

**Induction Of Neurotrophic and Differentiation Genes in Neural Stem
Cells by Valproic Acid**

M.Sc. Thesis

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**INDUCTION OF NEUROTROPHIC AND DIFFERENTIATION GENES IN
NEURAL STEM CELLS BY VALPROIC ACID**

By

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**A Thesis Submitted to the School of Graduate Studies in Partial
Fulfillment of the Requirements for the Degree**

Master of Science

McMaster University

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MASTER OF SCIENCE (2013)

McMaster University

(Medical Sciences)

Hamilton, Ontario

**TITLE: Induction Of Neurotrophic and Differentiation Genes in Neural
Stem Cells by Valproic Acid**

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NUMBER OF PAGES: xii, 105

ABSTRACT

The short-chain branched fatty acid, valproic acid (2-*propylpentanoic acid*), has long been in use clinically as an anticonvulsant and mood-stabilizing drug. Recently, VPA has been shown to inhibit the activity of histone deacetylases (HDACs), resulting in chromatin remodelling and changes in gene expression. Although the molecular mechanism for VPA action in the central nervous system (CNS) is not well understood, many signalling pathways have been suggested to serve as targets for this HDAC inhibitor. For instance, VPA was found to induce differentiation in adult hippocampal neural progenitor cells via the β -catenin-Ras-ERK pathway. VPA was also shown to up regulate Bcl-2, a neurotrophic/neuroprotective protein, with association of extracellular signal-regulated kinase (ERK-1) and phosphatidylinositol 3- kinase (PI3) pathway activation. In this study, C17.2 neural stem cells were used to examine the effects of VPA on the expression of the novel neurotrophic factors; cerebral dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF). Also the effect of VPA on other genes including; glial cell-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), the orphan nuclear receptor-related factor1 (Nurr-1), the early growth response protein 1(Egr-1), and the sex determining region Y-box-2 (Sox-2) were examined. Histone H3 acetylation and the ERK1/2 pathway were examined as possible targets for VPA action. Treatment with clinically relevant concentrations of VPA (1mM, and 3 mM) induced a significant increase of CDNF protein concentrations. Also, an increase in the mRNA expressions of GDNF, Nurr-1, and Egr-1 were detected following 24 hours VPA treatment at clinically relevant concentrations. Also, an increase of histone H3 acetylation was noticed, suggesting HDAC inhibition as a potential mechanism mediated by VPA to induce gene expression in C17.2 NSCs. These findings might support the role of VPA in neuronal development, differentiation and neuroprotection.

ACKNOWLEDGMENT

My sincere gratitude goes to Dr. Niles for his guidance, support, and patience for the past two years. Thank you Dr. Niles for giving me the opportunity to be a part of your lab. It has been a great privilege working with you. Many thanks go to my committee members Dr. Lobb and Dr. Werstiuk for the intellectual guidance offered during regular meetings. I would also like to thank Dr. Mishra for the use of his lab equipment. Many thanks go to my friends for their tremendous support. Finally, deep thanks go to my mother Afaf Telmesani and for my brother Abdulrahman Almutawaa for their encouragement and love.

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List of Abbreviation

| | |
|---------------------------------|---|
| AD | Alzheimer's Disease (AD) |
| AP-1 | Activator Protein-1 |
| ALS | Amyotrophic Lateral Sclerosis |
| Bcl-2 | B-cell lymphoma-2 |
| BDNF | Brain-Derived Neurotrophic Factor |
| CDNF | Conserved Dopamine Neurotrophic Factor |
| CNS | Central Nervous System |
| CREB | cAMP Response Element-Binding Protein |
| DA | Dopaminergic Neuron |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DMSO | Dimethyl Sulfoxide |
| Egr-1 | Early Growth Response Protein-1 |
| ERK-1/2 | Extracellular Signal-Regulated kinases1/2 |
| FBS | Fetal Bovine Serum |
| GABA | Gamma-Aminobutyric Acid |
| GAPDH | Glyceraldehyde-3-Phosphate Dehydrogenase |
| GDNF | Glial cell line-Derived Neurotrophic Factor |
| GSK-3 β | Glycogen Synthase Kinase-Beta |
| HATs | Histone Acetyltransferases |
| HDACs | Histone Deacetylases |
| HD | Huntington's Disease |

| | |
|----------------|--|
| HS | Horse Serum |
| HPS70 | Heat Shock Protein 70 |
| MANF | Mesencephalic Astrocyte-Derived Factor |
| MAPK | Mitogen- Activated Protein Kinase |
| MPTP | 1-Methyl-4-Phenyl-1,2,3,6- tetrahydropyridine |
| NSCs | Neural Stem Cells |
| Nurr-1 | Nuclear Receptor-Related Factor1 |
| NMDA | N-Methyl-D-Aspartate |
| PKA | Protein Kinase A |
| PKC | Protein Kinase C |
| PKB/AKT | Protein Kinase B |
| PIK3 | Phosphatidylinositol 3- kinase |
| PD | Parkinson's Disease |
| RGC | Retinal Ganglion Cell |
| SMA | Spinal Muscular Atrophy |
| Sox-2 | Sex determining region Y-box -2 Gene/ transcription factor |
| TH | Tyrosine Hydroxylase |
| VPA | Valproic Acid |
| 6-ODHA | 6-hydroxydopamine |
| 18sRNA | 18S ribosomal RNA |

CHAPTER 1. INTRODUCTION

Neural Stem Cells: an Overview

Recent evidence that has reported adult neurogenesis, a process by which new neurons are generated and migrate to integrate to existing circuitries (Abrous et al., 2005), following insults like stroke or trauma has reformed our understanding of central nervous system (CNS) functionality. Neural stem cells (NSCs) have been isolated from different regions of the brain and have been defined loosely as: (i) cells that can generate neural tissue or are derived from the nervous system; (ii) have the capacity to renew themselves, and give rise to cells other than themselves through asymmetric fate-committed division (Gage, 2000). Neural stem cell (NSC) has the capacity renew itself, but has shown to have a short survival time (Gage et al., 1995). Following division, progenitor cells undergo further lineage-restricted differentiation and give rise to either neuroblasts or glioblasts (Luskin et al., 1993; Gage et al., 1995), which subsequently differentiate into either astrocytes or oligodendrocytes (Figure 1.1).

The remarkable regenerative capacity of the adult NSCs holds promise for the repair of the damaged CNS. Several strategies have been proposed to induce and/or enhance the regeneration process including: cellular replacement, neurotrophic factor delivery, elimination of growth inhibitory mechanisms, and modifying the immune response (Horner et al., 2000).

NSCs can be isolated from either the embryonic CNS (Azari H et al., 2011), or from very restricted areas in the adult brain including the sub-ventricular zone (SVZ) lining the lateral ventricles, and the dentate gyrus of the hippocampus (Johansson et al., 1991; Ming et al., 2011). Moreover, lineage-restricted multi-potent neural progenitors cells (NPCs), that can give rise to both neuronal and glial progeny *in vitro*, have been isolated from different adult brain regions (Arsenijevic et al., 2001; Nunes et al., 2003).

Although NSCs have discrete advantages over primary tissues preparations, as they can be easily expanded, genetically modified and efficiently propagated into the desired cell type (Rossi et al., 2002; Reekmans et al., 2012), NSCs have shown a limited proliferation potential when propagated for a long time *in vitro*. NSCs limited proliferation capacity might be attributed to telomere shortening (Villa et al., 2004). However, immortalized NSC lines carrying an oncogene have shown proliferation *in vitro* (Bithell et al., 2005). NSC immortalization utilizing v-myc oncogene increases telomerase activity, thus preserving stable cell cycle length, mitotic potential, differentiation, and neuron generation capacity (Villa et al., 2004).

Hence, immortalized NSCs are excellent candidates for *in vitro* and *in vivo* developmental research, drug discovery and screening, and for cell therapeutics studies in the nervous system (Villa et al., 2004). The following section discusses the characteristics of the immortalized NSC cell line, C17.2, which was used in this study.

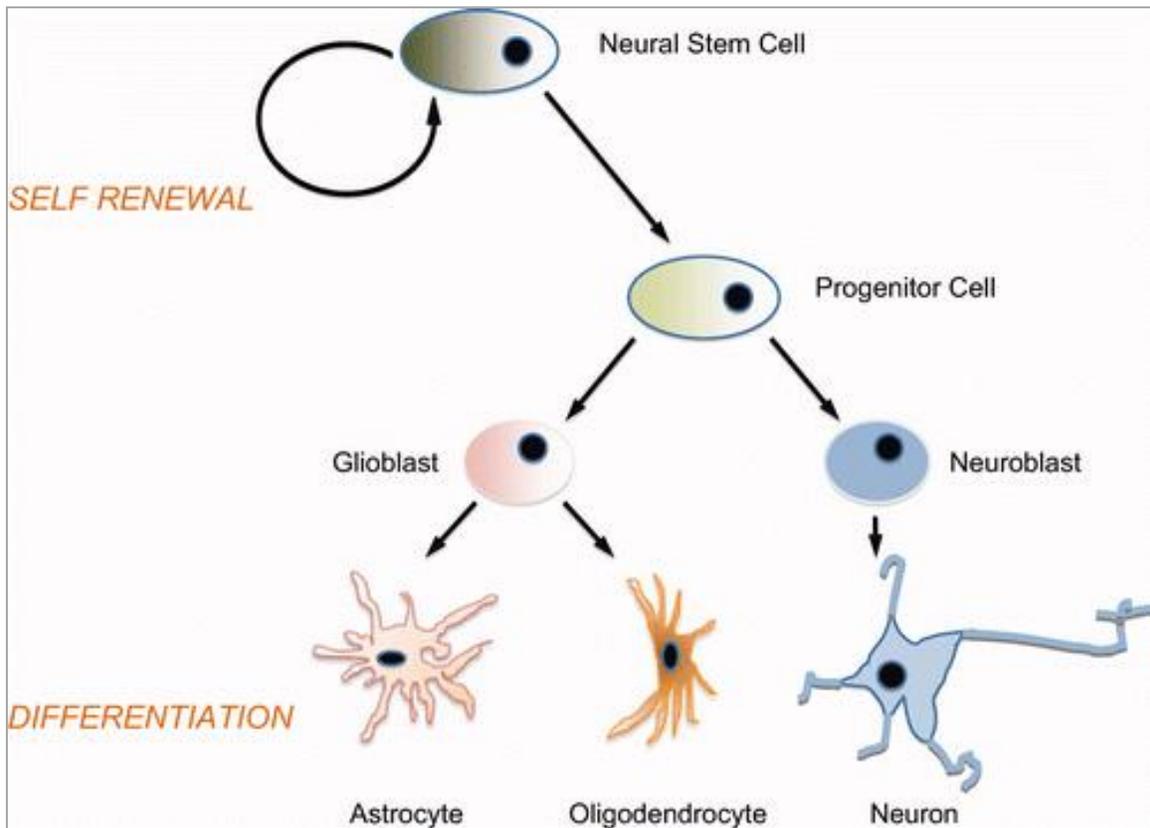


Figure 1.1 Neural stem cell (NSC) self-renewal and differentiation.

A NSC has the ability to renew itself and to differentiate into other neural cell types. NSCs undergo multiple divisions to give rise to intermediary progeny cells that subsequently differentiate further into astrocytes, oligodendrocytes, and glial cells.

From: Ahmed-et al., 2011. Stem cells in the adult human brain. Br J Neurosurg.(25):28-37

1.2. C17.2 Cells: a Neural Stem Cell Line

C17.2 is a multi-potent neural progenitor cell line that was generated by v-myc immortalization of neonatal mouse cerebellar precursor cells (Ryder et al., 1990; Snyder et al., 1992). C17.2 cells have demonstrated the capacity for self-renewal as well as its differentiation into neurons, astrocytes, and oligodendrocytes (Ryder et al., 1990; Snyder et al., 1992). In addition, C17.2 cells have the ability to differentiate into more specific neurons like dopaminergic (Wagner et al., 1999) and cholinergic neurons (Doering et al., 2000).

NSCs transplantations have been shown to be of therapeutic value in the treatment of several adult neurodegenerative disorders. Early studies have indicated the ability of C17.2 cells to successfully integrate into the CNS of several animal models to replace lost neurons and/or glia. (Ryder et al., 1990; Snyder et al., 1992; Snyder et al., 1995; Snyder et al., 1997; Park et al., 2002; Riess et al., 2002; Yang et al., 2002; Ryu et al., 2005). The most dramatic effects of NSC replacement were observed using immortalized neural stem cell lines (C17.2) (Mi et al., 2005). The apparent success of NSC replacement may in part be explained by the distinct properties of the cell line (Mi et al., 2005). Furthermore, C17.2 express several neurotrophins, including NGF, GDNF, and BDNF (Lu et al., 2003; Niles et al., 2004; Yan et al., 2004).

Interestingly, neuronal differentiation of C17.2 cells can be induced by hormone or drug treatment (Sharma et al., 2008; Li et al., 2011). The C17.2 cell line serves as promising source of multi-potent neural stem cells as these cells may differentiate into neurons or other cells, and be easily propagated and transplanted.

1.3. Neurotrophic Factors

Neurotrophins are a family of closely related proteins that were first identified as survival factors for sympathetic and sensory neurons in both central and peripheral nervous systems (Reichardt et al., 2006; Skaper et al., 2012). Neurotrophins demonstrate a differential distribution throughout the central and peripheral nervous systems (Dawbarn & Allen, 2003). These proteins have been shown to influence the proliferation, differentiation, survival, and death of neurons during both early development and adulthood (Chao et al., 2006). In addition, neurotrophins stimulate proliferation of neural stem cells, increase survival of host and graft cells, and induce differentiation as well as regenerative responses (Ito et al., 2002; Markus et al., 2002).

There are six distinct biological families of neurotrophic factors that are expressed by stem cells, and have been shown to directly impact stem cell development (Teng et al., 2011);

1. Classic neurotrophins that comprise nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4)
2. The transforming growth factor (TGF)-beta family, represented by glial cell line-derived neurotrophic factor (GDNF) and the bone morphogenic proteins (BMPs). Other members are neurturin, artemin, and persephin.
3. The cytokine growth factor family, including ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), and cardiotropin-1
4. The epidermal growth factor (EGF) family, consisting of EGF, transforming growth factor-alpha (TGF-alpha), neuregulins, and neural and thymus-derived activators for ErbB kinases (NTAK)
5. The insulin-like growth factors (IGFs), consisting of insulin-like growth factor I (IGF-I) and IGF-II
6. The fibroblast growth factor (FGF) family, consisting of at least 24 different proteins including acidic FGF (FGF1) and basic FGF (FGF2) (Teng et al., 2011).

Neurotrophins are still the focus of ongoing research and further protein identification can be expected in the near future. Meteorin, conserved dopamine neurotrophic factor (CDNF), and mesencephalic astrocyte-derived factors (MANF) are examples of neurotrophins that have been characterized lately (Nishino et al., 2004; Petrova et al., 2003; Lindholm et al., 2007).

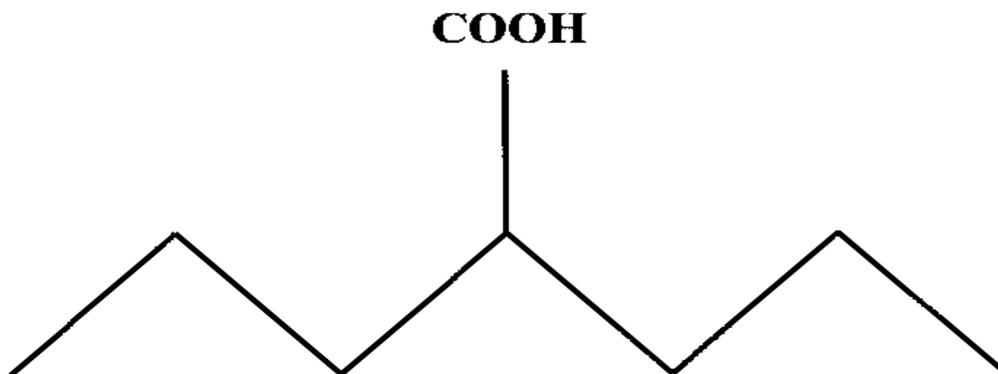
A number of hypotheses have been proposed to explain the cell and molecular pathogenesis of neurodegenerative diseases. Alterations in neurotrophins expression are widely implicated in the pathology of neurodegeneration. (Dawbarn & Allen, 2003). For instance, decreased BDNF mRNA has been reported in hippocampal and temporal cortex samples from Alzheimer's disease (AD) patients (Phillips et al., 1991; Connor et al., 1997). Similarly, changes in neurotrophins have been also reported in amyotrophic lateral sclerosis (ALS); mRNA and protein concentration of NGF, BDNF, NT-3 and NT-4/5 were increased in muscle tissue. BDNF was strongly up regulated in the early stage of the disease, whereas levels of NGF, NT-3, and NT-4/5 gradually increased during the course of the disorder, and peaked at later stages (Kust et al., 2002). Also, *post-mortem* ALS muscle biopsies showed increased NGF concentrations of 140% higher than normal (Stuerenburg et al., 1998). In Parkinson's disease, brain-derived growth factor and nerve growth factor concentrations are decreased in patients' brains (Moji et al., 1999; Howells et al., 2000).

