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THE CONTROL OF ADULT NEURAL STEM CELL PROLIFERATION AND COGNITION BY VALPROIC ACID

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CONTENTS

CHAPTER 3: The effect of Valproic acid on cell proliferation and Notch signalling expression of hippocampal neural stem cells

CHAPTER 4: The effect of Valproic acid on cell proliferation and Notch signalling expression in Epn1, Med1 and SHSY5Y cancer cell lines

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PUBLICATIONS

Abstracts

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ABSTRACT

Valproic acid (VPA) is extensively used for the treatment of epilepsy. This drug also functions as an inhibitor of histone deacetylase enzymes and causes the expression of growth arrest genes. It has been reported that mild to moderate cognitive impairments occur in adult patients taking VPA. This investigation aimed to examine the relationship between cognition and changes in cell proliferation within the rat hippocampus, a brain region where continued formation of new neurons is associated with learning and memory. Also, the antiproliferative function of VPA was investigated in rat and mouse hippocampal neural stem cells (NSCs) and cancer cell lines *in vitro*.

The cognitive and antiproliferative effects of VPA were determined in adult Hooded Lister rats treated with VPA (300mg/kg) by intraperitoneal injection twice daily for 10 days. Cognition was assessed by the Novel Object Location (NOL) and contextual fear conditioning tests. Cell proliferation within the subgranular zone (SGZ) of the dentate gyrus was determined by immunostaining for Ki67. Additionally, levels of the brain-derived neurotrophic factor (BDNF), doublecortin (DCX) and the receptor Notch1 expression were measured by Western blotting. The results showed that animals treated with VPA had a hippocampal specific cognitive impairment as shown by NOL, test but not contextual fear conditioning. This was linked to a significant reduction in cell proliferation within the SGZ. Moreover, VPA treatment statistically significantly decreased levels of BDNF and Notch1, but not DCX within the hippocampus.

The antiproliferative effect of VPA treatment (0.3 and 1 mM) for 24 hours was investigated in hippocampal neural stem cells (NSCs) *in vitro*. The numbers of neurosphere and cell proliferation assessed in VPA-treated rat and mouse hippocampal NSCs showed significantly decreased in a concentration-dependent manner. Levels of Notch1, Sox2, nestin and c-Myc gene expression were quantified using qPCR. This revealed that 1 mM VPA reduced expression

of Notch1, Sox2 and nestin but not c-Myc. However, there were no changes in levels of these gene expressions in 0.3 mM VPA.

The influence of VPA on cancer cell proliferation was examined in human Epn1, Med1 and SHSY5Y cell lines by treating with 1, 2 and 3 mM VPA for 72 hours. The data of cell proliferation showed that VPA produced a significant reduction of tumour cell growth in a dose-dependent manner in all three cell lines. VPA (1, 2 and 3 mM) induced levels of Notch1 expression in SHSY5Y cell lines. Additionally, the number of dividing cells of Epn1 and Med1 treated with 2.5 mM VPA was significantly decreased compared to untreated cells using Ki67 immunostaining. However, Notch1 expression was not detected in either Epn1 or Med1 cell lines.

These results indicate that VPA treatment induced cognitive deficits in rats and that this was associated with a reduction in hippocampal NSCs both *in vivo* and *in vitro*. In addition, VPA reduced BDNF and Notch1 expression in the hippocampus. Moreover, VPA induced a decrease of levels of Notch1, Sox2 and nestin gene expression in hippocampal NSCs. The present study also reveals that antiproliferative effect of VPA was associated with an increase of Notch1 expression in SHSY5Y. However, this action of VPA appeared to have no link with Notch1 expression in Epn1 and Med1 cell lines.

ABBREVIATION

AMDA   Metalloproteinase

AML   Acute myeloid leukemia

AMPA   α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

BDNF   Brain derived neurotrophic factor

BrdU   5-bromo-2-deoxyuridine

CA   Cornu amnonis

CaMK II   Ca2+/calmodulin-dependent kinase II

cAMP   Cyclic adenosine monophosphate

cDNA   Complementary deoxyribonucleic acid

CNS   Central nervous system

CoA   Coactivators

CoR   Corepressors

CREB   cAMP response element binding

CR   Conditional reaction

CS   Conditioned stimulus

CSF   Cerebrospinal fluid

ER   Endoplasmic reticulum

DCX   Doublecortin

DEPC   Diethylpyrocarbonate

DNA   Deoxyribonucleic acid

dNTP   Deoxyribonucleoside triphosphate

EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
FBS	Fetal bovine serum
FL	Familiar location
GABA	γ-Aminobutyric acid
GABA-T	γ-Aminobutyric acid transaminase
GAD	Glutamic acid decarboxylase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GFP	Green Fluorescent Protein
GMT	Greenwich Mean Time
Hes1	Hairy and enhancer of split 1
Hes5	Hairy and enhancer of split 5
HDAC	Histone deacetylase
HPLC	High-performance liquid chromatography
HIV	Human immunodeficiency virus
5-HT	5-hydroxytryptamine
IgG	Immunoglobulin G
i.p.	Intraperitoneal injection
ITL	Inter-trial interval
KDa	Kilo dalton

Kuz	Kuzbanian
LIF	Leokocyte inhibitory factor
LTD	Long-term depression
LTP	Long-term potentiation
MAML1	Mastermind-like proteins
mRNA	Messenger ribonucleic acid
MTC	Medullary thyroid cancer
NCAM	Neural cell adhesion molecule
NETs	Neuroendocreine tumours
NECD	Notch extracellular domain
NFL	Full length Notch receptor
NICD	Notch intracellular domain
NL	Novel location
NMDA	N-methyl-D-aspartic acid
NOL	Novel object location
NSC	Neural stem cell
PAG	Periaqueductal gray
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PNET	Primitive neuroectodermal tumour
PNS	Peripheral nervous system
qPCR	Quantitative real-time reverse transcription polymerase

RAM	RBP-JK-associated module
RBP-J	Recombination signal binding protein for immunoglobulin kappa J region
RMP	Rostral migratory path
RMS	Rostral migratory stream
RNA	Ribonucleic acid
SSA	Succinic semialdehyde
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SCLC	Small cell lung cancer
SDS-PAGE	Sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis
SGZ	Sub granular zone
SVZ	Sub ventricular zone
TACE	Tumour necrosis factor-α converting enzyme
TAD	Tranactivation domain
TAE	Tris-Acetate ethylenediaminetetraacetic acid
TLE	Temporal lobe epilepsy
TBST	Tris-buffered saline tween-20
US	Unconditioned stimulus
VEGF	Vascular endothelial growth factor
VPA	Valproic acid
VZ	Ventricular zone

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

CHAPTER 4

LIST OF TABLES

Chapter 1

Literature review

1. Introduction

It has been suggested that many chemicals and therapeutic drugs may have cognitive side-effects. These may be longstanding and debilitating for patients on these medications. For instance, chemotherapeutic drugs can cause memory loss and other cognitive problems (Schagen et al., 2006, Tannock et al., 2004). More recently, it has been suggested that the commonly used antiepileptic drug, valproic acid (VPA), may have both cognitive effects and direct adverse effects on the adult hippocampus (Aldenkamp, 2001, Gualtieri and Johnson, 2006). This project investigated the nature and cause of these effects.

