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DNA Methyltransferase Inhibitors Induce Cerebral Dopamine Neurotrophic Factor Expression in C6 Glioma Cells

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Abstract

Cerebral dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF) are involved in neuroprotection and mitigating endoplasmic reticulum (ER) stress in the brain and peripheral organs. In earlier work, an increase in histone acetylation, following treatment with an epigenetic modulator, valproic acid, was associated with induction of CDNF and MANF in cultured cells and rat brain. These findings prompted an investigation of the effects of DNA methyltransferase (DNMT) inhibitors, which can alter epigenetic function, on the expression of CDNF and MANF. Rat C6 glioma cells were treated with a micromolar range of DNMT inhibitors: 5-aza-2'-deoxycytidine (DAC or decitabine), 5-azacytidine (AZA) or zebularine (ZEB) for 24 h. Subsequently, qPCR analysis was used to examine the mRNA expression of DNMT1, ten-eleven translocation methylcytosine dioxygenase 2 (TET-2), CDNF and MANF. A significant dose-dependent decrease in DNMT1 mRNA levels, together with a significant increase in TET-2 expression, was observed following treatment with AZA or DAC. Importantly, DAC, AZA and ZEB caused a significant dose-dependent increase in CDNF mRNA levels. In contrast, MANF mRNA expression decreased following treatment with AZA, with no significant effects observed with DAC or ZEB. Western analysis revealed no significant changes in CDNF protein levels following treatment with DAC for 24 h. The significant increase in CDNF expression, following treatment with DNMT1 inhibitors, suggests that DNA methylation is involved in the regulation of this neurotrophic factor. Clarification of the epigenetic or other mechanisms underlying the regulation of CDNF may provide novel therapeutic approaches in neurodegenerative and ER stress-related disorders.

Keywords

CDNF, MANF, 5-Azacytidine, 5-Aza-2'-deoxycytidine, Zebularine,

Gene Expression

1. Introduction

The neurotrophic factors, cerebral dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF), have been observed to have a protective effect on central dopaminergic neurons, providing potential candidates in the treatment of neurodegenerative disorders [1] [2]. In addition to their effects in the central nervous system (CNS), CDNF and MANF have also been shown to be expressed and have protective effects in peripheral organs, including the heart, pancreas and enteric system [3] [4]. These atypical neurotrophic factors are located in the endoplasmic reticulum (ER) and they play an important role in modulating unfolded-protein response (UPR) signaling, protecting cells from ER stress-induced death, and exerting trophic activities in the extracellular space under pathological conditions [5].

There is evidence that the expression of CDNF and MANF may be regulated by epigenetic mechanisms. Valproic acid (VPA), an inhibitor of histone deacetylase (HDAC) activity, promotes gene transcription through the acetylation of histones that results in an open chromatin structure, facilitating access to DNA by transcription factors and related agents [6]. VPA has been shown to induce CDNF and MANF expression both *in vitro* and *in vivo*. In neural stem cells, the expression of CDNF and MANF was induced following treatment with VPA [7]. Furthermore, CDNF and MANF expression were elevated in rat hippocampus and striatum following chronic VPA treatment, suggesting epigenetic regulation of these proteins [8]. In addition to their effects on histone acetylation, HDAC inhibitors, such as VPA and trichostatin A, have been implicated in DNA methylation [9] [10], which is a major mechanism involved in the epigenetic regulation of gene expression [11]. Given the interplay between histone acetylation and DNA methylation [6], it is possible that the latter mechanism was also involved in the induction of CDNF and MANF by VPA, as observed in earlier studies [7] [8]. Therefore, this study examined the effects of epidrugs, which inhibit DNA methylation, on the expression of CDNF and MANF. The mRNA levels of DNA methyltransferase 1 (DNMT1) which encodes an enzyme with a major role in DNA methylation [12], were examined in order to assess the suppressive action of the inhibitors used. In addition, the expression of ten-eleven translocation methylcytosine dioxygenase 2 (TET-2), was assessed, as this enzyme is an essential mediator of DNA demethylation [13], and an upregulation target for DNMT inhibitors [14] [15].

2. Materials and Methods

2.1. Cell Culture and Drug Treatment

Rat C6 glioma cells, obtained from the American Type Culture Collection (Ma-

nassas, VA), were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, fungizone and penicillin/streptomycin, as reported previously [16]. 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.

5-Azacytidine (AZA) was obtained from Sigma Aldrich (Oakville, ON, CA), 5-aza-2'-deoxycytidine (decitabine; DAC) and zebularine (ZEB) were purchased from Abcam (Toronto, ON, CA). Cells were treated at a confluency of 60% - 70% with AZA (0.1 - 25 μM), DAC (0.1 - 20 μM) or ZEB (5 - 100 μM) for 24 h. Vehicle controls were 0.05% and 0.04% DMSO for AZA and DAC respectively, or PBS for ZEB. Dose-dependent and time-dependent cytotoxicity has been reported for the cytosine nucleoside analogs examined in this study after at least 48 - 72 h of treatment [17] [18]. At a concentration of 5 μM or lower, treatment of various cell lines with AZA or DAC produces slight non-significant effects on cell viability after 24 h [19] [20] [21]. Therefore, in the present study, cells were treated with a range from low to high drug concentrations for only 24 h.

