EFFECT OF VPA ON MELATONIN RECEPTORS, BDNF AND GDNF IN VIVO

EFFECTS OF VALPROIC ACID ON EXPRESSION OF THE MELATONIN RECEPTORS MT_1 AND MT_2, AND THE NEUROTROPHIC FACTORS BDNF AND GDNF IN VIVO

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TITLE: Effects of Valproic Acid on expression of the melatonin receptors MT_1 and MT_2, and the neurotrophic factors BDNF and GDNF *in vivo*

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Abstract:

Valproic acid (VPA) is clinically utilized as an anti-convulsant and mood stabilizer, though its mechanism of action has not been fully elucidated. Evidence suggests an interaction between VPA and the melatonergic system as VPA up-regulated the melatonin MT_1 receptor subtype in rat C6 glioma cells. To determine if the observed effects can translate to an *in vivo* model, we investigated the effects of chronic VPA administration in a rat model on the expression of MT_1 and MT_2 receptors in the hippocampus. We also investigated the effect of chronic VPA treatment on the expression of the neurotrophic factors BDNF and GDNF in the rat hippocampus and striatum.

(1) Animals were separated into two groups with the experimental group receiving VPA (4 mg/mL) for 17 days, and the control receiving vehicle. The hippocampus was dissected and MT_1, MT_2, BDNF and GDNF mRNA were analyzed with RT-PCR. (2) Animals were separated into three groups with the first group receiving VPA (4 mg/mL), the second receiving VPA (3 mg/mL) for 16 days, and the control receiving vehicle. MT_2 mRNA in the hippocampal subregions were analyzed with in situ hybridization.

VPA induced the expression of MT_1 and MT_2 mRNA in the hippocampus in the experimental group compared to the control group. VPA also increased MT_2 mRNA expression in the subregions of the hippocampus. Additionally, BDNF and GDNF mRNA expression were increased in the VPA treatment group.

These findings raise the interesting question of whether the diverse clinical effects of VPA involve an interaction with the melatonergic system.

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List of Abbreviations and Symbols

^{125}I-melaontin	^{125}-iodomelatonin
14C	Carbon-14
4P-PDOT	4-phenyl-2-propironamidotertralin
6-OHDA	6-hydroxydopamine
AANAT	Arylalkylamine-N-Acetyltransferase
AD	Alzheimer's Disease
AP-1	Activator protein 1
BCL-2	B-cell lymphoma 2
BDNF	Brain-derived Neurotrophic Factor
BrdU	5-bromo-2-deoxyuridine
CA1	Cornu Ammonis 1
CA2	Cornu Ammonis 2
CA3	Cornu Ammonis 3
cAMP	Cyclic adenosine 3', 5'-monophosphate
CBP	CREB binding protein
cDNA	Complementary DNA
CGMP	Cyclic guanine monophosphate
CHAP31	Cyclic Hydroxamic-Acid-Containing Peptide 31
CNS	Central Nervous System
CREB	cAMP response element-binding protein
CSF	Cerebrospinal fluid
CYP	Cytochrome P450
DAG	Diacylglycerol
DCX	Doublecortin
DG	Dentate Gyrus
ELISA	Enzyme-linked immune sorbent assay
ERK1/2	Extracellular signal-regulated kinase
GABA	Gamma-Aminobutyric Acid
GAD67	Glutamate Decarboxylase 67
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDNF	Glial-derived Neurotrophic Factor
GPCRs	G-protein coupled receptors
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
hsp70	Heat-Shock Protein 70
IP3	Inositol triphosphate
MAPK	Mitogen-activated protein kinase
MARCKS	Myristoylated alanine-rich C kinase substrate
MPTP	1-methyl-4-phenyl-1,2,3,6-tetra-hydropyridine
mRNA	Messenger RNA
MT1	Melatonin receptor subtype 1
MT2	Melatonin receptor subtype 2
NaB	Sodium butyrate
NE	Norepinephrine
NF-L	Light neurofilament subunit
NMDA	N-methy-D-aspartate
Nurr1	Nuclear receptor related -1 protein
OD	Optical density
PKA	Protein Kinase A
PKC	Protein kinase C
PTZ	Phentylenetetrazol
RSK	Ribosomal S6 kinase
RT-PCR	Reverse transcription polymerase chain reaction
SAHA	Suberoylanilide Hydroxamic Acid
SCN	Suprachiasmatic Nucleus
SGZ	Subgranular zone
TH	Tyrosine hydroxylase
TrkB	Tyrosine-related kinase B
TSA	Trichostatin A
VPA	Valproic Acid

Declaration of Academic Achievement

My supervisor Dr. Len Niles designed the experiments and performed the animal sacrifices. I performed and analyzed all the experiments presented in the RT-PCR section. Rachel Kang, our lab technician and Sarra Bahna, an undergraduate student in the lab helped with animal care and the PCR procedure. In the in situ hybridization section, I obtained the brain slices, provided cDNA for the probe and performed the final analysis. Dr. Foster's lab performed the in situ hybridization procedure.

1. Introduction

1.1 VPA: Profile

Valproic acid (N-dipropylacetic acid, or 2-propylpentanoic acid) is one of the mainstays of therapy for epilepsy and bipolar mood disorders, due to its anticonvulsant and mood-stabilizing effects (Blaheta & Cinatl, 2002). It is a branched short-chain fatty acid with a half-life of 9 to 16 hours. Clinically, VPA is usually administered as uncoated tablets, but may also be administered in the form of syrup, capsules and enteric-coated tablets. Ninety percent of VPA in the blood is bound to albumin (Cramer & Mattson, 1979), and despite its hydrophilic nature enters the CNS by crossing the blood brain barrier via passive diffusion and bidirectional carrier-mediated transport, such as an anion exchanger at the brain capillary endothelium (Perucca, 2002). VPA crosses into the brain parenchyma utilizing another set of transporters which results in higher neuronal and glial concentrations than interstitial fluid concentrations (Perucca, 2002). VPA, in addition to being an effective anticonvulsant and mood-stabilizing agent has been shown to be an effective anxiolytic (Lal et al., 1980), antidystonic (Fredow & Loscher, 1991), and antinociceptive (Loscher & Vetter, 1985) in animal studies. Clinically, VPA is effective in clinical depression (Delucchi & Calabrese, 1989), absence seizures (Coppola et al., 2004; Erenberg et al., 1982), tonic-clonic seizures, complex partial seizures (Dean & Penry, 1988; Egan et al., 2003), and juvenile myoclonic epilepsy (Calleja et al., 2001). VPA is metabolised in the liver via microsomal glucuronide conjugation, mitochondrial β-oxidation and cytochrome P450 (CYP)-dependent oxidation, specifically CYP2C9,

CYP2A6, CYP2B6 and CYP2C19 (Davis et al., 1994; Perucca, 2002; Zaccara et al., 1988). A small amount of VPA is excreted in the urine without undergoing metabolism.

1.2 VPA: Anticonvulsant Effects

The molecular mechanisms behind the anticonvulsant effect of VPA are yet to be fully elucidated however it is thought that due to the wide spectrum of anticonvulsant effects, a variety of molecular mechanisms may be involved. Most importantly is the modulation of GABAergic activity, as potentiation of the inhibitory activity of GABA (gamma-Aminobutyric acid) results in potent anticonvulsant effects (Loscher, 1993). VPA enhances central GABAergic activity via the inhibition of GABA transaminase, an enzyme involved in the degradation of GABA (Chateauvieux et al., 2010; Loscher, 1993). VPA also increases GABA synthesis, decreases turnover (Mesdjian et al., 1982), and induces expression of the rate limiting enzyme in the formation of GABA, GAD67 (glutamate decarboxylase), in GABAergic interneurons *in vitro* (Dong et al., 2005). VPA exerts inhibitory effects on several excitable membranes such as blocking voltage-dependent sodium channels, calcium channels, and potassium voltage channels and it also inhibits the release of β-hydroxybutyric acid, an excitatory amino acid (Gean et al., 1994; McLean & Macdonald, 1986). VPA protects rat cerebral cortical and cerebellar granule cells from glutamate-related excitotoxicity (Kanai et al., 2004) by attenuating neuronal excitation mediated by activation of N-methyl-D-aspartate (NMDA) glutamate receptors (Gean et al., 1994).

1.3 VPA: Anti-Bipolar Effects

The modulation of GABAergic activity is not thought to play a role in VPA's anti-manic effects as other anti-convulsants such as gabapentin, topiramate, and phenytoin do not demonstrate anti-manic effects (Coyle & Duman, 2003). Additionally, although the anticonvulsant effects of VPA are apparent after acute treatment, the anti-manic effects are only witnessed after chronic administration with a delayed onset of therapeutic response in the clinical setting of days to weeks (G. Chen et al., 1997). This suggests that VPA exerts these effects through the regulation of intracellular signalling pathways that regulate gene expression. It was demonstrated that chronic treatment with VPA decreased the brain volume reductions in bipolar disorder (Drevets, 2000). Imaging studies in mood disorders have shown decreased hippocampal and frontal cortex volume in patients with recurrent mood disorder and post-traumatic stress disorder (Bremner, 1999; Sheline et al., 1999). Additionally, degeneration in the frontal cortex during major depression has also been reported (Rajkowska, 1997; Rajkowska et al., 1999). In preclinical studies, acute stress as well as chronic stress, decreases the expression of BDNF (brain-derived neurotrophic factor) in rat hippocampus (M. A. Smith et al., 1995). Therefore, it is thought that neural degeneration caused by neurotrophic down regulation may play a role in the pathophysiology of mood disorder, and therefore, neurotrophic factors may be involved in the therapeutic action of mood stabilizers (Bown et al., 2002). As such,

chronic treatment of VPA has been shown to increase BDNF in the rat brain (Fukumoto et al., 2001).

1.4 VPA and Neuroprotection

It is also thought that induction of BDNF may play a role in the neuroprotective effects of VPA. Valproic acid has been shown to induce neurogenesis and stimulate neurotrophic factor action. For instance, chronic administration of valproic acid was shown to increase BDNF and GDNF expression in astrocytes (P. S. Chen et al., 2006), which protects dopaminergic neurons in midbrain neuron-glia cultures (X. Wu et al., 2008). Additionally, BDNF induction by chronic treatment of VPA was exhibited in the rat hippocampus and frontal cortex (Fukumoto et al., 2001). BDNF is reported to support the survival of various neurons such as dopaminergic neurons and GABAergic neurons in the embryonic rat ventral mesencephalon, cholinergic neurons in embryonic rat septal cells, and serotonergic neurons in the rat frontoparietal cortex (Alderson et al., 1990; Hyman et al., 1991; Hyman et al., 1994; Mamounas et al., 1995). BDNF and its receptor trkB mRNA are widely distributed in the CNS, but expression is mainly localized in the hippocampus (Conner, Lauterborn, Yan, Gall, & Varon, 1997). GDNF is the most potent trophic factor for dopaminergic neurons (Kirik et al., 2004; A. D. Smith et al., 2005), and this may be the mechanism through which VPA protects dopaminergic neurons from neurotoxicity (Peng et al., 2005). VPA has also been shown to protect against the apoptotic death of endoplasmic reticulum stress proteins (Bown et al., 2002).

1.5 VPA: HDAC Inhibitor

HDACs are a family of individual proteins grouped into four subfamilies (Class 1, Class II, Class III, and Class IV) that are recruited into larger multi-protein complexes with transcriptional co-repressors (Alland et al., 1997; Laherty et al., 1997). Histone deacetylases are enzymes that are involved in the remodelling of chromatin and have a key role in the regulation of gene expression (Jenuwein & Allis, 2001). DNA forms nucleosomes by wrapping around a histone octamer formed by four histone partners (a H3-H4 tetramer and two H2A-H2B dimers), and the structure is stabilized by H1 by electrostatic neutralization of the linker DNA segments through a positively charged carboxy-terminal domain (Luger et al., 1997). Histone acetylation status at a given locus appears to play a large role in the regulation of gene expression by modulating the accessibility of transcription factors to target DNA segments (Berger, 2002; Jenuwein & Allis, 2001; Kouzarides, 2000). Acetylation at lysine residues at the N-terminal of histones is associated with transcriptional activation as acetylation causes chromatin to become decondensed (euchromatin), and it is thought that euchromatin allows for greater access for transcription factors to chromatin. Deacetylation is associated with transcriptional silencing (Peterson & Laniel, 2004) as deacetylation causes chromatin to become condensed (heterochromatin) which creates a barrier for transcription factors. Histone acetylation has been shown to be an important regulatory mechanism for controlling transcription in at least 2% of transcribed genes (Van Lint et al., 1996).

As an HDAC inhibitor, VPA binds to the zinc-containing catalytic domain of Class 1 (a, b) and Class II (a) HDACs, and both these classes are implicated in neuronal

function (Chateauvieux et al., 2010; Drummond et al., 2005). For instance, VPA has been shown to promote neurogenesis in the dentate gyrus of the hippocampus and could be considered as a potential drug for treating some neurodegenerative diseases (Chuang, 2005; Hao et al., 2004). Recent evidence also shows that HDAC inhibitors such as suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA) are effective for treating neurodegenerative disorders or enhancing synaptic plasticity (Hockly et al., 2003; Vecsey et al., 2007). Also, when cells were cultured in the presence of HDAC inhibitors such as VPA, TSA, or sodium butyrate (NaB), neural progenitors reduced their proliferation and largely differentiated into neurons. These findings were also confirmed *in vivo* in rats after chronic administration. VPA also up-regulates neuroD in neural progenitor cells suggesting that VPA could induce neuronal differentiation through the transcriptional activation of neurogenic transcription factors (Hsieh et al., 2004). Additionally, VPA has also shown to have potent neuroprotective properties. VPA enhanced functional recovery following an intra-cranial haemorrhage through inhibition of inflammation, apoptosis and the modification of cell survival pathways. Concomitantly, VPA treatment causes hyperacetylation of histone H3 and the heat-shock protein 70 (Hsp70) promoter, and upregulated Hsp70. This indicates that VPA may exert its neuroprotective effects through transcriptional activation of cytoprotective factors following HDAC inhibition (Sinn et al., 2007; Zhang et al., 2012).

VPA via HDAC inhibition has also been shown to have tumour suppressor properties. Indeed, it is thought that discord in HDAC activity and DNA methylation can

cause transcriptional silencing of tumour suppressor genes, which may be one of the main epigenetic flaws in cancer cells. In preclinical studies, VPA was shown to suppress the growth and increase the differentiation of many different tumour cell lines such as the teratocarcinoma F9 cell line and neuroblastoma BE(2)-C cell line (Cinatl et al., 1997; Cinatl et al., 2002). Additionally, several other HDAC inhibitors such as TSA, cyclic hydroxamic-acid-containing peptide 31 (CHAP31) have exhibited anti-tumour effects. These effects include preventing proliferation and inducing differentiation in several transformed cell lines such as erythroleukemia, acute myelogenous leukemia, and carcinomas of the skin, breast, prostate, bladder, lung, colon, and cervix (Finzer et al., 2001; H. Huang et al., 1999; Inokoshi et al., 1999; Y. B. Kim et al., 2000; Kitamura et al., 2000; Komatsu et al., 2001; Rashid et al., 2001; Saunders et al., 1999; Vigushin et al., 2001). These anti-tumour effects of HDAC inhibitors may be mediated by the transcriptional restoration of tumour suppressor genes which are essential for regulating differentiation, DNA repair, apoptosis, and the cell cycle.