1.1 Valproic acid

The synthesis of valproic acid (VPA) was first reported by Burton in 1882 as a clear, colourless, eight-branched chain fatty acid which derived its name from chemical name of 2-propylvaleric acid (Fig. 1.1) (Blaheta and Cinatl, 2002, Henry, 2003). VPA is an oily liquid at room and body temperature, has high solubility in organic solvents but low solubility in water (Henry, 2003). In 1962, Carraz and colleagues investigated whether antiepileptic drugs were highly soluble in VPA instead of water or the usual organic solvents. It was discovered that VPA on its own was a potent anticonvulsant. This led to the therapeutic potential of VPA as an anticonvulsant, with the first epilepsy trials for VPA in humans reported the following year. VPA was approved for the treatment of epilepsy in France in 1967 and by the USA in 1978 (Blaheta and Cinatl, 2002, Henry, 2003, Lagace et al., 2004).
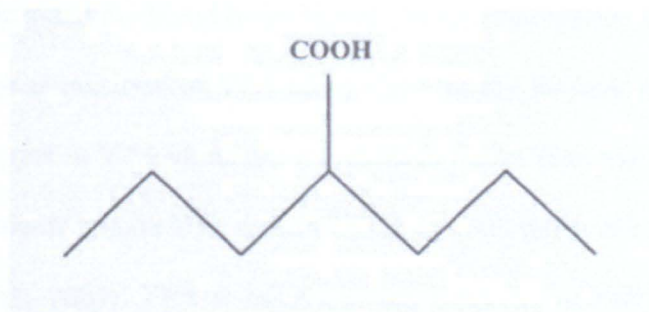
Fig 1.1 Chemical structure of VPA (Adapted from Blaheta and Cinalt 2002).

1.1.1 Properties of VPA

VPA administered orally is absorbed rapidly and effectively (96% to 100%) across intestinal mucosal membranes without any effects of first-pass metabolism in humans (Lagace et al., 2004). After oral administration, VPA serum concentration maximizes approximately 1-3 hours. In humans, VPA has a half-life of between 13 and 18 hours. However the half-life is dramatically different between species, for instance, 0.6 hours in rhesus monkeys, 0.8 hours in mice, 2.5 hours in rats and 9 hours in cats. The lipid soluble portion of VPA is distributed minimally into tissues by passive diffusion across membranes because VPA is highly ionized into a carboxylate moiety, valproate. VPA (70-94%) is bound to plasma proteins, albumin, but a low proportion of VPA is bound to albumin in elderly patients and pregnant women. The concentration of VPA reaches its peak within minutes in the cerebrospinal fluid (CSF) of rodents and monkeys after intravenous or intraperitoneal injection (Lagace et al., 2004). Studies in humans show a concentration of VPA in brain tissue and CSF less than 30% of the total serum concentration. The brain VPA concentration depends on the unbound plasma fractions (Henry, 2003). However, VPA can reduce epileptic symptoms within less than 20 minutes, since VPA reaches the brain rapidly. In the central nervous system (CNS), the

3

choroid plexus and the blood-brain barrier regulate the uptake and elimination of VPA. The elimination of VPA takes place mostly through the blood-brain barrier. The rate of VPA elimination in CNS is higher than the rate of uptake; therefore patients require high doses of VPA to accomplish effective treatment (Lagace et al., 2004). VPA is largely metabolized in the liver, consequently only a very small amount is found in urine (Henry, 2003, Lagace et al., 2004).

1.1.2 Mode of VPA action

1.1.2.1 VPA effects on epilepsy

Epilepsy is a disorder of the central nervous system characterized by recurrent seizures unprovoked by an acute systemic or neurogenic insult. A seizure is the sequence of a sudden imbalance between the excitatory and inhibitory inputs to a hyperexcitable network. It has been reported that pathological stimulation of seizures enhances neurogenesis and migration of type-3 progenitor cells in the hippocampal dentate gyrus in rodent models (Jessberger et al., 2005). There are several different types of epilepsy including temporal lobe epilepsy (TLE) which is the most frequent cause of partial seizures (Rho et al., 2004, Shin and McNamara, 1994). Experimental studies have led to two theories regarding the cellular network changes which cause the hippocampus (the most common sites of origin of partial seizures) to become hyperexcitable. The first theory suggests that a selective loss of interneurons decreases the normal inhibition of the dentate granule cells, an important group of principal neurons. Therefore, GABA-mediated inhibition of dentate granule cells is decreased. The second proposes that synaptic reorganization follows injury and creates recurrent

4

excitatory connections, via axonal sprouting, between neighbouring dentate granule cells. This investigation proposes an increase in hyperexcitation causing seizure (Shin and McNamara, 1994, Rho et al., 2004). Anticonvulsants can function to inhibit seizure by increasing synaptic GABA, by prolonging the open time of the chloride channel or by enhancing binding of GABA to GABA receptor, thus increasing the frequency of chloride channel openings (Treiman, 2001).

Researchers have discovered that one of the anti-epileptic therapeutic mechanisms of VPA is to increase levels of the inhibitory neurotransmitter γ-aminobutyric acid (GABA). As a consequence, acute uptake of VPA increases GABA in the brains of rodents and in the plasma and CSF of humans. There are also studies showing that GABA signalling potentiated to prevent seizures (Lagace et al., 2004). In contrast, GABA antagonists which reduce GABAergic inhibition in the cerebral cortex cause seizures. VPA increases GABA by inhibition of GABA transaminase (GABA-T) which degrades GABA to succinic semialdehyde (SSA). Furthermore, VPA may stimulate glutamic acid decarboxylase (GAD) to transform glutamic acid to GABA (Lagace et al., 2004). Action of VPA also depresses aspartate release within brain. Moreover, one of the primary actions of VPA have been postulated to block Na^+-channels and Ca^+-channels (Kwan et al., 2001).

1.1.2.2 VPA effects on cell proliferation and differentiation

As described above, VPA has been recognized as a long-term anti-epileptic drug. More recently, it has been shown to be associated with inhibition of histone deacetylase (HDAC) enzymes. However, VPA inhibition of HDACs in human has not been found yet (Blaheta and Cinatl, 2002, Kostrouchova et al., 2007, Lagace et al., 2004). The important function of HDAC enzymes is removal of acetyl groups from an ε-N-acetyl lysine amino acid on histone proteins. Deacetylation adds a positive electric charge to the lysine amino acid, which increases the affinity between histones and DNA. This results in the inhibition of transcription which requires a separation of the DNA strands and loosening in the DNA/histone attachment (Blaheta and Cinatl, 2002). A separate family of enzymes, the histone acetyl transferases (HAT), adds acetyl groups to the histone proteins, loosen the binding of DNA to histone proteins and so promote transcription. Both *in vitro* and *in vivo*, transcriptional regulation is a crucial event in determining cell function, including, growth arrest, differentiation, and apoptosis. Interestingly, VPA directly inhibits HDACs 1-5 and 7, causing hyperacetylation and so weakening the association of histones with DNA. Consequently, gene transcription is enhanced. Surprisingly, this enhanced transcriptional activity, specifically up regulates the expression of genes associated with cell differentiation, cell cycle arrest and apoptosis (Blaheta and Cinatl, 2002, Kostrouchova et al., 2007, Lagace et al., 2004). For example, studies have confirmed that VPA causes an increase in the expression of p21, a cyclin-dependent kinase inhibitor, which induces cell cycle arrest in both normal and cancer cells. In line with this, the site of VPA antiproliferative effects within the cell cycle is postulated to be in the mid G1

phase. VPA is also capable of inducing neuronal differentiation and suppressing glial differentiation by up-regulating neurogenic transcription factors including Neuro D in neural progenitor cells (Li et al., 2005, Martin and Regan, 1991, Kostrouchova et al., 2007, Hsieh et al., 2004). In neural stem cells, accordingly, VPA promotes neural production but attenuates glial formation (Laeng et al., 2004). *In vitro*, VPA reduces the number of neural cells migrating during epithelial-mesenchymal transitions, decreases their proliferation, and increases cell-cell adhesion. This causes a slowing of motility of neural cells and a change in neural cell morphology, making them more rounded (Fuller et al., 2002).