2.2. Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted with TRIzol as described by the supplier (Invitrogen Canada Inc.). Subsequently, cDNA was synthesized from 1 μg of DNase-treated RNA using the SensiFAST cDNA Synthesis Kit (FroggaBio, Toronto, ON, CA), and oligo (dT) primers. Following drug treatment, the relative expression levels of DNMT1, TET-2, MANF and CDNF were examined by RT-qPCR using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories Ltd, Mississauga, ON, CA) and primers (**Table 1**). Amplification was performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Ltd, Mississauga, ON, CA), as follows: initial heat activation at 95˚C for 30 sec, followed by denaturation at 95˚C for 15 sec and annealing/extension at 60˚C for 30 sec for 40 cycles, followed by 95˚C for 10 sec, as reported previously [22]. Internal controls, β2 microglobulin (B2M) and/or TATA-box binding protein (TBP), were used in each experiment. No template controls were included, along with melt curve analysis, to confirm specificity.

2.3. Western Blotting

Cytosolic protein samples were extracted in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Triton-X 100, 1 mM EDTA) with added protease inhibitors. The DC Protein Assay Kit II (Bio-Rad Laboratories Ltd, Mississauga, ON, CA) was used to assess protein concentrations, according to supplier instructions. SDS-PAGE gels were loaded with 20 μg protein and run at 100 V for 2 h. Following protein transfer, polyvinylidene difluoride (PVDF; 0.2 μM) membranes were blocked for 1 h and incubated with primary antibodies: CDNF (1:250; Novus Biologicals, Centennial, CO, USA) and β-Actin (1:10,000; Sigma-Aldrich, Oakville, ON, CA) for 72 h at 4˚C. Membranes were then probed with secondary antibody: 1:2000 of anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Dallas, TX, USA)

Table 1. Nucleotide sequences of primers used for RT-qPCR.

Gene	Primers (5'→3')	Nucleotides	Size (bp)
DNMT1	CCTGGAGAACGAACACTCT	113 - 132	163
	CATGGTCTCACTGTCCGACT	275 - 256	
TET-2	CAA CAT GGT CTC CCA CAC AG	6035 - 6054	206
	TGG AAG GAT CCT GGA AGT TG	6240 - 6221	
CDNF	AAAGAAAACCGCCTGTGCTA	289 - 308	199
	TCATTTTCCACAGGTCCACA	487 - 468	
MANF	GGCGACTGCGAAGTTTGTAT	133 - 152	137
	CGATTTTCTTTGCCTCTTGC	269 - 250	
B2M	CCC AAA GAG ACA GTG GGT GT	962 - 981	150
	CCC TAC TCC CCT CAG TTT CC	1111 - 1092	
TBP	CTCAGTTACAGGTGGCAGCA	1264 - 1283	80
	CTCAGTGCAGAGGAGGGAAC	1343 - 1324	

for CDNF blots, and 1:10,000 of anti-mouse IgG-HRP (Sigma Aldrich, Oakville, ON, CA) for β-Actin blots, at room temperature for 1 h, and bands were visualized by film autoradiography as reported previously [23].

2.4. Statistical Analysis

The delta-delta cycle threshold (ΔΔCq) method [24] was used to analyze relative fold changes in gene expression, following treatment with DNMT inhibitors or vehicle, as reported previously [8] [22]. Normalized optical density values for CDNF vs. β-actin were used to assess protein expression. One-way analysis of variance (ANOVA) was used to determine whether there were significant treatment effects on mRNA or protein levels. In addition, post-hoc analysis (Newman-Keuls) was used to determine significant differences between drug treatments and controls, as reported previously [7] [23]. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effects of DNMT Inhibitors on DNMT1 Expression

Following treatment with AZA for 24 h, a decrease of DNMT1 mRNA expression was observed, with a significant treatment effect ($F_{(9, 35)} = 9.257$, $P < 0.0001$) revealed by one-way ANOVA. A Newman-Keuls test showed significant decreases in DNMT1 mRNA levels at 0.5, 3 and 10 μM ($P < 0.01$) and at 1, 5, 20 and 25 μM AZA ($P < 0.001$), compared to control (**Figure 1A**). Treatment with another DNMT inhibitor, DAC, also resulted in a significant treatment effect ($F_{(5, 12)} = 7.035$, $P < 0.0002$) as revealed by one-way ANOVA. A significant decrease of DNMT1 mRNA levels at 0.5 - 20 μM ($P < 0.01$) was seen compared to control (**Figure 1B**). Treatment with ZEB at higher doses reduced DNMT1 mRNA levels, but these changes were not significant (**Figure 1C**).

