERK2 as a Transcriptional Repressor of Interferon Signaling. *Cell*, *139*(3), 610–622. https://doi.org/10.1016/j.cell.2009.08.037

Huang, J., Chen, C., Gu, H., Li, C., Fu, X., Jiang, M., Sun, H., Xu, J., Fang, J., & Jin, L. (2016). Mesencephalic astrocyte-derived neurotrophic factor reduces cell apoptosis via upregulating GRP78 in SH-SY5Y cells. *Cell Biology International*, *40*(7), 803–811. https://doi.org/10.1002/cbin.10621

Ikegame, T., Bundo, M., Murata, Y., Kasai, K., Kato, T., & Iwamoto, K. (2013). DNA methylation of the BDNF gene and its relevance to psychiatric disorders. *Journal of human genetics*, *58*(7), 434–438. https://doi.org/10.1038/jhg.2013.65

Ishimaru, N., Fukuchi, M., Hirai, A., Chiba, Y., Tamura, T., Takahashi, N., Tabuchi, A., Tsuda, M., & Shiraishi, M. (2010). Differential epigenetic regulation of BDNF and NT-3 genes by trichostatin A and 5-aza-2′-deoxycytidine in Neuro-2a cells. *Biochemical and Biophysical Research Communications*, *394*(1), 173–177. https://doi.org/10.1016/j.bbrc.2010.02.139

Kemppainen, S., Lindholm, P., Galli, E., Lahtinen, H. M., Koivisto, H., Hämäläinen, E., Saarma, M., & Tanila, H. (2015). Cerebral dopamine neurotrophic factor improves long-term memory in APP/PS1 transgenic mice modeling Alzheimer's disease as well as in wild-type mice. *Behavioural Brain Research*, *291*, 1–11. https://doi.org/10.1016/j.bbr.2015.05.002

Kim, B., Rincón Castro, L. M., 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*(1–3), 45–48. https://doi.org/10.1016/j.ejphar.2008.04.058

Kimura, S., Kuramoto, K., Homan, J., Naruoka, H., Ego, T., Nogawa, M., Sugahara, S., & Naito, H. (2012). Antiproliferative and Antitumor Effects of Azacitidine Against the Human Myelodysplastic Syndrome Cell Line SKM-1. *Anticancer Research*, *32*(3), 795–798. http://ar.iiarjournals.org/content/32/3/795.full

Kirik, D., Rosenblad, C., & Bjorklund, A. (2000). Preservation of a functional nigrostriatal

dopamine pathway by GDNF in the intrastriatal 6-OHDA lesion model depends on the site of administration of the trophic factor. *European Journal of Neuroscience*, *12*(11), 3871–3882. https://doi.org/10.1046/j.1460-9568.2000.00274.x

Kiziltepe, T., Hideshima, T., Catley, L., Raje, N., Yasui, H., Shiraishi, N., Okawa, Y., Ikeda, H., Vallet, S., Pozzi, S., Ishitsuka, K., Ocio, E. M., Chauhan, D., & Anderson, K. C. (2007). 5-Azacytidine, a DNA methyltransferase inhibitor, induces ATR-mediated DNA double-strand break responses, apoptosis, and synergistic cytotoxicity with doxorubicin and bortezomib against multiple myeloma cells. *Molecular Cancer Therapeutics*, *6*(6), 1718–1727. https://doi.org/10.1158/1535-7163.MCT-07-0010

Kornicka, K., Marycz, K., Marędziak, M., Tomaszewski, K. A., & Nicpoń, J. (2017). The effects of the DNA methyltranfserases inhibitor 5-Azacitidine on ageing, oxidative stress and DNA methylation of adipose derived stem cells. *Journal of Cellular and Molecular Medicine*, *21*(2), 387–401. https://doi.org/10.1111/jcmm.12972

Latge, C., Cabral, K. M. S., De Oliveira, G. A. P., Raymundo, D. P., Freitas, J. A., Johanson, L., Romão, L. F., Palhano, F. L., Herrmann, T., Almeida, M. S., & Foguel, D. (2015). The solution structure and dynamics of full-length human cerebral dopamine neurotrophic factor and its neuroprotective role against α-synuclein oligomers. *Journal of Biological Chemistry*, *290*(33), 20527–20540. https://doi.org/10.1074/jbc.M115.662254