1.6 Melatonin

Melatonin (N-acetyl-5-methoxytryptamine) is secreted during the dark hours at night from the pineal gland. As a naturally occurring hormone, it performs a wide array of activities in numerous types of cells (Bellon et al., 2007). It plays a role in the modulation of several biological functions such as neuroendocrine and immune function, control of seasonal reproduction, regulation of circadian rhythms and body temperature (Jimenez-Jorge et al., 2005; Moriya et al., 2007). Exogenous melatonin has been shown to target

cerebrovascular, reproductive, endocrine, and immune functions as well as tumour growth (Dubocovich et al., 2003). Melatonin has hypnotic action and has a role in sleep initiation as the trigger for opening the circadian sleep-gate (Krauchi & Wirz-Justice, 2001), inhibition of dopamine release from retina (Dubocovich, 1983; Dubocovich et al., 1997), vasoregulatory activity (Doolen et al., 1998; Ting et al., 1999), immunomodulatory roles, and effects on cell growth (Blask et al., 2002; Ram et al., 2002). It is controlled primarily by the hypothalamic suprachiasmatic nucleus (SCN) in the anterior ventral hypothalamus above the optic chiasm, and is the master clock that controls behavioural, metabolic, and physiological rhythms (Gillette & Mitchell, 2002) including the release of melatonin. The SCN regulates the release of melatonin through a polysynaptic network with descending fibers projecting through the medial forebrain bundle to the intermediolateral horns of the cervical spinal cord. The postganglionic sympathetic fibers then reach the pineal gland to regulate melatonin biosynthesis through the release of norepinephrine (NE) (Buijs et al., 1998). Endogenous melatonin is also thought to exert its effects by feeding back onto the master clock to regulate neuronal activity and circadian rhythms which in turn regulates many biological functions such as sleep and immune function (Dubocovich et al., 2003). In nocturnal animals, melatonin is associated with arousal and physical activity where administration of melatonin did not promote sleep and rest as it did in humans (Dollins et al., 1994; Huber et al., 1998; Mailliet et al., 2001). However, the SCN has similar functions in both nocturnal and

diurnal animals, suggesting that the differential effect of melatonin in these species must be downstream to the SCN.

Melatonin is produced from serotonin by the sequential action of the enzymes arylalkylamine-N-acetyltransferase (AANAT) and hydroxyindole-O-methyltransferase (Ganguly et al., 2002). Acetylation of serotonin by N-acetyltransferase produces N-acetylserotonin, which is then methylated by hydroxyindole-O-methytransferase to form melatonin (N-acetyl-5-methoxytryptamine) (Bernard et al., 1999). Melatonin is then released into the capillaries and into the cerebrospinal fluid where higher concentrations occur (Tricoire et al., 2003). Melatonin is removed from circulation as a result of 6-hydroxylation in the liver by cytochrome P450 monooxygenases, followed by excretion in a sulfatoxy-conjugated form. The half-life of melatonin in plasma is about 10 minutes due to the rapid hydroxylation to 6-sulfatoxymelatonin in the liver (von Gall et al., 2002).

Melatonin is released during the hours of darkness or subjective night and has important regulatory effects on light-dark entrainment of biological and physiological responses (Borjigin et al., 1999; Reiter, 1991; Reiter, 1993). Melatonin has been postulated to induce morphological and transcriptional changes that suggest a neuronal phenotype in C17.2 mouse neural stem cells (Sharma et al., 2008). This is further supported by studies that have demonstrated that melatonin influences cell growth and differentiation of neural blastoma and PC12 cells (Roth et al., 2001; Song & Lahiri, 1997). In mouse neural stem cells, melatonin has been shown to promote neuritogenesis and to have neurotrophic effects (Sharma et al., 2008). Specifically, melatonin can induce

GDNF (glial cell-line derived neurotrophic factor) expression (Niles et al., 2004). Melatonin at pharmacological doses has been shown to be neuroprotective in 6-hydroxydopamine (6-OHDA) and MPTP (1-methyl-4-phenyl-1,2,3,6-tetra-hydropyridine) models of Parkinson's disease (Joo et al., 1998; B. Thomas & Mohanakumar, 2004). Melatonin exerts its various effects through several mechanisms such as binding to intracellular proteins such as calmodulin, binding to nuclear receptors of the orphan family and binding to plasma membrane localized melatonin receptors (Ekmekcioglu, 2006). It is thought that melatonin's mechanism of action in inducing neuronal differentiation is through its interaction with two high affinity melatonin receptors, MT1 and MT2 (Dubocovich et al., 2003; Reppert et al., 1996; Sanchez-Hidalgo et al., 2007; Schuster et al., 2005). Melatonin is also thought to act through these receptors in regulating the main functions of the SCN, namely sleep promotion and phase shifting of sleep onset (Dubocovich et al., 2003).

1.7 Melatonin Receptors: MT_1 and MT_2

Melatonin induces multiple physiological effects via high-affinity G protein-coupled MT_1 and MT_2 receptors (GPCRs) with seven transmembrane-spanning domains (Liu et al., 1997). GPCRS are controllers of physiological processes such as neurotransmission, cellular metabolism, secretion, and cell differentiation and growth (Bockaert & Pin, 1999). MT_1 and MT_2 receptors are 350 and 362 amino acids long, respectively with calculated molecular weights of 39-40 kDa (Rivera-Bermudez et al., 2004; Song & Lahiri, 1997). The receptors have potential glycosylation sites in their N-

terminus, as well as protein kinase C (PKC), casein kinase 1 and 2, and protein kinase A (PKA) phosphorylation sites which may participate in the regulation of receptor function as demonstrated for other GPCRs (Ferguson, 2001). Both receptors are expressed in very low density even in tissues most sensitive to melatonin, however, this low density is most likely due to the high affinity of melatonin for its endogenous receptors. The human MT_2 receptor has a lower affinity than the MT_1 receptor, but both receptors have generally similar binding characteristics (Dubocovich et al., 2005). The pattern of MT_1 and MT_2 expression appears to be cell-type specific and species dependent. For instance, MT_1 receptors have been localized in the SCN in mice (Dubocovich & Markowska, 2005; Liu et al., 1997), human cerebellum (Al-Ghoul et al., 1998), human hippocampus (Savaskan et al., 2002), central dopaminergic pathways (substantia nigra, ventral tegmental area, nucleus accumbens, caudate-putamen) in humans (Uz et al., 2005), rat ovary (Clemens et al., 2001), hamster testis (Frungieri et al., 2005), human retina coronary blood vessels and aorta (Scher et al., 2002; Ekmekcioglu et al., 2001a; Ekmekcioglu et al., 2001b), mouse liver and kidney (Naji et al., 2004), human gallbladder(Aust et al., 2004), human skin (Slominski et al., 2003)and the human immune system (Pozo et al., 2004). As well, the developing human brain primarily expresses MT_1 with few or no MT_2 receptors (L. Thomas et al., 2002). However, MT_2 is expressed predominantly in the brain with more restrictive expression. It has been co-localized with MT_1 receptors in human cerebellar granule cells (Al-Ghoul et al., 1998), human retina (Reppert et al., 1995) and human hippocampus (Savaskan et al., 2002; Savaskan et al., 2005).

1.8 Melatonin Receptors: Signalling Cascade

The main functions of the melatonin receptors are inhibition of neuronal firing and phase-shifting circadian rhythmic activity of neurons in the suprachiasmatic muclei (Liu et al., 1997; Reppert et al., 1994; Reppert, 1997). Wan et al. (1999) showed that since $GABA_A$ receptor function is critical for neuronal inhibition, activation of the melatonin MT_1 receptors upregulated $GABA_A$ receptor function. However, in the hippocampus, melatonin inhibited $GABA_A$ receptor function via MT_2 (Wan et al., 1999). Thus melatonin can potentiate opposite effects through its receptors.

MT_1 and MT_2 signal by coupling to Gαi and Gαq receptors. Activation of these receptors promotes dissociation of G proteins to alpha and beta-gamma dimers, which interact with various effector molecules involved in the transmission of cell signaling (Gilman, 1995), such as the small G protein Ras (Wood et al., 1992). The conformational change in Ras allows it to transmit the signal through the recruitment and activation of Raf kinases. Raf activation takes place through the binding of Ras and phosphorylation of Raf. Raf in turn binds to and phosphorylates two serine residues at positions 217 and 221 found in the activation loop of the downstream dual specificity kinases, MEK1 and MEK2 (Alessi et al., 1994). MEK1/2 are 45kDa kinases which share approximately 80% sequence identity with each other (Brott et al., 1993), and which phosphorylate ERK1/2 within a conserved Thr-Glu-Tyr motif in their activation loop (Krishna & Narang, 2008). Once activated, ERK translocates from the cytoplasm into the nucleus where it phosphorylates various target molecules such as nuclear substrates, cytoskeletal proteins and transcription factors (Yoon & Seger, 2006). The ERK (extracellular signal regulated

kinase) 1/2 pathway is known to induce transcription factors and has been shown to be important in the induction of differentiation, proliferation and cell survival in neuronal cell cultures (Lewis et al., 1998). For instance, one study showed that a deficiency in ERK1 affected adipocyte differentiation and proliferation (Bost et al., 2002). As well, inhibition of ERK 1/2 activation through the blocking of its upstream regulator, MEK1, was shown to prevent neuronal differentiation in embryonic stem cell cultures (Krishna & Narang, 2008). Interestingly, cells expressing both MT_1 and MT_2 receptors, showed inhibition of the phosphorylation of the MAPK/ERK cascade (P. Cui et al., 2008), while cells expressing only MT_1 receptors showed that melatonin increased the phosphorylation of ERK (Bordt et al., 2001; A. S. Chan et al., 2002). The differential effect of the MT_2 receptor may be explained as the possible modulation of the melatonin signal by the MT_1 homodimeric receptor configuration and the MT_1/MT_2 dimerized receptor configuration (Imbesi et al., 2008).

MT_1 receptor activation leads to inhibitory effects on the forskolin-stimulated cAMP signal transduction cascade (Niles & Hashemi, 1990) which results in a reduction of protein kinase A activity (Morgan et al., 1994) and nuclear factor CREB (cAMP responsive element binding protein) phosphorylation (McNulty et al., 1994). MT_1 also stimulates c-Jun n-terminal kinase activity via both pertussis toxin-sensitive (Gi) and insensitive (Gs, Gz, and G16) proteins (A. S. Chan et al., 2002). Activation of MT_1 also causes inositol triphosphate accumulation (Brydon et al., 1999; Roka et al., 1999) which increases intracellular calcium and in turn may lead to activation of PKC-alpha (Gilad et

al., 1997). SCN multiunit activity is also inhibited through MT_1 receptors (Liu et al., 1997) and is more noticeable during daytime when SCN neuronal activity is high , via activation of the inward rectifier potassium channel KIR3 (Jiang et al., 1995).

The MT_2 receptor couples to several signal transduction pathways such as phosphoinositide production, inhibition of adenylyl cyclase and the inhibition of soluble guanylyl cyclase pathway (Boutin et al., 2005). Similar to MT_1 it too has been shown to inhibit forskolin-stimulated cAMP formation and cGMP accumulation (Petit et al., 1999; Reppert et al., 1995). Also it has been shown that the phase shifting abilities in the SCN are modulated by the MT_2 receptors through an increase in PKC activity (Hunt et al., 2001; McArthur et al., 1997).

1.9 Melatonin Receptors: Regulation

It has been reported that melatonin receptors undergo internal regulation (Gauer et al., 1993; Tenn & Niles, 1993). Melatonin binding and receptor mRNA levels have been shown to vary on a circadian basis, with light and plasma concentration of melatonin affecting receptor expression levels. Light exposure during the night increases ^{125}I-melatonin binding, but also suppressed melatonin synthesis (Guerrero et al., 1999; Masson-Pevet et al., 2000). Studies have also shown an inverse relationship between serum melatonin levels and receptor density (Gauer et al., 1993; Tenn & Niles, 1993). This indicates that melatonin down regulates some of its receptor population, but it is also capable of positively regulating its receptors (Gilad et al., 1997; Masson-Pevet et al., 2000; Recio et al., 1996; Schuster et al., 2000; Schuster et al., 2001). For instance,

melatonin activates the MAPK/ERK pathway and inhibits the formation of cAMP, both of which are implicated in the negative regulation of melatonin receptors. The activation of the cAMP second messenger pathway by forskolin was shown in a primary culture of pars tuberalis cells to increase MT_1 mRNA, however this is blocked by melatonin which acting through the melatonin receptors inhibits the cAMP pathway(Barrett et al., 1996). Additionally, Castro et al (2005) suggested that the MAPK-ERK pathway may be involved in the negative regulation of MT_1 mRNA expression as the MAPK-ERK inhibitor PD98059 was shown to not block the induction of MT_1 mRNA by VPA, but rather enhanced the inducing effect of VPA on MT1 mRNA expression. Another major regulatory process is desensitization, where receptor responsiveness wanes following persistent agonist challenge and is characterized by uncoupling of receptor and G protein, receptor internalization and receptor down regulation (Ferguson, 2001). For instance, MT_1 receptors in the ovine pars tuberalis desensitize following long exposure (>5 hr) to melatonin (1 microM) (Hazlerigg et al., 1993).

This receptor regulation is important as it is required to maintain cellular homeostasis through the regulation of associated signal transduction pathways. Also ageing and Alzheimer`s disease have been associated with decreased MT_1 receptor expression in human SCN and cortex (Brunner et al., 2006; Laudon et al., 1988), and keeping with that, evidence has shown decreased binding in the hypothalamus of aged rats by 2-[^{125}I]Iodomelatonin, a radioiodinated ligand that binds with high affinity to melatonin receptor sites (Laudon et al., 1988; Oaknin-Bendahan et al., 1995).

1.10 MT_1/MT_2 Neuroprotection

It is thought that the induction of expression of neurotrophic factors such as glial cell-line derived neurotrophic factor (GDNF) in C6 glioma cells and in rat neural stem cells may be through the activation of MT_1 receptors by melatonin (Armstrong & Niles, 2002; Niles et al., 2004). As well, melatonin also increases the expression of dopaminergic specific markers such as tyrosine hydroxylase (TH) (McMillan et al., 2007) , a rate-limiting enzyme involved in the synthesis of dopamine, in SH-SY5Y cells (Presgraves et al., 2004), and β-III-tubulin and nurr1 in C17.2 mouse neural stem cells possibly through MT_1 receptor mediated activation of the ERK cascade (Sharma et al., 2008). Additionally, nurr1 has been shown to regulate the expression of several other dopaminergic markers including TH (Jankovic et al., 2005). Nurr1 might also regulate neurotrophic factors such as GDNF and BDNF by acting as a transcription regulator (Jankovic et al., 2005). Thus, this suggests that melatonin receptors may play a direct and indirect role in the modulation of dopaminergic markers and/or neurotrophic factors.

1.11 VPA and Melatonin Receptors

Novel evidence from our group suggests an interaction between valproic acid treatment and expression of mammalian G-protein coupled melatonin receptors in rat C6 glioma cells (Castro et al., 2005). The melatonin MT_1 receptor subtype was upregulated following treatment with valproic acid at doses of 3mM and 5mM after 24 hour and 48 hour treatment, as shown by increases in mRNA and protein expression (Castro et al., 2005).

Further investigation revealed significant time-dependent increases in MT_1 mRNA expression at lower but clinically relevant concentrations (0.5mM or 1mM) of valproic acid (B. Kim et al., 2008).

It has been shown that VPA activates the MAPK-ERK pathway (Yuan et al., 2001) but this pathway seems to suppress MT_1 mRNA expression (Castro et al., 2005). However, the significant induction despite the activation of the inhibitory pathway suggests that another pathway is involved in the upregulation of melatonin receptors by VPA. It is likely that epigenetic regulation of gene transcription by VPA (Phiel et al., 2001) is involved in its upregulation of melatonin receptor expression, as we have observed a concentration-dependent induction of melatonin MT_1 receptor mRNA in C6 cells in response to a structurally distinct HDAC inhibitor, TSA (B. Kim et al., 2008).