VPA has been demonstrated as a potential novel therapeutic agent, due to its HDAC inhibitor effects, on transcriptional regulation in cancer cells (Gottlicher et al., 2001). Transcription, which is the primary target of HDAC inhibitors such as VPA, plays an crucial role in determining cell fate in response to treatment (Chen et al., 2006). Strikingly, VPA decreases cell proliferation of small cell lung cancer, human thyroid cancer and endometrial tumour cells by inducing cell cycle arrest and stimulating apoptosis (Platta et al., 2008, Shen et al., 2005, Blaheta et al., 2005). Similarly in neuroendocrine cancers, VPA induces cell cycle arrest, causing an inhibition of cell growth of carcinoid cells (Greenblatt et al., 2007). Therefore, VPA has been investigated as a new therapeutic approach for the treatment of cancers of the nervous system. This novel clinical use of VPA may cause milder side effects in comparison with the normal regimen of chemotherapy with or without radiotherapy.

1.1.2.3 VPA effects on brain development

As the use of VPA as an effective treatment for epilepsy became more wide spread it became apparent that VPA, if taken during pregnancy, could act as a teratogen. Specifically, it became associated with an increased incidence of neural tube defects. This ability to induce neural tube effects is found in both human and mouse embryos but the mechanism remains unknown (Kultima et al., 2004). The number of gross malformations induced by VPA is low (<1%); however recently, more subtle long-term cognitive and behavioural effects of VPA exposure *in utero* have been found. A study of exposure to VPA of children *in utero* showed a higher incidence of minor morphological abnormalities but some children had major cognitive and behavioural problems including autism syndrome (Viinikainen et al., 2006). During brain development, moreover, prenatal VPA treatment leads to alterations of the development of the hippocampal formation by interrupting lamination and cell migration (Manent et al., 2007). It is likely that these development effects of VPA are associated with its ability to inhibit cell proliferation and migration.

1.1.2.4 VPA effects on the adult brain including cognition

1.1.2.4.1 Human studies

Epilepsy develops in 1-1.5 % of the population and VPA is widely used as a long-term epileptic therapy ($>10^4$ individuals in the UK) (Wallace et al., 1998) and this has led to an investigation into its cognitive effects in adults. Several studies have reported that patients treated with VPA develop mild to moderate cognitive deficits (Aldenkamp, 2001, Easterford et al., 2004, Gualtieri and

Johnson, 2006, Jamora et al., 2007, Hommet et al., 2007). VPA caused impairment of memory function, psychomotor function and mental speed (Hommet et al., 2007). A study of 50 patients taking VPA has demonstrated that 6% of the patients develop Parkinsonism and cognitive impairment (Easterford et al., 2004). Six patients suffered from insidious cognitive impairment and Parkinsonism while being with treated VPA (Masmoudi et al., 2006). More recently, a side effect such as Parkinsonism appeared in six (5.04%) of 119 patients who had been on long-term high doses of VPA (Jamora et al., 2007). In contrast the previously mentioned studies at least one study has found that administration of VPA monotherapy in patients does not show cognitive and neurological symptoms (Gillham et al., 1990). In epileptic patients, withdrawal of VPA has been found to alleviate these cognitive deficits; however, it takes at least several months, after discontinuing drug treatment, to resolve these adverse effects (Goldberg and Burdick, 2001, Hessen et al., 2006, Masmoudi et al., 2006, Hommet et al., 2007). Although, VPA has been generally considered to have a good safety profile for several years, the drug-induced cognitive dysfunction and Parkinsonism must be considered in all patients treated with VPA. Recently, VPA is increasingly being used as a mood stabiliser in bipolar disorder which will increase the number of patients taking this drug (Rao et al., 2007).

Assessment of the cognitive effects of VPA is complicated by the fact that epilepsy, the prime reason for prescribing VPA, also causes cognitive impairments and changes to hippocampal function (Jessberger et al., 2007). For this reason an animal model of the behavioural effects of VPA independent

of pre existing conditions would be useful in understanding the effects of this drug.

1.1.2.4.2 Animal studies

Few studies have looked at cognitive effects of VPA in animals. One group has reported that animals receiving a single dose of 250 mg/kg VPA showed cognitive impairment in terms of long-term memory deficits using a passive avoidance test (Balakrishnan and Pandhi, 1997, Pandhi and Balakrishnan, 1999).

A more recent study treated adult rats with 300 mg/kg VPA injected i.p. twice daily for 14 days (Hsieh et al., 2004). This regime produced a significant decrease (~50%) in the numbers of BrdU positive cells in neurogenic region of the hippocampus in comparison to control animals. The same study treated neural progenitor cells from the adult hippocampus *in vitro*. Exposure to 1 mM VPA for 24 hours substantially decreased the number of BrdU positive cells from 50% to 20% compared to controls (Hsieh et al., 2004). Furthermore, VPA treatment increased apoptosis of the hippocampal progenitor cells as evaluated by staining propidium iodide. This result was dose-dependent with treatment with 0.3 and 1 mM VPA for 2 days producing approximately 2% and 7% apoptotic cells respectively. However, the amount of cell death in the lower dose was only slightly higher than untreated cells. These results show that VPA has similar effects on proliferation and cell death in adult neural progenitor cells as has been described for cancer cells.

Hsieh et al also postulated that VPA has the potential to affect chromatin modification which is important in determining stem cell fate between self-renewal and differentiation (Hsieh et al., 2004). Strikingly, the mechanism of VPA action leads to increases in the level of a neural transcription factor (NRSF/REST) which activates gene expression to induce neuronal differentiation (Hsieh et al., 2004, Jessberger et al., 2007).

An animal study has reported that the serum concentrations of rats, after administration subcutaneously with 400 mg/kg of VPA for 1, 3, 6 and 9 hours, were approximately 2.5, 1.2, 0.4 and 0.3 mmol/L, respectively. Moreover, the serum concentration almost dropped to zero within 24 hours (Ubeda et al., 2002).

In addition, metabolites of VPA have been found to accumulate in the hippocampus (Masmoudi et al., 2000) suggesting that this brain region may be particularly affected by this drug. The results of this investigation suggest that VPA may be having a direct effect on hippocampal neurogenesis and this might be the mechanism for the cognitive deteriorations found in human studies. However, behavioural testing was not done in this investigation and it remains to be determined to what extent changes in neuronal stem cells proliferation and differentiation correlate with behavioural changes after VPA treatment.

1.2 Anatomy of hippocampus

The hippocampal formation comprises the hippocampus, the presubiculum, the subiculum, and the dentate gyrus (Kempermaan, 2006, FitzGerald et al., 2007). The hippocampus is located at the floor of the inferior horn of the lateral ventricle and its external surface is continuous through the subiculum with the parahippocampal gyrus. In coronal section, the appearance of the hippocampus is an s-shape. The gross anatomy of the human hippocampal formation and fornix is similar to a sea horse; from which it derives its name - hippocampus. The anterior extremity of hippocampus forms the *pes hippocampi* which resemble a paw (Wikinson, 1998). Part of the hippocampus is also called Ammon's horn or cornu ammonis (CA) and is subdivided into four CA fields: CA1, CA2, CA3 and CA4 (Kempermaan, 2006, FitzGerald et al., 2007, Anderson et al., 2007). CA3 and CA2 fields (region inferior) are a larger area compared to CA1, also called region superior. CA3 and CA2 pyramidal cell bodies are large and quite variable but CA2 is not innervated by mossy fibers. CA1 pyramidal cells are more substantially homogeneous and smaller than CA3 cells. CA4 is defined as the deep layer of dentate gyrus (polymorphic layer) (Anderson et al., 2007). The thin white matter on the ventricular surface of hippocampus is known as the alveus in which these fibres run medially to form the fimbria of fornix. After the hippocampus terminates beneath the splenium of corpus collosum, the fimbria continues as the crus of fornix, forming commissural and associational connections with the limbic and cortical systems (Con, 2003).