NSCs express neurotrophic factors that influence the growth and hemostasis of their own and surrounding tissues via autocrine and paracrine mechanisms (Teng et al., 2011). Neurotrophic factors delivered by stem cells might be used as a novel approach to treat CNS insults or degeneration (Dawbarn and Allen, 2003).

1.4. Valproic Acid

Valproic acid (VPA) (2-propylpentanoic acid) is widely used as an anticonvulsant and mood stabilizer. VPA was first synthesized by Burton in 1882, but was not utilized clinically until Pierre Eymard serendipitously discovered its anticonvulsant properties in 1962 (Löscher et al., 2002). Since then, VPA has been used to treat a wide range of neurologic conditions ranging from epilepsy to bipolar depression. Due to its simple structure, an eight-carbon branched-chain fatty acid (Figure 1.2), VPA penetrates the blood-brain barrier within 1 minute of intravenous injection (Phiel et al., 2001).

Accumulating evidence has demonstrated VPA's ability to influence multiple targets at the level of gene expression. Studies over the past decade have reported anti-neoplastic properties of VPA in various cancer cell lines (Blaheta & Cital, 2002). More recent studies have reported VPA's neuroprotective properties *in vivo* and *in vitro* (Monti et al., 2009). Although the molecular mechanisms underlying VPA action in these settings are not fully understood, recent reviews have suggested histone deacetylase (HDAC) inhibition as a key mechanism (Phiel et al., 2001; Marchion et al., 2005).



2-propylpentanoic acid

Figure 1.2. Chemical structure of VPA.

Short chain fatty acid with eight carbon atoms. Molecular Formula:
 $C_8H_{16}O_2$.

From: Blaheta RA, Cinatl J Jr.(2002). Anti-tumor mechanisms of valproate: a novel role for an old drug. *Med Res Rev.* 22:492-511.

1.4.1 VPA Implications in neurological disorders

Valproic acid has been a well-established drug for epilepsy since the late 1960s, and it is now used for the treatment of a variety of psychiatric and neurological disorders, including clinical depression, bipolar disorder, seizures, schizophrenia, migraines, and headaches (Blaheta & Cital, 2002; Chateauvieux et al., 2010; Löscher et al., 2002). The mechanism of action of VPA in any of these settings is not fully understood. However, the anti-epileptic activity of VPA has been linked to mechanisms altering neuronal excitability (Löscher, 1999). These mechanisms include increase of GABA-ergic activity (Johannessen et al., 2003), attenuation of NMDA-mediated excitatory neurotransmission (Zeise et al., 1991; Gean et al., 1994), and negative regulation of voltage gated ion channels: mainly Na⁺, and possibly Ca⁺⁺ and K⁺ (Monti et al., 2009). More recently, VPA has been suggested as an effective agent in treating spinal muscular atrophy (SMA), which is a common autosomal recessive disease caused by loss of the homozygous survival motor neuron gene SMN1. The severity of the disease is dependent on the nearly identical copies of the SMN1 gene, SMN2. VPA was reported to increase the level of functional SMN protein through the activation of SMN2 transcription (Brichta, 2003; Wirth et al., 2006; Tsai et al., 2008).

Furthermore, VPA has been proposed to be of therapeutic value in treating Huntington's disease (HD) (Chiu et al., 2011). A combination of VPA and lithium treatment to transgenic mouse models of Huntington's disease (N171-82Q) significantly prolonged their median survival, without any side effects on their behavior or the striatal dopamine content (Chiu et al., 2011).

1.4.2. VPA Implications in neuronal differentiation

HDAC inhibitors have the ability to alter the differentiation state of NSCs (Sun et al., 2011). VPA has shown to induce NSCs differentiation through multiple mechanisms. For instance, VPA was reported to induce neuronal-like differentiation in C6 glioma cells associated with histone 4 (H4) (Benítez et al., 2008). Similarly, in neuroblastoma cell lines as well as cultured cortical and dentate gyrus neurons, VPA has been reported to promote neurite growth and up-regulate neuronal markers through activating ERK /mitogen-activated protein kinase (MAPK) and the Notch signaling cascades (Yuan et al., 2001; Hao et al., 2004; Stockhausen et al., 2005). In addition, chronic exposure of hippocampal HN33 cells to therapeutic concentrations of VPA induced neuronal differentiation, associated with a reduction in MARCKS expression and an increase in GAP-43 expression (Watterson et al., 2002).

MARCKS and GAP-34, which are implicated in actin-membrane plasticity and neurite outgrowth during neuronal differentiation, were associated with alteration in PKC activity and temporal changes in PKC isozyme expression (Watterson et al., 2002). Furthermore, VPA was reported to induce neuronal differentiation of adult and embryonic hippocampal progenitor cells *in vitro* and *in vivo*. In such settings, VPA was reported to exert its effect via activation of neurogenic basic helix loop helix (bLHLH) transcription factors like Ngn-1, Math1, and Neuro D, associated with histone H3 and histone H4 hyperacetylation (Hsieh et al., 2004; Yu et al., 2009). More recently, VPA has been demonstrated to have an inductive effect on neurite outgrowth in neuroblastoma N1E-115 cells through interaction with the small GTPas proteins Arl4D and Arf6 (Yamauchi et al., 2009). Both proteins are thought to regulate neurite formation as well as cytoskeletal and morphological changes.

The involvement of VPA in multiple important pathways underlies the enormous potential of this drug to exert its differentiation effect in neuronal tissues. The principle mechanisms and potential neuronal targets of VPA are discussed in section 1.5.

1.4.3. VPA Implications in neuroprotection

VPA has been reported to play a neuroprotective role in several *in vitro* and *in vivo* models of neurodegenerative disease. A report by Mora et al. (1999) showed that VPA protected cerebellar granule cells from apoptosis induced by low potassium. Likewise, VPA protected cultured rat hippocampal neurons against β -amyloid excitotoxicity and glutamate-induced injuries by balancing intracellular Ca^{++} concentration (Mark et al., 1995). Additionally, VPA has been shown to exert a neuroprotective effect in animal models of Parkinson disease PD, characterized by a marked loss of dopaminergic (DA) neurons in the substantia nigra, VPA treatment prevents apoptosis, induced by a neurotoxin in human SH-SY5Y neuroblastoma cells, a well-established model for PD (Pan et al., 2005). In addition, VPA has been shown to protect midbrain DA neurons from LPS or 1-methyl-4-phenylpyridinium (MPP⁺)-induced neurotoxicity through increased neurotrophic factor release from astrocytes, inhibiting microglia-mediated inflammation, and decreasing microglial cell activity (Peng et al., 2005; Chen et al., 2006). Furthermore, VPA has been shown to protect cerebellar granule neurons exposed to toxic concentrations of 6-hydroxydopamine (6-OHDA) by increasing alpha-synuclein expression and preventing its nuclear translocation (Monti et al., 2007).

In a rat PD-like model, long-term administration of VPA counteracted the death of substantia nigral neurons, prevented the decrease of the dopaminergic marker tyrosine hydroxylase (TH) in the substantia nigra and striatum, and suppressed the drop of striatal dopamine levels (Monti et al., 2010).

As VPA-HDAC inhibition activity was noticed, several studies documented VPA neuroprotective effects in association with increased histone acetylation. For example, VPA was shown to protect mature cerebellar granule cell cultures from excitotoxicity induced by 2S, 4R-4-methylglutamate (or SYM 2081), an inhibitor of excitatory amino acid transporters and an agonist of low-affinity kainate receptors. SYM-induced apoptosis was associated with instant and strong nuclear accumulation of pro-apoptotic glyceraldehyde-3-phosphate dehydrogenase (GAPDH). VPA treatment caused a time-dependent decrease in the expression of nuclear GAPDH, accompanied by an increase in acetylated histone H3 (Kanai et al., 2004). Similarly, VPA induced neuroprotection in rat cerebellar granule cell cultures as well as *in vivo* by elevating α -synuclein mRNA and its protein with concomitant hyperacetylation of histone H3 (Monti et al., 2007). Alpha-synuclein is suggested to be protective against certain neurotoxins such as glutamate and 6-hydroxydopamine (Leng & Chuang, 2006; Monti et al., 2007).

A number of alternative neuroprotective mechanisms for VPA have also been proposed. In neuroblastoma cell lines, VPA attenuates rotenone-induced apoptosis through the induction of heat shock protein and activation of AKT (Pan et al., 2005). Similarly, VPA delayed retinal ganglion cell (RGC) death and enhanced axonal regeneration after optic nerve crush (ONC) through activation of extracellular signal-regulated kinase (pERK) 1/2 phosphorylation in addition to increased histone acetylation (Biermann et al., 2010; Biermann et al., 2011).

Observations from the above studies and reports highlight the tremendous neuroprotective efficacy of VPA; however, the precise mechanism of action remains unclear.

1.5. Possible Mechanisms of VPA Action

1.5.1 GABA signaling

The effect of VPA on the GABAergic system has been reported in earlier studies. VPA has been reported to elevate GABA levels in several rat brain regions (Patsalo et al., 1981). VPA has been shown to influence several enzymes connected to the synthesis and degradation of GABA. VPA elevates GABA levels by increasing its precursor α -ketoglutarate through α -ketoglutarate dehydrogenase inactivation (Luder et al., 1990). Also, VPA might inhibit GABA decay by decreasing its degradation, which is mediated by GABA transaminase and succinate semi-aldehyde dehydrogenase (Whittle et al., 1978; Löscher et al., 1993). Moreover, VPA may also act to directly stimulate GABA neuro-transmission by enhancing the responses of GABA-A and GABA-B receptors (Harrison et al., 1982; Motohashi et al., 1992). Lastly, VPA has been shown to enhance the activity of glutamate decarboxylase (GAD), a key enzyme for GABA synthesis that converts glutamate to GABA (Löscher 1993; Dong et al., 2007).

1.5.2. Ion channels

A number of studies have demonstrated the inhibitory effect of VPA on a variety of ion channel activities. The observed anticonvulsant activity of VPA might be linked to its action on Na⁺ ion channels.

VPA was shown to lessen neuronal excitability by inhibiting voltage-dependent Na⁺ activity and expression (Zona et al., 1990; Tian et al., 1994; Macdonald & Kelly, 1995; Stahl et al., 2004). Similarly, VPA-mediated neuroprotection against NMDA-dependent neurotoxicity has been linked to Na⁺ current blockage by VPA (Farber et al., 2002).

Despite the clear association between VPA and Na⁺ ion channel regulation, the effect on K⁺ ion channels is less consistent. VPA was reported to lessen neuronal excitability by reducing voltage-dependent potassium conductance (Vandongen et al., 1986). Conversely, VPA was also reported to affect neural activity by increasing the amplitude of the late potassium outward currents (Wlden et al., 1993). The role of VPA in ion channel regulation and neuroprotection is seemingly unresolved and requires further investigation.

1.5.3. Inhibition of histone deacetylation

Transcriptional regulation is a major process in a wide range of cellular functions ranging from development to differentiation to cell death. Transcription is tightly regulated by several factors including chromatin accessibility (Hsieh et al., 2004).

In eukaryotic cells, double-stranded DNA is wrapped around an octamer of histones to form a nucleosome that subsequently folds into high order chromatin. Modifications of histones through acetylation or deacetylation can alter the interaction between DNA and histone and consequently control the expression of genes (Marchion et al., 2005). In the acetylated state, the nucleosome structure becomes more open and relaxed due to increased protein acetylation at the histone-tails by histone acetyltransferases (HATs). This in turn reduces steric forces and facilitates binding of transcription factors and polymerase enzymes to target gene promoters, resulting in transcriptional initiation and an increase in gene expression (Chuang et al., 2009). Conversely, in the hypo-acetylated state, nucleosomes become tightly compacted and relatively non-accessible to transcription factors resulting in gene repression. In particular, histone deacetylases (HDACs) are nuclear and cytoplasmic enzymes that modulate the acetylation activity of histones and thus indirectly modulate transcriptional activity (Menegola et al., 2006; Carafa et al., 2013). Earlier studies on 293T cells, Neuro2A cells, and teratocarcinoma F9 cells have shown that VPA directly inhibits HDAC causing hyperacetylation of histones in these cell lines (Gottlicher et al., 2001; Piel et al., 2001). Recent studies have supported these results by detecting an increase in histone H4 acetylation of adult hippocampal neuronal progenitor cells treated with VPA (Hsieh et al., 2004; Yu et al., 2009).

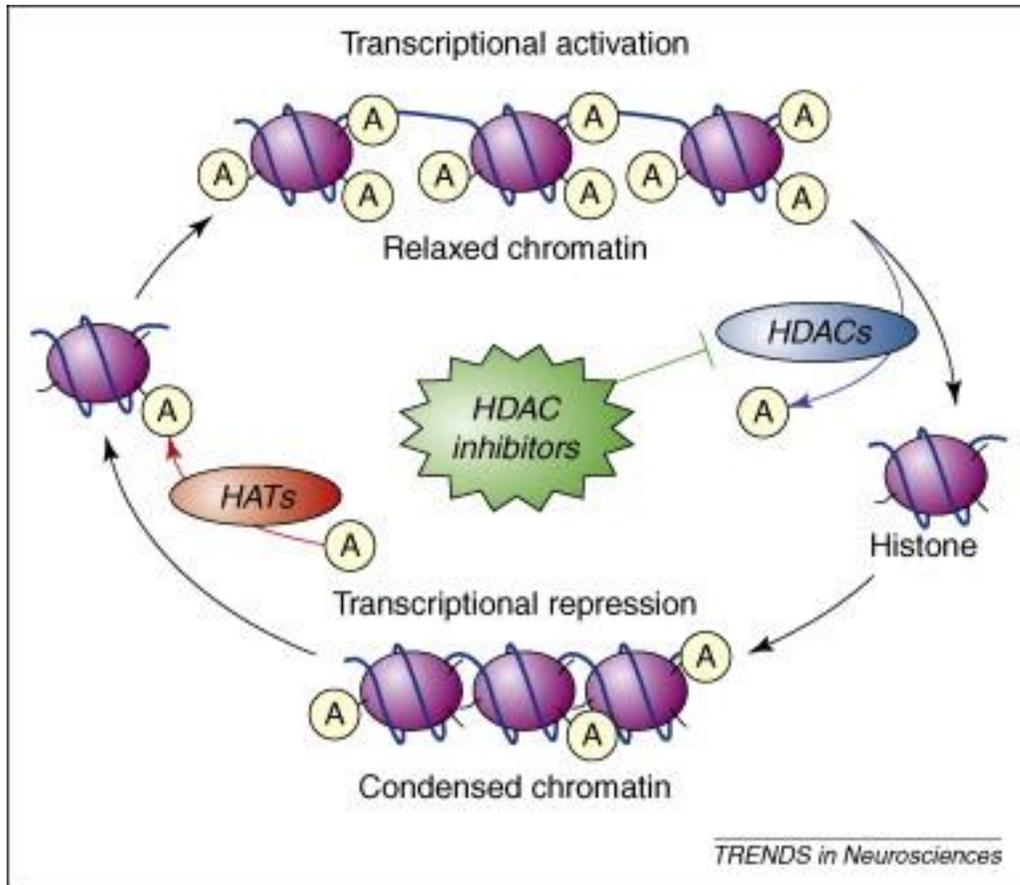


Figure 1.3. Histone acetylation/Deacetylation.

DNA is wrapped around a core of histones to form the nucleosome (chromatin). Nucleosomal histones are subject to acetylation by histone acetyltransferases (HAT) that leads to a more open state of chromatin resulting in increased gene expression. In a deacetylated state, nucleosomal histones become condensed into compact chromatin through histone deacetylase (HDAC) activity, resulting in decreased transcription. VPA inhibits HDAC activity causing histone hyperacetylation, chromatin relaxation, and gene expression.

From Chuang et al., 2009. Multiple roles of HDAC inhibition in neurodegenerative conditions. Trends Neurosci 32: 591–601

1.5.4. Kinase pathways

Mitogen-activated protein kinases pathway (MAPKs)

MAPKs have been proposed as an attractive target for therapeutic approaches in neurodegenerative diseases (Harper et al., 2003; Miloso et al., 2008). The MAPK family of intracellular signal transducers includes extracellular regulated kinase (ERKs), c-jun N-terminal kinase (JNK), and p38. A large body of evidence has demonstrated the ability of VPA to activate the ERK1/2 pathway both *in vitro* and *in vivo*, thus regulating transcription factors and gene expression. *In vitro* studies have shown that VPA activates ERK pathways in several neural cell lines. In human SH-SY5Y neuroblastoma, VPA at therapeutic concentrations has been shown to stimulate ERK pathways, increase growth cone-associated protein 43 (GAP-43) and B-cell lymphoma 2 (Bcl-2) protein levels, promote neurite growth and cell survival, and increase norepinephrine uptake, suggesting VPA's role in exerting a neurotrophin-like effect (Yuan et al., 2001). In cerebral cortex neural progenitor cells (NPCs), VPA simultaneously induced differentiation and reduced proliferation in part through activation of the ERK pathway (Jung et al., 2008). Recently, VPA has been shown to enhance Schwann cell proliferation possibly by activation of the mitogen-activated protein kinase (MAPK) or extracellular signal-regulated kinase (ERK1/2) pathway (Fei et al., 2011). Also, VPA was shown to increase phosphorylated c-Fos and increased c-Jun level in SH-SY5Y cells (Asghari et al., 1998).