1.12 Hypothesis

Therefore, in light of the *in vitro* evidence, it is hypothesized that VPA upregulates the expression of the mammalian G-protein coupled melatonin receptors, MT_1 and MT_2 *in vivo.* VPA should also induce *in vivo* expression of BDNF and GDNF, as observed *in vitro*.

1.13 Objectives

a) To investigate the effects of chronic VPA administration on MT_1 and MT_2 receptor mRNA expression in rat brain.
b) To investigate the effects of chronic VPA administration on BDNF mRNA expression in rat brain.

c) To investigate the effects of chronic VPA administration on GDNF mRNA expression in rat brain.

2. Materials and Methods

2.1 RT-PCR

2.11 Animals

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

2.12 VPA Treatment

Subjects were randomly divided into 2 groups: treatment group receiving VPA via drinking water (4 mg/mL drinking water; n=4) and control group receiving vehicle (water; n=4) for 17 days.

2.13 Brain Section Collection

All animals were decapitated following 17 days of treatment via drinking water between the hours of 11 am and 3 pm. Frontal cortex, cerebellum, striatum, and hippocampus were dissected rapidly on ice from 6 animals (3 treatment and 3 control), and stored in RNALater at 4°C until used for RNA isolation, cDNA synthesis and PCR amplification.

2.14 RT-PCR Detection

Total RNA was isolated from homogenized tissue with TRIzol as described by the supplier (Invitrogen Canada Inc., Burlington, ON). DNAse treatment was performed with approximately 10 µg of RNA, and cDNA was synthesized from 2.2 µL of RNA using the Omniscript reverse transcriptase kit (Qiagen Inc., Mississauga, Ontario, Canada) and oligo dT primers. Changes in MT_1 and MT_2 mRNA expression following VPA treatment were assessed using 10 µL of the RT product along with the primers for MT_1 or MT_2 (Table 1) at the following PCR parameters; 94°C for 30s, 57°C for 30s and 72°C for 1min for 40 cycles followed by a final incubation at 72°C for 10min. Changes in BDNF and GDNF mRNA expression following VPA treatment were assessed by amplifying 2µL of the RT with the appropriate primers (Table 1) for 30 cycles as follows: 94°C for 30s, 55°C for 30s, 72°C for 1 min, followed by a final incubation at 72°C for 10min.

Gene	**Primers (5' → 3')**	**Nucleotides**	**Size (bp)**
MT_1	TTG TGG CGA GTT TAG CTG TG	184-203	472
	TTT ACC CTC CGT CTG ACC TG	655-636	
MT_2	TAC ATC AGC CTC ATC TGG CTT	64-84	297
	CAC AAA CAC TGC GAA CAT GGT	340-360	
GDNF	ATG GGA TGT CGT GGC TGT CTG	58–98	643
	TCT CTG GAG CCA GGG TCA GAT	700–680	
BDNF	GGA TGA GGA CCA GAA GGT TGC	2342–2362	390
	TTG TCT ATG CCC CTG CAG CCT	2711–2731	
GAPDH	TTC ACC ACC ATG GAG AAG GC	1147–1166	237
	GGC ATG GAC TGT GGT CAT GA	1383–1364	

Table 1 - PCR Primers

2.15 Data Analysis

Amplified cDNA bands were run on 1.5% agarose gels stained with ethidium bromide, visualized under UV light and digitally scanned using an AlphaImager 2200 system (Alpha Innotech Corp.). Optical density (OD) ratios of each target gene over the internal control (GAPDH) were obtained for semi-quantitative analysis.

2.2 In Situ Hybridization

2.21 Animals

Adult male Sprague-Dawley rats (150-200 g) were housed in a 12-h light-dark cycle, with lights on at 7 am. Animals had free access to a standard diet and water. All experiments were carried out according to the guidelines set by the Canadian Council for Animal Care and approved by the McMaster University Animal Research Ethics Board (AREB)

2.22 VPA Treatment

Subjects were randomly divided into 3 groups: treatment group receiving VPA via drinking water (3 mg/mL drinking water; n=12), treatment group receiving VPA drinking water (4 mg/mL drinking water; n=12) and control group receiving vehicle (water; n=12) for 16 days.

2.23 Brain Section Collection

All animals were decapitated following 16 days of treatment via drinking water between the hours of 3 and 6 pm in the afternoon on two consecutive days. Brains were removed and rapidly frozen by immersion in 2-methylbutane which was chilled on dry ice (-60°C), and stored at −80 °C. Cryostat-cut 10-µm-thick coronal sections were thaw-mounted onto gelatin-coated slides, dried, and stored at −35°C. Levels collected included the hippocampus (Bregma -3.14mm). (Paxinos and Watson, 1998)

2.24 Riboprobe

The riboprobe for MT_2 was generated in our laboratory (See Table 1 for primer sequence and the product size obtained). Complimentary DNA was generated through polymerase chain reaction and ligated into the pGEM T-easy expression vector (Promega, Mississauga, ON, Canada). Target sequences were confirmed by DNA sequencing (Mobix Lab, McMaster University). The resultant sequence was then blasted using the NCBI Blast tool to ensure specificity of the riboprobe for the gene of interest and to rule out the potential for cross-reactivity. In addition, no hybridization signal was detected for sense probes (Amath et al., 2012).

2.25 In Situ Hybridization

In situ hybridization procedures were performed using ribonucleotide (cRNA) probes. Tissue sections were fixed with 4% formaldehyde, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine–HCl, pH 8.0, dehydrated, and delipidated with chloroform. Radiolabeled probes were diluted in a hybridization buffer and applied

to brain sections (approximately 500,000 CPM/section). Slides were incubated overnight at 55 °C in a humidified chamber. To reduce nonspecific binding of the probe, slides were washed in 20 μg/ml RNase solution for 30 min at room temperature, followed by 1 h each in 2× SSC at 50 °C, 0.2× SSC at 55 and 60 °C. Slides were dehydrated and air-dried for autoradiography (Foster et al., 2002).

2.26 Autoradiography

Slides and ^{14}C plastic standards containing known amounts of radioactivity (American Radiochemicals, St. Louis, MO) were placed in X-ray cassettes, apposed to film (BioMax MR; Eastman Kodak, Rochester, NY) for 1 week, and developed in an automatic film developer (X-OMAT; Eastman Kodak) (Foster et al., 2002).

2.27 Data analysis

Autoradiographic film images of brain sections and standards were digitized with a solid-state camera (Qiacam, Quorum Technologies) and a Macintosh computer-based image analysis system with NIH Image software (Wayne Rasband, National Institute of Mental Health). Light transmittance through the film was measured by outlining the structure on the monitor. Transmittance was converted to radioactivity levels using the Rodbard curve applied to the standards (Foster et al., 2002).

2.3 Statistical Analysis

Following conversion to percentage values, PCR data were analyzed by an unpaired Student's t-test, with $p<0.05$ as the level of significance. In situ hybridization data were analyzed by ANOVA, with $p<0.05$ as the level of significance. Data are shown as means ± SEM.

3.0 Results

3.1 Effects of VPA on MT_1 mRNA expression in the hippocampus

The effects of VPA on MT1 receptor expression was evaluated in vitro in C6 rat glioma cells and shown to upregulate MT1 receptor expression following acute treatment with VPA(Castro et al., 2005). However, there has yet to be evidence showing this relationship in vivo with chronic administration of VPA at clinically therapeutic doses. To investigate the effect of VPA on the expression of MT_1 receptor mRNA in the hippocampus, 7 week old rats were treated with 0.4% VPA via drinking water. Following a 17 day treatment period, rats were decapitated and the hippocampus was dissected for RT-PCR analysis (n=2). Following RT-PCR analysis, MT_1 mRNA levels were converted to optical density (OD) values and normalized to their respective GAPDH OD levels as reported previously (Armstrong & Niles, 2002). After conversion of MT_1 /GAPDH OD ratios to percentage values, a t-test showed a significant increase in MT1 mRNA expression following VPA treatment ($t(4) = 3.285$, $p<0.03$) (Figure 1).

3.2 Effects of VPA on MT2 mRNA expression in the hippocampus

To evaluate the effect of VPA on the expression of MT_2 receptor mRNA in the hippocampus, 7 week old rats were treated with 0.4% VPA via drinking water. Following a 17 day treatment period, rats were decapitated and the hippocampus was dissected for RT-PCR analysis (n=3). Following RT-PCR analysis, MT_2 mRNA levels were converted to OD values and normalized to their respective GAPDH OD levels as reported previously (Armstrong & Niles, 2002). After conversion of MT_2 /GAPDH OD ratios to

percentage values, statistical analysis revealed a significantly higher expression of MT_2 receptor mRNA in the hippocampus of VPA treated animals compared with controls ($t(4) = 4.932$, $p<0.007$) (Figure 2).

3.3 Effects of VPA on MT2 mRNA expression in subregional hippocampal areas.

To evaluate and localize the effect of VPA on the expression of MT_2 receptor mRNA in the hippocampus and its subregions, 4 week old rats were treated with 0.3% VPA, 0.4% VPA via drinking water or water. Following a 16 day treatment period, rats were decapitated and whole brains were removed for in situ hybridization. Following autoradiographic analysis, MT_2 mRNA OD values were converted to DPM values. Subregion analysis of the CA1, CA2, CA3 and dentate gyrus revealed a significant difference in MT_2 receptor mRNA expression between treatment groups and the control group. Statistical analysis reveals a significant increase in MT_2 receptor mRNA expression in the CA1 ($n=12$) ($F(2,15)=4.66$, $p<0.02$), CA3 ($n=12$) ($F(2,15)=6.07$, $p<0.01$) ,and dentate gyrus ($n=12$) ($F(2,15)=6.15$, $p<0.01$) in both treatment groups as compared to the control (Figure 3, 5 , 6). Analysis of the CA2 region found a significant difference between treatment groups and the control group of MT_2 receptor mRNA expression ($n=12$) ($F(2,17)=9.73$, $p<0.001$) (Figure 4).

3.4 Effects of VPA on BDNF mRNA expression in the hippocampus and striatum

Recent evidence has shown that VPA upregulates BDNF in rat astrocytes (Chen et al., 2006) and C6 rat glioma cells (Castro et al., 2005). Also, chronic administration of VPA was shown to induce BDNF in the hippocampus and cerebral cortex of rats

(Fukumoto et al., 2001). However, there is no evidence citing the induction of BDNF in the rat striatum following chronic VPA administration. To evaluate the effect of chronic administration of VPA via drinking water on the expression of BDNF mRNA in the hippocampus and striatum *in vivo,* 7 week old rats were treated with 0.4% VPA via drinking water. Following a 17 day treatment period, rats were decapitated and the hippocampus and striatum were dissected for RT-PCR analysis. Following RT-PCR analysis, BDNF mRNA levels were converted to OD values and normalized to their respective GAPDH OD levels as reported previously (Armstrong & Niles, 2002). After conversion of BDNF /GAPDH OD ratios to percentage values, statistical analysis showed a significantly higher expression of BDNF mRNA in the hippocampus (n=2) ($t(2) = 12.59$, $p<0.006$) and striatum (n=3) ($t(4) = 3.513$, $p<0.01$) of VPA treated animals compared with controls (Figures 7 and 8).

3.5 Effects of VPA on GDNF mRNA expression in the hippocampus and striatum

Castro et al. (2005) demonstrated the induction of GDNF mRNA in C6 rat glioma cells following clinically relevant doses of VPA. GDNF induction following acute treatment of VPA at clinically relevant doses was also shown in rat astroglia (P. S. Chen et al., 2006). Fukumoto et al., (2001) assessed the levels of GDNF *in vivo* following chronic administration of VPA and found no significant change. However, ELISA assays were used to analyze brain tissue homogenates which may not have been sensitive enough to detect cell-specific changes in GDNF expression. Therefore, to evaluate the effect of VPA on the expression of GDNF mRNA in the hippocampus and striatum *in*

vivo, 7 week old rats were treated with 0.4% VPA via drinking water. Following a 17 day treatment period, rats were decapitated and the hippocampus and striatum were dissected for RT-PCR analysis. Following RT-PCR analysis, GDNF mRNA levels were converted to OD values and normalized to their respective GAPDH OD levels as reported previously (Armstrong & Niles, 2002). After conversion of GDNF /GAPDH OD ratios to percentage values, statistical analysis showed a significantly higher expression of GDNF mRNA in the hippocampus (n=3) ($t(4) = 5.404$, $p<0.005$) and striatum (n=2) ($t(2) = 11.32$, $p<0.007$) of VPA treated animals compared with controls (Figures 9 and 10).

Experimental Group	Weight (g)	Average Daily Water Consumption (mL)
Control	528 ± 11 g	55 ± 3 mL
VPA (Treatment)	568 ± 37 g	54 ± 2 mL

Table 2 - Control and Treatment Group Average Weights and Water Consumption

Average weight of control animals (n=4) was 528 ± 11g and the average weight of VPA (treatment) animals (n=4) was 568 ± 37 g. Daily water consumption was measured over 17 days. Average daily water consumption for control animals was 55 ±3 mL. Daily water consumption for 0.4%VPA (treatment) animals was 54 ±2 mL.

Experimental Group	Weight (g)	Average Daily Water Consumption (mL)
Control	162 ± 4 g	39 ± 2 mL
VPA 0.3%	158 ± 3 g	37 ± 2 mL
VPA 0.4%	156 ± 4 g	36 ± 2 mL

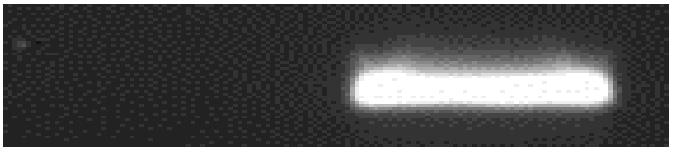
Table 3 - Control and Treatment Group Average Weights and Water Consumption

Average weight of control animals (n=12) was 162 ± 4 g. Average weight of 0.3% VPA (treatment) animals (n=12) was 158 ± 3 g. Average weight of 0.4% VPA (treatment) animals (n=12) was 156 ± 4 g. Daily water consumption was measured over 17 days. Average daily water consumption for control animals was 39 ± 2 mL. Daily water consumption for 0.3% VPA (treatment) animals was 37 ± 2 mL. Daily water consumption for 0.4% VPA (treatment) animals was 36 ± 2 mL.

Figure 1 - Induction of MT_1 mRNA by VPA in the rat hippocampus

(A) Gel images of RT-PCR amplification of MT_1 (472 bp) in the hippocampus of control and treatment groups after 17 day VPA treatment show increased expression of MT_1 mRNA in VPA treated rats in comparison to controls. Lanes 1, 2: Control, VPA. (B) The histogram represents the means ± S.E.M. for percentage (%) values of MT_1/GAPDH optical density (OD) ratios and indicates a significant 8-fold increase in MT_1 mRNA expression in the treatment group as opposed to the control group. **$p<0.05$ vs control. These results are in keeping with in vitro evidence suggesting VPA is involved in the positive expression of the melatonin receptor subtype MT_1 in rat C6 cells.