12

The hippocampus is recognized as a trilaminar cortex. The external layer, called molecular layer, continues with the molecular layers of the dentate gyrus and neocortex. The pyramidal layer, the middle layer, consists of large pyramidal shape neurons which are the principal cells of hippocampus. The internal layer, called the polymorphic layer, is beneath the alveus. The dentate gyrus also has three layers (molecular layer, granule cell layer and plexiform layer) and covers the distal tip of the CA region. Granule cells are the principal cells of the middle layer of the dentate gyrus which is consequently named a granule cell layer. The hilus of the dentate gyrus is continuous with the polymorphic layer of area CA3 (FitzGerald et al., 2007, Kiernan, 1998).

The perforant pathway which projects from the entorhinal cortex contains the largest afferent fibres of the hippocampus. These afferent fibres terminate on the dendrites of the granule cells of the dentate gyrus in the molecular layer which surrounds the external surface of the dentate gyrus. The axons from the dentate gyrus granule cells are known as mossy fibres, project to the pyramidal cells in the CA3 field (Figure 1.2). These CA3 pyramidal cells project axons into the fimbria; they give off *Schaffer* collaterals which run to CA1 where the third synapse in this circuit is found. CA1 pyramidal cells project axons to synapse on the subiculum. The subiculum then returns a projection to the entorhinal cortex. The subiculum is the other source of efferent fibres, via the alveus, to end in the fimbria. The largest efferent pathway of hippocampus is the fornix which originates from the fimbria. In addition, the efferent pathway from the hippocampus is also a projection via the entorhinal cortex to the

association areas of the neocortex, the diencephalon, and brain stem (Kempermaan, 2006, FitzGerald et al., 2007, Kieman, 1998).
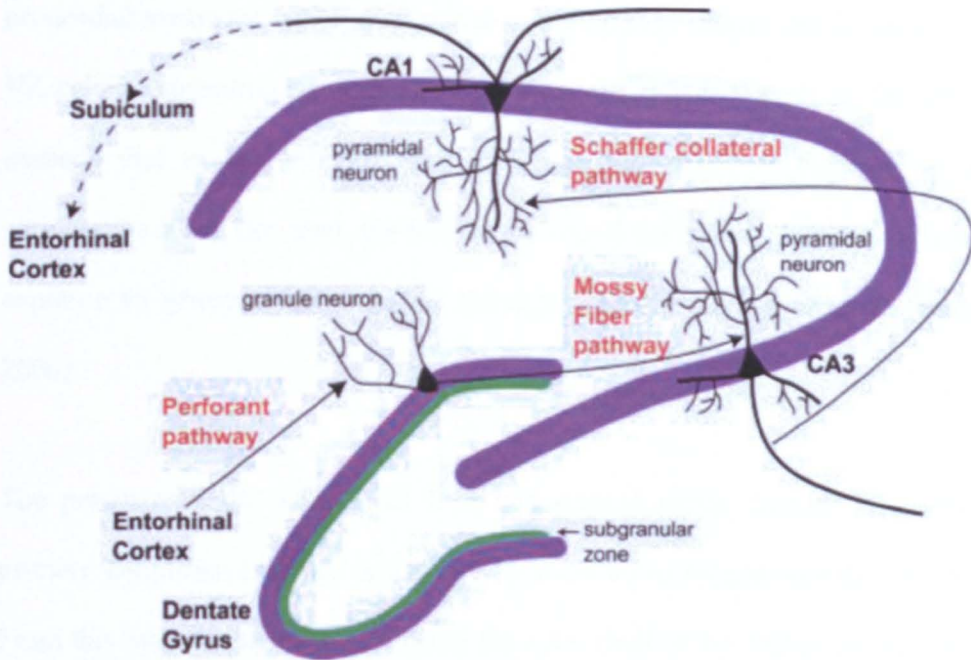
Figure 1.2 Diagram shows hippocampal circuits. CA1 and CA3 of the hippocampus are shaded purple. Subgranular zone (SGZ; shaded green) of the dentate gyrus (shaded purple) can generate new neurons throughout life. This pathway is essential for acquisition of memory and has three steps, called tri-synaptic circuit. Afferent fibers from the sensory association cortex project to the dentate gyrus via the enthorinal cortex which is called the perforant pathway. Then, mossy fibers are given off from granule cells to pyramidal cells in the CA3. After that, CA3 pyramidal cells project axons, which are known as Schaffer collaterals, to synapse with CA1 pyramidal cells. CA1 pyramidal cells return a projection to the entorhinal cortex via the subiculum. Abbreviation: CA1, cornu ammonis 1; CA3, cornu ammonis 3 (Adapted from McCaffery et al., 2006).

1.3 Development of hippocampal dentate gyrus and subgranular zone

Hippocampal development originates from the ventricular zone (VZ) in the dorsal forebrain. The VZ is derived from the neuroepithelial cells lining the primordial ventricles which give rise to a proliferative matrix just beneath the VZ called subventricular zone (SVZ). The proliferation of cells in the SVZ exceeds that in the VZ and after birth the VZ does not function as a germinative layer. However, the SVZ in the lateral walls of the lateral ventricle continue to generate new neurons throughout postnatal life (Kempermaan, 2006).

The precursor cells, which will form the dentate gyrus, migrate from the primary germinative matrix in the SVZ to form a second germinative matrix. From this matrix cells migrate to form the outer shell of the dentate gyrus. The secondary germinative matrix gives rise to a tertiary germinative matrix around E22. During the early postnatal period, the secondary matrix has nearly finished developing and is replaced by the tertiary matrix. This is most active during postnatal day 3 to postnatal day 10. The tertiary germinative matrix forms the hilus or plexiform layer of the dentate gyrus in the adult. The tertiary germinative matrix generates the inner layer of the granule cell layer. The newly generated granule cells migrate from this inner layer to the outer shell of the dentate gyrus. Consequently, the youngest granule cells are located internally and the oldest granule cells are located externally. The tertiary germinative matrix becomes increasingly confined to the subgranular zone (SGZ), from which the new neurons of adult hippocampal neurogenesis are continuously generated. This postnatal phenomenon (hippocampal adult neurogenesis) has recently generated significant interest (Kempermaan, 2006).

1.4 Memory and hippocampus

1.4.1 Memory

From prospective investigations, memory is formed and retrieved in three mains stages: encoding, storage and retrieval, respectively. Encoding or registration is receiving, processing and combining of received information. Storage is a creation of a permanent record of the encoded information. Retrieval or recall is calling back the stored information in response to some cue for use in a process or activity (Anderson et al., 2007). Memory can be divided into two types; short term and long term memory. Short term memory also known as working memory, has a strictly limited capacity and new information is temporarily stored for a certain period of time. Short-term memory allows recall for a period of seconds to a minute without rehearsal, but is not retained indefinitely (Fig. 1.3). By contrast, long-term memory has a much larger capacity and is capable of storing information for potentially unlimited duration (sometimes a whole life span) (Bear et al., 2007, FitzGerald et al., 2007). In human and non-human animals, working memory has an important role in intellectual function (Smith et al., 1996). The frontal cortex (particularly the dorsolateral prefrontal cortex) and the parietal lobe are essential for transient patterns of neuronal activity involved in short-term memory. While long-term memories are sustained by more stable and permanent changes in neural connections widely spread throughout the brain. Short-term memory is consolidated and transferred into long-term memory by the hippocampus (Kempermaan, 2006).