Lawrence T. (2009). The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harbor perspectives in biology*, *1*(6), a001651. https://doi.org/10.1101/cshperspect.a001651

Lee, A.-H., Iwakoshi, N. N., & Glimcher, L. H. (2003). XBP-1 Regulates a Subset of Endoplasmic Reticulum Resident Chaperone Genes in the Unfolded Protein Response. *MOLECULAR AND CELLULAR BIOLOGY*, *23*(21), 7448–7459. https://doi.org/10.1128/MCB.23.21.7448-7459.2003

Li, C., Grider, J. R., Murthy, K. S., Bohl, J., Rivet, E., Wieghard, N., & Kuemmerle, J. F. (2020). Endoplasmic Reticulum Stress in Subepithelial Myofibroblasts Increases the TGF-β1 Activity That Regulates Fibrosis in Crohn's Disease. *Inflammatory Bowel Diseases*, *26*(6), 809–819. https://doi.org/10.1093/IBD/IZAA015

Lin, J. H., Walter, P., & Yen, T. S. B. (2008). Endoplasmic Reticulum Stress in Disease Pathogenesis. *Annual Review of Pathology: Mechanisms of Disease*, *3*(1), 399–425. https://doi.org/10.1146/annurev.pathmechdis.3.121806.151434

Lindahl, M., Chalazonitis, A., Palm, E., Pakarinen, E., Danilova, T., Pham, T. D., Setlik, W., Rao, M., Võikar, V., Huotari, J., Kopra, J., Andressoo, J. O., Piepponen, P. T., Airavaara, M., Panhelainen, A., Gershon, M. D., & Saarma, M. (2020). Cerebral dopamine neurotrophic factor–deficiency leads to degeneration of enteric neurons and altered brain dopamine neuronal function in mice. *Neurobiology of Disease*, *134*, 104696. https://doi.org/10.1016/j.nbd.2019.104696

Lindahl, M., Danilova, T., Palm, E., Lindholm, P., Võikar, V., Hakonen, E., Ustinov, J., Andressoo, J. O., Harvey, B. K., Otonkoski, T., Rossi, J., & Saarma, M. (2014). MANF Is Indispensable for the Proliferation and Survival of Pancreatic β Cells. *Cell Reports*, *7*(2), 366–375. https://doi.org/10.1016/j.celrep.2014.03.023

Lindahl, M., Saarma, M., & Lindholm, P. (2017). Unconventional neurotrophic factors CDNF and MANF: Structure, physiological functions and therapeutic potential. *Neurobiology of Disease*, *97*, 90–102. https://doi.org/10.1016/j.nbd.2016.07.009

Lindholm, P., Peränen, J., Andressoo, J. O., Kalkkinen, N., Kokaia, Z., Lindvall, O., Timmusk, T., & Saarma, M. (2008). MANF is widely expressed in mammalian tissues and differently regulated after ischemic and epileptic insults in rodent brain. *Molecular and Cellular Neuroscience*, *39*(3), 356–371. https://doi.org/10.1016/j.mcn.2008.07.016

Lindholm, P., & Saarma, M. (2010). Novel CDNF/MANF family of neurotrophic factors. *Developmental Neurobiology*, *70*(5). https://doi.org/10.1002/dneu.20760

Lindholm, P., Voutilainen, M. H., Laurén, J., Peränen, J., Leppänen, V. M., Andressoo, J. O., Lindahl, M., Janhunen, S., Kalkkinen, N., Timmusk, T., Tuominen, R. K., & Saarma, M. (2007). Novel neurotrophic factor CDNF protects and rescues midbrain dopamine neurons in vivo. *Nature*, *448*(7149), 73–77. https://doi.org/10.1038/nature05957

Liu, H., Tang, X., & Gong, L. (2015a). Mesencephalic astrocyte-derived neurotrophic factor and cerebral dopamine neurotrophic factor: New endoplasmic reticulum stress response

proteins. *European Journal of Pharmacology*, *750*, 118–122. https://doi.org/10.1016/j.ejphar.2015.01.016

Liu, H., Zhao, C., Zhong, L., Liu, J., Zhang, S., Cheng, B., & Gong, L. (2015b). Key subdomains in the C-terminal of cerebral dopamine neurotrophic factor regulate the protein secretion. *Biochemical and Biophysical Research Communications*, *465*(3), 427–432. https://doi.org/10.1016/j.bbrc.2015.08.029