A)



B)

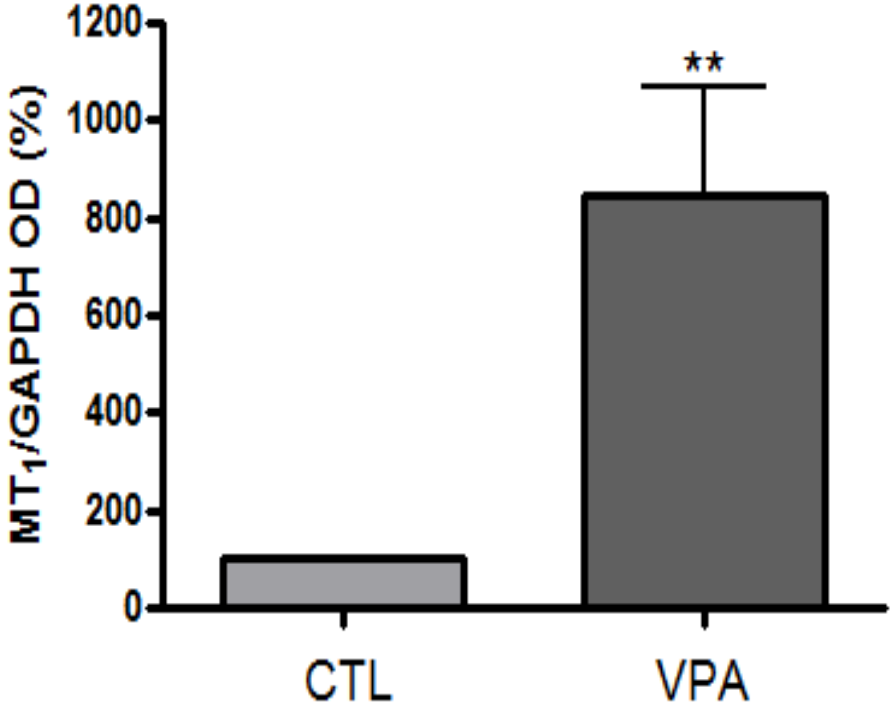
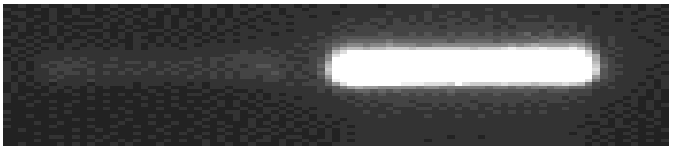


Figure 2 - Induction of MT_2 mRNA by VPA in the rat hippocampus

(A) Gel images of RT-PCR amplification of MT_2 (297 bp) in the hippocampus of control and treatment groups after 17 day VPA treatment show increased expression of MT_1 mRNA in VPA treated rats in comparison to controls. Lanes 1, 2: Control, VPA. (B) The histogram represents the means ± S.E.M. for percentage (%) values of MT_2/GAPDH optical density (OD) ratios and indicates a significant 14-fold increase in MT_2 mRNA expression in the treatment group as opposed to the control group. **$p<0.01$ vs control. These results are in keeping with in vitro evidence suggesting VPA is involved in the positive expression of the melatonin receptor subtype MT_2 in rat C6 cells.

A)


1
2

B)

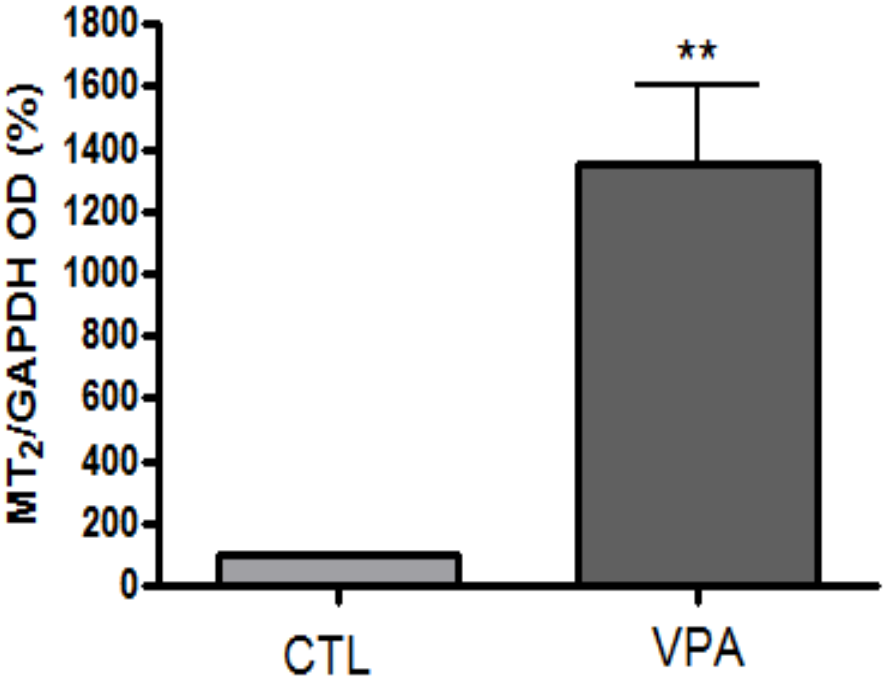
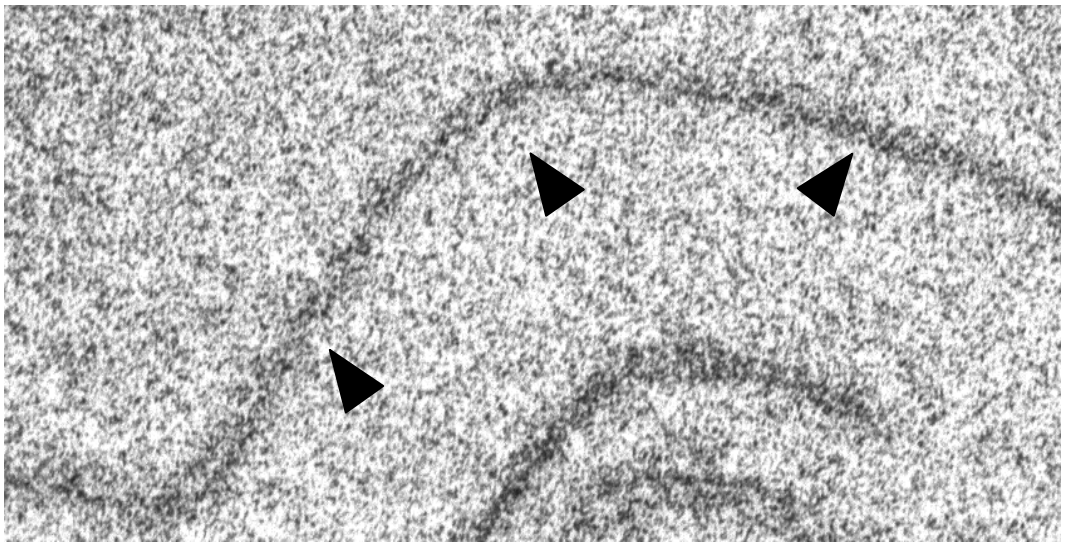

MT2/GAPDH OD (%)
1800
1600
1400
1200
1000
800
600
400
200
0
**
CTL
VPA

Figure 3 - Induction of MT_2 mRNA by VPA in the rat hippocampus CA1 region

(A) Representative film autoradiographs of densitometric analysis of basal MT_2 mRNA expression in the CA1 region of the rat hippocampus of control animals. Representative images show significantly increased MT_2 mRNA expression following chronic VPA treatment in comparison to control animals in (B) VPA 0.3% treated animals, and (C) VPA 0.4% treated animals. (D) The histograms represents the means ± S.E.M. for DPM values of MT_2 expression and indicate a significantly greater expression of MT_2 mRNA for both VPA 0.3% and VPA 0.4% treatment in comparison to the vehicle. *$p<0.05$ vs control. These results are in keeping with RT-PCR evidence that showed increased MT_2 expression in the whole hippocampus, and further localizes the effect of VPA on the melatonin receptor subtype MT_2 expression.

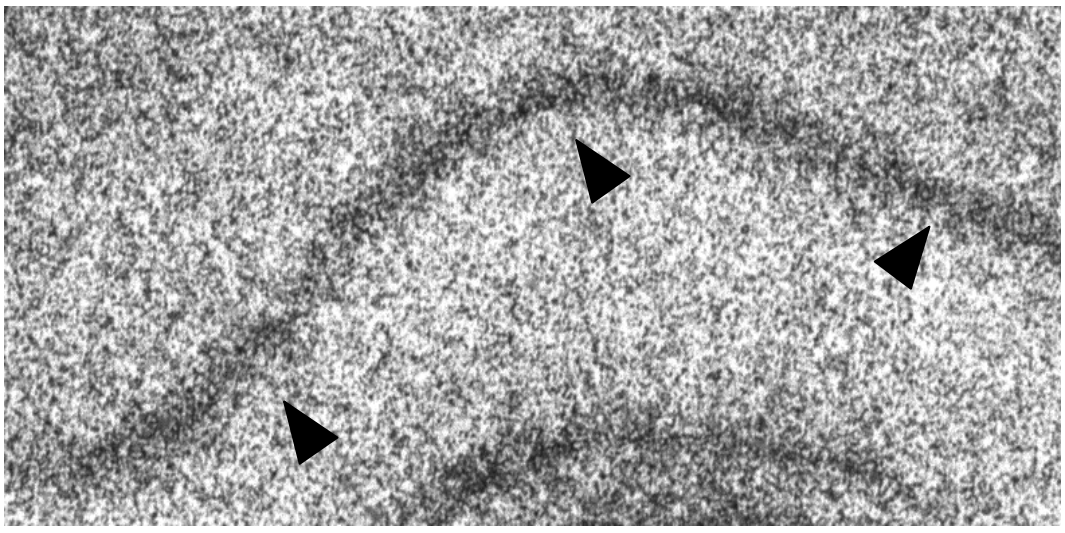
CTL

A)

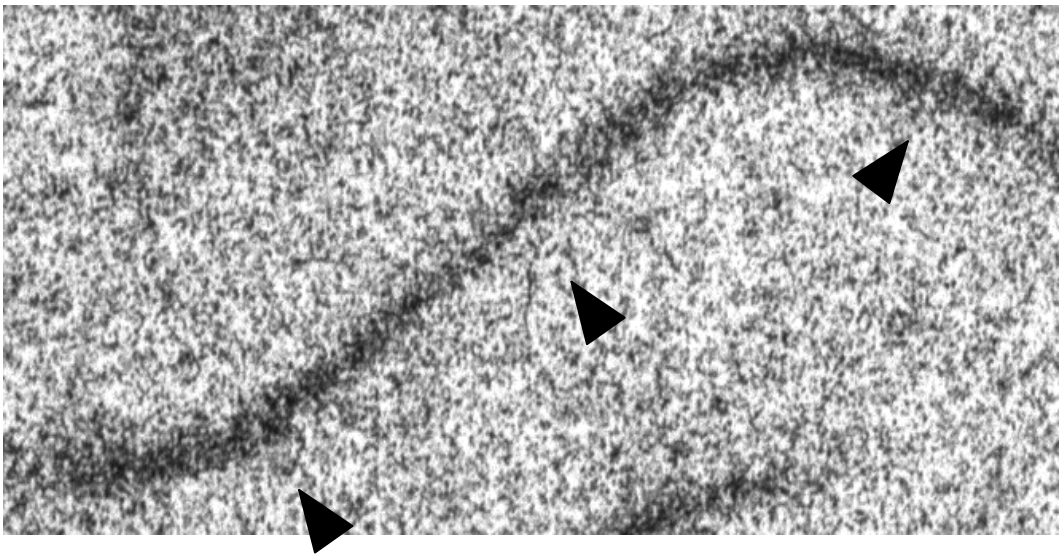


VPA 0.3%

B)



C)


VPA 0.4%

D)

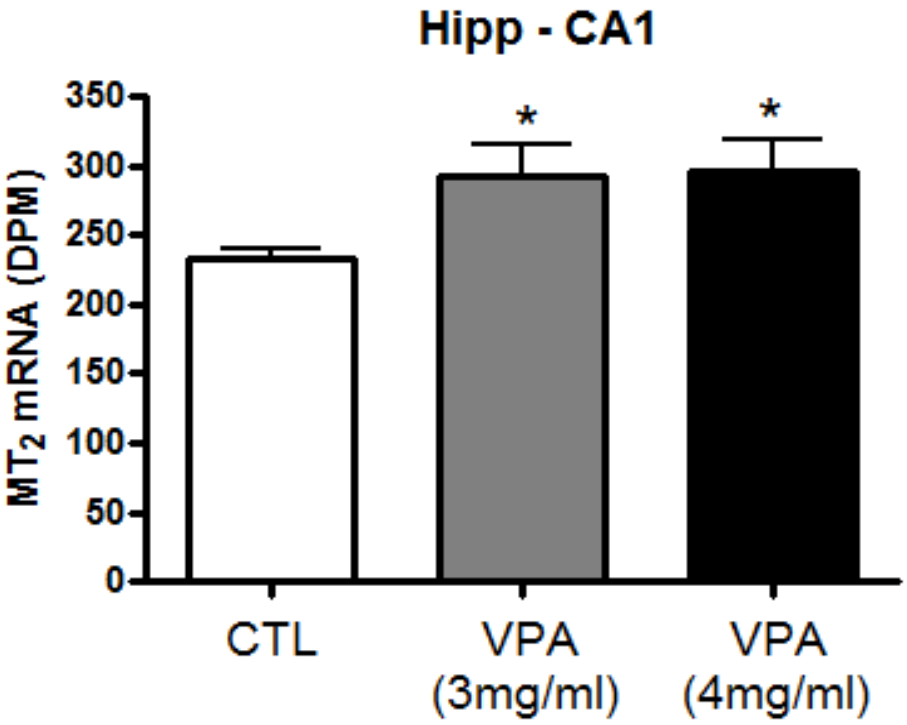
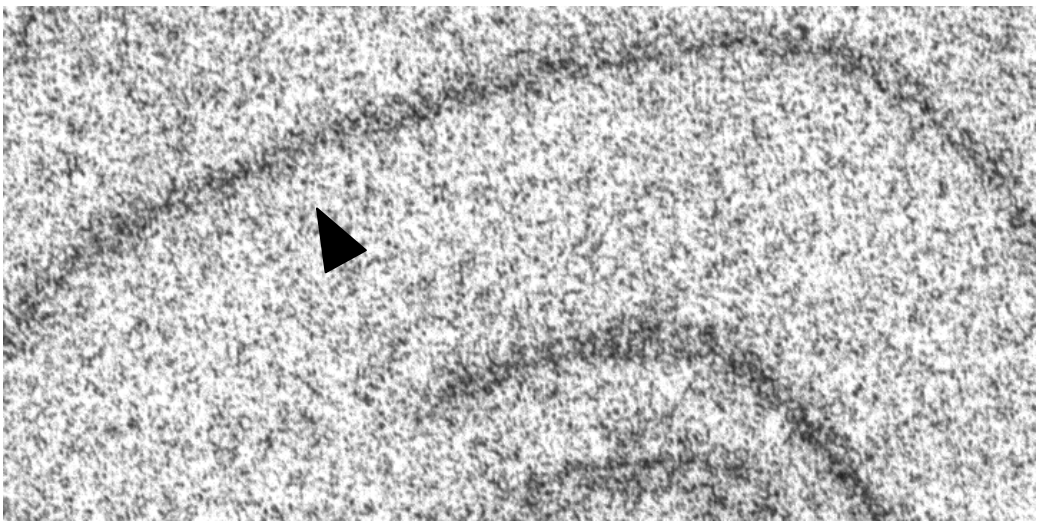

Hipp - CA1
MT2 mRNA (DPM)
350
300
250
200
150
100
50
0
*
*
CTL
VPA (3mg/ml)
VPA (4mg/ml)

Figure 4 - Induction of MT_2 mRNA by VPA in the rat hippocampus CA2 region

(A) Representative film autoradiographs of densitometric analysis of basal MT_2 mRNA expression in the CA2 region of the rat hippocampus of control animals. Representative images show significantly increased MT_2 mRNA expression following chronic VPA treatment in comparison to control animals in (B) VPA 0.3% treated animals, and (C) VPA 0.4% treated animals. (D) The histograms represents the means ± S.E.M. for DPM values of MT_2 expression and indicate a significantly greater expression of MT_2 mRNA for both VPA 0.3% and VPA 0.4% treatment in comparison to the vehicle. **$p<0.01$ vs control. These results are in keeping with RT-PCR evidence that showed increased MT_2 expression in the whole hippocampus, and further localizes the effect of VPA on the melatonin receptor subtype MT_2 expression.