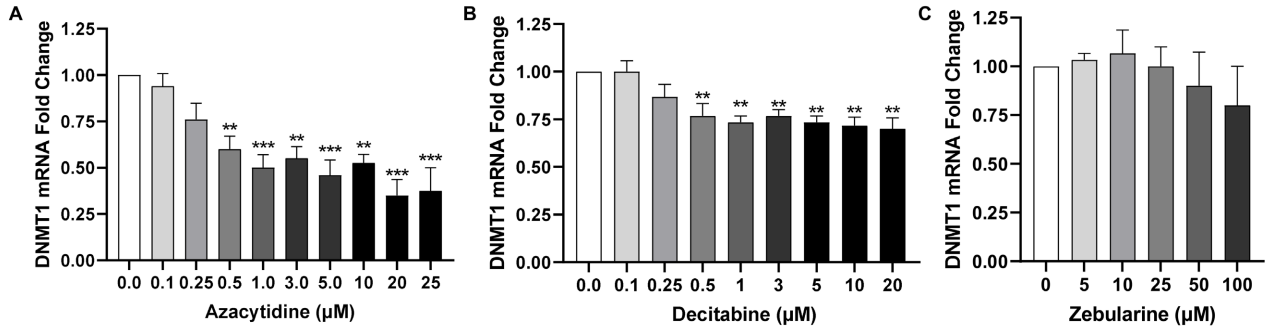

Figure 1. Concentration-dependent effects of DNA methyltransferase inhibitors on DNMT1 expression. C6 cells were treated with (A) Azacytidine (0.1 - 25 μM), (B) Decitabine (0.1 - 20 μM), and (C) Zebularine (5 - 100 μM) for 24 h, and RT-qPCR used to quantify relative mRNA levels. Data shown are the means ± SEM (n = 3 - 5). $^{**}p < 0.01$, $^{***}p < 0.001$ versus control.

3.2. Effects of DNMT Inhibitors on TET-2 Expression

Treatment with AZA for 24 h resulted in a significant treatment effect ($F_{(9, 25)} = 4.061$, $P < 0.026$) on TET-2 expression. There appeared to be a concentration-dependent increase in TET-2 mRNA levels after treatment with AZA (0.1 - 5 μM) for 24 h, followed by a decrease at higher doses, 10, 20 and 25 μM. However, a significant increase in mRNA levels was only observed at the 5 μM dose ($P < 0.05$) compared to control and higher doses, 10, 20 and 25 μM (**Figure 2A**). DAC treatment for 24 h resulted in a significant treatment effect ($F_{(5, 12)} = 3.783$, $P < 0.0091$), with significant concentration-dependent increases seen at doses 1, 5, 10 μM ($P < 0.05$) and 20 μM ($P < 0.01$) compared to control (**Figure 2B**). Similar to DNMT1 results, treatment with ZEB for 24 h resulted in no significant effects on TET-2 mRNA levels (**Figure 2C**).

3.3. Effects of DNMT Inhibitors on MANF Expression

Treatment with AZA for 24 h caused a decrease in MANF mRNA expression. One-way ANOVA showed a significant treatment effect ($F_{(9, 27)} = 10.35$, $P < 0.0001$), and a Newman-Keuls test revealed significant decreases at 5 μM ($P < 0.05$), and at 10 - 25 μM ($P < 0.01$) compared to control (**Figure 3A**). Treatment with other DNMT inhibitors, DAC or ZEB, for 24 h, had no significant effects on MANF mRNA levels (**Figure 3B**, **Figure 3C**).

3.4. Effects of DNMT Inhibitors on CDNF Expression

A concentration-dependent increase in CDNF mRNA expression was observed after treatment with AZA (0.1 - 25 μM) for 24 h. One-way ANOVA revealed a significant treatment effect ($F_{(9, 33)} = 5.326$, $P < 0.0002$), with significant increases in CDNF mRNA levels at 20 and 25 μM AZA ($P < 0.01$) compared to control (**Figure 4A**). Treatment with DAC also resulted in a significant treatment effect ($F_{(8, 18)} = 4.977$, $P < 0.0023$), with dose-dependent increases in CDNF expression, after 24 h. Significant increases were seen at doses 3, 5 and 20 μM ($P < 0.05$), and 10 μM ($P < 0.01$), compared to control (**Figure 4B**). Similarly, treatment with ZEB for the same time period resulted in a significant treatment effect ($F_{(5, 12)} =$

5.997, $P < 0.0052$). A significant increase in CDNF mRNA levels was observed at 100 μM ZEB ($P < 0.05$) compared to control (Figure 4C). DAC, which appeared to be more potent than AZA or ZEB in altering CDNF mRNA expression, was selected for western analysis of CDNF. However, there were no significant changes in CDNF protein levels following treatment with DAC for 24 h (Figure 4D, Figure 4E).

4. Discussion

Previously, treatment with VPA, which can alter epigenetic function via inhibition of HDAC activity, was found to induce CDNF and MANF expression together with an increase in histone acetylation in mouse C17.2 cells [7]. Given the functional interaction between histone acetylation and DNA demethylation [6], it is reasonable to infer that changes in the methylation status of DNA may also play a role in regulating these neurotrophic factors. In keeping with this view, an increase in CDNF expression was observed following treatment with AZA or DAC, which also suppressed DNMT1 mRNA levels. In addition, as observed in mouse T cells [15] and human skin fibroblasts [14], DAC and AZA induced expression of TET-2, which catalyzes the conversion of 5-mC (5-methylcytosine) to 5-hmC (5-hydroxymethylcytosine), an intermediate product in TET-mediated