Lipid kinase phosphatidylinositol-3 kinase (PI3K)/ AKT pathway (or Protein kinase B (pKB) / AKT)

The PI3K pathway is required for cell survival signaling in neurons (Brunet et al., 2001; Barnabe-Heider et al., 2003). PI3K converts phosphatidylinositol (4, 5)-biphosphate(PIP-2) to phosphatidylinositol (3, 4, 5)-triphosphate (PIP-3) through phosphorylation (Monti et al., 2009). The exact action of VPA on AKT is not clear yet. VPA is purported to increase phosphorylation-dependent AKT activation by enhancing the PI3K pathway phosphorylation (Mora et al. 2002; De sarno et al., 2002; Pan et al., 2005). The neuroprotective effect of VPA might be linked to PI3K/PKB pathway activation (Mora et al., 1999; Pan et al., 2005).

Protein kinase C

PKC comprises a family of structurally related isoforms of phospholipid-dependent serine/threonine kinases. PKC isozymes are detected in nearly all cell types and are implicated in regulation of cell growth and differentiation (Black et al., 2000; Shen et al., 2003). In the brain, PKC modulates pre- and post-synaptic neurotransmission, neurotransmitter release, and long-term remodeling of gene expression (Robinson et al., 1991; DiazGranados et al., 2008).

The PKC family has long been recognized as a possible target for VPA action. Chronic exposure of rat C6 glioma cells to clinically relevant concentrations of VPA was reported to diminish PKC α and PKC ϵ activity and expression in membranes and cytosolic fractions (Chen et al., 1994). Also, VPA was reported to induce a concentration- and time-dependent reduction of myristoylated alanine-rich C kinase substrate (MARCKS), a vital PKC substrate implicated in neuronal development and adult neural plasticity, in immortalized hippocampal cells (Watson et al., 2002). *In vivo*, VPA prevented cognitive impairment in rats by decreasing PKC α phosphorylation-dependent activity (Birnbaum et al., 2004).

Protein kinase A

Cyclic AMP-dependent protein kinase (PKA) signaling has been shown to be a critical regulator for neuronal or glial differentiation in several neuronal cell lines (Vogt Weisenhorn et al., 2001). The effect of VPA on PKA activity is controversial. VPA reported to cause accumulation of cAMP in glioma cells and in rat hippocampal (Chen et al., 1996) but not in human SH-SY5Y neuroblastoma cells (Wang et al., 1999).

Glycogen synthase kinase-3 β

Glycogen synthase kinase-3 β (GSK-3 β), a serine-threonine kinase, is a key negative intermediate of the Wnt signaling pathway. GSK-3 β is regulated by several kinases, such as AKT, MAPKs, PKC, and PKA (Monti et al., 2009). Accumulating evidence has identified GSK-3 as a key regulator in multiple neuron developmental processes, including neurogenesis, neuronal migration, neuronal survival, and axonal growth (Nadri et al., 2003; Hur et al., 2010).

The canonical pathway of Wnt signalling acts through balancing of β -catenin causing its accumulation in the cytoplasm and translocation into the nucleus to act as a transcriptional co-activator of transcription factors that belong to the TCF/LEF family. In absence of Wnt signaling, GSK-3 constantly phosphorylates β -catenin, a well-established marker for Wnt signaling, leading to its rapid degradation thereby targeting it for ubiquitination and digestion by proteasome (Valkenburg et al., 2011). When Wnt bind to the Frizzled protein receptors, GSK-3 β is inhibited leading to the stabilization of the β -catenin protein. Then, β -catenin translocates into the nucleus and activates the transcription of Wnt-dependent genes. *In vitro* studies has shown that VPA at can inhibit GSK-3 β , and increase the cytosolic and the nuclear concentrations of β -catenin (Chen et al., 1999).

Conversely, VPA was reported to have no effect on GSK-3 β activity or expression *in vivo* (Kozlovsky et al., 2003).

However, VPA has been shown to increase the soluble fractions of β -catenin protein *in vivo* (Gould et al., 2004).

Protein kinase induced-phosphorylation is involved in many cascades in the cell. VPA involvement in kinase phosphorylation/activation or inhibition might affect the key cellular mechanisms. The main kinase pathways impacted by VPA and discussed above are summarized in Figure 1.4.

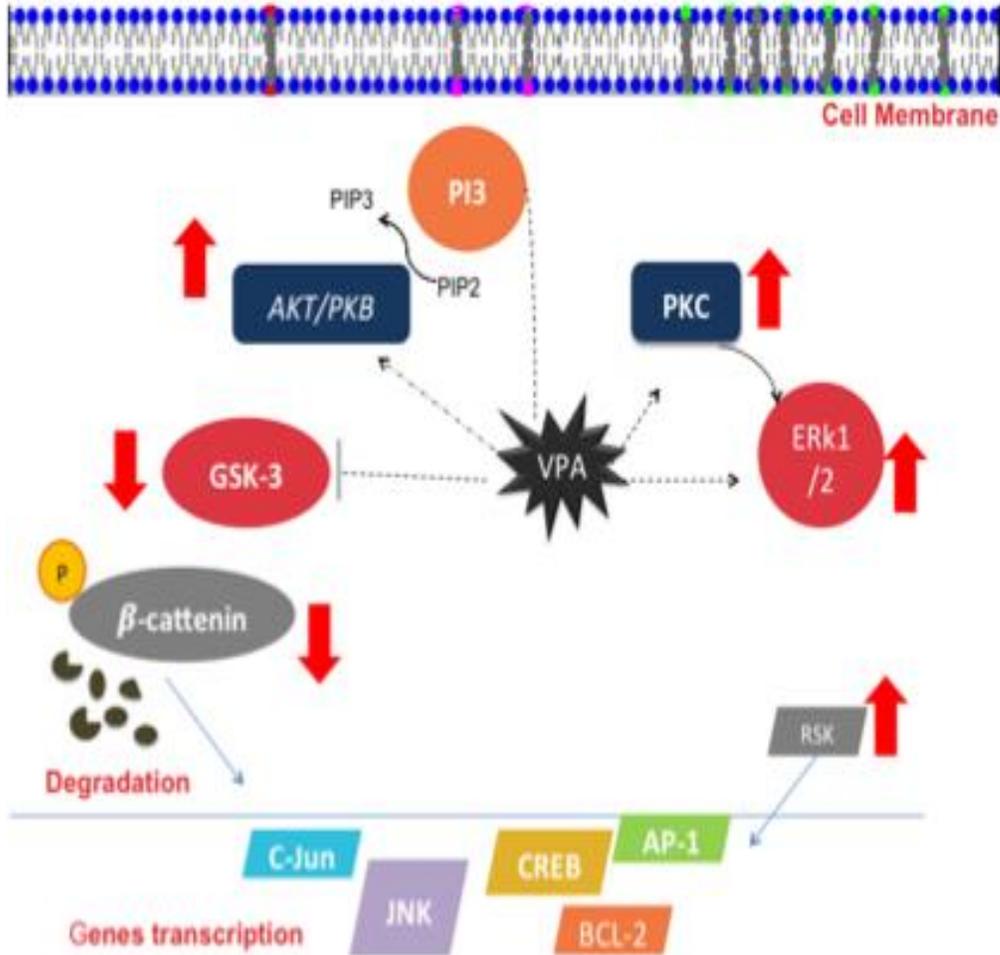


Figure 1.4. Possible Kinase pathways targets for VPA. Scheme for protein kinase pathways influenced by VPA. The dashed lines indicate positive or negative regulation by VPA. The bold arrows represent increase or decrease VPA impact on enzyme/transcription activity. P= Phosphorylation.

Adapted and modified from: Monti et al., 2009. Biochemical, molecular and epigenetic mechanisms of valproic acid neuroprotection. *Curr Mol Pharmacol.*(2:95-109.)

1.6. Effects of VPA on Transcription Factors

1.6.1 Activator protein AP-1

AP-1 is a transcription factor involved in many aspects of brain physiology including development, plasticity and neurodegeneration (Ryski and Kaczmarek, 2004; Raivich et al., 2006). AP-1 is composed of dimeric transcription factor subunits consisting of Jun, Fos, and activating transcription factor (ATF) (Karin et al., 1997). These factors bind to a common DNA site in the promoter region of the gene and activate gene transcription/expression. The AP-1 family of transcription factors regulates many different genes involved in neurotransmitter, neurotrophin, and receptor biosynthesis (Hughes et al., 1995). Notably, VPA-treatment at therapeutic doses was reported to increase DNA binding activity of AP-1 transcription factors as well as the increase expression of genes regulated by AP-1 in cultured cells and brain regions involved in mood regulation (Chen et al., 1997; Asghari et al., 1998; Chen et al., 1999; Yuan et al., 2001). VPA might induce AP-1 activation via the PKC–MAPK pathways or via inhibiting GSK-3 β (Chen et al., 1999; Yuan et al., 2001). In addition, VPA increased the expression of genes regulated by AP-1 protein, including B-cell lymphoma-2 (Bcl-2), and growth associated protein-43 (GAP-43) (Yuan et al., 2001; Creson et al., 2009). Bcl-2 has been implicated in the regulation of CNS functions, including brain development (Shacka and Roth, 2005), neuronal processes (Chen et al., 1997), and adult hippocampal neurogenesis (Kuhn et al., 2005).

GAP-43 plays a key role in guiding the growth of axons and modulating the formation of new neuronal connections (Benowitz et al., 1997).

1.6.2. cAMP response element-binding protein (CREB)

Cyclic-AMP response element-binding protein (CREB) is a cellular transcription factor that is activated through phosphorylation and is involved in a wide range of brain functions, including development, synaptic plasticity, and memory (Lonze et al., 2002). In PC12 neuronal cultures, VPA has been shown to regulate tyrosine hydrolase (TH) gene expression through cAMP/CREB dependent pathways (Decastro et al., 2005). In addition, VPA enhanced retinal ganglion cells RGC survival and regeneration following optic nerve crush partially through the activation of CREB (Biermann et al., 2010).

The exact mechanism by which VPA exerts its initial effects is currently unknown. No VPA cell surface receptors have yet been identified. Nonetheless, VPA participation in many important mechanisms underlies the potential of this drug to influence key functions and processes of neuronal cells.

1.7. Hypothesis

Given the neuroprotective properties of valproate, it was my hypothesis that treating C17.2 cells with VPA might modify the mRNA expression of: neurotrophic factors, such as CDNF, MANF, GDNF, BDNF, the differentiation markers Egr-1, Sox2, and the early DA marker Nurr-1. The mechanisms underlying the neuroprotective effect of VPA is unclear, however the inhibition of histone deacetylase is thought to be involved. Other pathways like ERK1/2, AKT and GSK-3 β might also be implicated. This hypothesis was tested experimentally by treating C17.2 cells with VPA at variable times (24-, 48-, and 72-hour) and with different concentrations (0.5 mM, 1mM, and 3 mM), followed by mRNA analysis of the targeted genes (CDNF, MANF, BDNF, Nurr-1, Egr-1, and Sox-2). Histone H3 acetylation and ERK1/2 pathway inhibition were studied as possible mechanisms involved in VPA action. Parallel western blots were also performed for the novel neurotrophic factors, CDNF and MANF, to study the effect of VPA on their protein expression.

CHAPTER 2. MATERIALS and METHODS

2.1 Culturing C17.2 Cells from Frozen Stock

C17.2 cells were stored in 2ml cryovial tubes in liquid nitrogen. Tubes of C17.2 cells were obtained and thawed by mixing the tube gently by hand. 1ml of cells was transferred to a sterile 15ml conical tube. Nine ml of Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) plus 5% horse serum (Invitrogen Canada Inc., Burlington, ON, Canada), Fungizone (1.25 μ g/ml or 0.5% GIBCO), and Penicillin/Streptomycin (100IU/ml/100 μ /ml, GIBCO) was preheated at 37°C for 30 minutes in a water bath and added to the conical tube. Cells were mixed repetitively using a glass pipette to disperse the cells. Subsequently, cells and the media were dispensed into 10-cm Corning culture dishes (Fisher Scientific Ltd., Nepean, ON, Canada) and incubated in a 5% CO₂–95% air incubator at 37°C. DMEM media with 10% FBS and 5% horse serum were replaced every other day until the cells reached 90% confluence for subculture. It took between 4 to 5 days to reach 90% confluence.

2.2 Subculturing C17.2 Cells

Cells were sub-cultured into 10-cm Corning culture dishes as they reached 90% confluence. The previous media was removed using an electronic pipette.

Cells were scraped, collected and re-suspended in 1ml of DMEM media with 10% FBS. Cells were diluted in a range of 1:13 to 1:20 dilution in DMEM media with 10% FBS. Cells were mixed repeatedly using a glass pipette. 1ml of diluted cells was added to 9ml of DMEM with 10% FBS in the culture dishes and allowed to incubate for 1 to 2 days at 37°C and 5% CO₂–95% air until they reached 60-70% confluence. As cells reached 60-70% confluence the media was changed to DMEM with 1% FBS and cells were kept for 24 hours prior to treatment.

2.3 Valproic Acid Treatment

VPA (Sigma-Aldrich, Canada Ltd. Oakville, Ontario) was prepared as a 50mM stock in sterile DMEM. From this stock, 100µl, 200µl, 600µl was directly added to cell cultures (in DMEM 1% FBS) to give final concentrations of 0.5, 1, and 3mM VPA, respectively. For VPA treatment, old media was removed and prepared VPA concentrations were added to new DMEM 1% FBS in culture dishes. Cells were maintained in VPA for 24h, 48h, or 72h, and then RNA was extracted.

2.4. PD98059 Treatment

PD98059 is a potent selective inhibitor of the mitogen activated protein kinase (MAPK) pathway (also known as MAPK/ERK kinase or MEK kinase). PD98059 (Sigma-Aldrich Canada Ltd., Oakville, Ontario.) was prepared as 100mM stock in 187 μ l 100% Dimethyl sulfoxide (DMSO), aliquoted, and kept at - 20°C. From this stock, 2 μ L were directly added to cultured cells (maintained in 1% FBS/DMEM) to give a final concentration of 20 μ M PD98059. Control culture dishes were treated with 2 μ L DMSO (100%) for a final concentration of 0.02 % DMSO. For PD treatment, old media was removed and PD90859 added to new 1% FBS/DMEM media in cell cultures and left for 20 min. Then, 0.5 or 1mM VPA was added to culture dishes and incubated for 24h. After 24h incubation, RNA was extracted.

2.5. RNA Extraction

Total RNA was isolated from treated C17.2 cells using Trizol (Invitrogen Canada Inc., Burlington, ON, Canada) according to the manufacturer's instructions. Media was removed from culture dishes using an electronic pipette. 1ml of Trizol was added per 10cm culture dish and then cells were scraped and transferred to a 1ml Eppendorf tube.

Samples were homogenized by continuous pipetting for at least 30 seconds and then were incubated for 5 minutes at room temperature.

After that, 200 μ l of chloroform (ACP, Montréal) was added and samples were incubated again at room temperature for 15 minutes. Samples were centrifuged at 13,000 rpm for 15 minutes at 4°C in order to separate the aqueous phase (RNA floats in the upper aqueous layer) and the organic phase (contains DNA and proteins). The RNA containing aqueous phase from each sample was transferred to a new Eppendorf tube. To precipitate the extracted RNA, 500 μ l of isopropyl alcohol was added to the aqueous solution and incubated for 15 minutes at room temperature. Samples were kept overnight at - 20°C. The next day, samples were centrifuged for 10 minutes at 13,000 rpm at 4°C and the supernatant was discarded. RNA remained as a white pellet attached to the bottom of the Eppendorf tube. RNA pellets were re-suspended with 1ml of 75% ethanol, vortexed for 5 minutes and centrifuged for 5 minutes at 10,000 rpm at 4°C. The supernatant from each sample was removed by careful pipetting and the RNA pellet was dissolved in 35 μ l of RNase free water and incubated for 10 minutes in a water bath at 55-60°C. Finally, samples were kept at - 80°C.

2.6. Quantifying RNA Concentration

RNA concentrations were determined using Beckman spectrophotometer. Diethylpyrocarbonate (DEPC) water (Invitrogen, Carlsbad, CA, USA) was used to set the spectrophotometer reading to zero. RNA samples were diluted to 1:200 (1 μ l of RNA sample was added to 199 μ l RNase free water). Absorbance readings at 260nm (A₂₆₀) were obtained to determine RNA yield. RNA concentration was calculated as follows, based on an absorbance of 1 unit at 260nm being equivalent to 40 μ g of RNA/ml. An estimate of purity of RNA was obtained by UV light with respect to absorbed contamination, such as protein, was provided by the ratio of readings at 260nm and 280nm (A₂₆₀/A₂₈₀). Values ranging from about 1.8-2.0 were considered acceptable.