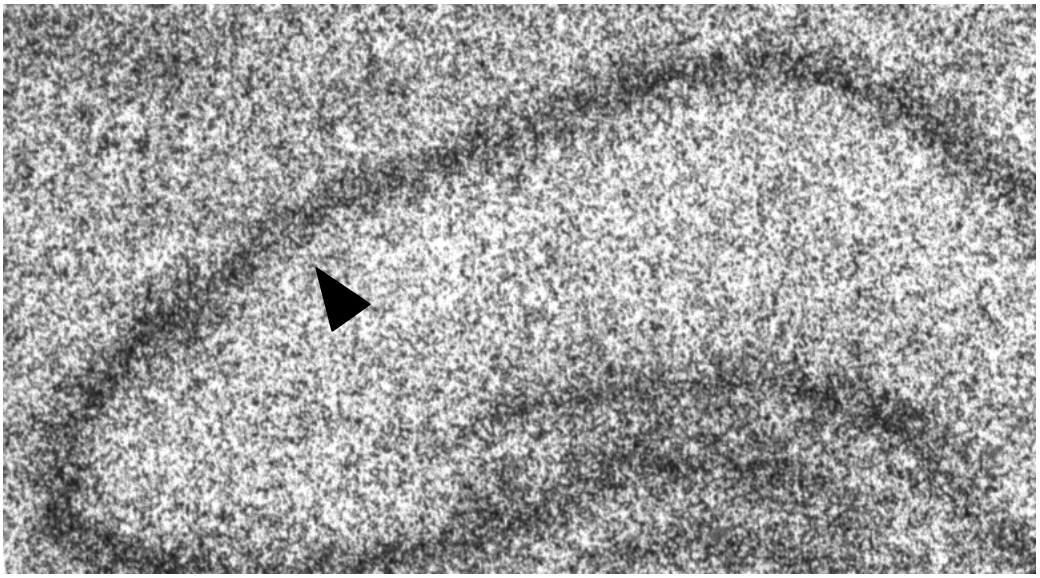
CTL

A)



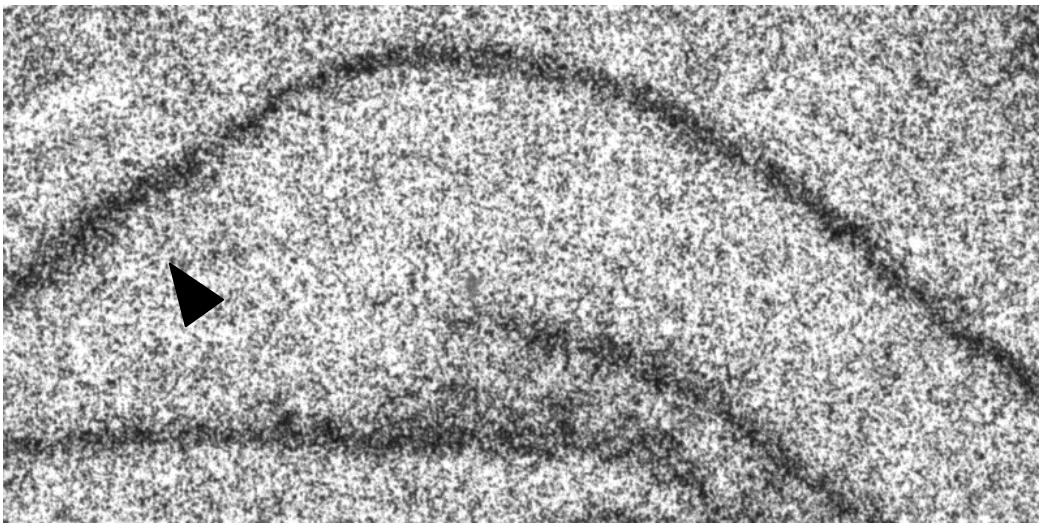
VPA 0.3%

B)



VPA 0.4%

C)



D)

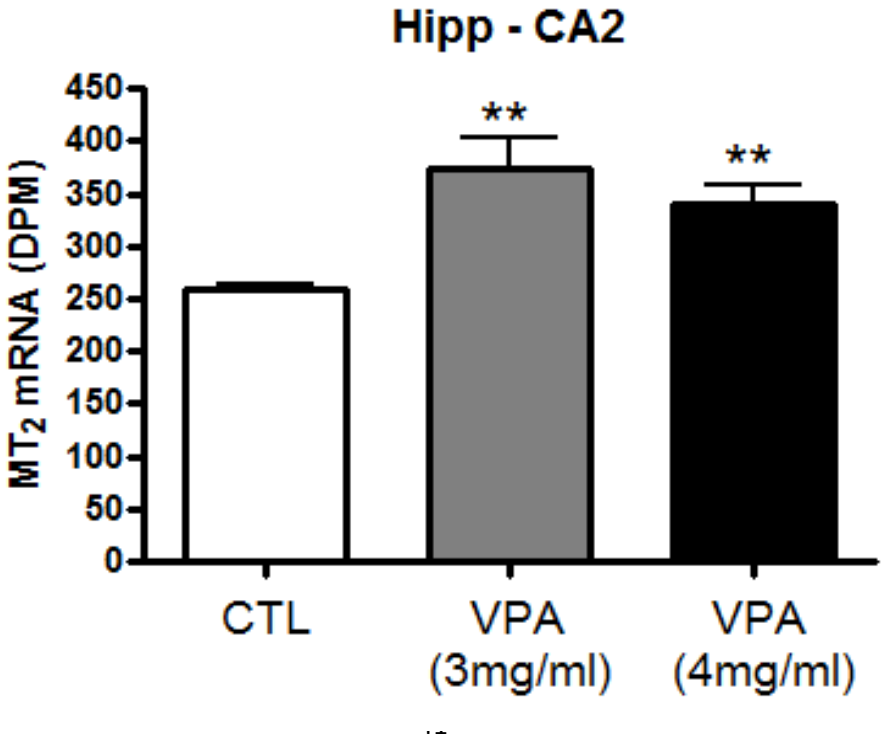
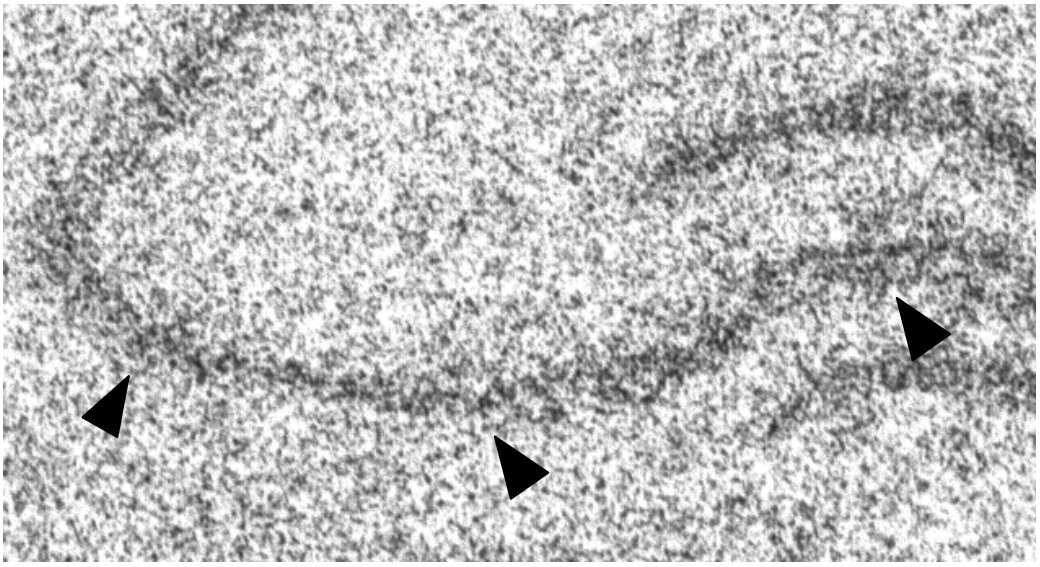

Hipp - CA2
MT2 mRNA (DPM)
450
400
350
300
250
200
150
100
50
0
**
**
CTL
VPA (3mg/ml)
VPA (4mg/ml)

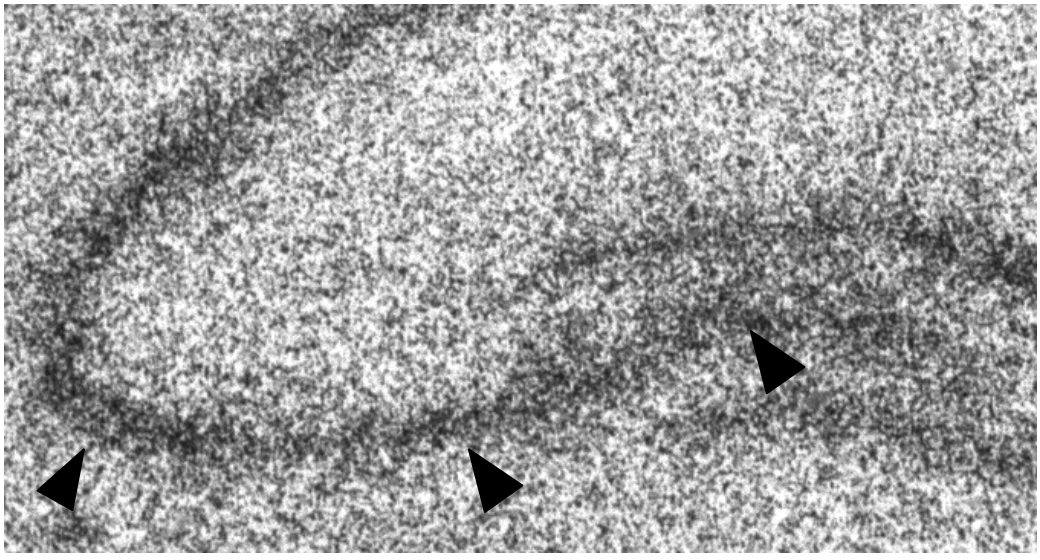
Figure 5 - Induction of MT_2 mRNA by VPA in the rat hippocampus CA3 region

(A) Representative film autoradiographs of densitometric analysis of basal MT_2 mRNA expression in the CA3 region of the rat hippocampus of control animals. Representative images show significantly increased MT_2 mRNA expression following chronic VPA treatment in comparison to control animals in (B) VPA 0.3% treated animals, and (C) VPA 0.4% treated animals. (D) The histograms represents the means ± S.E.M. for DPM values of MT_2 expression and indicate a significantly greater expression of MT_2 mRNA for both VPA 0.3% and VPA 0.4% treatment in comparison to the vehicle. *$p<0.05$ vs control. These results are in keeping with RT-PCR evidence that showed increased MT_2 expression in the whole hippocampus, and further localizes the effect of VPA on the melatonin receptor subtype MT_2 expression.

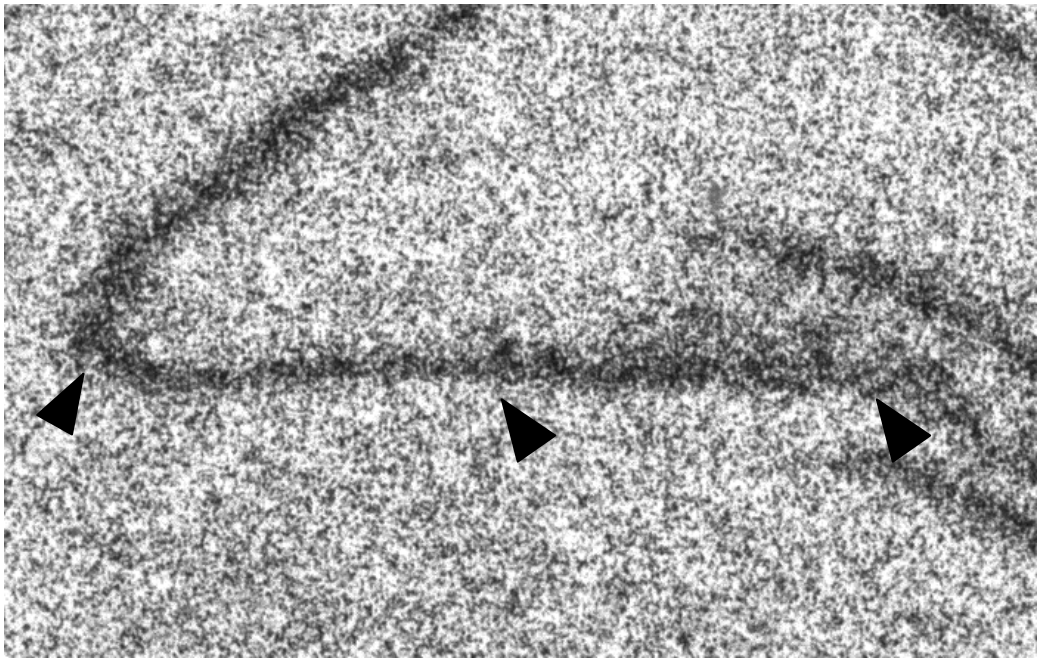
A) **CTL**



B) **VPA 0.3%**



C)


VPA 0.4%

D)

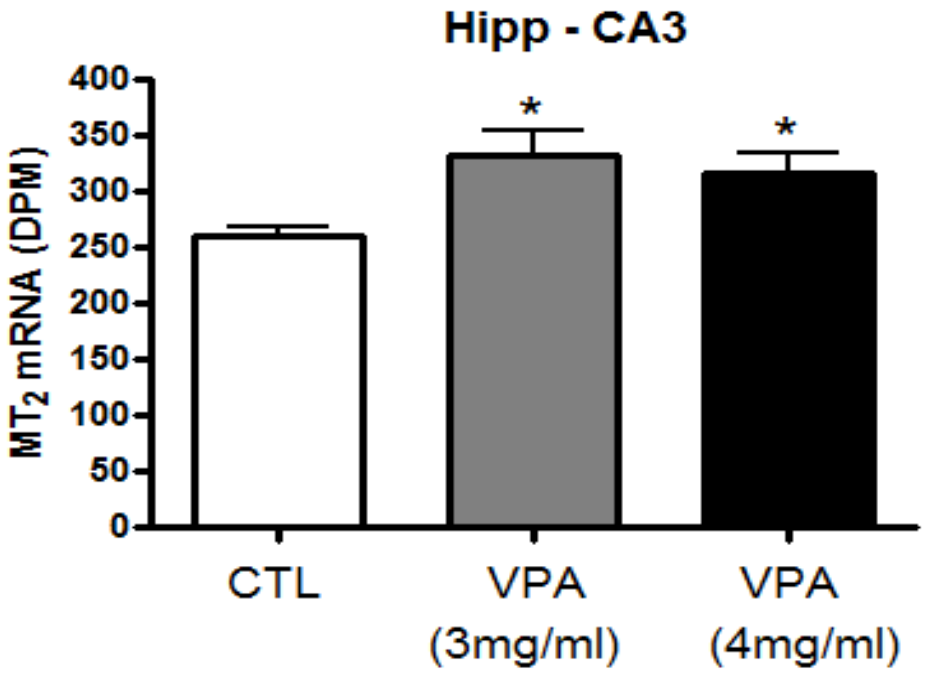
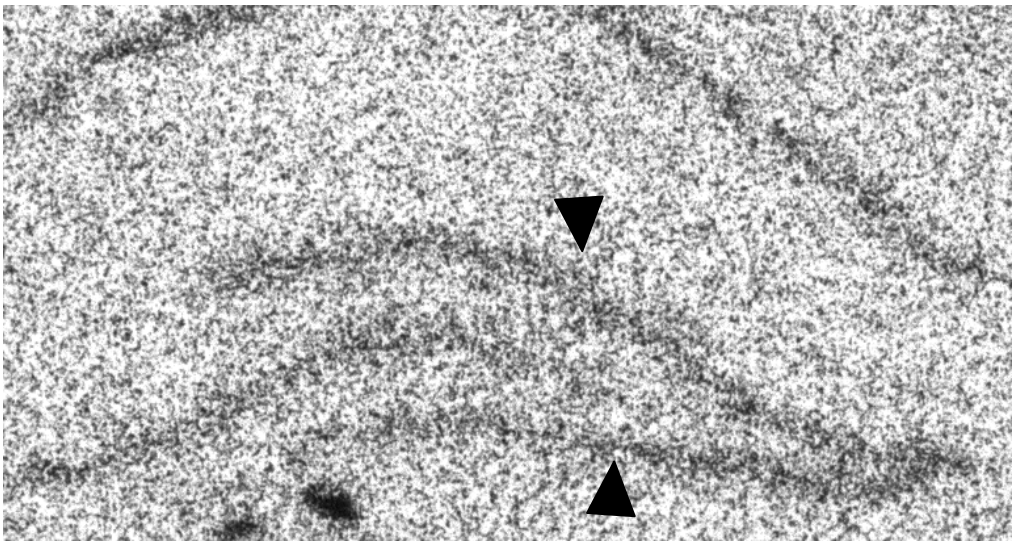