16

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repetition    │    Short-term memory    │───────► forgetting
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                           │ Encoding                │
retrieval                  │ consolidation           │
                           ▼                         │
         ┌──────────────────────────────┐           │
         │      Long-term memory        │───────────┘
         │    (days, months, years)     │
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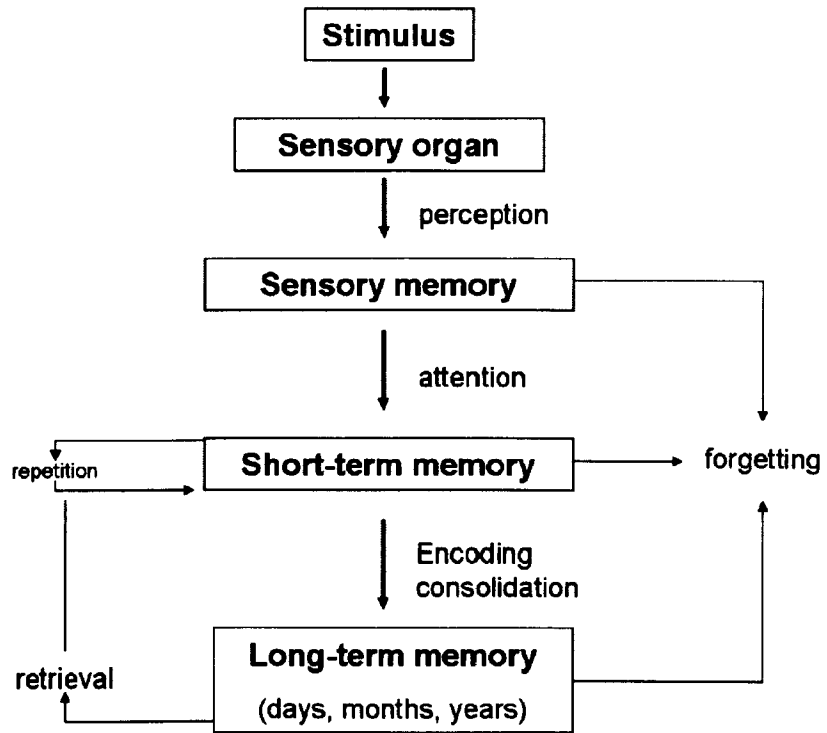
Figure 1.3 Short-term memory. Short-term memory is a system which is required to temporarily store and manage new information. Short-term memory is involved in the selection, initiation, and termination of information-processing functions such as encoding, storing, and retrieving data. Several recalls/rehearsals of short-term memory are needed for long-term memory to last long for a year (Modified from http://www.aboutmind.com/memory-brain-neurons-1.shtml).

Long-term memory

Explicit (declarative)   Implicit (procedural)

Episodic   Semantic   skills   Priming   conditioning
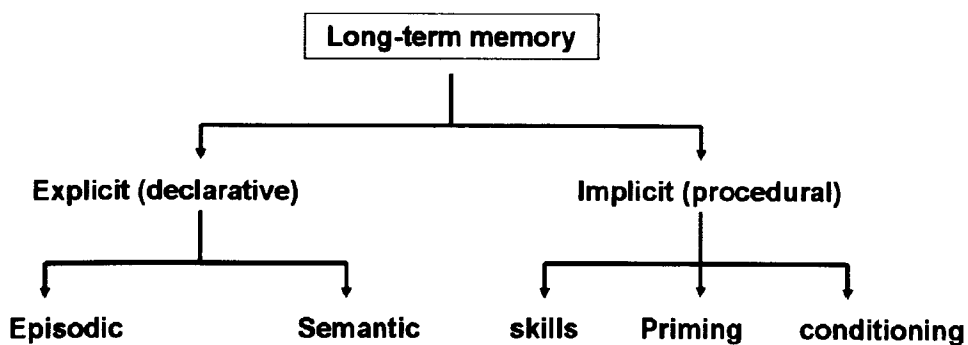
Figure 1.4 Long-term memory is divided into declarative and procedural memories. There are two types of declarative memory: semantic (i.e. words, ideas, concepts) and episodic (i.e. biographical events) memory. Procedural memory is composed of skills, priming and conditioning (Modified from Milner et al., 1998).

Long-term memory is divided into declarative and procedural memories (Fig. 1.4). Declarative memory, which is also called explicit memory, involves the knowledge and recall of facts and events that can be consciously recollected. There are two major sub-types of declarative memory; semantic and episodic memory. Semantic memory is the memory of meanings, understanding and knowledge. Episodic memory, on the other hand, involves in information specific to a particular context, such as a time and place (FitzGerald et al., 2007). The formation of new episodic memories requires the medial temporal lobe, a structure that includes the hippocampus. In addition, the hippocampus is believed to be involved in spatial learning and declarative learning. In contrast to declarative memory, procedural or implicit memory is primarily described as the memory for learning skills of performed motor tasks (i.e. riding a bicycle) and mental activities (i.e. using common vocabulary and grammatical rules of language), which does not depend on the conscious recall of information. This

memory is stored gradually by practice and repetition (FitzGerald et al., 2007, Kieman, 1998). Procedural memory involved in motor learning depends on the cerebellum and basal ganglia (Lynch, 2004).

1.4.2 Long-term potentiation, long-term depression and memory

Long-term potentiation (LTP) is a process in which synapses are strengthened. After intense stimulation of the presynaptic neuron, the amplitude of the post-synaptic neuron's response increases. Nerve impulses trigger release of glutamate, from the pre-synaptic cell. Glutamate is the most abundant excitatory neurotransmitter in the mammalian nervous system. Glutamate receptors in the opposing post-synaptic cell, such as the NMDA receptor, bind glutamate and then these receptors are activated. In the postsynaptic neuron, this stimulus causes sufficient depolarization to evacuate the magnesium ions that are blocking the NMDA receptor, thus allowing large numbers of calcium ions to enter the dendrite (Lynch, 2004, Anderson et al., 2007, Bear et al., 2007). This rise in calcium activates protein kinase C and Ca^{2+}/camodulin-dependent protein kinase II (CaMK II). Either protein kinase C or CaMK II phosphorylate the AMPA receptors to remain open longer. Additionally, new AMPA receptors are delivered and inserted into the postsynaptic membrane. As a result, the postsynaptic neurons become further depolarized, thus contributing to LTP (Bear et al., 2007, Anderson et al., 2007).

In contrast to LTP, long-term depression (LTD) may be regarded as a complementary mechanism to LTP. In the hippocampus, LTD plays an important role to bring synapses that have been potentiated back to an ordinary

level in order to have synapses available for new information. LTD develops when a presynaptic neuron is active without the postsynaptic neuron's being subjected to strong depolarization, as it is with LTP. This lack of association between the two neurons raises the concentration of calcium in the postsynaptic neuron, but much less than in LTP (Duman et al., 2001b, Duman et al., 2001a). Consequently, phosphatases are activated instead of CaMK II being activated. This enzyme dephosphorylates the AMPA receptors by removing certain phosphate groups from them. This depolarization of the AMPA receptor reduces the amplitude of the postsynaptic potential to the normal level and decrease the number of AMPA receptors during LTD. The mechanism of LTD is opposite to LTP showing that AMPA receptors are removed from the postsynaptic membrane to a reserve in LTP while these receptors are inserted in to the membrane in LTD (Bear et al., 2007, Anderson et al., 2007).