2.7. DNase Treatment

RNA samples were treated with DNase1 (Invitrogen) to get rid of any DNA impurities. A total of 25 μ g of RNA/sample was treated with 2.5 μ l of DNase1 enzyme, 2.5 μ l of 10x DNase1 buffer and RNase free-water to bring the volume up to 25 μ l. The samples were heated in a water bath for 40 minutes at 37°C. 2 μ l of 25mM EDTA (pH 8.0) were added to deactivate the DNase 1 enzyme. If there was less than 25 μ g of RNA available, the reaction was scaled down accordingly.

Lastly, the samples were incubated in the water bath for 10 minutes at 65°C and samples were preserved at - 80°C.

2.8. Reverse Transcription (RT)

Following DNase treatment, cDNA was synthesized from 2µg of total DNase treated RNA using the Omniscript Reverse Transcriptase Kit (Qiagen Inc., Mississauga, ON) as instructed by the supplier. The master mix was prepared by adding 2µl of 10 X buffer RT, 2µl of 5mM dNTP mix (0.5 mM final), 2µl of Oligo dT (0.5µg/ml, MBI Fermentas), 1µl of RNase inhibitor (10 Units/µl) (Invitrogen), 1µl Omniscript (4 Units final), and a volume of RNA (2.2-3.2µl) required to bring the final volume to 20 µl after the addition of RNA (9.8µl). The reaction mixture was vortexed for 5 seconds, then incubated for 60 minutes at 37°C, heated for 5 minutes at 93°C to deactivate the enzyme, and finally cooled on ice. The RT product or cDNA was preserved at - 20°C.

2.9. Polymerase Chain Reaction (PCR)

To detect the genes of interest, cDNA was amplified using the HotStarTaq Master Mix Kit (Qiagen). 2µl of RT product was used with appropriate primers. The parameters and conditions of semi-quantitative PCR were established in preliminary cycle-dependent experiments (Armstrong et al., 2002; Sharma et al., 2008) or as indicated in Table 2.1.

The thermal cycler was programmed with a pre-heat activation step at 95°C for 5 minutes to activate the HotstarTaq DNA polymerase. RT products were amplified according to the following conditions: 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute and a final incubation period of 72°C for 10 minutes. For Nurr-1, conditions were specifically altered as follows; 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 1 minute and a final incubation period of 72°C for 10 minutes. Primers for the amplification of the target genes are indicated in Table 2.2. In order to verify RT-PCR product specificity and absence of DNA, negative controls (lacking cDNA) were amplified using appropriately diluted RNA without reverse transcriptase treatment. The housekeeping gene 18s ribosomal RNA was used as an internal control for all genes examined.

2.10. Semi-Quantitative Analysis

Amplified cDNA/PCR products were run on 1.5 % agarose gel in 1X Tris/Borate/EDTA (TBE) buffer and stained with 3ml of ethidium bromide (10mg/ml). 10µl of RT-PCR products were loaded per lane. Gels were run at 87V for 1 hour and scanned digital images for gene bands on the gel were obtained using Alphamager[®] 2200.

The relative optical density (OD) ratio of each gene (i.e. CDNF, MANF, GDNF, BDNF, Nurr-1, Sox-2, and Egr-1) over the internal control 18S rRNA was obtained for semi-quantitative analysis.

2.11. Protein Extraction

Media was removed and cultured cells were washed twice with 10 ml of ice-cold 1x PBS. Cells were scraped in 1ml of 1x PBS and centrifuged for 3 minutes at 4000 RPM at 4°C. Cytosolic protein was extracted using 500µl of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) with Complete Protein Inhibitor Cocktail (1:7 dilution, Roche Diagnostic, Indianapolis, IN, USA). The samples were incubated on ice for 1 hour, vortexed every 15 minutes and then centrifuged for 10 minutes at 14,000 RPM. Supernatant from each sample was discarded carefully to obtain a protein pellet. For nuclear extraction, cells were homogenized in ice-cold homogenizing buffer (pH 7.4) containing 10mM Tris-HCl, 5mM NaF, 1mM Na₃VO₄, 1mM EDTA, 1mM EGTA, and 320mM sucrose. The homogenate was centrifuged (13,000 RPM) for 10 minutes at 4°C after incubation on ice for 10 minutes. The pellet was re-suspended in 500µL of 0.4 N H₂SO₄ and incubated on ice for 30 minutes. The supernatant containing nuclear protein was collected by centrifugation (13,000 RPM) again for 10 minutes at 4°C and transferred to a fresh tube.

Protein from each sample was precipitated with 250 μ L of 100% trichloroacetic acid (containing 4mg/mL deoxycholic acid) and incubated on ice for 1 hour. Protein precipitates were pelleted by 10 minutes of centrifugation (13,000 RPM) at 4°C. The pellet was washed with 500 μ L of ice-cold acidified acetone (0.1% HCl) followed by ice-cold acetone for 5 minutes. The protein pellets were dried for 30 minutes and the dried pellets were re-suspended in 10mM Tris HCl (pH 8.0).

2.12. Protein Quantification

Protein concentration for each sample was determined by using the DC Protein Assay Kit (Bio-Rad Laboratories, Mississauga, ON). Readings were obtained on a spectrophotometer at a wavelength of 750nm and concentrations of samples were calculated using absorbency readings and the standard curve equation. Samples were then aliquoted and stored at -20°C

2.13. Western Blot Analysis of CDNF and MANF Protein Expression and Histone H3 Acetylation

Solubilized cytoplasmic or nuclear samples were separated by sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Gels with 80 µg cytoplasmic protein or 10 µg nuclear proteins per lane were run at 200V for one hour at 4°C. Proteins were transferred to PVDF membranes (0.2µm) overnight at 25V, 4°C.

Blots were blocked for an hour at room temperature with 5% skim milk in TBS buffer (50mM Tris-HCl, 150mM NaCl; 0.1%Tween-20; pH 8.5) and then incubated in rabbit anti-CDNF (1:300; ProSci, Poway, CA, USA) over three nights, or rabbit anti-MANF (1:1000; ProSci, Poway, CA, USA) overnight. Nuclear proteins were incubated with rabbit anti-acetyl histone H3 (1:2500) over two nights at 4°C. After washing, blots were incubated in 5% skim milk-TBS Containing horseradish peroxidase-conjugated second antibody (1:3000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 hours at room temperature. The blots were illuminated with enhanced chemiluminescence reagents (Amersham Biosciences, Inc., Baie d'Urfe, Quebec, Canada) to detect CDNF, MANF and acetylated histone H3. Films were developed for 5-15 minutes and Optical density ratios were determined against β-actin, which is constitutively expressed housekeeper protein, or total histone H3 for semi-quantification of targeted protein expression. Antibodies used to detect β-actin were as follows: anti- β-actin mouse monoclonal primary antibody 1:10000 and HPR- conjugated whole mouse IgG secondary antibody (1:200000; both from Sigma-Aldrich, Oakville, ON).

2. 14. Statistical Analysis

The optical density (OD) values for genes of interest were calculated by dividing the ratio of each gene's OD value over the OD value of the internal control 18S rRNA (for mRNA), or β -actin or total histone H3 (for protein expression). Obtained ratios were further normalized to account for inter-experimental variations by conversion to percentage values followed by one-way analysis of variance (ANOVA). A Neuman–Keuls multiple comparison test was used to determine significant differences between treatments, with $p < 0.05$ as the level of significance. The data presented are expressed as mean \pm standard error of the mean.

Table 2.1. PCR conditions for amplification of genes

| Gene | cDNA (μ L) | Annealing Temperature | Number of Cycles |
|--------|-----------------|-----------------------|------------------|
| CDNF | 2 | 55° C | 33 |
| MANF | 2 | 55° C | 33 |
| GDNF | 2 | 55° C | 30 |
| BDNF | 2 | 55° C | 30 |
| Nurr-1 | 1.5 | 52° C | 30 |
| Egr-1 | 2 | 55° C | 30 |
| Sox-2 | 2 | 55° C | 28 |
| 18sRNA | 2 | 55° C | 27 |

Table 2.2. Primer nucleotide sequences

| Gene | Primer 5'-3' | Size of PCR product (bP) |
|----------------|--|--------------------------------|
| CDNF | 5' GGT CGC TAA AAT TGC AGA GC 3' 5' AAG GTA GCC CAG CCC ACT AT 3' | 244 |
| MANF | 5' CTT TCT CCT CCT GCT GGT TG 3' 5' CTG CTG AGC CAA GGG TAA AG 3' | 248 |
| GDNF | 5' ATG GGA TGT CGT GGC TGT CTG 3' 5' TCT CTG GAG CCA GGG TCA GAT 3' | 643 565 |
| BDNF | 5' GGA TGA GGA CCA GAA GGT TGC 3' 5' TTG TCT ATG CCC CTG CAG CCT 3' | 390 |
| Nurr-1 | 5' GST CCC TTC ACA ACT TCC AC 3' 5' CAA GTG CGA ACA CCG TAA TG 3' | 400 |
| Egr-1 | 5' GAC GAG TTA TCC CAG CCA AA 3' 5' GGC AGA GGA AGA CGA TGA AG 3' | 202 |
| Sox-2 | 5' AAG GGT TCT TGC TGG GTT TT 3' 5' AGA CCA CGA AAA CGG TCT TG 3' | 150 |
| 18SrRNA | 5' GCA ATT ATT CCC CAT GAA CG 3' 5' GGC CTC ACT AAA CCA TCC AA 3' | 123 |

CHAPTER 3. RESULTS

3.1 Effects of VPA on Gene Expressions and Protein Concentrations in C17.2 Cells

3.1.1. Effects of VPA on MANF mRNA and protein expression

The bands observed at 248 bp and 123 bp, corresponded to the predicted transcript size of the MANF and 18S rRNA, the internal control, respectively (Figure 3.1). Treatment of C17.2 cells with VPA at clinically relevant concentrations (0.5 mM, 1 mM) for 24-hour caused an up-trend in MANF mRNA expression, but no significant differences were detected by the one-way ANOVA (as shown in Figure3.1). The ubiquitously expressed housekeeping gene 18S rRNA, which was used as an internal control, did not change during the treatment. To verify RT-PCR product specificity and absence of DNA, a negative control for MANF (lacking cDNA) was amplified using diluted RNA without reverse transcriptase treatment, and run in parallel to samples.

Preliminary Western blotting analysis showed a clear up-trend following treatment with VPA at 1mM and 3m M for 24- and 48-hour (Figure 3.2). In contrast, VPA treatment at 3 mM for 72-hour caused a slight non-significant decrease in MANF protein expression (see Figure3.2). The bands observed at 20kDA and 40kDa, corresponded to MANF and the housekeeping protein β -actin, which was used as an internal control.

3.1.2. Effects of VPA on CDNF mRNA and protein expression

The bands observed at 244 bp and 123 bp, corresponded to the predicted transcript size of CDNF and the internal control 18S rRNA (Figure 3.3). Treatment of C17.2 cells with VPA (0.5, 1, 3mM) for 24-hour caused a dose-dependent uptrend in CDNF mRNA expression. Increases of 120% and 75% were observed in cells treated for 24 hours with VPA at clinically relevant concentrations of 0.5mM 1mM, respectively, compared to control and 3mM (as shown in Figure 3.3). However, the one-way ANOVA has shown no statistical significance possibly due to small sample size (n=3). The housekeeping gene 18S rRNA did not change during the treatment. To verify RT-PCR product specificity and absence of DNA contamination, a negative control for CDNF (lacking cDNA) was amplified using diluted RNA without reverse transcriptase treatment, and run in parallel to samples.

Parallel western blotting suggested that treating C17.2 cells with clinically significant VPA (0.5 and 1mM) or slightly above (3mM), for 24, 48, and 72-hour might up-regulate CDNF protein expression (see Figure 3.4). The bands observed at 18 kDa and 40 kDa, corresponded to CDNF protein and the housekeeping protein β -actin, respectively. The one-way ANOVA indicated a significant treatment effect ($p < 0.05$) in cells treated for 24 hours at 1mM or 3 mM VPA (see Figure 3.4).

For 48 hours treatment, VPA caused an uptrend in CDNF protein expression of about 25% at 0.5 mM and up to 50% at 1.0 or 3 mM drug concentrations.

Preliminary results from western showed a clear dose-dependent uptrend in CDNF protein expression in C17.2 cells treated with VPA for 72-hour to reaching about a 100% increase at 3mM. However, ANOVA analysis did not show significant differences, presumably due to the low value (n=2), and the high variance observed at higher treatment concentrations. The housekeeping protein β -actin did not change during the treatment.

3.1.3 Effects of VPA on GDNF mRNA expression

Treatment of C17.2 cells for 24 hours with VPA (0.5, 1, and 3mM) caused a dose-dependent increase of GDNF mRNA expression. The observed bands at 643 bp and 565 bp corresponded to the predicted larger and smaller GDNF transcripts, respectively. The observed band at 123 bp corresponds to 18S rRNA, the internal control (Figure 3.5). GDNF transcripts obtained from alternative splicing of GDNF were consistently detected, as reported previously by our laboratory (Armstrong & Niles, 2002).

The one-way ANOVA indicated a significant treatment effect ($p < 0.001$ vs. control) observed after treatment with clinically relevant concentrations (0.5 and 1mM) of VPA for 24 hours, compared to the control and 3mM (as shown in Figure3.5). Maximal induction of GDNF mRNA by 90% was seen at a concentration of 0.5mM VPA. The house keeping gene 18S rRNA did not change during the treatment. The negative control for GDNF (lacking cDNA) was amplified using diluted RNA without reverse transcriptase treatment and run in parallel to samples to verify RT-PCR product specificity and absence of DNA.

3.1.4. Effects of VPA on Nurr-1 mRNA expression

Treatment of C17.2 cells for 24 hours with VPA (0.5, 1, and 3mM) demonstrated a dose-dependent increase of Nurr-1 mRNA expression. The bands observed at 400 bp and 123 bp, corresponded to the predicted transcript size of Nurr-1 and the internal control 18S rRNA, respectively (figure 3.6). The one-way ANOVA reflected a significant increase ($p < 0.01$) in Nurr-1 mRNA expression of about 75% at 0.5mM compared to control. In addition, a significant induction ($p < 0.05$) in Nurr-1 mRNA expression of about 40% was observed at 1mM VPA compared to control. Interestingly, there was a significant ($p < 0.01$) decrease in Nurr-1 mRNA expression by 50% at 3mM VPA, which is considerably above the clinical concentrations of 0.5mM and 1mM. The housekeeping gene 18S rRNA, which was used as control, did not change during the treatment.

To verify RT-PCR product specificity and absence of DNA, a negative control for Nurr-1 was amplified using diluted RNA without reverse transcriptase treatment and run in parallel to samples.

3.1.5 Effects of VPA on Egr-1 mRNA expression

Treatment of C17.2 cells with clinically significant concentrations of VPA for 24 hours caused a dose-dependent increase of Egr-1 mRNA. The bands observed at 202 bp and 123 bp, corresponded to the predicted transcript size of Egr-1 and the internal control 18S rRNA, respectively (Figure 3.7). The one-way ANOVA indicated a significant ($p < 0.05$) increase in Egr-1 mRNA by 60% at 0.5mM VPA, and by 50% at 1mMVP (as shown in Figure3.7). The housekeeping gene 18S rRNA, did not change during the treatment. The negative control for Egr-1 was amplified using diluted RNA without reverse transcriptase treatment and run in parallel to samples to verify RT-PCR product specificity and absence of DNA.

3.1.6. Effects of VPA on BDNF mRNA expression

BDNF mRNA transcripts were detected after the treatment of C17.2 cells for 24 hours with VPA (0.5, 1, and 3mM). The one-way ANOVA showed no significant changes in BDNF mRNA expression (Figure3.8).

BDNF transcripts were highly expressed in both treated and non-treated C17.2 cells (Figure 3.8). The bands observed at 390 bp and 123 bp corresponded to the predicted transcript size of BDNF and the internal control 18S rRNA, respectively. The housekeeping gene 18S rRNA did not change during VPA treatment. To verify RT-PCR product specificity and absence of DNA, a negative control for BDNF (lacking cDNA) was amplified using diluted RNA without reverse transcriptase and run in parallel to samples.

3.1.7. Effects of VPA on Sox-2 expression in C17.2 cells

Sox-2 mRNA transcripts were robustly expressed in both controls and C17.2 cells treated with VPA at 0.5, 1, or 3mM, for 24 hours. The one-way ANOVA showed no significant changes in Sox-2 mRNA expression (Figure 3.9). The bands observed at 150 bp and 123bp corresponded to the predicted transcript size of Sox-2 and 18S rRNA, respectively. The control gene 18S rRNA did not change during the treatment. The negative control of Sox-2 (lacking cDNA) was synthesized using diluted RNA without reverse transcriptase treatment and run in parallel to samples to verify RT-PCR product specificity and absence of DNA.

3.1.8 Effects of VPA on histone H3 acetylation

Preliminary western blot analysis showed that 1mM VPA increases histone H3 acetylation in C17.2 cells by more than 900% for 24-hour VPA treatment, and by 50% after 48 hours treatment, as compared to cells exposed to vehicle (DMEM/FBS). After 72 hr of treatment with VPA, the acetylation levels of histone H3 had returned to normal (Figure 3.10). Histone H3 has a molecular weight of 17kDa.