Hipp - CA3
MT2 mRNA (DPM)
400
350
300
250
200
150
100
50
0
*
*
CTL
VPA (3mg/ml)
VPA (4mg/ml)

Figure 6 - Induction of MT_2 mRNA by VPA in the rat dentate gyrus

(A) Representative film autoradiographs of densitometric analysis of basal MT_2 mRNA expression in the DG region of the rat hippocampus of control animals. Representative images show significantly increased MT_2 mRNA expression following chronic VPA treatment in comparison to control animals in (B) VPA 0.3% treated animals, and (C) VPA 0.4% treated animals. (D) The histograms represents the means ± S.E.M. for DPM values of MT_2 expression and indicate a significantly greater expression of MT_2 mRNA for both VPA 0.3% and VPA 0.4% treatment in comparison to the vehicle. *$p<0.05$ vs control. These results are in keeping with RT-PCR evidence that showed increased MT_2 expression in the whole hippocampus, and further localizes the effect of VPA on the melatonin receptor subtype MT_2 expression.

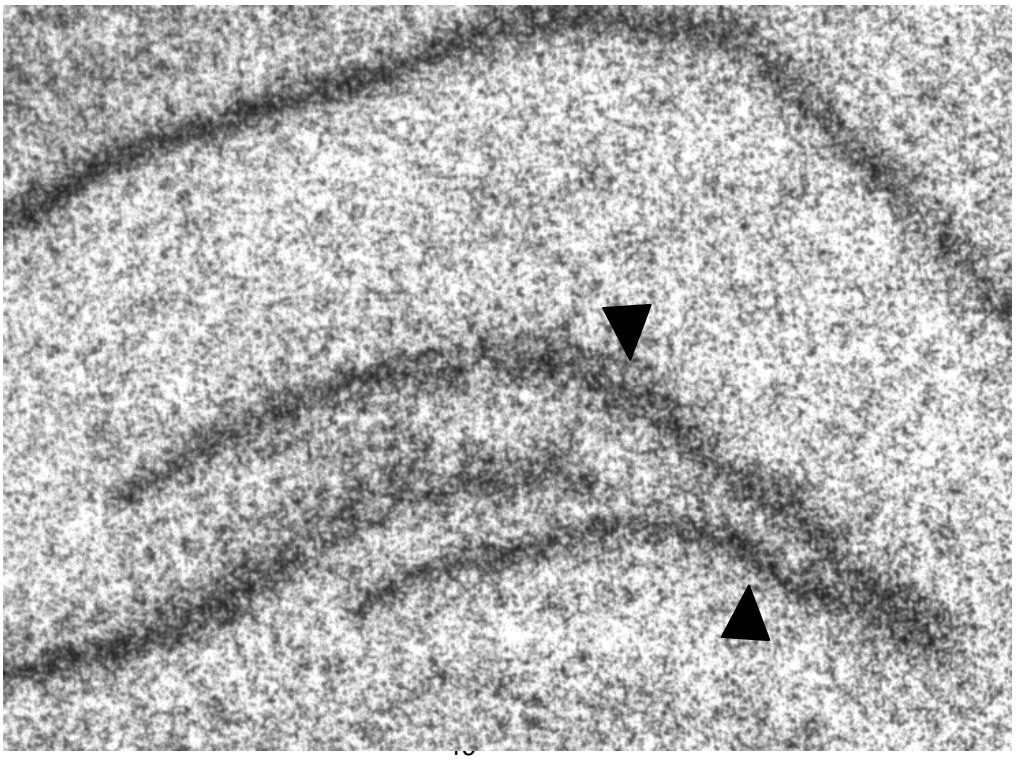
CTL

A)



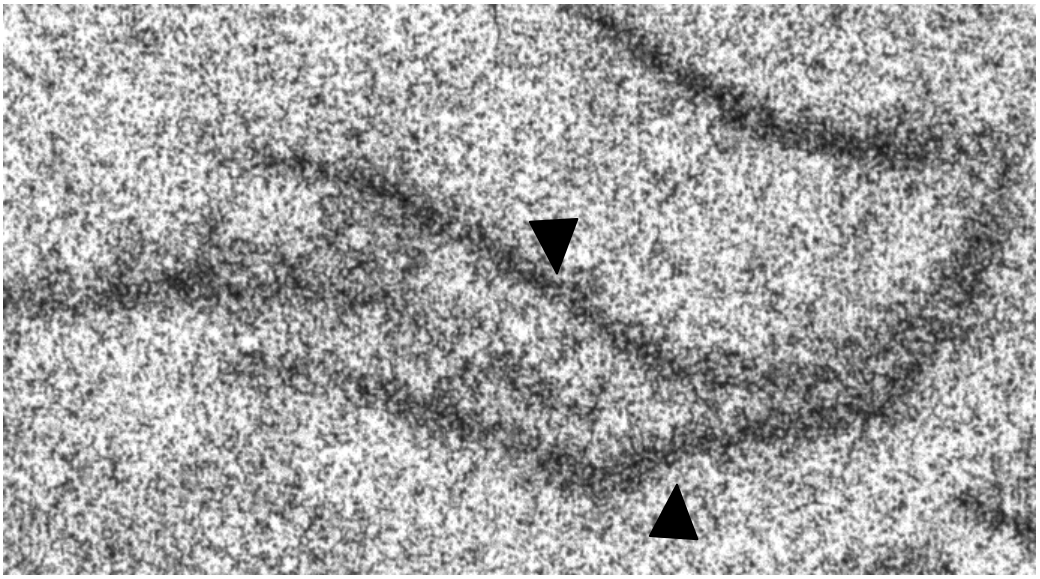
VPA 0.3%

B)



VPA 0.4%

C)



D)

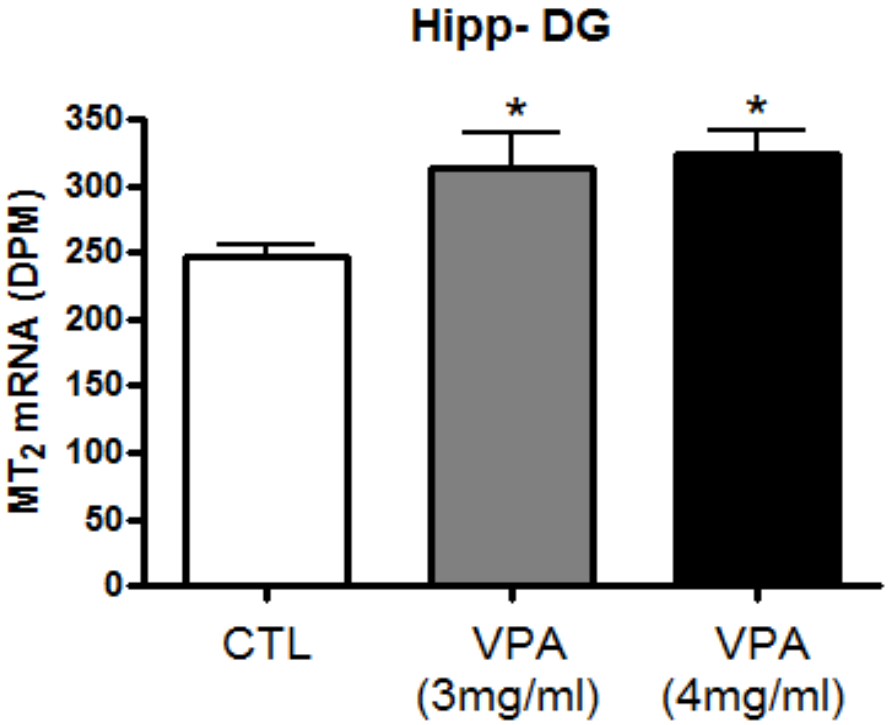
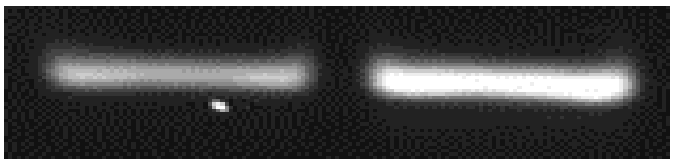

Hipp- DG
MT2 mRNA (DPM)
350
300
250
200
150
100
50
0
*
*
CTL
VPA (3mg/ml)
VPA (4mg/ml)

Figure 7 - Induction of BDNF mRNA by VPA in the rat hippocampus

(A) Gel images of RT-PCR amplification of BDNF (390 bp) in the hippocampus of control and treatment groups after 17 day VPA treatment show increased expression of BDNF mRNA in VPA treated rats in comparison to controls. Lanes 1, 2: Control, VPA. (B) The histogram represents the means ± S.E.M. for percentage (%) values of BDNF/GAPDH optical density (OD) ratios and indicates a significant increase in BDNF mRNA expression in the treatment group as opposed to the control group. **$p<0.01$ vs control. These results suggest that VPA is involved in the positive expression of the neurotrophic factor BDNF in the rat hippocampus.

A)



B)

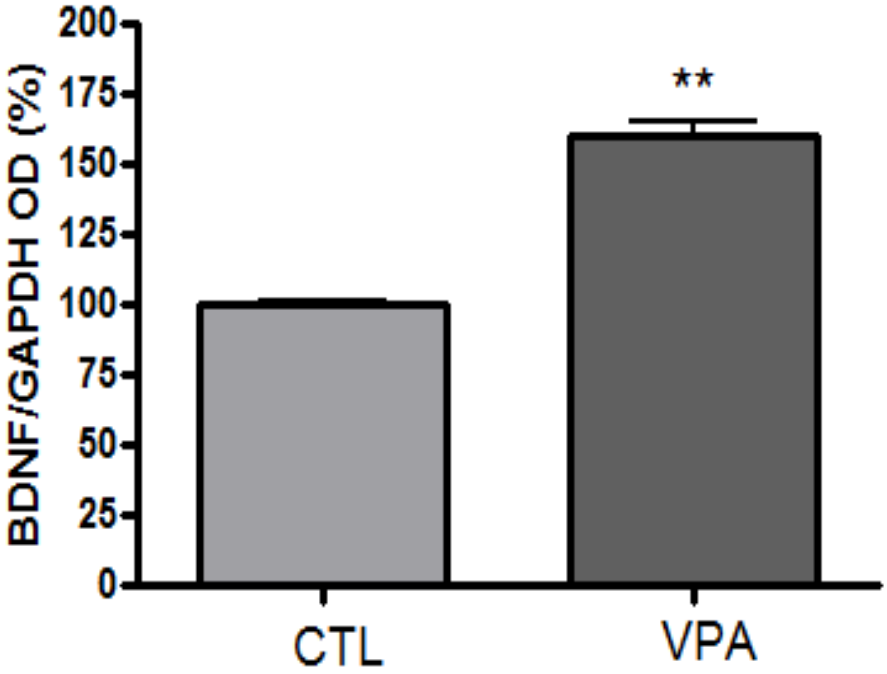


Figure 8 - Induction of BDNF mRNA by VPA in the rat striatum

(A) Gel images of RT-PCR amplification of BDNF (390 bp) in the striatum of control and treatment groups after 17 day VPA treatment show increased expression of BDNF mRNA in VPA treated rats in comparison to controls. Lanes 1, 2: Control, VPA. (B) The histogram represents the means ± S.E.M. for percentage (%) values of BDNF/GAPDH optical density (OD) ratios and indicates a significant increase in BDNF mRNA expression in the treatment group as opposed to the control group. **$p<0.01$ vs control. These results suggest that VPA is involved in the positive expression of the neurotrophic factor BDNF in the rat striatum.

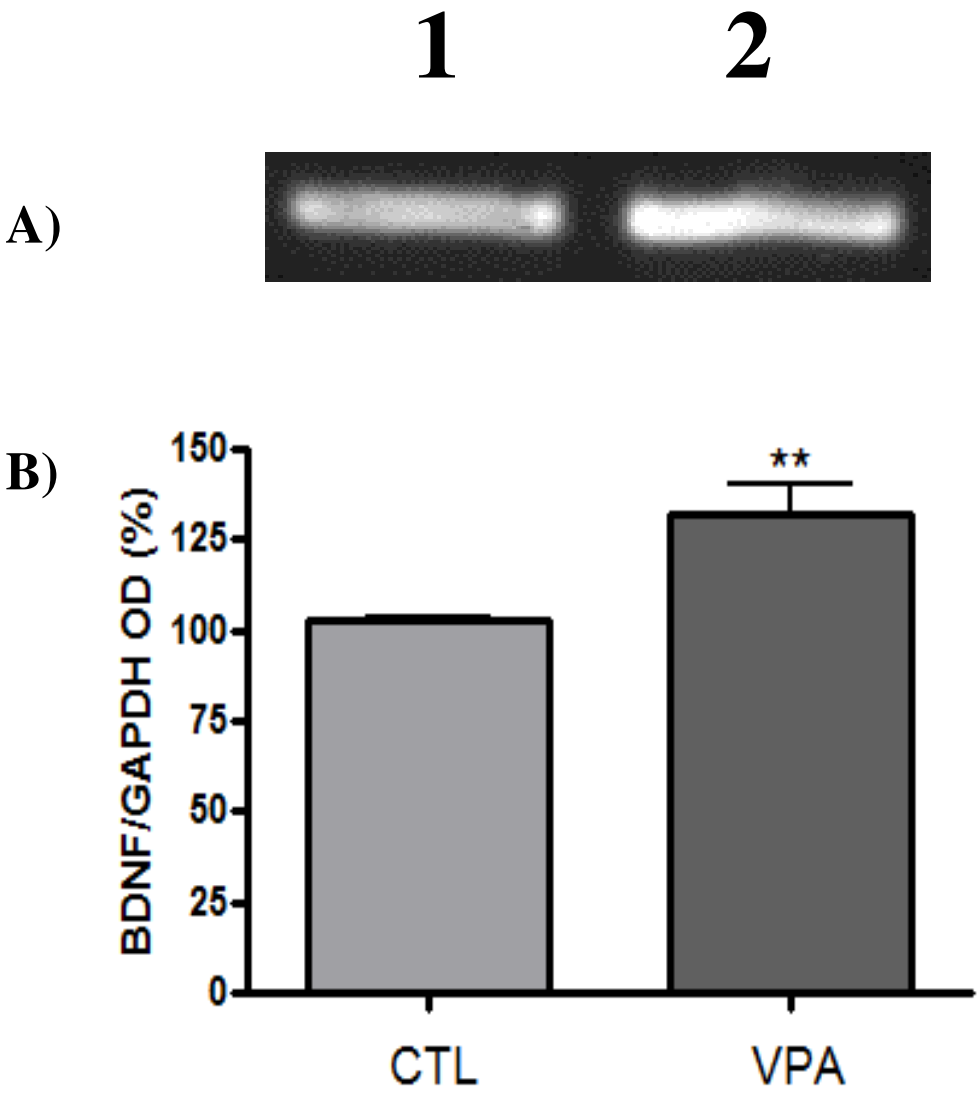

1
2
A)
B)
BDNF/GAPDH OD (%)
150
125
100
75
50
25
0
**
CTL
VPA

Figure 9 - Induction of GDNF mRNA by VPA in the rat hippocampus

(A) Gel images of RT-PCR amplification of GDNF (643 bp) in the hippocampus of control and treatment groups after 17 day VPA treatment show increased expression of GDNF mRNA in VPA treated rats in comparison to controls. Lanes 1, 2: Control, VPA. (B) The histogram represents the means ± S.E.M. for percentage (%) values of GDNF/GAPDH optical density (OD) ratios and indicates a significant increase in GDNF mRNA expression in the treatment group as opposed to the control group. **$p<0.01$ vs control. These results suggest that VPA is involved in the positive expression of the neurotrophic factor GDNF in the rat hippocampus.