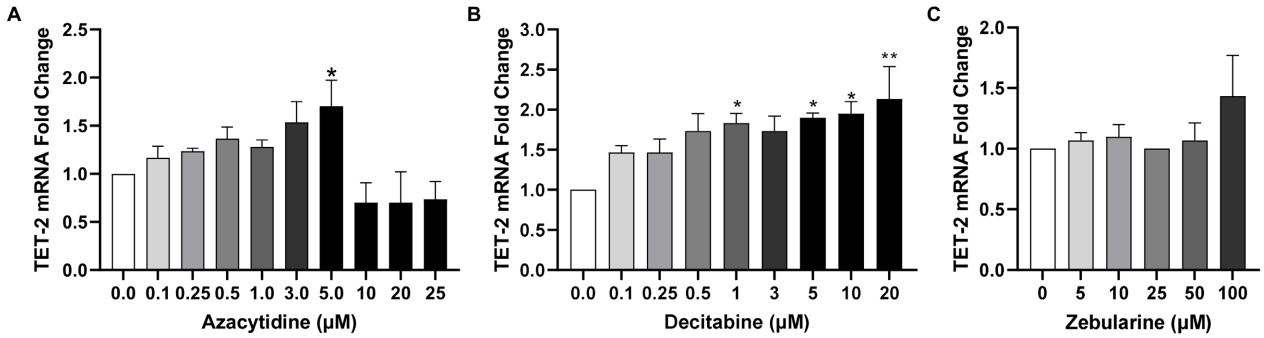

Figure 2. Concentration-dependent effects of DNA methyltransferase inhibitors on TET-2 expression. C6 cells were treated with (A) Azacytidine (0.1 - 25 μM), (B) Decitabine (0.1 - 20 μM), and (C) Zebularine (5 - 100 μM) for 24 h, and RT-qPCR used to quantify relative mRNA levels. Data shown are the means ± SEM (n = 3 - 5). *$p < 0.05$, **$p < 0.01$ versus control.

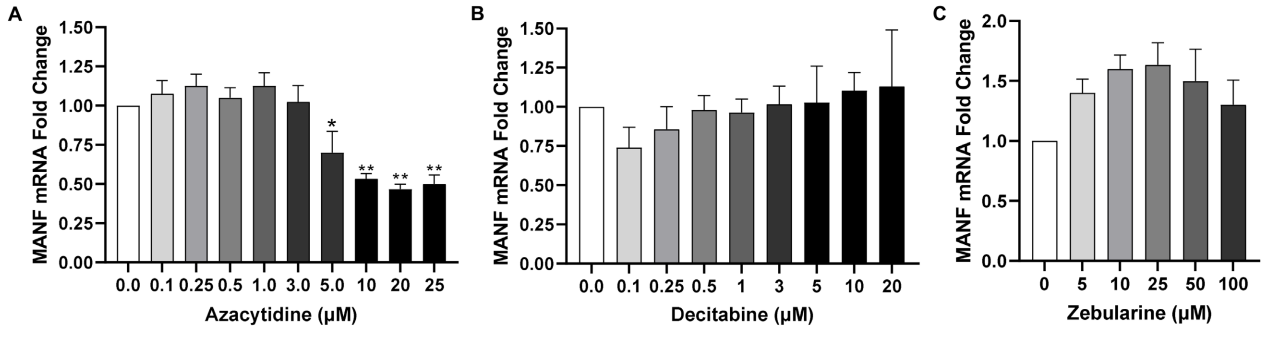

Figure 3. Concentration-dependent effects of DNA methyltransferase inhibitors on MANF expression. C6 cells were treated with (A) Azacytidine (0.1 - 25 μM), (B) Decitabine (0.1 - 20 μM), and (C) Zebularine (5 - 100 μM) for 24 h, and RT-qPCR used to quantify relative mRNA levels. Data shown are the means ± SEM (n = 3 - 4). *$p < 0.05$, **$p < 0.01$ versus control.