3.2 Effects of PD98059 Treatment on CDNF and MANF mRNA and Protein Expression

In order to investigate whether the ERK1/2 pathway is involved in modulating VPA action on CDNF and MANF mRNA/protein expression, the synthetic inhibitor PD98059 was used to block the ERK1/2 pathway. The following sections discuss the preliminary results.

3.2.1. Effects of PD98059 treatment on CDNF and MANF protein concentrations in C17.2 cells treated with VPA (24 h treatment)

Preliminary data from western blotting suggests that treating C17.2 cells with PD98059 did not block the effect of VPA (Figures 3.11 and 3.12). 24-hour PD 98059 was observed to cause an increase in both MANF and CDNF protein expression by nearly 25% and nearly 50%, respectively (Figures 3.11 and 3.12).

Interestingly, a combination of both drugs, VPA and PD98059, showed a trend toward higher induction by 40% and 60% for MANF and CDNF protein expression, respectively (Figures 3.11 and 3.12).

3.3 Special Remarks on High Control Expression of Targeted Genes

In this study, transcripts of targeted genes (CDNF, MANF, BDNF, GDNF, Nurr-1, Egr-1, and Sox-2) from non-treated cells have been used as a control.

High expressions of targeted genes (CDNF, MANF, BDNF, GDNF, Nurr-1, Egr-1, and Sox-2) from non-treated cell were noticed in some 48-hour experiment (not shown), some 72-hour experiment (not shown), and the late replications of 24-hour experiment (not shown). Also, the expression of the amplified cDNA of targeted genes (CDNF, MANF, BDNF, GDNF, Nurr-1, Egr-1, and Sox-2) for cells treated with VPA for late replications of the 24-hour, the 48-hour, the-72-hour, and in cells treated with PD998059 for 24-hours (Figure 3.13) encountered the same problems. Therefore, the replications of 48-hour, and 72-hour of the VPA experiments, and PD998059 were discontinued while this problem is being examined. High basal expression might be attributed to several factors, as follows.

Cell stress and media replacement

Cell culture media is changed frequently in order to optimize nutrition and to maintain homeostasis. Media were changed for C17.2 cell cultures in this study every 48 hours before they reached 90-100% confluence and were changed 24 hours prior to treatment. The primary technique used for media processing was removed by vacuum either by electronic or glass pipette. In some iterations of the current study, media was not changed at exact 48 hours intervals. Instead media was changed either before or after 48 hours. The fluctuations in cell culture processing may translate into variable media conditions. It has been demonstrated experimentally that intermittent changes in culture media causes synchronized changes in gene expression of neural progenitor cells (Kim et al, 2012). This might suggest a role for abrupt media changes in influencing gene expression and is a possible explanation for the high levels of control gene expression seen in this study.

Repetitive passaging

High basal expression of targeted genes in non-treated C17.2 cells was noticed in this study and in a previous study by the *Niles* group (not published) with a higher passage number and/or repeated passaging. Repeated sub-culturing is known to impact cell line properties over time.

Cell lines at high passage numbers experience alterations in cellular morphology/behavior, growth rates, response to stimulants and protein expression compared to lower-passage cells (Briske-Anderson et al., 1996; Wenger et al., 2004). Late replications of the 24-hour, the 48-hour, and the 72-hour experiments involved cells beyond 25 passages, whereas normal control expression observed in earlier replications of this study involved cell ranging from 15-20 passages.

Figure 3.1. Concentration-dependent effects on MANF mRNA expression in C17.2 cells following 24-hour VPA treatment

A) Representative gel image of RT-PCR detection of MANF (248 bp) and 18S rRNA (123 bp)

B) Histogram of MANF/ 18S rRNA optical density ratios as function of VPA concentration

Lanes 1-4: control, 0.5mM VPA, 1mM VPA, 3mM VPA.

Lane 5: control without reverse transcription. M= marker.

Data shown are the mean \pm s.e.m, for percentage values of optical density ratios. (n= 3)

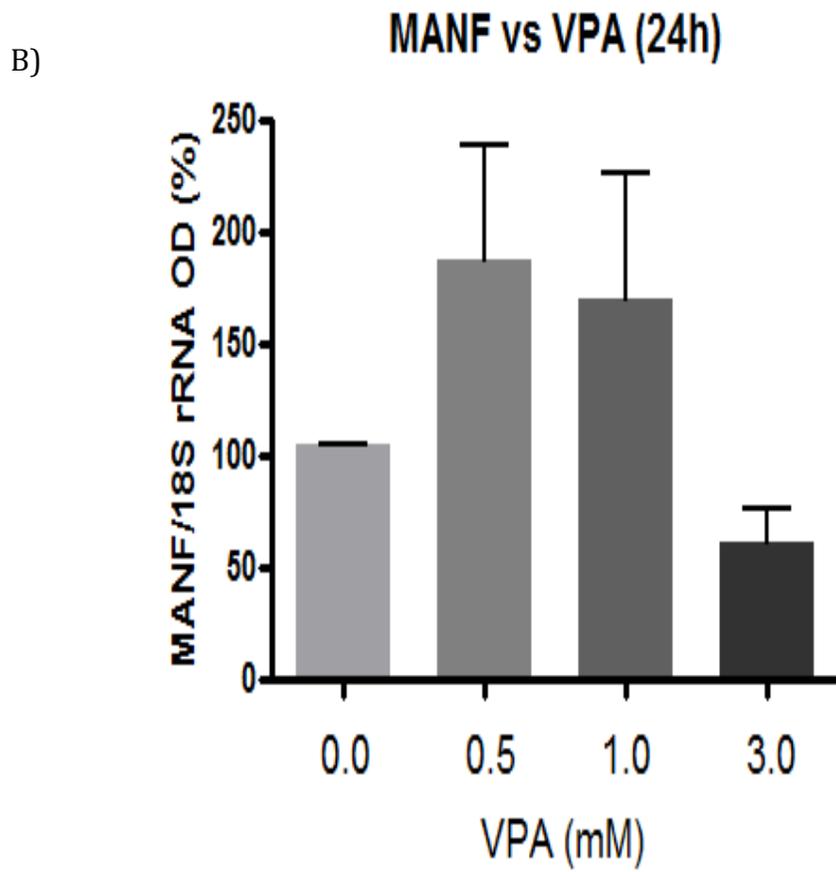
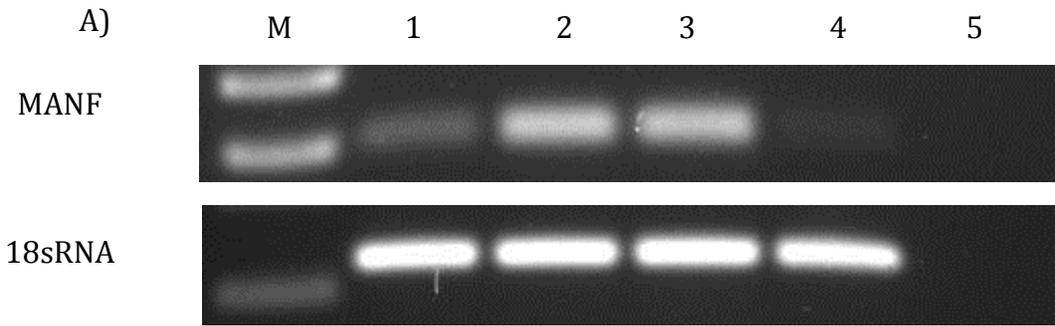


Figure3.2. Concentration-dependent effects on MANF protein concentrations in C17.2 cells following 24-, 48-, and 72-hour

VPA treatment

- A) Immuno-blot of MANF/ β -actin protein at 24-h VPA treatment
- B) Histogram for MANF / β -actin optical density ratios as function of VPA concentrations for 24-h treatment
- C) Immuno-blot of MANF/ β -actin protein at 48-h VPA treatment
- D) Histogram for MANF / β -actin optical density ratios as function of VPA concentrations for 48-h treatment
- E) Immuno-blot of MANF/ β -actin protein at 72-h VPA treatment
- F) Histogram for MANF / β -actin optical density ratios as function of VPA concentrations for 72-h treatment

Lanes 1-4: control, 0.5mM VPA, 1mM VPA, 3mM VPA
MANF protein is 20 kDa and β -actin protein is 42 kDa

Data shown are the mean \pm s.e.m., for percentage values of optical density ratios. (n= 2).

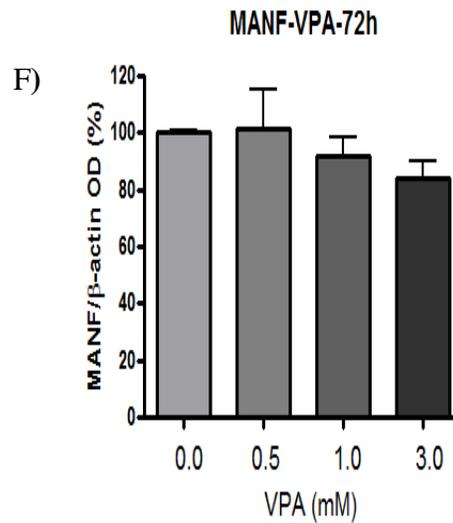
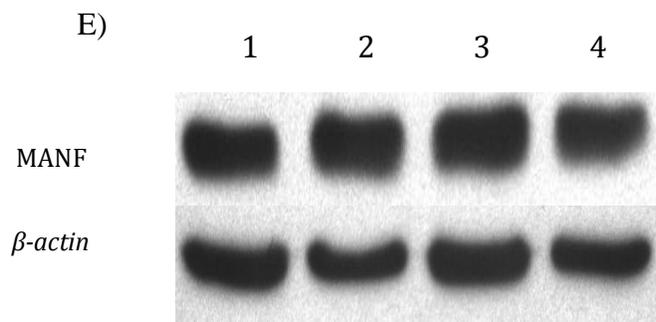
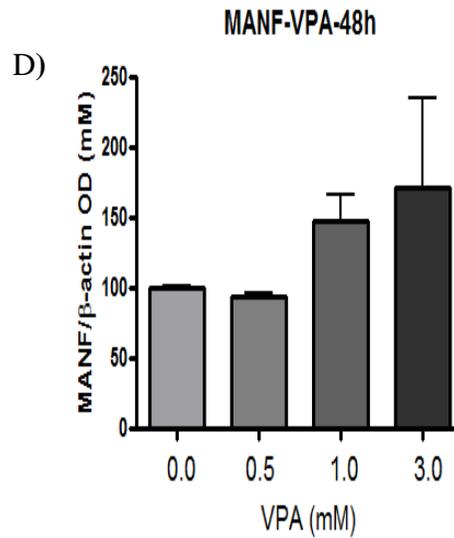
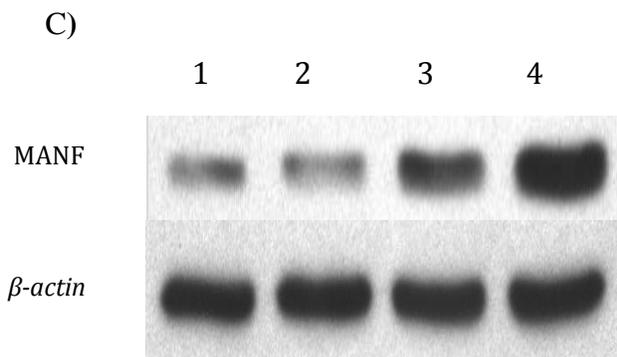
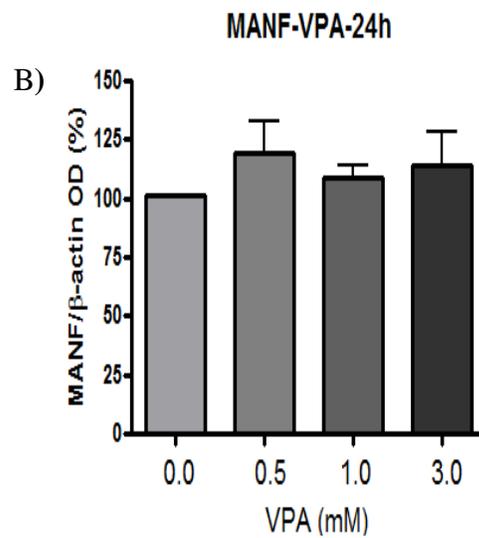
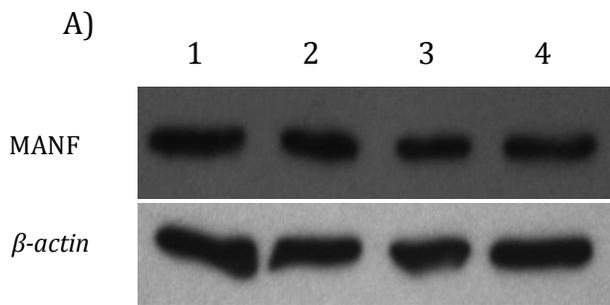


Figure 3.3. Concentration-dependent effects on CDNF mRNA expression in C17.2 cells following 24-hour VPA treatment

A) Representative gel image of RT-PCR detection of CDNF (244pb) and 18S rRNA (123 bp)

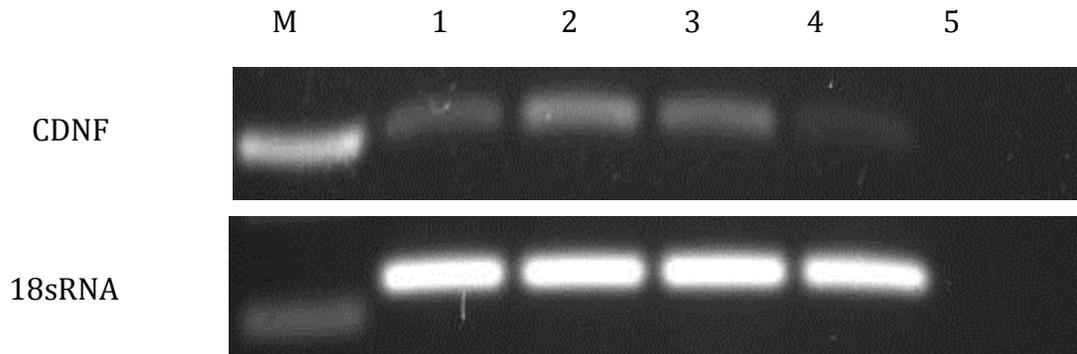
B) Histogram of CDNF/ 18S rRNA optical density ratios as function of VPA concentration

Lanes 1-4: control, 0.5mM VPA, 1mM VPA, 3mM VPA.

Lane 5: control without reverse transcription. M=marker

Data shown are the mean \pm s.e.m., for percentage values of optical density ratios. (n= 3)

A)



B)

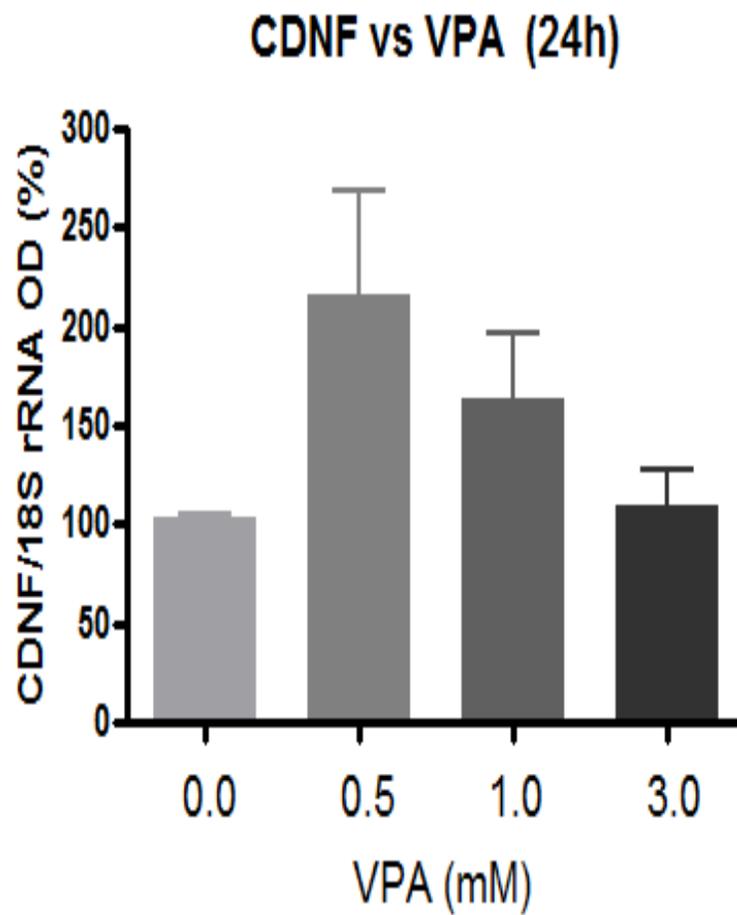


Figure 3.4. Concentration-dependent effects on CDNF protein concentrations in C17.2 cells following 24-, 48-, and 72-hour VPA treatment

A) Immuno-blot of CDNF / β -actin protein at 24h VPA treatment

B) Histogram for CDNF / β -actin optical density ratios as function of VPA concentrations treatment for 24-h

C) Immuno-blot of CDNF / β -actin protein at 48-h VPA treatment

D) Histogram for CDNF / β -actin optical density ratios as function of VPA concentrations treatment for 48-h

E) Immuno-blot of CDNF / β -actin protein at 72-h VPA treatment

F) Representative Histogram for CDNF / β -actin optical density ratios as function of VPA concentrations treatment for 72-h

Lanes 1-4: control, 0.5mM VPA, 1mM VPA, 3mM VPA.
CDNF protein is 18 kDa and β -actin protein is 42 kDa.