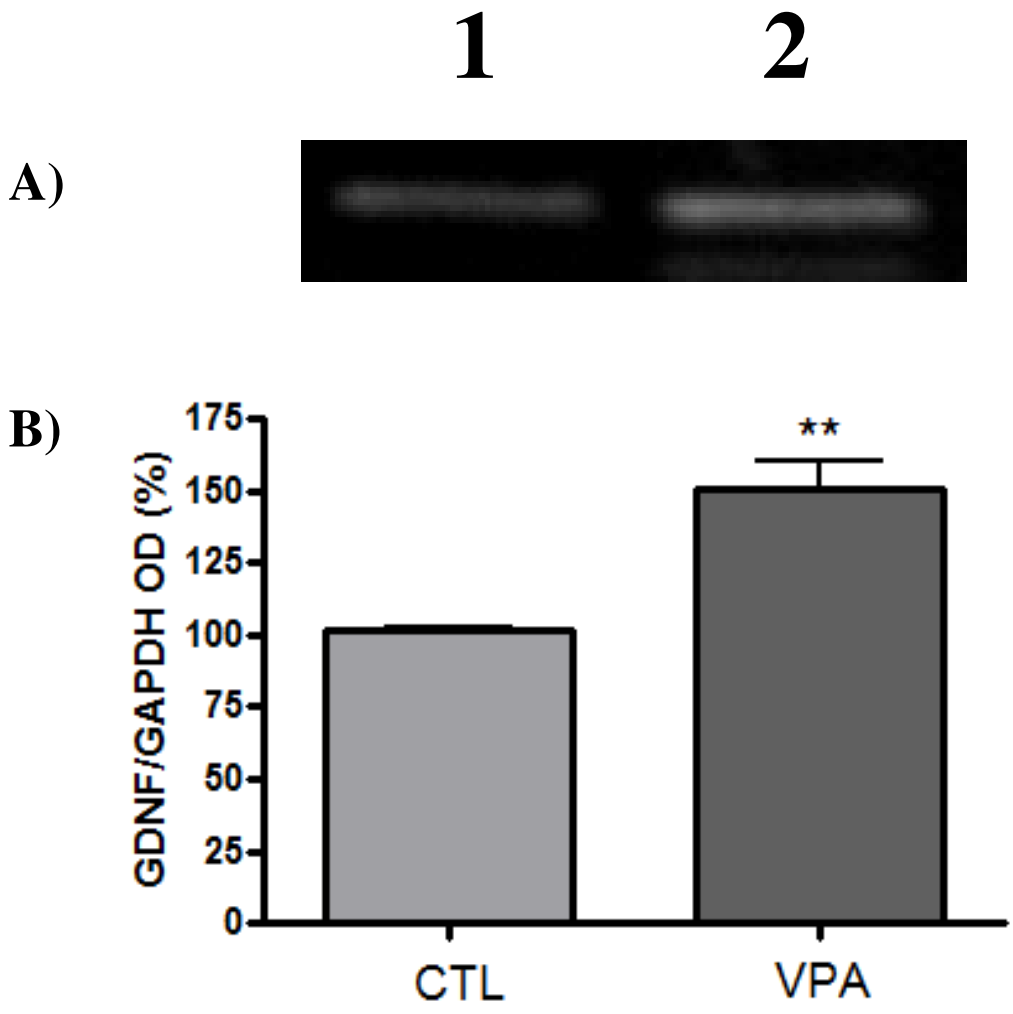
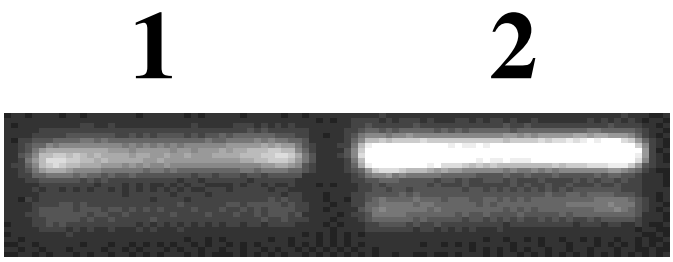

1
2
A)
B)
GDNF/GAPDH OD (%)
175
150
125
100
75
50
25
0
**
CTL
VPA

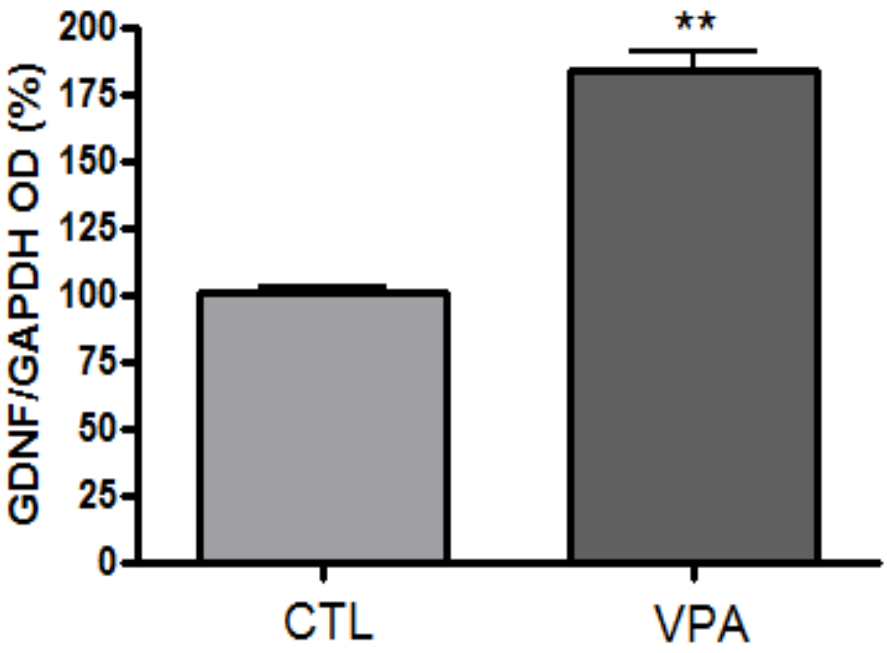
Figure 10 - Induction of GDNF mRNA by VPA in the rat striatum

(A) Gel images of RT-PCR amplification of GDNF (643 bp) in the striatum of control and treatment groups after 17 day VPA treatment show increased expression of GDNF mRNA in VPA treated rats in comparison to controls. Lanes 1, 2: Control, VPA. (B) The histogram represents the means ± S.E.M. for percentage (%) values of GDNF/GAPDH optical density (OD) ratios and indicates a significant increase in GDNF mRNA expression in the treatment group as opposed to the control group. **$p<0.01$ vs control. These results suggest that VPA is involved in the positive expression of the neurotrophic factor GDNF in the rat striatum.

A)



B)



4.0 Discussion

Common VPA dosage in *in vivo* models of rodents have used 300-400 mg/kg, the same dose at which seizure control was seen in rats (Nissinen & Pitkanen, 2007). Additionally, it must be mentioned that VPA metabolism and kinetics differ in humans and rodents, in whom VPA metabolism is increased ten times versus humans (Loscher, 1999). Therefore, to reach therapeutic ranges of 50-100 ug/ml (Sackellares et al., 1981; Vasudev et al., 2001), administration of higher doses than in humans are required (Loscher, 1993).

Frisch et al, 2009, showed that high doses of VPA (825 mg/kg) in drinking water resulted in cognitive teratogenicity indicated by memory impairment and delayed motor learning, and decreased brain-volume reduction in distinct brain areas in offspring of dams treated with VPA. However, at medium doses of VPA (500 mg/kg) it was shown that this dose increased the learning process and increased hippocampal volumes (Frisch et al., 2009). Neuroprotective effects were also seen at this dose in rats after cerebral ischemia/reperfusion (Ren et al., 2004) and also to protect neurogenesis in the dentate gyrus (Hsieh et al., 2004). Therefore, doses of 0.3% and 0.4% VPA in drinking water, which produce plasma levels of VPA within a therapeutic range (S. S. Cui et al., 2003; Frisch et al., 2009) were chosen in our study.

In vitro evidence has shown that VPA upregulates the mRNA and protein expression of the melatonin G-protein coupled MT_1receptors in rat C6 glioma cells, but inconsistent detection of the MT2 subtype precluded analysis of its expression in this cell

line (Castro et al., 2005). This study investigated if this relationship could be replicated in an *in vivo* model. Our results indicate a marked increase in both MT_1 and MT_2 receptor mRNA expression in the hippocampus of VPA treated animals versus control animals. The significant increases of up to 8-fold for the MT_1 and 14-fold for the MT_2 indicate that VPA up-regulates the expression of both melatonin receptor subtypes in the hippocampus. Further analysis conducted by the lab with real-time PCR showed a significant 7-fold and 10-fold increase in MT1 and MT2 mRNA expression in the hippocampus of VPA treated rats (Niles et al., 2012). Melatonin receptors are differentially distributed within the CNS, with the widely distributed MT_1 receptor subtype expressed in areas such as the suprachiasmatic nuclei of the hypothalamus, cerebellum and hippocampus, while the MT_2 receptor subtype is mainly found to be enriched in the hippocampus with restricted expression in the whole brain, (Ishii et al., 2009).

Furthermore, our novel results from in situ hybridization show that MT_2 receptor mRNA is significantly upregulated in the CA1, CA2, CA3 and dentate gyrus of the hippocampus in treatment groups in comparison to the control group. The hippocampus plays an important role in learning and long-term memory formation and consolidation (Eichenbaum et al., 1992; Squire & Cave, 1991). Functionally and histologically the hippocampus is composed of sub-regions with discrete functions that form an interconnected network; these sub-regions include the cornu ammonis 1 (CA1), cornu ammonis 2 (CA2), cornu ammonis 3 (CA3) and the dentate gyrus (DG).

CA1 pyramidal neurons are involved in spatial learning and in the consolidation and retrieval of recent contextual memory (Morris et al., 1982). CA3, due to its dense excitatory projections within its own pyramidal cells, is thought to have auto-associative properties and to also play a role in contextual memory acquisition (Daumas et al., 2005).Importantly, both CA1 and CA3 are central in higher-order spatial and contextual learning tasks, and are thought to communicate with other sub-regions and the association cortex to allow integration of information through a hippocampal loop (Jones & McHugh, 2011).

Another sub-region in the hippocampus, the dentate gyrus, is thought to contribute to learning and memory through adult neurogenesis in its subgranular zone (SGZ) (Altman & Das, 1965; Eriksson et al., 1998; Gould et al., 1998). The ability of neural stem cells to proliferate and differentiate is considered an important component of cognitive health. For instance, environmental stressors such as irradiation have been shown to inhibit neurogenesis and consequently have also been associated with the onset of cognitive impairments. Ablation of new neurons have also shown an impairment in learning tasks dependent on the hippocampus, which indicates that adult neurogenesis may play a role in the learning mechanisms of the hippocampus (Shors et al., 2001; Shors et al., 2002).

Learning impairments in zebrafish caused by pentylenetetrazol (PTZ), a proconvulsant known to induce learning deficits, were mitigated by VPA (Y. Lee et al., 2010). Additionally, zebrafish chronically treated with VPA (1-3 weeks) learned the

passive-avoidance response in comparison to acutely treated zebrafish. VPA treatment also led to behavioural and cognitive functional improvements of visuospatial memory and learning, and fine motor fluency (Bolanos et al., 1998; Siren et al., 2007). Taken together, the improvements in learning and memory suggest that VPA may have a neuroprotective role in the hippocampus, though the mechanism is yet to be elucidated. Hao, 2004 found that chronic VPA treatment for 6 weeks promoted neurogenesis within the granular layer of the dentate gyrus of mice as seen by increased BrdU-positive cells. Lithium was also shown to regulate hippocampal neurogenesis via the ERK pathway and consequently improve spatial learning and memory deficits in rats following ischemia (Yan et al., 2007). It is thought VPA induced neurogenesis is similarly mediated through the ERK pathway (Hao et al., 2004), which suggests that VPA may also improve spatial learning and memory deficits through hippocampal neurogenesis.

The induction of MT2 mRNA in the CA1, CA2, CA3 and DG by VPA is suggestive of involvement of the melatonergic system in the neurogenic and cognitive effects of VPA. MT2 has been localized to the hippocampus, specifically in the pyramidal neurons of all hippocampal sub-regions (Savaskan et al., 2005). It has also been shown that the MT2 receptor inhibits $GABA_A$ receptor-induced membrane currents (Wan et al., 1999), which may account for the melatonin-induced increase in the firing of CA1 hippocampal neurons (Musshoff et al., 2002). This indicates that hippocampal activity may be influenced by melatonin and play a role in regulating memory processes.

Additionally, (Argyriou et al., 1998) showed that melatonin facilitated short-term memory and had permanently assisted memory processes.

Melatonin has been shown to modulate the differentiation and survival of adult hippocampal neural precursor cells in vivo and in vitro (Ramirez-Rodriguez et al., 2009). Specifically, increased neurogenesis as witnessed by increases in BrdU and DCX immunoreactive cells was exhibited during the active period of melatonin (Crupi et al., 2011; Holmes et al., 2004). Furthermore, exogenous melatonin increases new cell formation in the hippocampus and modulates neurogenesis in the rat dentate gyrus following pinealectomy, as well as in ischemic-stroke mice (Chern et al., 2012; Rennie et al., 2009). However, these effects were compromised when pre-treated with the MT2 receptor antagonists, luzindole and 4-phenyl-2-propironamidotertralin (4P-PDOT) (Chern et al., 2012). Ramirez (2009) also found that pre-treatment with luzindole partially blocked the neuronal differentiation induced by melatonin, suggesting a key function for the MT2 receptor. The role of the MT2 receptor in neurogenesis and subsequent hippocampal cognitive processes is further compounded by the localization of both MT1 and MT2 receptor transcripts in adult mice hippocampal neuronal precursor cells and dentate gyrus tissue homogenate (Ramirez-Rodriguez et al., 2009).