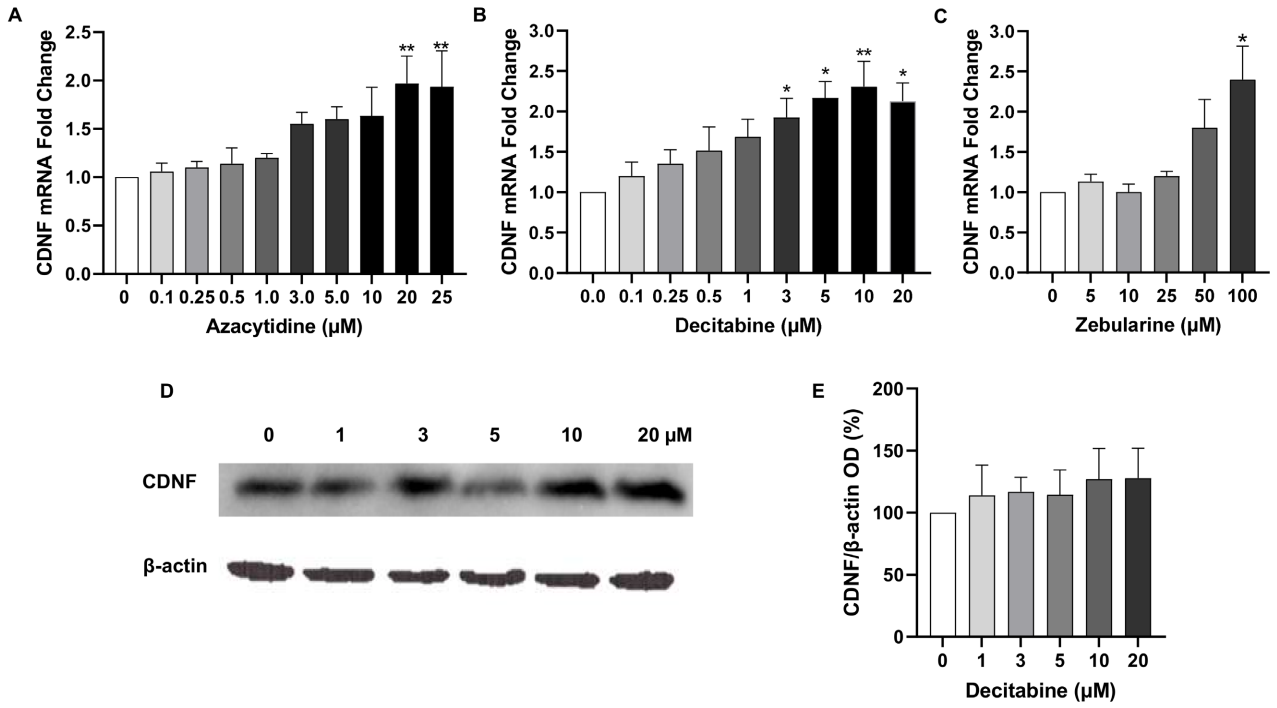

Figure 4. Induction of CDNF expression by DNA methyltransferase inhibitors. C6 cells were treated with (A) Azacytidine (0.1 - 25 μM), (B) Decitabine (0.1 - 20 μM), and (C) Zebularine (5 - 100 μM) for 24 h, and RT-qPCR used to quantify relative mRNA levels. Data shown are the means ± SEM (n=3 - 5). *$p < 0.05$, **$p < 0.01$ versus control. (D) Immunoblots of CDNF (18 kDa) and β-actin (42 kDa) following decitabine treatment. (E) Histograms showing the means ± SEM (n = 4) for percentage (%) values of CDNF/β-actin optical density ratios.

DNA demethylation [13]. The induction of TET-2 was similar up to 5 μM DAC or AZA treatment, but the sudden decrease in TET-2 mRNA levels observed at higher AZA (but not DAC) concentrations, may be related to structural and pharmacological differences between these drugs. In contrast to DAC and ZEB which are incorporated only into DNA, AZA is primarily incorporated into RNA allowing it to induce effects, such as destabilization and degradation of RNA, which do not involve DNA hypomethylation [25].

DNMT1 inhibition by DAC has been associated with hypomethylation of gene promoters and increases in gene expression [26] [27]. While it is possible that demethylation of the CDNF promoter accounts for its induction by DAC, other sites or mechanisms may be involved. There is increasing evidence that DAC can alter gene expression via direct or indirect mechanisms [28]. The direct actions of DAC result in changes in DNA methylation in the promoter and/or gene body of a particular target, while its indirect actions could involve the methylation of upstream transcription factors and other regulatory elements which can modulate expression of the target gene [28]. For example, DAC caused a significant increase in COX-2 expression in fibrotic lung fibroblasts by demethylation of the transcription factor C8orf4 (chromosome 8 open reading frame 4), which can regulate COX-2 expression [29]. In view of the foregoing, a major weakness of the present study is the lack of mechanistic information on

potential changes in the DNA methylation of the CDNF promoter and/or gene body. Future work in this area should include DNA methylation analysis such as bisulfite sequencing in order to determine the methylation status of the CDNF gene following DAC treatment.

Although a wide range of low to high concentrations of DAC increased CDNF mRNA expression, there were no changes in CDNF protein levels following similar treatment with this cytosine nucleoside analog for 24 h. Several factors could account for the discordance between mRNA and protein levels, which has been observed in other studies [30]. While a correlation is generally seen between mRNA and protein levels under steady state conditions, this relationship can be altered by multiple factors including the rate of translation (which is influenced by the mRNA sequence), regulatory proteins or micro-RNAs binding to the transcript, the time required for protein synthesis and cellular disruptions such as ER stress [31] [32]. Another shortcoming in the present study was the examination of the effects of DAC treatment after only a 24 h period, which could have missed earlier or later changes in the expression of CDNF protein. Future time course studies, over a range of treatment periods, should help to clarify the relationship between the CDNF transcript and protein expression.

Interestingly, in contrast to its induction of CDNF, DAC did not alter MANF expression, suggesting differential epigenetic regulation of these proteins. It is known that ER stress, which is caused by the accumulation of unfolded or misfolded proteins, can induce MANF expression [33] [34]. Recent *in vivo* studies have shown that CDNF levels are increased in mouse tissues following injection of tunicamycin (an ER stress inducer), indicating that like MANF, it can be induced by ER stress [35]. Furthermore, emerging reports have suggested that microRNAs (miRNAs) are involved in the regulation of endogenous CDNF and MANF levels. Recently, miR-144 was shown to directly suppress human MANF mRNA and protein levels in HEK293-T cells without affecting ER stress markers [36]. Thus, the suppression of MANF could involve induction of miR-144, which AZA was found to upregulate in HepG2 cells [37]. AZA and DAC were also seen to modulate other miRNAs [38] [39] that were linked to CDNF expression [36]. The possible role of these miRNAs in the observed epidrug-induced changes in CDNF and MANF expression should be examined in future studies.

It is well known that ER stress can activate the UPR (unfolded protein response), an adaptive signaling cascade, which mediates the restoration of ER proteostasis [40]. CDNF was shown to increase the expression of an early UPR regulator, glucose-regulated protein 78 (GRP78), following ER stress induction by thapsigargin in HEK293-T cells, indicating an activation of this cytoprotective signaling pathway [41]. Furthermore, activation of early UPR signaling was associated with a suppression of pro-apoptotic signals, such as active caspase-3 and C/EBP homologous protein (CHOP), suggesting that CDNF may act to mitigate ER stress through early activation of the UPR pathway, as well as provide protection from apoptotic signaling [41]. In addition, CDNF has been shown to

have neuroprotective and neurorestorative effects in neurodegenerative models. Pre-treatment with CDNF was found to inhibit α-synuclein aggregation in H4 human neuroglioma cells, as well as protecting cells from toxicity caused by α-synuclein oligomers [42]. *In vivo*, CDNF was seen to recover a number of tyrosine hydroxylase (TH)-positive cells in the substantia nigra following infusion of 6-hydroxydopamine, demonstrating a neurorestorative effect [43]. In the peripheral system, high levels of CDNF were especially seen in tissues with high metabolic function, including the skeletal muscle, heart and exocrine tissue of the pancreas [3]. CDNF was also detected in the submucosal and myenteric plexuses of the enteric system, where emerging evidence suggests that it is involved in the development and maintenance of this system [4].