Löscher W. (1999). Valproate: a reappraisal of its pharmacodynamic properties and mechanisms of action. *Progress in neurobiology*, *58*(1), 31–59. https://doi.org/10.1016/s0301-0082(98)00075-6

Malhotra, J. D., & Kaufman, R. J. (2007). The endoplasmic reticulum and the unfolded protein response. *Seminars in cell & developmental biology*, *18*(6), 716–731. https://doi.org/10.1016/j.semcdb.2007.09.003

Marycz, K., Houston, J. M. I., Weiss, C., Röcken, M., & Kornicka, K. (2019). 5-Azacytidine and Resveratrol Enhance Chondrogenic Differentiation of Metabolic Syndrome-Derived Mesenchymal Stem Cells by Modulating Autophagy. *Oxidative Medicine and Cellular Longevity*, *2019*. https://doi.org/10.1155/2019/1523140

Mätlik, K., Yu, L. ying, Eesmaa, A., Hellman, M., Lindholm, P., Peränen, J., Galli, E., Anttila, J., Saarma, M., Permi, P., Airavaara, M., & Arumäe, U. (2015). Role of two sequence motifs of mesencephalic astrocyte-derived neurotrophic factor in its survival-promoting activity. *Cell Death & Disease*, *6*(12), e2032. https://doi.org/10.1038/cddis.2015.371

Maurel, M., & Chevet, E. (2013). Endoplasmic reticulum stress signaling: the microRNA connection. *American journal of physiology. Cell physiology*, *304*(12), C1117–C1126. https://doi.org/10.1152/ajpcell.00061.2013

Mei, J. M., & Niu, C. S. (2014). Effects of CDNF on 6-OHDA-induced apoptosis in PC12 cells via modulation of Bcl-2/Bax and caspase-3 activation. *Neurological Sciences*, *35*(8), 1275–1280. https://doi.org/10.1007/s10072-014-1700-1

Mizobuchi, N., Hoseki, J., Kubota, H., Toyokuni, S., Nozaki, J., Naitoh, M., Koizumi, A., & Nagata, K. (2007). ARMET is a soluble ER protein induced by the unfolded protein

response via ERSE-II element. *Cell structure and function*, *32*(1), 41–50. https://doi.org/10.1247/csf.07001

Mnasri, N., Mamarbachi, M., Allen, B. G., & Mayer, G. (2018). 5-Azacytidine engages an IRE1α-EGFR-ERK1/2 signaling pathway that stabilizes the LDL receptor mRNA. *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms*, *1861*(1), 29–40. https://doi.org/10.1016/j.bbagrm.2017.11.010

Monti, B., Polazzi, E., & Contestabile, A. (2010). Biochemical, Molecular and Epigenetic Mechanisms of Valproic Acid Neuroprotection. *Current Molecular Pharmacology*, *2*(1), 95–109. https://doi.org/10.2174/1874467210902010095

Moore, L. D., Le, T., & Fan, G. (2013). DNA methylation and its basic function. *Neuropsychopharmacology* (Vol. 38, Issue 1, pp. 23–38). https://doi.org/10.1038/npp.2012.112

Nagaraju, G. P., Wu, C., Merchant, N., Chen, Z., Lesinski, G. B., & El-Rayes, B. F. (2017). Epigenetic effects of inhibition of heat shock protein 90 (HSP90) in human pancreatic and colon cancer. *Cancer Letters*, *402*, 110–116. https://doi.org/10.1016/j.canlet.2017.05.021

Nakayama, H. (1999). Possible involvement of DNA methylation in 5-azacytidine-induced neuronal cell apoptosis. *Histology and Histopathology*, *14*(1), 143–150. https://doi.org/10.14670/HH-14.143

Nathanson, N. M. (2012). Regulation of neurokine receptor signaling and trafficking. *Neurochemistry International*, *61*(6), 874–878. https://doi.org/10.1016/j.neuint.2012.01.018

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. *The International Journal of Neuropsychopharmacology*, *15*(09), 1343–1350. https://doi.org/10.1017/S1461145711001969

Norisada, J., Hirata, Y., Amaya, F., Kiuchi, K., & Oh-Hashi, K. (2016). A comparative analysis of the molecular features of MANF and CDNF. *PLoS ONE*, *11*(1). https://doi.org/10.1371/journal.pone.0146923