Furthermore, the promotion of neurogenesis in the adult rat dentate gyrus by chronic administration of agomelatine, a melatonin receptor agonist and serotonin receptor subtype 2C antagonist, may also provide further evidence that the melatonin receptors are involved (Banasr et al., 2006). However, further research is required to

validate that the neurogenic properties of agomelatine are exclusively due to its melatonin receptor agonist function.

Evidence suggests that the melatonergic system, and more specifically the MT2 receptor, is strongly implicated in hippocampal cognitive processes that are relevant to adult neurogenesis. It also suggests that this may be a possible target of VPA in its neuroprotective and neurogenic properties.

The mechanisms of the induction of MT2 receptor in the hippocampus by VPA is not well understood, though several mechanisms are postulated to play a role. It has been reported that chronic treatment of rats with valproate or lithium increases in a concentration-dependent manner, the downstream effectors of the ERK pathway such as ribosomal S6 kinase 1, CREB, BDNF, and BCL-2 in total homogenates of rat prefrontal cortex and hippocampus, which suggests that both mood stabilizers activate the MAPK-ERK pathway (Einat et al., 2003; Yuan et al., 2001). However, ERK appears to be involved in suppressing MT_1 mRNA expression. As previously mentioned, 24 hour treatment of 3 mM VPA increased MT_1 receptor mRNA 14-fold in C6 cells, while 24 hour treatment of 5 mM VPA only increased MT_1 receptor mRNA 5-fold. The higher induction of MT_1 at a lower dose of VPA may be explained by an increased activation of the inhibitory pathway by the 5mM dose of VPA (Castro et al., 2005). Furthermore, co-treatment with a pharmacological inhibitor of the MAPK/ERK pathway, PD98059 enhanced the induction of MT_1 mRNA receptor expression by VPA. Taken together, this

suggests that despite the activation of the inhibitory MAPK/ERK pathway, the potent induction of MT_1 by VPA may be due to another pathway entirely.

One such pathway may be the inhibition of phorbol 12-myristate 12-acetate (PMA)-responsive isoforms of PKC (α, δ, ε). It is known that the activation of PKC can negatively regulate MT1 mRNA expression in the pars tuberalis in sheep (Barrett et al., 1998). VPA can decrease PKC activity (specifically the α and ε isoforms) by decreasing the transport of myo-inositol into cells which in turn depletes the activators of PKC, inositol triphosphate (IP3) and diacylglycerol (DAG)(Coyle & Duman, 2003). Another possible pathway through which VPA may regulate the expression of melatonin receptor mRNA is through epigenetic regulation as a histone deacetylase (HDAC) inhibitor, which modifies gene expression without an alteration of nucleotide sequence (Phiel et al., 2001). We have previously observed a concentration-dependent induction of melatonin MT_1 receptor mRNA in C6 cells in response to a structurally distinct HDAC inhibitor, trichostatin A (TSA) (B. Kim et al., 2008). This suggests that the method of MT1 receptor induction involves epigenetic regulation, and it is possible that VPA may exert its observed effects through similar HDAC inhibitory activity.

Both the PKC and epigenetic pathways have potent hippocampal neuroprotective roles. The expression of myristoylated alanine-rich C kinase substrate (MARCKS), a substrate of PKC is decreased due to the inhibition of PKC by VPA in the hippocampus (Manji & Lenox, 1999). MARCKS is implicated in brain development and in the regulation of synaptic efficiency via membrane cytoskeletal restructuring. Elevated

MARCKS expression in the hippocampus has been shown to impair hippocampus-dependent learning and long-term potentiation (LTP) and direct hippocampal infusions of MARCKS peptides showed dose-dependent memory impairment (McNamara et al., 2005; Timofeeva et al., 2010).

VPA has also shown induction of beta-catenin, which has anti-apoptotic effects, through the inhibition of histone deacetylase (Phiel et al., 2001). Another HDAC inhibitor, trichostatin A (TSA) has shown enhancement of hippocampus-dependent memory due to phosphorylation of CREB and recruitment of CREB-binding protein (CBP) which is a transcriptional coactivator that has been shown to possess histone acetyltransferase (HAT) activity (H. M. Chan & La Thangue, 2001; Impey et al., 2002; Tao et al., 1998). Furthermore, Jessberger et al., (2007) found that valproate IP injections inhibit impairments in hippocampus-dependent learning and block seizure-induced aberrant hippocampal dentate gyrus neurogenesis by inhibiting HDACs.

Our novel finding, that chronic treatment with VPA induces a significant induction of melatonin MT_1 and MT_2 receptors in the rat hippocampus, suggests that the melatonergic system is an important target for this psychopharmacological agent.

Previously, we had shown induction of BDNF mRNA by acute VPA treatment in the rat C6 glioma cell line (Castro et al., 2005). Presently, we have shown an increase in BDNF mRNA expression in the rat hippocampus and striatum following 17 day treatment of 4mg/mL VPA via drinking water.

This proposes potentially interesting clinical outcomes as VPA is clinically used to treat mood disorders and where a deficiency in neurotrophic factors are thought to play a role in their pathophysiology. For instance, patients with bipolar disease exhibited a loss of brain grey-matter volume and decreases in cellular density. Additionally, brain-imaging and post-mortem morphometric studies showed CNS volume reductions, specifically in numbers of glia and neurons in discrete brain regions of manic-depressive patients (Harrison, 2002). This strongly implicates a deficiency in neurotrophic factors such as BDNF which is an important factor in new neuron formation and cell survival (Manji & Duman, 2001; Babu et al., 2009; Barnabe-Heider & Miller, 2003; J. Lee et al., 2002). BDNF binds to the TrkB receptor to activate the Ras-Raf-MeK pathway which activates ERK and its downstream effectors ribosomal S6 kinase (RSK) and transcription factors cyclic AMP response element binding protein (CREB) and AP-1 (Yuan et al., 2001) to produce cellular neurotrophic actions such as neurite growth, regeneration, and neurogenesis (Coyle & Duman, 2003; E. J. Huang & Reichardt, 2003; Kaplan & Miller, 2000; Marinissen & Gutkind, 2001; Weeber & Sweatt, 2002).

Evidence also shows that reductions may be prevented or even reversed when patients are treated with mood stabilizers such as lithium and divalproex (Drevets et al., 1997; Drevets, 2000). VPA was also shown to correct depressive-like behaviour and reverse the loss of the light neurofilament subunit (NF-L) in depressive patients with hippocampal atrophy (Ferrero et al., 2007).

Neurotrophic factors such as BDNF have also been implicated for the treatment of mood disorders. VAL66Met BDNF polymorphism causes reductions in activity-dependent BDNF secretions in cultured cells (Egan et al., 2003), which is associated with a higher risk for bipolar disorder (Neves-Pereira et al., 2002). Additionally, it has been reported that decreases in neurotrophins cause apoptosis in the central nervous system (E. J. Huang & Reichardt, 2003). Together, this suggests that neurotrophic signaling pathway dysfunction may be a pathogenic factor for bipolar disorder.

The mechanism through which VPA may upregulate BDNF has yet to be fully elucidated. However, evidence suggests that VPA modulates the intracellular pathways implicated in the action of neurotrophins, since chronic treatment of rats with VPA activated the ERK pathway as seen through increases in phosphorylated CREB, BDNF and BCL-2 (Einat et al., 2003; Yuan et al., 2001). AP-1 and CREB are transcription factors, downstream of ERK, that bind to DNA sequences in the regulatory domain of genes (Sheng & Greenberg, 1990) and they modulate the expression of key proteins. CREB is a key element in the transcription of BDNF in the rat brain (Shieh & Ghosh, 1999; Tao et al., 1998), and an AP-1 site is present upstream of exon 1 in the rat BDNF gene (Nakayama et al., 1994). This suggests that the mood-stabilizing agent, VPA may alter the activities of these key transcription factors. Indeed, evidence shows a gradual increase in DNA-binding activity of AP-1 in cultured rat C6 glioma cells, human neuroblastoma cells and in the rat cerebral cortex and hippocampus following treatment of VPA at clinically relevant doses (G. Chen et al., 1997; McElroy et al., 1992).

Additionally, it has been shown that through the activation of the ERK 1/2 signal transduction pathway, VPA promotes neural progenitor cell differentiation into neurons, neurite growth, cell re-emergence, hippocampal neurogenesis and mediates neuronal protection (Coyle & Duman, 2003; Hao et al., 2004; Hsieh et al., 2004; Yuan et al., 2001). However the precise mechanisms by which VPA affects these intracellular pathways is unclear as VPA has no known cell surface receptors. VPA is not known to bind to any G-protein-coupled receptors that activate MAP kinases (Gutkind, 1998). Additionally, it was reported that VPA induction of ERK 1/2 phosphorylation is not correlated to HDAC inhibition, as PD98059 prevented VPA induced ERK 1/2 phosphorylation (Michaelis et al., 2006). Furthermore, the time course for the VPA-induced activation of ERK was much slower than when provoked by factors that interact directly with a cell surface receptor, such as insulin-like growth factor-1 or BDNF in SH - SY5Y cells (Encinas et al., 1999; B. Kim et al., 1997) which suggests that the activation of this pathway by VPA is not due to direct interactions with cell surface receptors. Taken together, VPA may induce the ERK pathway by up-regulating endogenous receptors whose activities are linked to this pathway. One such possible receptor group could be the G-protein coupled plasma membrane receptors MT_1 and MT_2, which once activated causes ERK translocation from the cytoplasm into the nucleus where it phosphorylates various target molecules such as nuclear substrates, cytoskeletal proteins and transcription factors (Yoon & Seger, 2006).

Recently, it was shown that melatonin increases the levels of BDNF in culture neurons and in the hippocampus (Imbesi et al., 2008; Soumier et al., 2009) and is also known to treat depression by stimulating adult hippocampal neurogenesis and synaptic plasticity (Crupi et al., 2010). However, despite reported ERK 1/2 activation, non-involvement of the ERK/MAP kinase pathway was supported by the observation that U0126, a specific ERK/MAP kinase pathway blocker, did not reduce VPA-induced BDNF exon 1-IX mRNA expression. Hence this suggests that VPA also induces BDNF directly via another pathway (Fukuchi et al., 2009)

Additionally, it has previously been shown that VPA increases the expression of both GDNF and BDNF in primary cortical astrocytes (P. S. Chen et al., 2006). Furthermore, Wu et al. (2008) demonstrated the induction of GDNF and BDNF mRNA in astrocytes by three distinct HDAC inhibitors, VPA, sodium butyrate (an HDAC inhibitor structurally similar to VPA) and trichostatin A (an HDAC inhibitor structurally different from VPA). This strongly suggests that VPA may increase BDNF and GDNF mRNA expression through epigenetic mechanisms.

Previously, we had showed induction of GDNF mRNA by nanomolar concentrations of melatonin in the rat C6 glioma cell line (Armstrong & Niles, 2002) and also by VPA (Castro et al., 2005). GDNF induction following acute treatment of VPA at clinically relevant doses was also shown in rat astroglia (P. S. Chen et al., 2006). However, Fukumoto et al., 2011 found that there was no significant induction of GDNF following chronic VPA administration *in vivo*. However, since ELISA assays were used

to analyze brain tissue homogenates, this approach may not have been able to pick up cell-specific changes in GDNF expression. Presently, we have shown an increase in GDNF mRNA expression in the rat hippocampus and striatum following 17 day treatment of 4mg/mL VPA via drinking water. GDNF is known to have restorative effects on dopaminergic neurons and act as a survival factor. It has been reported to increase the firing rate (Pothos et al., 2000) and excitability (Wang et al., 2003) of dopaminergic neurons. GDNF also promotes axonal regeneration and confers complete protection of dopaminergic neurons in face of the dopaminergic toxin 6-OHDA (Rosenblad et al., 1999). Additionally, clinical trials and animal studies have shown that GDNF can be therapeutically beneficial in Parkinson's disease (Gill et al., 2003; S. S. Wu & Frucht, 2005). BDNF and GDNF have both been implicated in several neurodegenerative diseases (Zuccato & Cattaneo, 2009). For instance, both GDNF and BDNF have been implicated in Parkinson's disease, while BDNF has also been implicated in Huntington's disease where the mutated protein huntingtin leads to a loss of BDNF transcription (Zuccato et al., 2001). Reduction of BDNF mRNA in the hippocampus has also been reported in Alzheimer's disease (Ferrer et al., 1999; Phillips et al., 1991) indicating that loss of trophic support can lead to neurodegenerative disease. It has been well established that melatonin concentrations decrease during aging, and patients with AD have a more profound melatonin reduction in the pineal gland, plasma and CSF (Y. H. Wu & Swaab, 2005). Additionally, the pineal gland also exhibits age-related changes such as calcification which disturbs circadian rhythmicity and decline in melatonin production.

Also it has been reported that the density of binding sites in the hypothalamus was significantly lower in old male rats versus that in mature animals (Laudon et al., 1988). Thus, the correlation between decreased melatonin and progression of AD pathogenesis indicates that melatonin may play a neuroprotective role in AD (Y. H. Wu & Swaab, 2005). Since loss of trophic support and a decrease in melatonin receptor density may play a role in the pathogenesis of neurodegenerative diseases, Valproate, as an HDAC inhibitor which targets the melatonergic system, may be an appropriate alternative or complementary neurotrophic treatment for brain trauma, ischemia, and neurodegenerative diseases (Loy & Tariot, 2002). In keeping with this view, other HDAC inhibitors such as SAHA and TSA have been shown to be effective in treating neurodegenerative disorders and enhancing synaptic plasticity (Hockly et al., 2003; Vecsey et al., 2007). Since GDNF and BDNF are rapidly degraded and do not cross the blood brain barrier, the ability to increase endogenous levels of these neurotrophins by agents such as melatonin may provide a safe alternative to other invasive methods of direct delivery into the CNS. Also the site of upregulation also plays a key role as it has been shown that GDNF delivery into the striatum in Parkinson's disease preserves dopamine terminals and promotes functional striatal dopamine innervation (Nestler, 2001) and we have shown (see Figure 10) that chronic treatment of VPA increases GDNF mRNA in the striatum.

It is recognized that circadian rhythm desynchronization plays a key role in mood disorders, as many circadian rhythms such as body temperature, cortisol, thyrotropin, prolactin, growth hormone, and melatonin are disrupted in depressed patients. Recurrent

mood disorders as opposed to isolated episodes of depression involve disruptions in circadian rhythms. However, these circadian rhythms return to normal upon patient recovery, suggesting that restoration of normal circadian rhythms may prove to be therapeutic. Mood stabilizers may normalize these rhythms in addition to their effects on mood, and this combination of effects on mood as well as on circadian rhythms distinguish mood stabilizers from antidepressants (Coyle & Duman, 2003). Agomelatine, an agonist of melatonin MT_1 receptor has been shown to play a key role in battling depression and mood disorders much like VPA (Popoli, 2009). Additionally, VPA, as previously examined, though not a direct agonist, has been shown to increase melatonin MT_1 receptor expression *in vitro* in both rat C6 glioma cells (Castro et al., 2005)and human MCF-7 breast cancer cells. A synergistic inhibition of MCF-7 cell proliferation was exhibited in response to the combination of VPA and melatonin (Jawed et al., 2007). These findings suggest that the therapeutic effects of VPA may involve the melatonergic system, and by extending research into an *in vivo* model to elucidate the mechanisms of this relationship, we hope to shed more light onto the psychotropic, neuropharmacological and neuroprotective effects of VPA and the melatonergic system.

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