Changes in neuronal synapses are capable of being used to form the basis of learning and memory, and these changes are believed to be brought about by LTP and LTD. Several lines of studies show that LTP and LTD are correlated to memory. For instance, activation of NMDA receptors needed for synaptic plasticity in both LTP and LTD is involved in learning (Bear et al., 2007). Researchers have found that rats receiving an NMDA-receptor antagonist injected into the hippocampus spend a longer time finding the location of the hidden platform in the Morris water maze test, than normal rats (Lynch, 2004, Bear et al., 2007). This finding reveals that NMDA receptors are necessary for memory. In parallel with the role of the NMDA receptor in hippocampal LTP,

it has been found that CaMK II knockout mice shows deficits in hippocampal LTP as well as memory (Bear et al., 2007). In short, NMDA receptors are essential for hippocampal LTP and LTD as well as learning and memory.

1.4.3 The hippocampus and spatial memory

Assessment of preference between a new and a familiar object or picture can be used to investigate recognition memory using the visual paired comparison task. In both human and animal studies, localised damage to the hippocampus impairs memory (Con, 2003). For example, Clark and his colleagues (2000) have demonstrated that direct hippocampal damage in rats produced recognition memory impairment. In cognitive psychology and neuroscience, spatial memory is a form of working memory, which is used to define position within the environment. In non-humans, spatial memory is responsible for describing the environment, including distances and directions such as learning the location of food at the end of the maze (Abrahams et al., 1997). In humans, similarly, spatial memory is needed for navigating around a familiar city. It is still controversial how spatial memories are formed and then stored in a spatial map both in human and animals. Prior to spatial memories being formed; sensory information (particularly vision and propioceptive) of environments is gathered and processed in CA1. Several studies have demonstrated that the hippocampus is strongly involved in spatial memory and spatial information (spatial mapping) is particularly processed in the right hippocampus of the brain (Abrahams et al., 1997, Nunn et al., 1999). Accordingly, the hippocampus is also considerably implicated in spatial memory (Morris water maze) and recognition memory, but spatial memory requires more of the

hippocampus than recognition memory (Broadbent et al., 2004). Moreover, investigation of hippocampal lesions in rodents and primates has produced impairments of spatial memory. Human, patients who have had the right hippocampus removed, show deficits in object location memory (Nunn et al., 1999, Broadbent et al., 2004). Similarly, spatial memory deficits have been demonstrated in patients who have right hippocampal injury or resection (Abrahams et al., 1997).

The hippocampus is not a storage area of memory, but functions as the gateway to memory. It prepares and transfers information (i.e. short-term memory) before long-term memory is stored permanently in neocortical areas (Kempermaan, 2006). The hippocampus converts information into explicit and implicit memory (FitzGerald et al., 2007, Kieman, 1998). John O'Keefe and his colleagues have demonstrated that many neurons in the hippocampus selectively exhibit a high rate of firing when an animal is in a specific location in an environment corresponding to the cell's place field. These neurons, which are distinct from other neurons with spatial firing properties, are called place cells (Anderson et al., 2007, Bear et al., 2007). These place cells in the hippocampus are specifically active in this spatial navigation with humans as well as with rats. Interestingly, alteration of the spatial environment demonstrates that place cells are responsive to new location more than old location (Bear et al., 2007). Further evidence from transgenic studies show that the plasticity inherent in long-term place field stability depends on NMDA receptors. For example, mutant mice with blocked NMDA receptors take longer time than control mice to find a hidden platform in the Morris water

22

maze (Anderson et al., 2007). Additional studies have reported that NMDA receptors are required in consolidation of spatial memory and AMPA receptors in involved in the retrieval of this memory (Liang et al., 1994).

1.5 Neural stem cells

The term stem cell describes undifferentiated cells that have unlimited proliferative capacity, long-term self-renewal and pluripotency. Stem cells may be pluripotent – in which case they can give to all cell types (e.g. embryonic stem cells) or can be multipotent and be able to give rise to a limited number of cell types but not all body tissues (Rao, 2006). Self-renewal is considered to be the most important definition of stemness and is defined as the ability to go through numerous cycle of cell division while maintaining the undifferentiated state and not becoming senescent (Kempermaan, 2006). Self-renewal is also important to sustain stem cell developmental potency and proliferative capacity (Rao, 2006). Embryonic stem cells are true stem cells in that they are pluripotent and show unlimited capacity for self-renewal. In contrast, adult stem cells would be better described as progenitor cells because their capabilities for self-renewal are not unlimited and their degree of potency can be multipotent, bipotent or unipotent (Kempermaan, 2006). The most primitive cells, cell produced by the first few division of the fertilized egg, are totipotent that can produce all organisms and tissues. The potency can be limited within stem cell lineage; therefore, stem cells may be pluripotent or multipotent (generating various cell types, but not entire system), oligopotent (capability of differentiating into a few cell types) or unipotent (producing only one cell type) (Rao, 2006).

Embryonic stem (ES) cells are derived from the inner cell mass of the blastocyst. In cell culture, ES cells are capable to produce functioning neurons and glial cells. Adult stem cells which also known as somatic stem cells generate another cell like itself and also divide and create a cell more differentiated than itself (Kempermaan, 2006). Stem-like cells have been discovered in the embryonic CNS and PNS. The neural stem cells are derived from neuroectoderm and are multipotent (Kempermaan, 2006). In adult rodent, primate and human, interestingly, neural stem cells (NSCs) can be most easily isolated from two known neurogenic regions; the sub ventricular zone (SVZ) of the lateral walls of the lateral ventricle and SGZ of the dentate gyrus (which produces granule cells in the hippocampus) (Potten et al., 2006). Recently, stem cells have been shown to be a more widespread feature of the adult nervous system than previously considered as they have been found in the non-neurogenic areas such as the spinal cord and neocortex (Potten et al., 2006).

During embryonic development, symmetric division of stem cells within the neuroepithelium forming the neural tube can produce either two identical stem cells or by asymmetrical division create one stem cell and one transit-amplifying or transit amplifying progenitor cell (Fig. 1.5) (Kempermaan, 2006, Potten et al., 2006). Transit amplifying progenitor cells are rapidly dividing progenitors which lack the self-renewing ability of their stem cells but which are the majority of dividing progenitor in the tissue (Diaz-Flores et al., 2006). In adult tissues, only multipotent progenitor cells rather than pluripotent or totipotent cells are found. As in embryonic tissues these can divide symmetrically to generate two identical daughters which are similar to the