Data shown are the mean \pm s.e.m., for percentage values of optical density ratios. * $p < 0.05$. (n= 2)

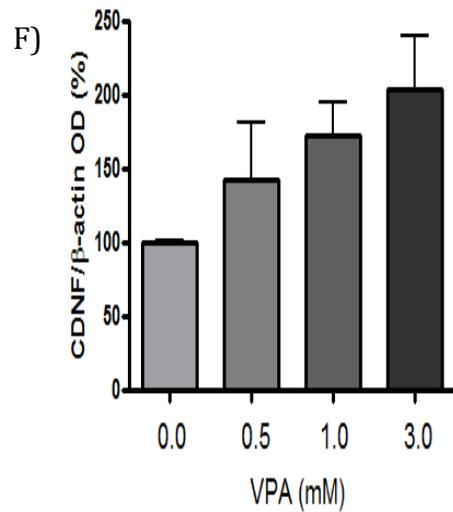
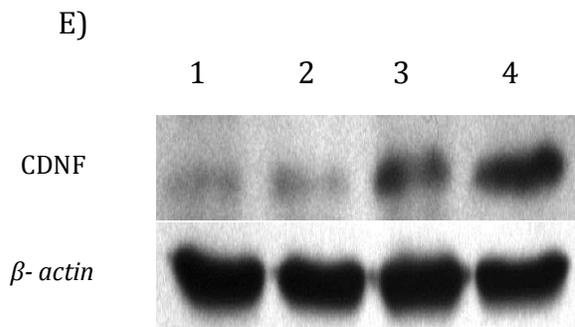
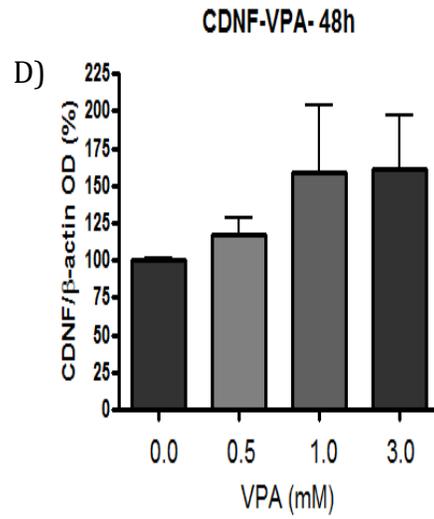
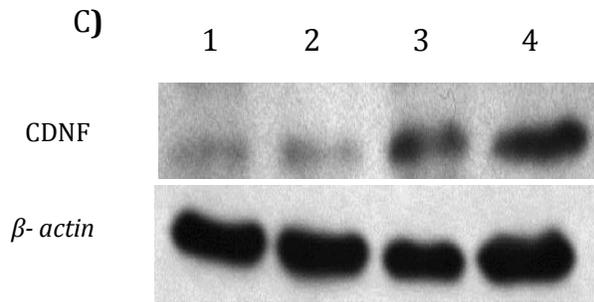
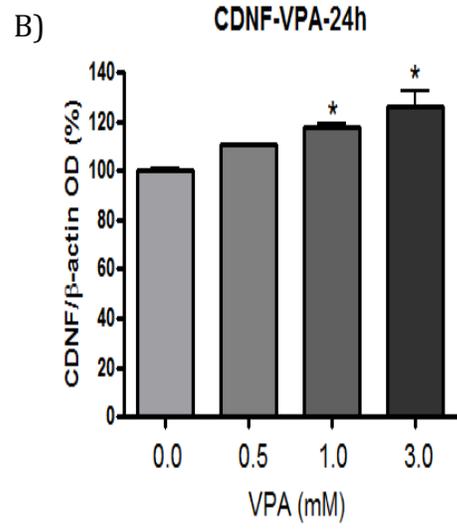
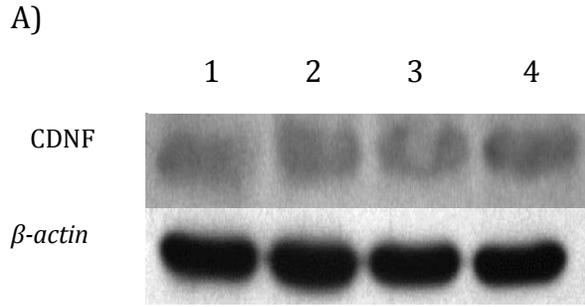


Figure 3.5 Concentration-dependent effects on GDNF mRNA expression in C17.2 cells following 24-hour VPA treatment

A) Representative gel image of RT-PCR detection of GDNF (643 and 565 bp) and 18S rRNA (123 bp)

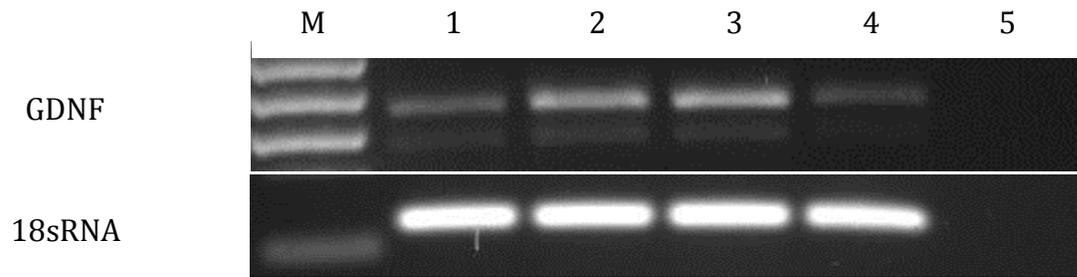
B) Histogram of GDNF/18S rRNA optical density ratios as function of VPA concentrations.

Lanes 1-4: control, 0.5mM VPA, 1mM VPA, 3mM VPA.

Lane 5: control without reverse transcription. M=marker

Data shown are the mean \pm s.e.m. for percentage values of optical density ratios. ** $p < 0.001$ vs. control. (n= 3)

A)



B)

GDNF vs VPA (24h)

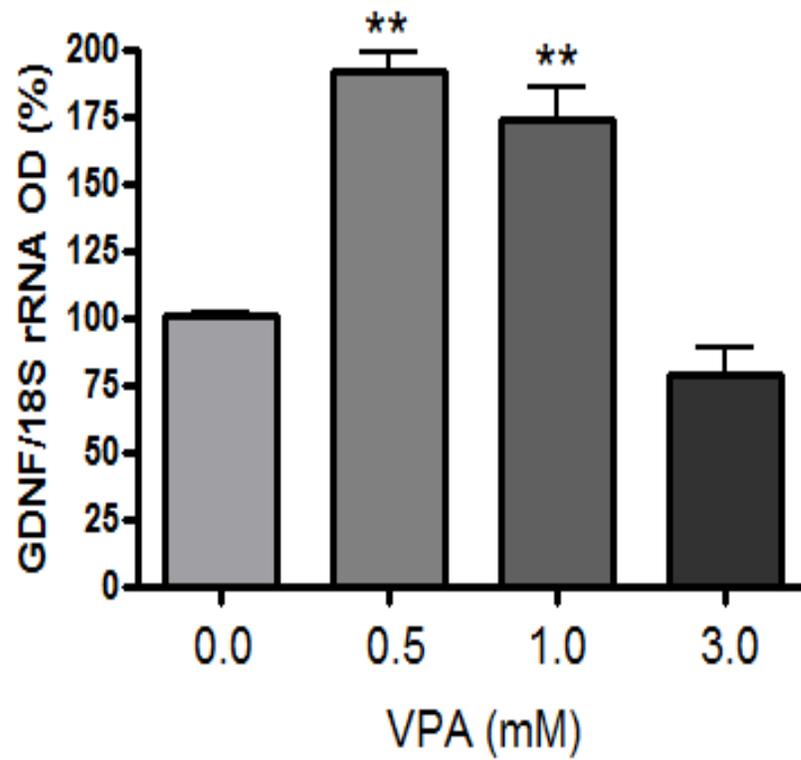


Figure 3.6 Concentration-dependent effects on Nurr-1 mRNA expression in C17.2 cells following 24-hour VPA treatment

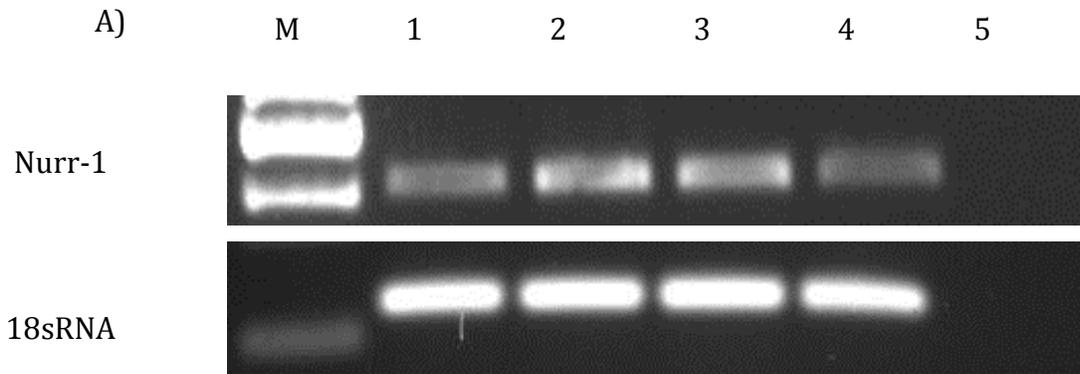
A) Representative gel image of RT-PCR detection of Nurr-1 (400bp) and 18S rRNA (123bp)

B) Histogram of Nurr-1/18S rRNA optical density ratios as function of VPA concentrations

Lanes 1-4: control, 0.5mM VPA, 1mM VPA, 3mM VPA.

Lane 5: control with reverse transcription. M= marker.

Data shown are the mean \pm s.e.m. for percentage values of optical density ratios. *** $p < 0.01$ vs. control, ** $p < 0.05$ vs. control, **# $p < 0.01$ vs. 0.5 and 1mM). (n= 3)



B)

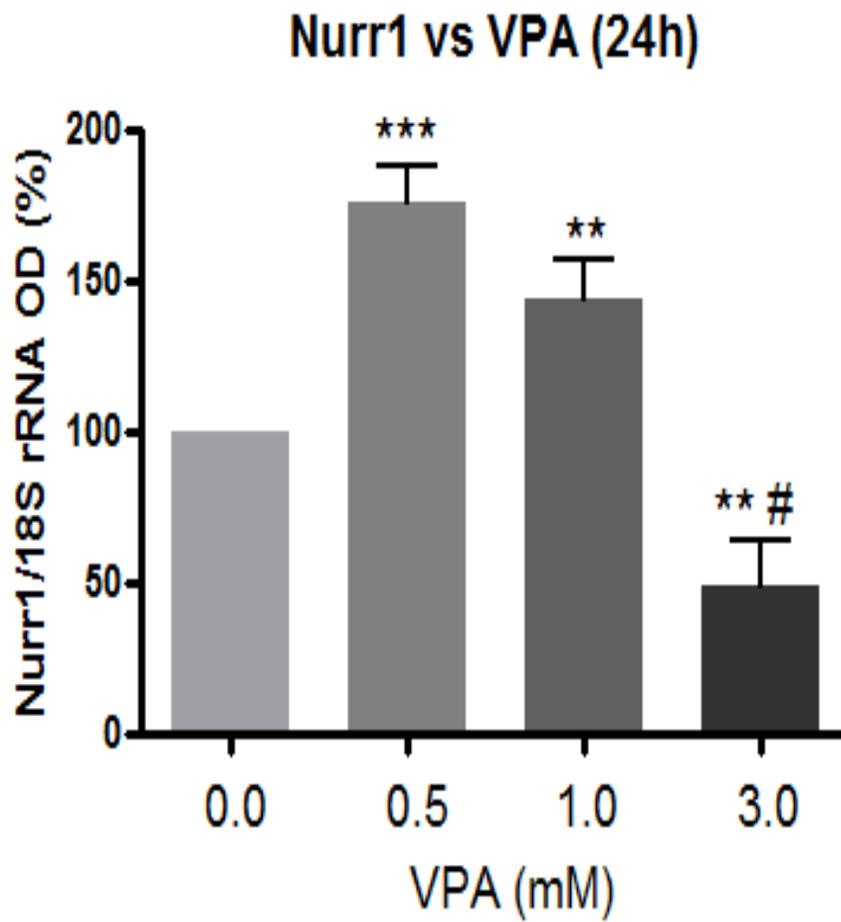


Figure 3.7 Concentration-dependent effects on Egr-1 mRNA expression in C17.2 cells following 24-hour VPA treatment

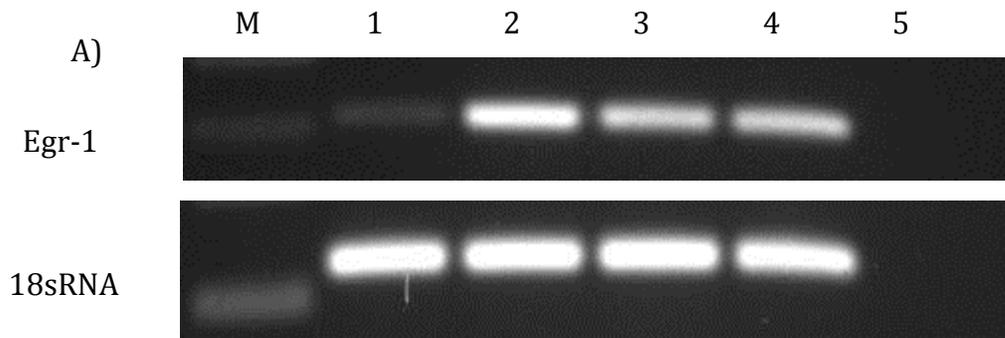
A) Representative gel image of RT-PCR detection of Egr-1 (202 bp) and 18S rRNA (123 bp)

B) Histogram of Egr-1/18S rRNA optical density ratios as function of VPA concentrations.

Lanes 1-4: control, 0.5mM VPA, 1mM VPA, 3mM VPA.

Lane 5: control with reverse transcription. M= marker.

Data shown are the mean \pm s.e.m., for percentage values of optical density ratios. * $p < 0.05$ vs. control. (n= 3)



B)

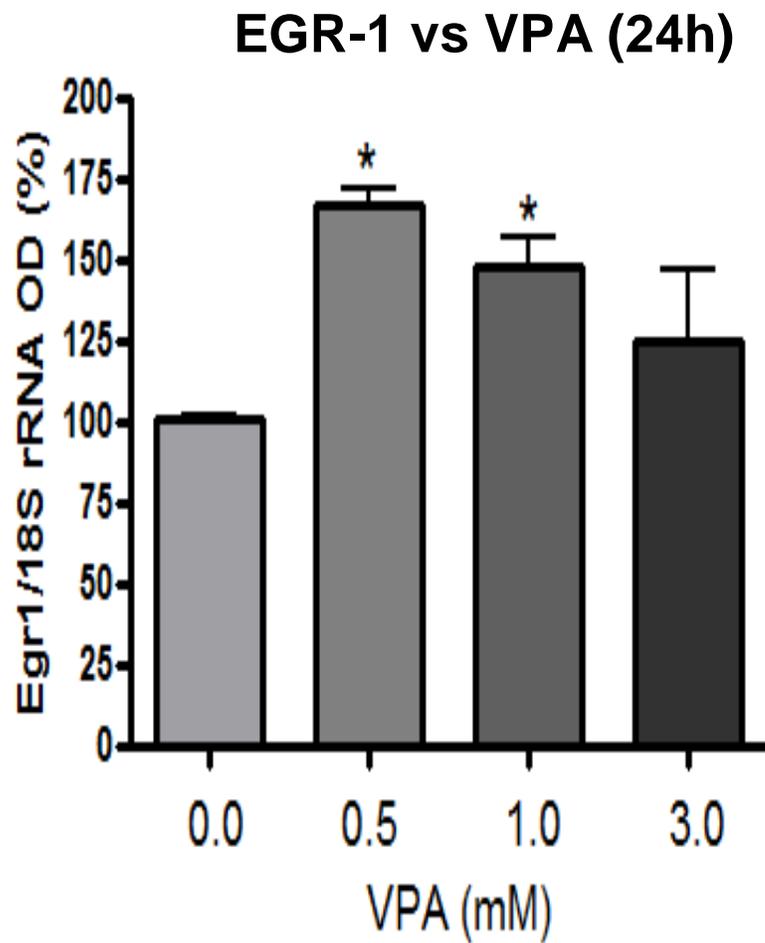


Figure 3.8. Concentration-dependent effects on BDNF mRNA expression in C17.2 cells following 24-hour VPA treatment

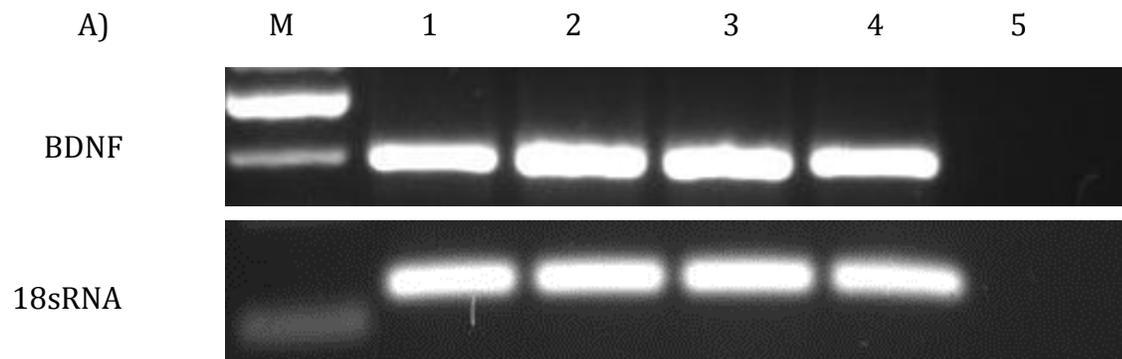
A) Representative gel image of RT-PCR detection of BDNF (390bp) and 18S rRNA (123 bp)

B) Histogram of BDNF/ 18S rRNA optical density ratios as function of VPA concentrations.