Nussbaum, R. L., & Ellis, C. E. (2003). Alzheimer's Disease and Parkinson's Disease. *New England Journal of Medicine*, *348*(14), 1356–1364. https://doi.org/10.1056/NEJM2003ra020003

Oh-Hashi, K., Hirata, Y., & Kiuchi, K. (2013). Transcriptional regulation of mouse mesencephalic astrocyte-derived neurotrophic factor in Neuro2a cells. *Cellular and Molecular Biology Letters*, *18*(3), 398–415. https://doi.org/10.2478/s11658-013-0096-x

Oh-Hashi, K., Tanaka, K., Koga, H., Hirata, Y., & Kiuchi, K. (2012). Intracellular trafficking and secretion of mouse mesencephalic astrocyte-derived neurotrophic factor. *Molecular and Cellular Biochemistry*, *363*(1–2), 35–41. https://doi.org/10.1007/s11010-011-1155-0

Pakarinen, E., Danilova, T., Võikar, V., Chmielarz, P., Piepponen, P., Airavaara, M., Saarma, M., & Lindahl, M. (2020). MANF Ablation Causes Prolonged Activation of the UPR without Neurodegeneration in the Mouse Midbrain Dopamine System. *ENeuro*, *7*(1). https://doi.org/10.1523/ENEURO.0477-19.2019

Parkash, V., Lindholm, P., Peränen, J., Kalkkinen, N., Oksanen, E., Saarma, M., Leppänen, V. M., & Goldman, A. (2009). The structure of the conserved neurotrophic factors MANF and CDNF explains why they are bifunctional. *Protein Engineering, Design and Selection*, *22*(4), 233–241. https://doi.org/10.1093/protein/gzn080

Petrova, P. S., Raibekas, A., Pevsner, J., Vigo, N., Anafi, M., Moore, M. K., Peaire, A. E., Shridhar, V., Smith, D. I., Kelly, J., Durocher, Y., & Commissiong, J. W. (2003). MANF: A new mesencephalic, astrocyte-derived neurotrophic factor with selectivity for dopaminergic neurons. *Journal of Molecular Neuroscience*, *20*(2), 173–187. https://doi.org/10.1385/JMN:20:2:173

Phiel, C. J., Zhang, F., Huang, E. Y., Guenther, M. G., Lazar, M. A., & Klein, P. S. (2001). Histone Deacetylase is a Direct Target of Valproic Acid, a Potent Anticonvulsant, Mood Stabilizer, and Teratogen. *Journal of Biological Chemistry*, *276*(39), 36734–36741. https://doi.org/10.1074/jbc.M101287200

Primeau, M., Gagnon, J., & Momparler, R. L. (2003). Synergistic antineoplastic action of DNA methylation inhibitor 5-aza-2′-deoxycytidine and histone deacetylase inhibitor depsipeptide

on human breast carcinoma cells. *International Journal of Cancer*, *103*(2), 177–184. https://doi.org/10.1002/ijc.10789

Rasmussen, K. D., & Helin, K. (2016). Role of TET enzymes in DNA methylation, development, and cancer. *Genes and Development* (Vol. 30, Issue 7, pp. 733–750). https://doi.org/10.1101/gad.276568.115

Romanowska, J., & Joshi, A. (2019). From genotype to phenotype: Through chromatin. *Genes* (Vol. 10, Issue 2). https://doi.org/10.3390/genes10020076

Sajadian, S. O., Ehnert, S., Vakilian, H., Koutsouraki, E., Damm, G., Seehofer, D., Thasler, W., Dooley, S., Baharvand, H., Sipos, B., & Nussler, A. K. (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). https://doi.org/10.1186/s13148-015-0133-x

Sales, A. J., Biojone, C., Terceti, M. S., Guimarães, F. S., Gomes, M. V. M., & Joca, S. R. L. (2011). Antidepressant-like effect induced by systemic and intra-hippocampal administration of DNA methylation inhibitors. *British Journal of Pharmacology*, *164*(6), 1711–1721. https://doi.org/10.1111/j.1476-5381.2011.01489.x

Santiago, M., Antunes, C., Guedes, M., Sousa, N., & Marques, C. J. (2014). TET enzymes and DNA hydroxymethylation in neural development and function - How critical are they? *Genomics*, *104*(5), 334–340. https://doi.org/10.1016/j.ygeno.2014.08.018