24

mother cell or divide asymmetrically to produce two daughter cells in which one is different from the mother cell while the other retains the characteristics of the mother cell (Kempermaan, 2006, Potten et al., 2006). Additionally, terminally symmetric division of progenitor cells can also generate two identical cells that are both different from the mother cell and then these progeny differentiate into neurons (Kempermaan, 2006).
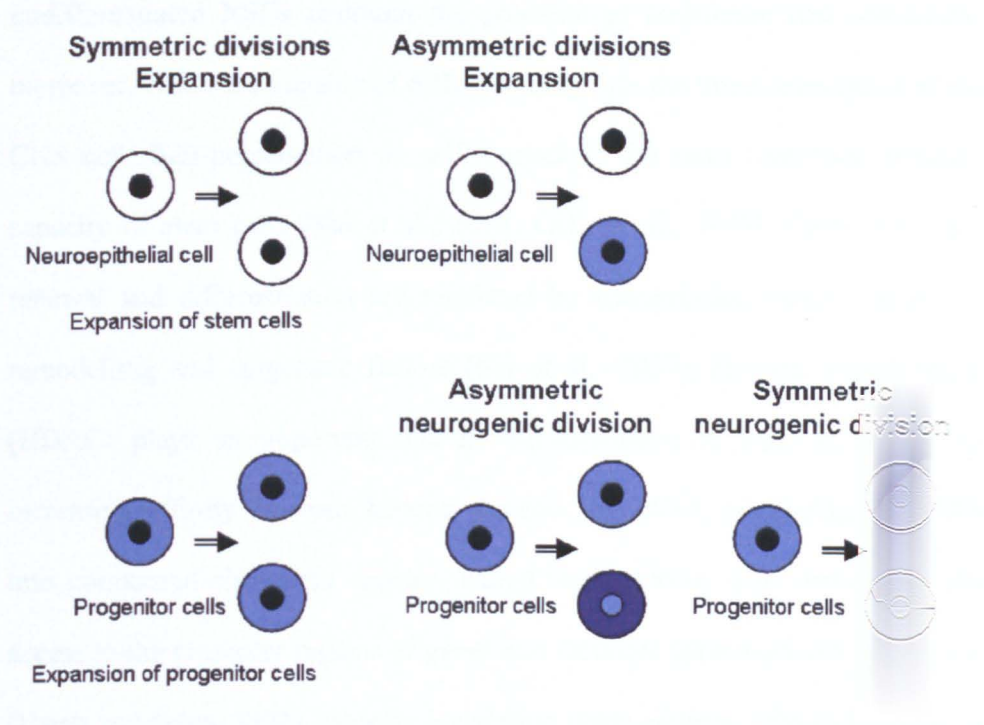
Figure 1.5 Symmetric and asymmetric divisions of stem and progenitor cells. The principal fundamental of stem cells is self-renewal. Accordingly, division of every stem cell has to produce at least one new stem cell. Asymmetric division generates one new stem cell progeny and one progenitor cell. Stem cell properties are limited in progenitor cells, especially self-renewal. Expansion of progenitor cells can produce two differentiating progenies (Adapted from Kampermann, 2006).

1.5.1 Neural stem cell self-renewal

Currently, two main neurogenic regions, SGZ and SVZ have been identified which continue to produce neurones and glia throughout life. In addition some non-neurogenic regions, i.e. the spinal cord, have been found to contain adult NSCs which appear to be quiescent in normal tissue. NSCs in the mammalian CNS have been extensively used to investigate a model of the molecular mechanisms that control cell-fate specification (Hsieh and Gage, 2004). The undifferentiated NSCs maintain the potential to proliferate and self-renew, moreover, NSCs are capable of differentiating into the three main types of the CNS cell. Self-perpetuation or self-renewal is the most important intrinsic capacity of stem cells (Shi et al., 2008, Galli et al., 2003). Stem cells self-renewal and differentiation are regulated by transcription factor, chromatin remodelling and epigenetic factors (Shi et al., 2008). Histone deacetylation (HDAC) plays an important role in the regulation of gene activation by increasing affinity between histone proteins and DNA, packaging the DNA into condensed chromatin regions, called nucleosomes. This activity blocks access to the promoter regions of genes and facilitate transcriptional repression (Hsieh and Gage, 2004). Histone acetylation is the addition of acetyl groups in histone proteins; consequently, the interaction between the positively charged histone tails and the negatively charged phosphate backbone of DNA is decreased, causing relaxation of the nucleosomes. This relaxation enables RNA polymerase and transcription factors to access transcriptional regions, thus resulting in transcriptional expression (Hsieh and Gage, 2004). Treatment of adult NSCs with HDAC inhibitors, i.e. mood stabilizers and anti-epileptics,

induced neuronal differentiation by activating REST-regulated neuronal-specific genes (Shi et al., 2008).

1.5.2 Stem cell niche

The immediate environment of stem cells, called the stem cell niche, has been postulated to be essential for controlling the behaviour of stem cells. This local environment influences the stem cell fate as stem cells are given signals from the niche to determine proliferation and differentiation into different types of progeny. During development of the mammalian brain, neural stem cells are generated and reside in the ventricular zone (VZ) and the SVZ, whereas there are two specific areas, the SVZ and the hippocampus of the dentate gyrus, which continue to generate stem cells in the adult brain. These regions have been found to contain many extracellular molecules and growth factors, i.e., EGF and FGFs. It is interesting that the region-specific niche molecules can control the ability of cell differentiation in a particular region. Stem cells isolated from non-neurogenic regions such as spinal cord are capable of making only glial after transplantation back into spinal cord although they can produce neurons and glial in culture. However, these spinal cord stem cells can generate neurons as well as glial in a neurogenic region such as SVZ. This indicates that the ability of stem cell proliferation and differentiation are influenced by environmental molecules within different adult CNS niches (Rao, 2006). The stem niche will be described in section 1.9.1.

1.5.3 Neural stem cells in the adult hippocampus

Proliferating cells have been shown in the SGZ of the dentate gyrus since 1960s (Altman, 1962); however, neurogenesis is still controversial (Abrous et al., 2005). On the basis of *in vivo* studies, two different precursor cell types have been found in the SGZ. Type-1 cells express glial fibrillary acidic protein (GFAP) and nestin and are equivalent to type B cells (the putative stem cells). Whereas type-2 cells are derived from type B cells and express nestin but not GFAP. These cells act as transient precursors and make new granule neurons (Abrous et al., 2005, Kempermaan, 2006). The type-1 cells are analogous to radial glia-like morphology and show astrocytic properties. In mice, approximately 50% of the GFAP-positive cells with radial glia morphology account for nestin-expressing cells (Kempermaan, 2006). Type-1 cells can be detected constantly with the proliferation marker bromodeoxyuridine (BrdU) for a long period of time. Type-1 cells produce type-2 and type-3 cells which are also called fast proliferating intermediate precursor cells. Type-2 cells are divided into subtypes. Type-2a cells are positive for nestin and negative for doublecortin neuronal marker (DCX) whereas type-2b cells are DCX positive (Kronenberg et al., 2003, Ehninger and Kempermann, 2008). Type-3 cells, show only little proliferative activity, are DCX-positive cells, but do not express nestin. Cells in type-3 stage migrate into the granule cell layer. Type-2b and type-3 cells, which show DCX expression, are equivalent to D cells or astrocyte-like precursor cells (Kempermaan, 2006, Ehninger and Kempermann, 2008). According to the classical morphology of stem cell biology, it can be concluded that type-1, type-2 and type-3 cells correspond to the stem cells,

transiently amplifying progenitor cells and the (migrating) neuroblasts, respectively (Kempermaan, 2006).

The stem cells in the SGZ are comparable to the astrocyte-like cells (B cells) that generate the transiently amplifying progenitor cells (D cells) which in turn give rise to migrating neuroblasts or neuronal progenitor cells (G cells) (Doetsch, 2003). In rodent, precursor cells can be isolated from the hippocampus by proliferating effect of FGF-2. These FGF-2 responsive precursor cells can be subcultured, frozen, thawed and cultured for over a period of time (Ray et al., 1993).

1.5.4 Neural stem cells in vitro

Neural stem cells can be isolated in cell culture to investigate the vital function such as the developmental potential and maintaining cell homeostasis. Neural stem cells can be grown either as free-floating neurospheres or as monolayer cultures under appropriate condition and in the presence of mitogens (Table 1.1). Growth factors and serum-free condition are used similarly in both methods (Kempermaan, 2006). These methods are beneficial models to investigate the action of epigenetic mechanisms (Hsieh and Gage, 2004). In monolayer culture, the putative stem cells are cultured in the presence of mitogens on coated surfaces as adherent cultures, whereas in neurosphere procedure, the putative stem cells are grown as cell suspension and form floating cluster of cells without adhering to the substrate. Lately, the neurosphere method has been exploited widely to rapidly produce a greater

number of dividing cells and it provides an easier method to prepare of single-cell suspensions (Kempermaan, 2006).