Lanes 1-4: control, 0.5mM VPA, 1mM VPA, 3mM VPA

Lane 5: control with reverse transcription. M= marker.

Data shown are the mean \pm s.e.m, for percentage values of optical density ratios. (n= 3)



B)

BDNF vs VPA (24h)

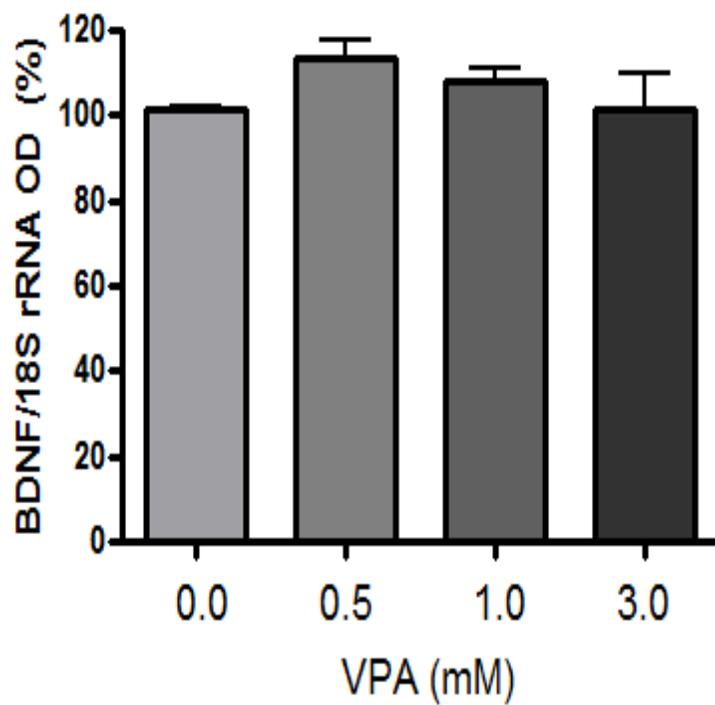


Figure 3.9. Concentration-dependent effects on Sox-2 mRNA expression in C17.2 cells following 24-hour VPA treatment

A) Representative gel image of RT-PCR detection of Sox-2 (150 bp) and 18S rRNA (123 bp)

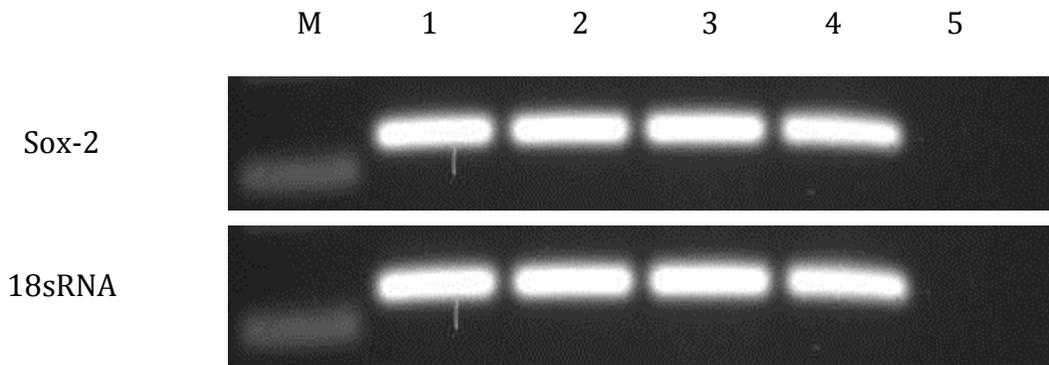
B) Histogram of Sox-2/18S rRNA optical density ratios as function of VPA concentration.

Lanes 1-4: control, 0.5mM VPA, 1mM VPA, 3mM VPA.

Lane 5: control with reverse transcription. M= marker

Data shown are the mean \pm s.e.m , For percentage values of optical density ratios. (n= 3)

A)



B)

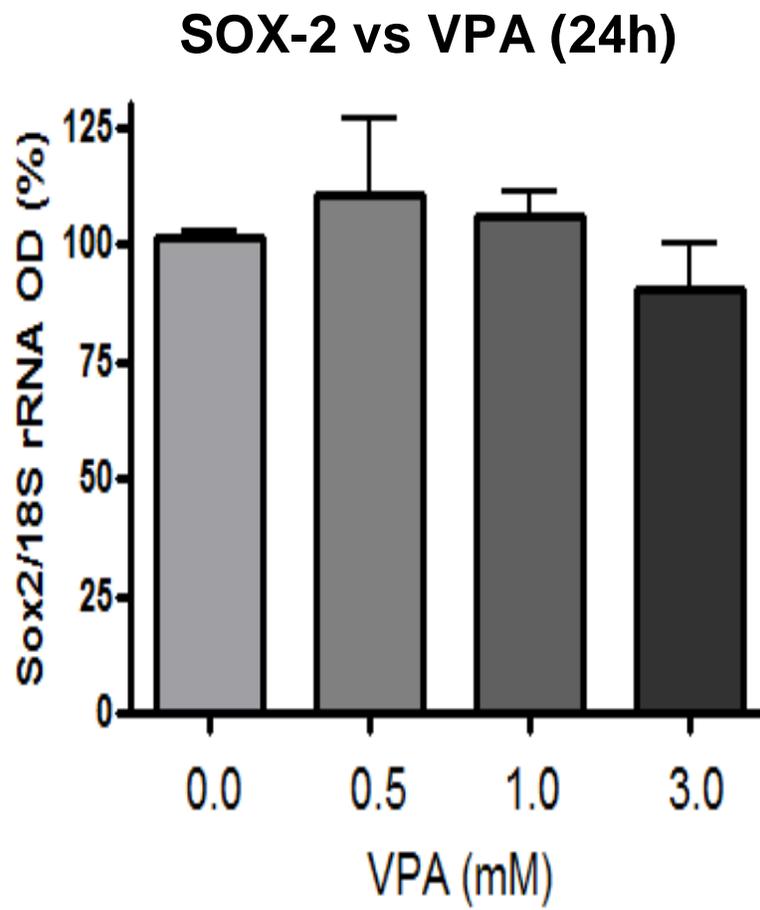


Figure 3.10. Effects of VPA on histone H3 acetylation in C17.2 cells

- A) Acetylated histone H3 in cells treated for 24-h
- B) Histogram of Ach3/Total H3 optical density ratios as a function of 1mMVPA for 24-h treatment
- C) Acetylated histone H3 in cells treated for 48-h
- D) Histogram of Ach3/Total H3 optical density ratios as a function of 1mM VPA for 48-h treatment
- E) Acetylated histone H3 in cells treated for 72-h
- F) Histogram of Ach3/Total H3 optical density ratios as a function of 1mMVPA for 72-h treatment
- G) Total acetylated histone H3 collected from treated C17.2 cells

Lanes 1-2: control, 1mM VPA.

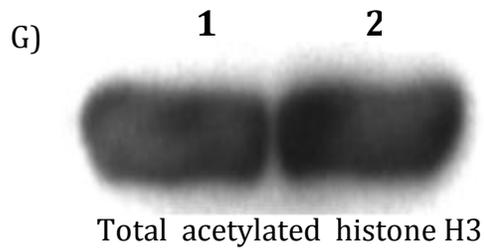
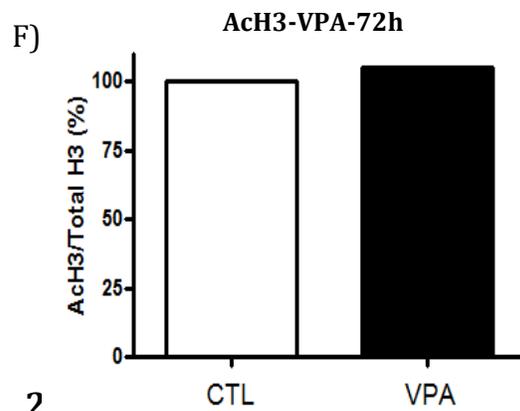
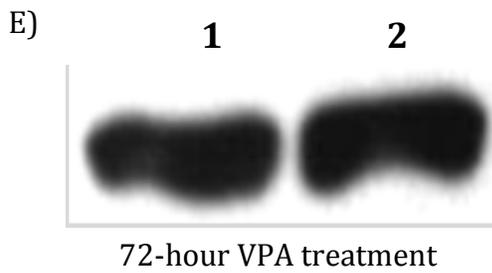
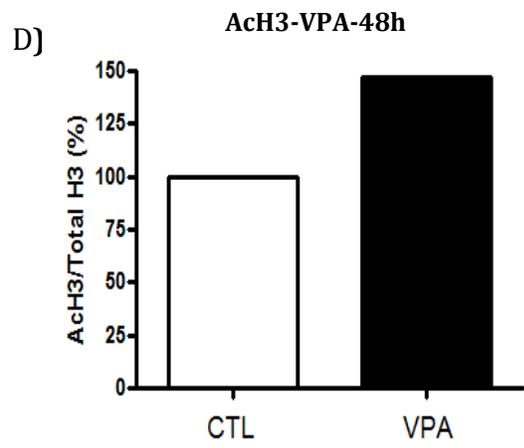
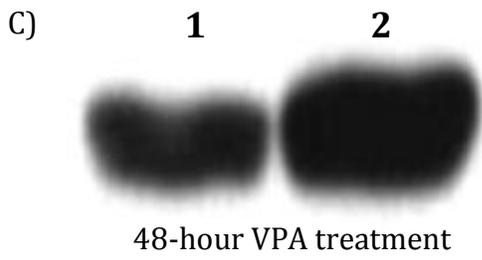
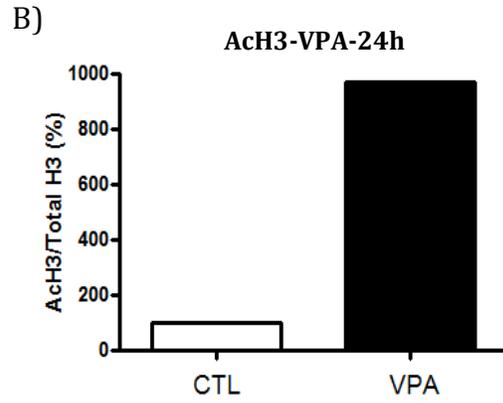
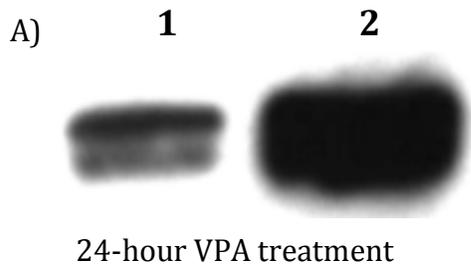


Figure 3.11. Effects of PD 98059 on MANF protein concentrations in C17.2 cells following 24-hour VPA treatment

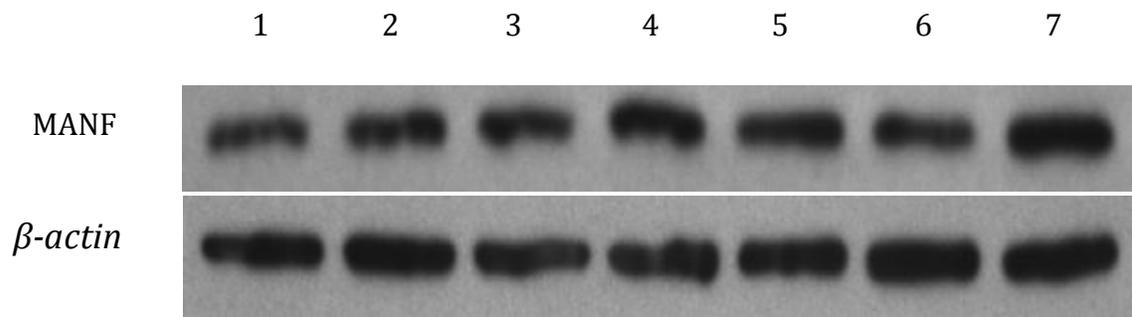
A) Immuno-blot of MANF and β -Actin protein

B) Histogram for MANF/ β -actin optical density ratios as function of VPA concentration treatment

Lanes 1-7: control, DMSO (0.2%), 0.5 mM VPA, 1 mM VPA, 20 μ M PD+0.5 mM VPA, 20 μ M PD+ 1mM VPA. M= marker
MANF is 20kDa and β -actin protein is 42 kDa.

Data shown are the mean \pm s.e.m., for percentage values of optical density ratios. (n= 2)

A)



B)

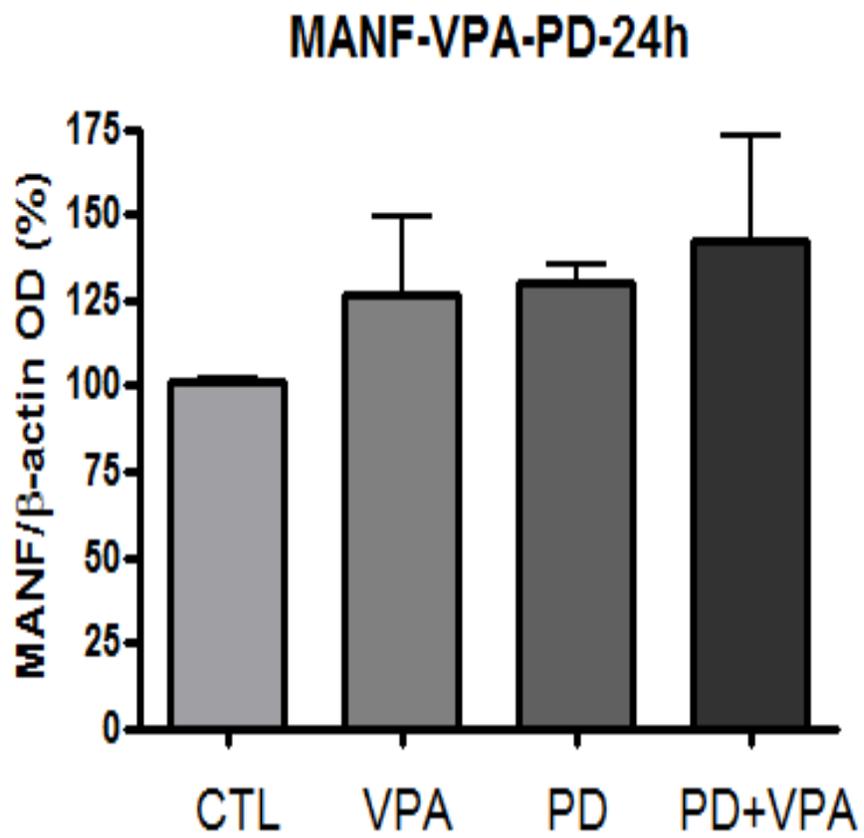


Figure 3.12. Effects of PD98059 on CDNF protein concentrations in C17.2 cells following 24-hour VPA treatment

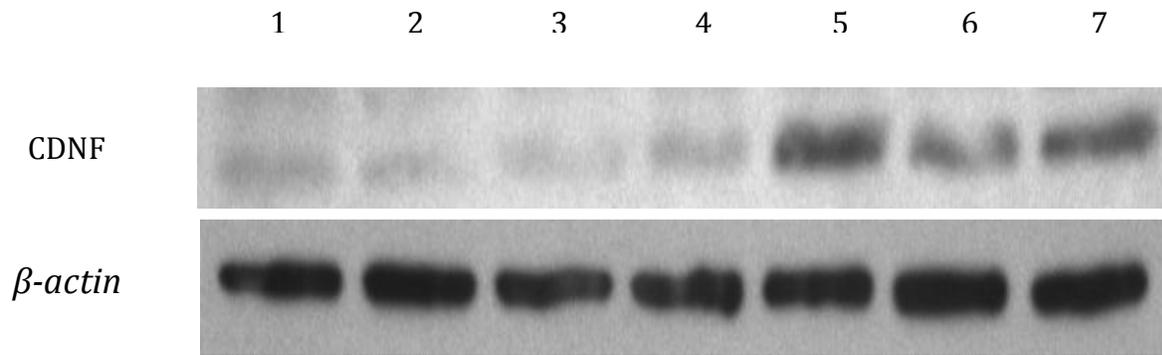
A) *Immuno-blot of CDNF and β -actin protein*