5. Conclusion

In conclusion, earlier evidence that HDAC inhibition by VPA induces MANF and CDNF expression in rat brain and cultured cells [7] [8] suggested that these neurotrophic factors may be subject to epigenetic modulation. The present preliminary findings partially corroborate this view, as CDNF expression was induced by inhibitors of DNMT activity, although the picture is less clear for MANF which showed a decrease in expression after similar treatment. In view of the neuroprotective and cytoprotective properties of CDNF and MANF, future studies of the epigenetic and/or other mechanisms underlying their regulation may provide a therapeutic avenue for neurodegenerative and metabolic/ER-stress disorders.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Lindahl, M., Saarma, M. and 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

[2] Huttunen, H.J. and Saarma, M. (2019) CDNF Protein Therapy in Parkinson's Disease. *Cell Transplantation*, **28**, 349-366. https://doi.org/10.1177/0963689719840290

[3] Danilova, T., Galli, E., Pakarinen, E., Palm, E., Lindholm, P., Saarma, M. and Lindahl, M. (2019) Mesencephalic Astrocyte-Derived Neurotrophic Factor (MANF) Is Highly Expressed in Mouse Tissues with Metabolic Function. *Frontiers in Endocrinology* (*Lausanne*), **10**, Article No. 765. https://doi.org/10.3389/fendo.2019.00765

[4] Chalazonitis, A., Li, Z., Pham, T.D., Chen, J., Rao, M., Lindholm, P., Saarma, M., Lindahl, M. and Gershon, M.D. (2020) Cerebral Dopamine Neurotrophic Factor Is

Essential for Enteric Neuronal Development, Maintenance, and Regulation of Gastrointestinal Transit. *Journal of Comparative Neurology*, **528**, 2420-2444. https://doi.org/10.1002/cne.24901

[5] Jäntti, M. and Harvey, B.K. (2020) Trophic Activities of Endoplasmic Reticulum Proteins CDNF and MANF. *Cell and Tissue Research*, **382**, 83-100. https://doi.org/10.1007/s00441-020-03263-0

[6] Vaissière, T., Sawan, C. and Herceg, Z. (2008) Epigenetic Interplay between Histone Modifications and DNA Methylation in Gene Silencing. *Mutation Research—Reviews in Mutation Research*, **659**, 40-48. https://doi.org/10.1016/j.mrrev.2008.02.004

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

[8] Niles, L.P., Sathiyapalan, A., Bahna, S., Kang, N.H. and Pan, Y. (2012) Valproic Acid Up-Regulates Melatonin MT1 and MT2 Receptors and Neurotrophic Factors CDNF and MANF in the Rat Brain. *International Journal of Neuropsychopharmacology*, **15**, 1343-1350. https://doi.org/10.1017/S1461145711001969

[9] Detich, N., Bovenzi, V. and Szyf, M. (2003) Valproate Induces Replication Independent Active DNA Demethylation. *Journal of Biological Chemistry*, **278**, 27586-27592. https://doi.org/10.1074/jbc.M303740200

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

[11] Ma, L., Li, C., Yin, H., Huang, J., Yu, S., Zhao, J., Tang, Y., Yu, M., Lin, J., Ding, L. and Cui, Q. (2023) The Mechanism of DNA Methylation and miRNA in Breast Cancer. *International Journal of Molecular Sciences*, **24**, Article No. 9360. https://doi.org/10.3390/ijms24119360

[12] Takebayashi, S., Tamura, T., Matsuoka, C. and Okano, M. (2007) Major and Essential Role for the DNA Methylation Mark in Mouse Embryogenesis and Stable Association of DNMT1 with Newly Replicated Regions. *Molecular and Cellular Biology*, **27**, 8243-8258. https://doi.org/10.1128/MCB.00899-07

[13] Santiago, M., Antunes, C., Guedes, M., Sousa, N. and Marques, C.J. (2014) TET Enzymes and DNA Hydroxymethylation in Neural Development and Function—How Critical Are They? *Genomics*, **104**, 334-340. https://doi.org/10.1016/j.ygeno.2014.08.018

[14] Manzoni, E.F.M., Pennarossa, G., deEguileor, M., Tettamanti, G., Gandolfi, F. and Brevini, T.A.L. (2016) 5-Azacytidine Affects TET2 and Histone Transcription and Reshapes Morphology of Human Skin Fibroblasts. *Scientific Reports*, **6**, Article No. 37017. https://doi.org/10.1038/srep37017

[15] Wang, X., Wang, J., Yu, Y., Ma, T., Chen, P., Zhou, B. and Tao, R. (2017) Decitabine Inhibits T Cell Proliferation via a Novel TET2-Dependent Mechanism and Exerts Potent Protective Effect in Mouse Auto- and Allo-Immunity Models. *Oncotarget*, **8**, 56802-56815. https://doi.org/10.18632/oncotarget.18063