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*(9), 2723–2732. Shaulian, E., & Karin, M. (2001). AP-1 in cell proliferation and survival. *Oncogene* (Vol. 20, Issue 19 REV. ISS. 2, pp. 2390–2400). https://doi.org/10.1038/sj.onc.1204383

Sidorova, Y. A., & Saarma, M. (2016). Glial cell line-derived neurotrophic factor family ligands and their therapeutic potential. *Molecular Biology* (Vol. 50, Issue 4, pp. 521–531). https://doi.org/10.1134/S0026893316040105

Stresemann, C., & Lyko, F. (2008). Modes of action of the DNA methyltransferase inhibitors

azacytidine and decitabine. *International Journal of Cancer* (Vol. 123, Issue 1, pp. 8–13). https://doi.org/10.1002/ijc.23607

Sun, Z.-P., Gong, L., Huang, S.-H., Geng, Z., Cheng, L., & Chen, Z.-Y. (2011). Intracellular trafficking and secretion of cerebral dopamine neurotrophic factor in neurosecretory cells. *Journal of Neurochemistry*, *117*(1), 121–132. https://doi.org/10.1111/j.1471-4159.2011.07179.x

Vaissière, T., Sawan, C., & Herceg, Z. (2008). Epigenetic interplay between histone modifications and DNA methylation in gene silencing. *Mutation Research - Reviews in Mutation Research* (Vol. 659, Issues 1–2, pp. 40–48). https://doi.org/10.1016/j.mrrev.2008.02.004

Voutilainen, M. H., Arumäe, U., Airavaara, M., & Saarma, M. (2015). Therapeutic potential of the endoplasmic reticulum located and secreted CDNF/MANF family of neurotrophic factors in Parkinson's disease. *FEBS Letters*, *589*(24 Pt A), 3739–3748. https://doi.org/10.1016/j.febslet.2015.09.031

Voutilainen, M. H., Bäck, S., Peränen, J., Lindholm, P., Raasmaja, A., Männistö, P. T., Saarma, M., & Tuominen, R. K. (2011). Chronic infusion of CDNF prevents 6-OHDA-induced deficits in a rat model of Parkinson's disease. *Experimental Neurology*, *228*(1), 99–108. https://doi.org/10.1016/j.expneurol.2010.12.013

Voutilainen, M. H., De Lorenzo, F., Stepanova, P., Bäck, S., Yu, L. Y., Lindholm, P., Pörsti, E., Saarma, M., Männistö, P. T., & Tuominen, R. K. (2017). Evidence for an additive neurorestorative effect of simultaneously administered CDNF and GDNF in hemiparkinsonian rats: Implications for different mechanism of action. *ENeuro*, *4*(1). https://doi.org/10.1523/ENEURO.0117-16.2017

Wakabayashi, K., Tanji, K., Odagiri, S., Miki, Y., Mori, F., & Takahashi, H. (2013). The Lewy body in Parkinson's disease and related neurodegenerative disorders. *Molecular neurobiology* (Vol. 47, Issue 2, pp. 495–508). https://doi.org/10.1007/s12035-012-8280-y

Wang, C. H., Jiang, T. C., Qiang, W. M., Zhang, L., Feng, L. J., Shen, Y. J., & Shen, Y. X. (2018a). Activator protein-1 is a novel regulator of mesencephalic astrocyte-derived

neurotrophic factor transcription. *Molecular Medicine Reports*, *18*(6), 5765–5774. https://doi.org/10.3892/mmr.2018.9601

Wang, D., Hou, C., Cao, Y., Cheng, Q., Zhang, L., Li, H., Feng, L., & Shen, Y. (2018b). XBP1 activation enhances MANF expression via binding to endoplasmic reticulum stress response elements within MANF promoter region in hepatitis B. *International Journal of Biochemistry and Cell Biology*, *99*, 140–146. https://doi.org/10.1016/j.biocel.2018.04.007

Wang, H., Ke, Z., Alimov, A., Xu, M., Frank, J. A., Fang, S., & Luo, J. (2014). Spatiotemporal expression of MANF in the developing rat brain. *PLoS ONE*, *9*(2). https://doi.org/10.1371/journal.pone.0090433