B) *Histogram for CDNF/ β -actin optical density ratios as function of VPA concentration treatment*

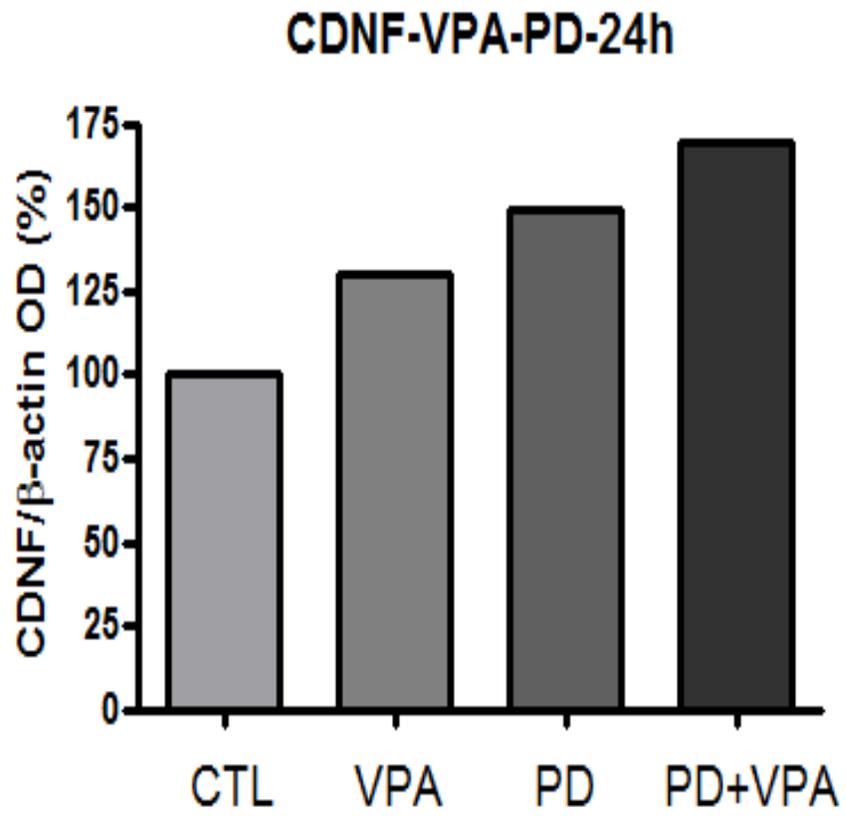
*Lanes 1-7: control, DMSO (0.2%), 0.5 μ M VPA, 1 μ M VPA, 20 μ M PD+0.5 μ MVPA, 20 μ M PD+ 1 μ M VP. M= marker
CDNF is 18 kDa and β -actin protein is 42 kDa.*

Data shown are for percentage values of optical density ratios.(n= 1)

A)



B)



CHAPTER 4. DISCUSSION

In this study, VPA (HDACc inhibitor) was used to treat C17.2 cells to examine its effect on the expression of novel conserved neurotrophic factors CDNF and MANF and other neurotrophic factors; BDNF and GDNF. We have also examined the effect of VPA on differentiation markers Egr-1, Sox2, and the early DA marker Nurr-1. In addition, we have investigated the involvement of HDAC inhibition and Erk-1/2 pathways in the regulation gene expression/transcription by VPA in C17.2 cells.

4.1 VPA enhances the expression of the novel neurotrophic factor CDNF

CDNF and MANF are proteins that have just come to light as members of a novel evolutionary conserved neurotrophic family. Research on MANF and CDNF remains in its infancy. Therefore, induction of MANF and CDNF by HDACs has not yet been adequately evaluated. A recent study has shown that chronic VPA treatment significantly increased MANF and CDNF transcript and protein levels in rat hippocampi and striata (Niles et al., 2012). Similarly, results from the present study suggest that VPA might influence CDNF mRNA expression in C17.2 cells following 24 hours of VPA treatment at clinically significant concentrations (0.5 and 1mM). Parallel Western blot analysis confirmed a significant ($p < 0.05$) increase in CDNF protein expression at 1mM and 3 mM VPA.

Also, a gradual concentration and time-dependent uptrend was observed in CDNF protein concentrations following 48- and 72-hour VPA treatment, suggesting a degree of sensitivity of CDNF to VPA concentrations over time. A similar uptrend in MANF mRNA and protein levels following 24 or 48 hours of treatment at clinically relevant concentrations of VPA, suggest a possible role of VPA in increasing MANF expression in C17.2 cells. Further study is required to substantiate these preliminary results.

CDNF and MANF are expressed in different mouse and human tissues (Lindholm et al., 2007; Lindholm et al., 2008). MANF was first purified from the conditioned medium of a ventral mesencephalic cell line (VMCL1), and CDNF was identified by bioinformatics and has been biochemically characterized (Lindholm et al., 2007). Both factors, MANF and CDNF, have been shown to be neuroprotective and neurorestorative in a 6-ODHA animal model of PD (Airavaara et al., 2012). CDNF was shown to protect and restore DA neuronal phenotypes and enhance behavioral measurements in a mouse MPTP model (Airavaara et al., 2011). Moreover, recent evidence has shown that MANF reduces infarction volume, promotes behavioral recovery, and inhibits cell necrosis/apoptosis in the cerebral cortex, in an experimental model of stroke in rats. In addition, MANF is suggested to regulate neuronal survival and plasticity in the cortex and hippocampus (Lidholm et al 2008; Niles et al, 2012).

These findings suggest a possible neuroprotective role for MANF in the setting of cerebral ischemia (Airavaara et al., 2009). MANF has also been shown to prevent cell death induced by simulated ischemia when added to cardiac myocyte cultures (Tadimalla et al., 2008).

4.2. VPA enhances the mRNA expression of the neurotrophic factor GDNF, but has no effect on BDNF

Earlier *in vivo* studies showed that chronic VPA treatment had no effect on GDNF expression in rat brain tissue (Fukumoto et al., 2001), while recent *in vitro* studies suggest a significant increase in GDNF mRNA after 48 hours of VPA treatment at 1, 3, and 5mM (Castro et al., 2005). Similarly, it has been reported that chronic treatment with clinically relevant doses of VPA significantly increases GDNF mRNA expression in the rat hippocampus and striatum, implicating GDNF as a potential target for VPA (Niles et al., 2012). A novel finding in the current study was a statistically significant induction ($p < 0.001$) of GDNF mRNA expression in C17.2 cells following 24 hours of VPA at clinically relevant concentrations (0.5mM and 1mM). GDNF was first isolated from a glioma cell line and shown to promote the survival of embryonic DA neurons. GDNF was also shown to promote the survival of different neurons of the central and peripheral nervous system (Airavaar et al. 2012).

Earlier studies have shown the neurorestorative effect of GDNF in mouse (Hoffer et al., 1994), rat (Rosenbald et al., 1999), and non-human primate models of Parkinson's disease (PD) (Gash et al., 1996). The neuroprotective and neurorestorative effects of GDNF were also shown by utilizing astrocytes (Safi et al., 2012) or viral vectors for its delivery into targeted neurons, in a number of animal models (Connor, 2001; Georgievska et al., 2002; McBride et al., 2002; McBride et al., 2003). Although GDNF was found to be promising in animal models of PD, the results from clinical trials were inconsistent. Despite showing considerable promise, the possible benefits of GDNF and MANF in neuroprotection and neurorestoration awaits further investigation (Dauer, 2007).

With regards to BDNF, several *in vitro* studies have reported BDNF up-regulation following VPA treatment. For example, increased BDNF mRNA expression was observed in C6 glioma cells and cortical neurons, as well as cultured human astrocytoma cells in response to VPA treatment (Castro et al, 2005; Fukuchi et al., 2009; Nishino et al., 2012). Although VPA can up-regulate BDNF in the CNS and other cell lines, no changes were observed in C17.2 cells, probably due to the high expression of BDNF transcripts. BDNF up-regulation by VPA was also observed in a previous study performed by our group (Niles et al., 2004).

Notably, significant induction of BDNF by VPA in previous studies was noticed mostly with chronic administration or high concentrations/doses of VPA.

4.3. 24h -VPA Treatment Enhances the mRNA Expression of Nurr-1 and Egr-1

Regulation of Nurr-1 and Egr-1 by VPA has not yet been examined in C17.2 cells. However, the expression of Nurr-1 in C17.2 cells was previously reported to be altered by melatonin, a molecule known to up-regulate histone H3 acetylation in these cells (Sharma et al., 2008).

Egr-1 has been shown to be activated by a variety of mitogenic, growth and differentiation stimuli (Milbrandt et al., 1988). In the present study, the significant up-regulation of Nurr-1 ($p < 0.01$ at 0.5 mM and $p < 0.05$ at 1mM) and Egr-1 ($p < 0.05$) mRNA expression following treatment with clinically relevant VPA concentrations might support VPA's role in promoting NSC differentiation.

Nurr-1, a transcription factor belonging to the orphan nuclear receptor super-family, is highly expressed in the developing and adult ventral midbrain (Jankovic et al., 2005). Within the CNS, Nurr-1 is expressed in many areas, including the cortex, hippocampus, brain stem and spinal cord.

The precise biological function of this protein, however, has not yet been elucidated (Perlmann & Wallén-Mackenzie, 2004).v Nurr-1 is required for early differentiation and maintenance of midbrain DA neurons (Chu et al., 2006). In the absence of Nurr-1, developing ventral midbrain neurons fail to express the dopaminergic neuronal marker tyrosine hydroxylase (TH), dopamine transporter (DAT), and the receptor tyrosine kinase signaling subunit *Ret* (Zetterstrom et al., 1997; Wallén et al., 2001; Sacchetti et al 200). Interestingly, a decrease in Nurr-1 expression has been observed in Parkinson's disease models (Chu et al., 2006; Le et al., 2008; Liu et al., 2012), and mutations in the Nurr-1 gene have been identified in rare cases of PD (Xu et al., 2002; Le et al., 2003; Grimes et al., 2006). More recently, a decrease in nigral Nurr-1 immunofluorescence was observed in other neurodegenerative diseases such as Alzheimer's disease (AD) and progressive supranuclear palsy disorder (PSPD) (Chu et al, 2006). *In vitro* over-expression of Nurr-1 in neuronal precursor cell cultures was observed to promote cellular differentiation into mature and functional DA neurons (Wagner et al. 1999; Kim et al., 2003; Shim et al., 2007).

Egr-1, also known as Krox24, Zif268, and NGFI-A, is expressed abundantly in several CNS and PNS areas of adult and developing animals, including the corpus callosum, striatum, cortex, and midbrain. (Lee et al., 1995; Beck et al., 2008).

Egr-1 plays a role in cellular growth, development, differentiation and apoptosis (Pignatelli et al., 2003; Pagel & Deindl , 2011). In N2 neuroblastoma cells, Egr-1 was shown to induce neuronal differentiation and cause neurite outgrowth (Pignatelli et al., 1999). Also, induction of Egr-1 has been linked to neuronal plasticity (Harada et al., 2001; Bozon et al., 2003; Knapska et al., 2004; James et al., 2006).

4.4. 24h-VPA Treatment Has No Effect on Sox-2 mRNA Expression in C17.2 Cells.

Sox-2, an established pluripotency factor, is a universal marker for neural stem cells and progenitor cells of vertebrate CNS (Graham et al., 2003). Sox-2 is expressed in many stem cells and precursor cells during development, including neural stem cells (NSCs), and therefore it is likely to be involved in self-renewal and precursor differentiation (Episkopou et al., 2005; Fauquier et al., 2008; Adachi et al., 2010). Recent studies suggest that low levels of histone deacetylase (HDAC) inhibitors promote pluripotency in many cell lines (Huangfu et al., 2008; Ware et al., 2009; Hezroni et al., 2011).

In this study, the HDAC inhibitor VPA did not alter Sox-2 mRNA expression in C17.2 cells treated with clinically significant concentrations for 24 hours.

Nevertheless, Sox-2 transcripts were highly expressed in C17.2 cells, which might support the ability of these cells to generate several neural cell types.

4.5. Possible Mechanisms of VPA Action

4.5.1. Histone (H3) hyperacetylation

HDAC inhibition might up-regulate neurotrophic and/or differentiation genes. The mechanism by which VPA alters the expression of CDNF, MANF, GDNF, Nurr-1, and Egr-1 is not fully understood.

However, earlier experiments on 293 T, neuro 2A, and treatocarcinoma F9 cells have shown that VPA directly inhibits HDAC, causing hyperacetylation of histones in these cell lines (Gottlicher et al., 2001; Phiel et al., 2004). Histone acetylation is an important regulatory mechanism for controlling transcription in approximately 2% of transcribed genes (Van Lint et al., 1996; Phiel et al., 2001). Therefore, alterations in histone acetylation by VPA would represent a significant perturbation in cellular regulation.

Growing evidence indicates that VPA regulates genes involved in neuronal protection and differentiation through HDAC inhibition (histone hyperacetylation). VPA has been suggested to be responsible for the increased expression of neurotrophins like BDNF and GDNF, through an HDAC inhibitory mechanism.

In C6 glioma cells, for instance, VPA mediated the expression of neurotrophic factors BDNF and GDNF in association with increased HDAC mRNA after 24 and 48 hours (Castro et al., 2005). Also, VPA was shown to up-regulate GDNF and BDNF expression in astrocytes and to protect DA neurons, at least in part through HDAC inhibition, in rat mesencephalic neuron-glia cultures and primary cerebral cortical astrocytes (Wu et al., 2008). Moreover, VPA has been reported to induce adult hippocampal neuronal progenitors to differentiate into neurons and induce proneuronal factors associated with histone H3 and H4 hyperacetylation (Hsieh et al., 2004; Yu et al., 2009).

Histone H3 acetylation was examined in this study to assess the involvement of HDAC inhibition as a possible mechanism modulating gene expression in C17.2 cells. The preliminary results from this study supports previous studies and shows that 1mM VPA increases histone H3 in C17.2 cells following 24-, 48-, and 72- hour VPA treatments.

4.5.2. Additional mechanisms and pathways implicated in the neuroprotection and differentiation effects of VPA

VPA has been shown to promote neuronal differentiation through the activation of ERK-1/2 pathways *in vivo* and *in vitro* (refer back to section 1.5.4). To investigate whether the ERK pathway is activated in C17.2 cells by VPA, PD 98059 was used as an inhibitor of the ERK-1/2 pathway.

Preliminary results showed that PD 98059 failed to block CDNF and MANF protein induction by VPA in C17.2 cells. Nevertheless, PD 98059 by itself increased CDNF protein levels compared to cells treated with VPA only. These preliminary findings do not support ERK/MAPK activation as the mechanism underlying CDNF and /or MANF induction by VPA, but are supportive of HDAC inhibition as a key regulatory mechanism involved in altering neurotrophin gene expression in C17.2 cells. VPA has been reported to activate PI3K/PKB pathways in several cell lines. For instance, VPA was shown to activate the PI3/PKB pathway to exert a neuroprotective action in cerebellar granule cells (Mora et al., 2002). In rat cerebral cortical neurons, the PI3-kinase/AKT pathway activity was shown to be required for the induction of the neuroprotective and anti-inflammatory protein HSP70 by VPA. In addition, long-term VPA treatment regulates the PI3K/PKB pathway effector AP-1 (Marte & Downward, 1997), which induces cell differentiation when activated.

VPA might regulate neurotrophic/differentiation factors in the C17.2 cell line, possibly via PI3/AKT activation. Future studies tackling PI3-kinase/AKT are needed to support the involvement of this pathway in the neuroprotective action of VPA.

Conclusion

Neurotrophic factors are potent agents, ameliorating neuronal degeneration in many models of neurological disease. Therefore, administering these factors could represent an alternative strategy for the treatment of acute and chronic brain disorders. The delivery of neurotrophic factors to the brain remains a primary challenge in the development of effective therapies for neurodegenerative disorders. Transplantation of NSCs has emerged as a promising approach to serve as a source of secreted neuroprotective factors. A better understanding of the mechanisms governing the expression of neurotrophic factors and differentiation genes in stem cells is essential. In the current study, VPA has been shown to increase mRNA and protein expression of the novel neurotrophic factors CDNF, MANF, and GDNF. VPA has also been shown to induce mRNA expression of the differentiation markers Egr-1 and Nurr-1. The induction of these genes was seen at clinical and supra-clinical concentrations of VPA. These findings suggest that CDNF, MANF, GDNF, Egr-1, and Nurr-1 are pharmacological targets for VPA.

The exact pathways through which VPA induces the expression of the above genes remain to be elucidated, but preliminary results may rule out the involvement of the ERK1/2 pathway for CDNF and MANF. VPA might be useful to enhance NSC differentiation and neurotrophin expression, with applications in cell-based replacement therapies for neurodegenerative disorders.

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