[16] Kim, B., Castro, L.M.R., Jawed, S. and Niles, L.P. (2008) Clinically Relevant Concentrations of Valproic Acid Modulate Melatonin MT1 Receptor, HDAC and MeCP2 mRNA Expression in C6 Glioma Cells. *European Journal of Pharmacology*, **589**, 45-48. https://doi.org/10.1016/j.ejphar.2008.04.058

[17] Hollenbach, P.W., Nguyen, A.N., Brady, H., Williams, M., Ning, Y., Richard, N., Krushel, L., Aukerman, S.L., Heise, C. and MacBeth, K.J. (2010) A Comparison of Azacitidine and Decitabine Activities in Acute Myeloid Leukemia Cell Lines. *PLOS ONE*, **5**, e9001. https://doi.org/10.1371/journal.pone.0009001

[18] Najem, S.A., Khawaja, G., Hodroj, M.H., Babikian, P. and Rizk, S. (2019) Adjuvant Epigenetic Therapy of Decitabine and Suberoylanilide Hydroxamic Acid Exerts Anti-Neoplastic Effects in Acute Myeloid Leukemia Cells. *Cells*, **8**, Article No. 1480. https://doi.org/10.3390/cells8121480

[19] Wang, L., Guo, X., Guo, X., Zhang, X. and Ren, J. (2019) Decitabine Promotes Apoptosis in Mesenchymal Stromal Cells Isolated from Patients with Myelodysplastic Syndromes by Inducing Reactive Oxygen Species Generation. *European Journal of Pharmacology*, **863**, Article ID: 172676. https://doi.org/10.1016/j.ejphar.2019.172676

[20] Brodská, B., Holoubek, A., Otevřelová, P. and Kuželová, K. (2013) Combined Treatment with Low Concentrations of Decitabine and SAHA Causes Cell Death in Leukemic Cell Lines but Not in Normal Peripheral Blood Lymphocytes. *BioMed Research International*, **2013**, Article ID: 659254. https://doi.org/10.1155/2013/659254

[21] Sanaei, M., Kavoosi, F. and Pourahmadi, M. (2021) Effect of Decitabine (5-aza-2'-deoxycytidine, 5-aza-CdR) in Comparison with Vorinostat (Suberoylanilide Hydroxamic Acid, SAHA) on DNMT1, DNMT3a and DNMT3b, HDAC 1-3, SOCS 1, SOCS 3, JAK2, and STAT3 Gene Expression in Hepatocellular Carcinoma HLE and LCL-PI 11 Cell Lines. *The Asian Pacific Journal of Cancer Prevention*, **22**, 2089-2098. https://doi.org/10.31557/APJCP.2021.22.7.2089

[22] Bahna, S.G. and Niles, L.P. (2017) Epigenetic Induction of Melatonin MT1 Receptors by Valproate: Neurotherapeutic Implications. *European Neuropsychopharmacology*, **27**, 828-832. https://doi.org/10.1016/j.euroneuro.2017.06.002

[23] Pan, Y. and Niles, L.P. (2015) Epigenetic Mechanisms of Melatonin Action in Human SH-SY5Y Neuroblastoma Cells. *Molecular and Cellular Endocrinology*, **402**, 57-63. https://doi.org/10.1016/j.mce.2015.01.003

[24] Livak, K.J. and Schmittgen, T.D. (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-ΔΔCT Method. *Methods*, **25**, 402-408. https://doi.org/10.1006/meth.2001.1262

[25] Aimiuwu, J., Wang, H., Chen, P., Xie, Z., Wang, J., Liu, S., Klisovic, R., Mims, A., Blum, W., Marcucci, G. and Chan, K.K. (2012) RNA-Dependent Inhibition of Ribonucleotide Reductase Is a Major Pathway for 5-Azacytidine Activity in Acute Myeloid Leukemia. *Blood*, **119**, 5229-5238. https://doi.org/10.1182/blood-2011-11-382226

[26] Kim, J.T., Li, J., Song, J., Lee, E.Y., Weiss, H.L., Townsend Jr., C.M. and Evers, B.M. (2015) Differential Expression and Tumorigenic Function of Neurotensin Receptor 1 in Neuroendocrine Tumor Cells. *Oncotarget*, **6**, 26960-26970. https://doi.org/10.18632/oncotarget.4745

[27] Sayar, N., Karahan, G., Konu, O., Bozkurt, B., Bozdogan, O. and Yulug, I.G. (2015) Transgelin Gene Is Frequently Downregulated by Promoter DNA Hypermethylation in Breast Cancer. *Clinical Epigenetics*, **7**, Article No. 104. https://doi.org/10.1186/s13148-015-0138-5

[28] Seelan, R.S., Mukhopadhyay, P., Pisano, M.M. and Greene, R.M. (2018) Effects of 5-Aza-2'-deoxycytidine (Decitabine) on Gene Expression. *Drug Metabolism Reviews*, **50**, 193-207. https://doi.org/10.1080/03602532.2018.1437446

[29] Evans, I.C., Barnes, J.L., Garner, I.M., Pearce, D.R., Maher, T.M., Shiwen, X., Renzoni, E.A., Wells, A.U., Denton, C.P., Laurent, G.J., Abraham, D.J. and McAnulty, R.J. (2016) Epigenetic Regulation of Cyclooxygenase-2 by Methylation of c8orf4 in Pulmonary Fibrosis. *Clinical Science* (*London*), **130**, 575-586. https://doi.org/10.1042/CS20150697