Wei, Y., Melas, P. A., Wegener, G., Mathé, A. A., & Lavebratt, C. (2014). Antidepressant-like effect of sodium butyrate is associated with an increase in TET1 and in 5-hydroxymethylation levels in the Bdnf gene. *The international journal of neuropsychopharmacology*, *18*(2), pyu032. https://doi.org/10.1093/ijnp/pyu032 Weinhold, B. (2006). Epigenetics: the science of change. *Environmental Health Perspectives*, *114*(3), A160. https://doi.org/10.1289/ehp.114-a160

Wu, X., Chen, P. S., Dallas, S., Wilson, B., Block, M. L., Wang, C.-C., Kinyamu, H., Lu, N., Gao, X., Leng, Y., Chuang, D.-M., Zhang, W., Lu, R. B., & Hong, J.-S. (2008). Histone deacetylase inhibitors up-regulate astrocyte GDNF and BDNF gene transcription and protect dopaminergic neurons. *The International Journal of Neuropsychopharmacology*, *11*(08), 1123. https://doi.org/10.1017/S1461145708009024

Xu, C., Bailly-Maitre, B., & Reed, J. C. (2005). Endoplasmic reticulum stress: Cell life and death decisions. *Journal of Clinical Investigation* (Vol. 115, Issue 10, pp. 2656–2664). https://doi.org/10.1172/JCI26373

Xu, S., Di, Z., He, Y., Wang, R., Ma, Y., Sun, R., Li, J., Wang, T., Shen, Y., Fang, S., Feng, L., & Shen, Y. (2019). Mesencephalic astrocyte-derived neurotrophic factor (MANF) protects against Aβ toxicity via attenuating Aβ-induced endoplasmic reticulum stress. *Journal of Neuroinflammation*, *16*(1), 35. https://doi.org/10.1186/s12974-019-1429-0

Yan, Y., Rato, C., Rohland, L., Preissler, S., & Ron, D. (2019). MANF antagonizes nucleotide

exchange by the endoplasmic reticulum chaperone BiP. *Nature Communications*, *10*(1), 1–15. https://doi.org/10.1038/s41467-019-08450-4

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. https://doi.org/10.1016/j.leukres.2004.11.022

Yu, S. M., & Kim, S. J. (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*(4), 1469–1475. https://doi.org/10.3892/ijo.2015.3110

Yu, S. M., & Kim, S. J. (2016). 5-Azacytidine regulates matrix metalloproteinase9 expression and the migration and invasion of human fibrosarcoma HT1080 cells via PI3-kinase and ERK1/2 pathways. *International Journal of Oncology*, *49*(3), 1241–1247. https://doi.org/10.3892/ijo.2016.3612

Yu, Z. Q., Zhang, B. Le, Ren, Q. X., Wang, J. C., Yu, R. T., Qu, D. W., Liu, Z. H., Xiong, Y., & Gao, D. S. (2013). Changes in transcriptional factor binding capacity resulting from promoter region methylation induce aberrantly high GDNF expression in Human glioma. *Molecular Neurobiology* (Vol. 48, Issue 3, pp. 571–580). https://doi.org/10.1007/s12035-013-8443-5

Zhang, J., Tong, W., Sun, H., Jiang, M., Shen, Y., Liu, Y., Gu, H., Guo, J., Fang, J., & Jin, L. (2017). Nrf2-mediated neuroprotection by MANF against 6-OHDA-induced cell damage via PI3K/AKT/GSK3β pathway. *Experimental Gerontology*, *100*, 77–86. https://doi.org/10.1016/j.exger.2017.10.021

Zhao, H., Cheng, L., Liu, Y., Zhang, W., Maharjan, S., Cui, Z., Wang, X., Tang, D., & Nie, L. (2014). Mechanisms of anti-inflammatory property of conserved dopamine neurotrophic factor: Inhibition of JNK signaling in lipopolysaccharide-induced microglia. *Journal of Molecular Neuroscience*, *52*(2), 186–192. https://doi.org/10.1007/s12031-013-0120-7

Zhou, Q., Agoston, A. T., Atadja, P., Nelson, W. G., & Davidson, N. E. (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. https://doi.org/10.1158/1541-7786.MCR-07-0330

Zhou, W., Chang, L., Fang, Y., Du, Z., Li, Y., Song, Y., Hao, F., Lv, L., & Wu, Y. (2016). Cerebral dopamine neurotrophic factor alleviates Aβ25-35-induced endoplasmic reticulum stress and early synaptotoxicity in rat hippocampal cells. *Neuroscience Letters*, *633*, 40–46. https://doi.org/10.1016/j.neulet.2016.09.008