[30] Koussounadis, A., Langdon, S.P., Um, I.H., Harrison, D.J. and Smith, V.A. (2015) Relationship between Differentially Expressed mRNA and mRNA-Protein Correlations in a Xenograft Model System. *Scientific Reports*, **5**, Article No. 10775. https://doi.org/10.1038/srep10775

[31] Liu, Y., Beyer, A. and Aebersold, R. (2016) On the Dependency of Cellular Protein Levels on mRNA Abundance. *Cell*, **165**, 535-550. https://doi.org/10.1016/j.cell.2016.03.014

[32] Cheng, Z., Teo, G., Krueger, S., Rock, T.M., Koh, H.W., Choi, H. and Vogel, C. (2016) Differential Dynamics of the Mammalian mRNA and Protein Expression Response to Misfolding Stress. *Molecular Systems Biology*, **12**, Article No. 855. https://doi.org/10.15252/msb.20156423

[33] Apostolou, A., Shen, Y., Liang, Y., Luo, J. and Fang, S. (2008) Armet, a UPR Upregulated Protein, Inhibits Cell Proliferation and ER Stress-Induced Cell Death. *Experimental Cell Research*, **314**, 2454-2467. https://doi.org/10.1016/j.yexcr.2008.05.001

[34] Mizobuchi, N., Hoseki, J., Kubota, H., Toyokuni, S., Nozaki, J.-I.I., Naitoh, M., Koizumi, A. and Nagata, K. (2007) ARMET Is a Soluble ER Protein Induced by the Unfolded Protein Response via ERSE-II Element. *Cell Structure and Function*, **32**, 41-50. https://doi.org/10.1247/csf.07001

[35] Eesmaa, A., Yu, L.Y., Göös, H., Danilova, T., Nõges, K., Pakarinen, E., Varjosalo, M., Lindahl, M., Lindholm, P. and Saarma, M. (2022) CDNF Interacts with ER Chaperones and Requires UPR Sensors to Promote Neuronal Survival. *International Journal of Molecular Sciences*, **23**, Article No. 9489. https://doi.org/10.3390/ijms23169489

[36] Konovalova, J., Gerasymchuk, D., Arroyo, S.N., Kluske, S., Mastroianni, F., Pereyra, A.V. and Domanskyi, A. (2021) Human-Specific Regulation of Neurotrophic Factors MANF and CDNF by microRNAs. *International Journal of Molecular Sciences*, **22**, Article No. 9691. https://doi.org/10.3390/ijms22189691

[37] Zhao, J., Li, H., Zhao, S., Wang, E., Zhu, J., Feng, D., Zhu, Y., Dou, W., Fan, Q., Hu, J., Jia, L. and Liu, L. (2021) Epigenetic Silencing of miR-144/451a Cluster Contributes to HCC Progression via Paracrine HGF/MIF-Mediated TAM Remodeling. *Molecular Cancer*, **20**, Article No. 46. https://doi.org/10.1186/s12943-021-01343-5

[38] Bian, E.B., Ma, C.C., He, X.J., Wang, C., Zong, G., Wang, H.L. and Zhao, B. (2016) Epigenetic Modification of miR-141 Regulates SKA2 by an Endogenous "Sponge" HOTAIR in Glioma. *Oncotarget*, **7**, 30610-30625. https://doi.org/10.18632/oncotarget.8895

[39] Go, H., Jang, J. Y., Kim, C.W., Huh, J., Kim, P.J. and Jeon, Y.K. (2019) Identification of microRNAs Modulated by DNA Hypomethylating Drugs in Extranodal NK/T-Cell Lymphoma. *Leukemia & Lymphoma*, **61**, 66-74. https://doi.org/10.1080/10428194.2019.1654096

[40] Hetz, C., Zhang, K. and Kaufman, R.J. (2020) Mechanisms, Regulation and Functions of the Unfolded Protein Response. *Nature Reviews Molecular Cell Biology*, **21**, 421-438. https://doi.org/10.1038/s41580-020-0250-z

[41] Arancibia, D., Zamorano, P. and Andrés, M.E. (2018) CDNF Induces the Adaptive Unfolded Protein Response and Attenuates Endoplasmic Reticulum Stress-Induced

Cell Death. *Biochimica et Biophysica Acta* (*BBA*)—*Molecular Cell Research*, **1865**, 1579-1589. https://doi.org/10.1016/j.bbamcr.2018.08.012

[42] Albert, K., Raymundo, D.P., Panhelainen, A., Eesmaa, A., Shvachiy, L., Araújo, G.R., Chmielarz, P., Yan, X., Singh, A., Cordeiro, Y., Palhano, F.L., Foguel, D., Luk, K.C., Domanskyi, A., Voutilainen, M.H., Huttunen, H.J., Outeiro, T.F., Saarma, M., Almeida, M.S. and Airavaara, M. (2021) Cerebral Dopamine Neurotrophic Factor Reduces α-Synuclein Aggregation and Propagation and Alleviates Behavioral Alterations *in Vivo*. *Molecular Therapy*, **29**, 2821-2840. https://doi.org/10.1016/j.ymthe.2021.04.035

[43] 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. and Saarma, M. (2007) Novel Neurotrophic Factor CDNF Protects and Rescues Midbrain Dopamine Neurons *in Vivo*. *Nature*, **448**, 73-77. https://doi.org/10.1038/nature05957