Table 1.1 Neural precursor cell cultures from the adult brain

	Neurospheres	Monolayers
Synonym	Aggregate cultures	Adherent cultures
	Cell culture on nonadherent	Grown on surface with
	surface as floating cells	adhesive substrates such as
		laminin, fibronectin
Growth factors		
Mouse	EGF and/or FGF-2	
Rat	EGF or FGF-2	FGF-2
Human	EGF and/or FGF2 and/or LIF	EGF, FGF-2, LIF

EGF, epidermal growth factor; FGF, fibroblast growth factor; LIF, leukocyte inhibitory factor (Modified from Kempermann, 2006).

The neurosphere culture is a selective technique in a heterogeneous primary culture. The undifferentiated NSCs are effectively selected and induced to an active proliferating state, as differentiating/differentiated cells rapidly die (Galli et al., 2003). Spherical clusters in cell suspension, called neurospheres, are generated from proliferating NSCs to form floating clusters of cells that adhere to each other (Galli et al., 2003, Kempermaan, 2006). Neurospheres can be generated from the astrocyte-like B cells (Rao, 2006). In neurosphere formation, two new identical NSCs are produced by symmetrical neurogenic division of NSCs, however, not all of the NSC progeny in neurospheres are identical to stem cells (Galli et al., 2003). The majority of proliferating cells are located on the outside of the three-dimensional neurospheres, in contrast, the differentiating cells are found within the spheres (Kempermaan, 2006).

NSCs

Primary
neurosphere

Dissociation

Selection   Death cells

Single cells

Self-renewal

Secondary
neurosphere

Differentiation
conditions

Multipotency   Neurons

Astrocytes

Oligodendrocytes
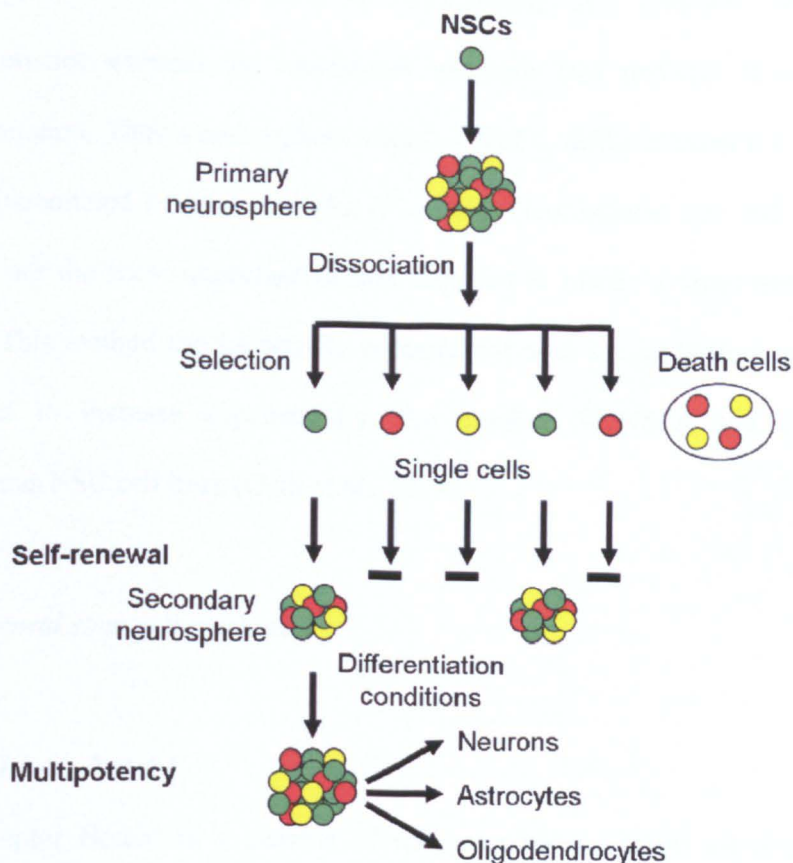
Figure 1.6 Schematic outline of NSC expansion in culture. Initially, a single NSC forms a clonal cluster of cells called neurosphere which contains a mixture of numerous NSCs, differentiating progenitors and differentiated neurons and glia. A neurosphere is dissociated and plated as single cells under serum-free conditions and in the sustained presence of mitogens such as EGF and/or FGF2. After neurospheres are dissociated, some of the cells die because of the stringent culture conditions; however, the surviving NSCs continue to proliferate and form more secondary neurospheres. NSCs in secondary neurospheres are capable of differentiating into neurons, astrocytes and oligodendrocytes by removing the mitogens (Modified from Galli et al., 2003; Kempermann, 2006).

Actually, 10-50% of the cells in neurospheres still conserve stem cell characteristics whereas the remainders are cells that undergo spontaneous differentiation. Thus a neurosphere contains NSCs, differentiating progenitors and differentiated neurons and glia (Fig. 1.6). Neurosphere size and time in culture are the most important factors required to obtain a large number of NSCs. This method can be serially repeated for over a year through multiple passages to increase exponentially, the number of NSCs and establish continuous NSC cell lines (Galli et al., 2003).

1.5.5 Neural stem cell markers

1.5.6.1 Notch1

The receptor Notch1 is a member of transmembrane proteins (Wang et al., 2004). Notch1 is involved in stem cell proliferation and also maintains neural precursor and stem cell proliferation (Breunig et al., 2007, Wang et al., 2004, Hitoshi et al., 2002). Moreover, Notch1 regulates NSC self-renewal and stem cell population *in vivo* and *in vitro* (Hitoshi et al., 2002, Potten et al., 2006). The receptor notch1 will be described in section 1.10.1.

1.5.6.2 Sox2

Sox2, which is a member of Sry related box family of transcription factor. Sox2 genes are defined as containing approximately 90% sequence homology of the male sex determining gene Sry. In common with Sox1, Sox2 and Sox3 which are a member of SoxB1 subfamily, it is needed to inducing pluripotent stem cells and maintaining self-renewal of undifferentiated embryonic stem

cells (Masui et al., 2007, Zhao and Daley, 2008, Kempermaan, 2006, Miyagi et al., 2006, Collignon et al., 1996, Avilion et al., 2003). Sox2 is expressed in embryonic stem cells in the inner cell mass of the blastocyst and exists in many multipotent cell lineages (Ferri et al., 2004). Besides embryonic stem cells, Sox2 is required for neural stem cell proliferation and maintenance of stemness in adult neurogenesis (Episkopou, 2005). Conversely, Sox2 heterozygous mice show a decrease of precursor cell proliferation in neurogenic regions. Furthermore, Sox2 expression is present in adult mouse neurospheres derived from neurogenic regions, for instance olfactory bulb and SGZ of the hippocampal dentate gyrus. This indicates that Sox2 is produced in adult NSCs (Brazel et al., 2005). Sox2 can be detected in neurospheres derived from either the embryonic CNS or the adult CNS (Ellis et al., 2004, Brazel et al., 2005). Moreover, Sox2-positive cells are capable of forming neurospheres and differentiating into neurons, astrocytes and oligodendrocytes in culture. Nevertheless, expression of Sox2 in adult mouse neural stem cells is greater than in embryonic stem cells (Kim et al., 2008). Taken together, these findings implicate Sox2 as a neural stem cell marker to investigate cells that have a potential of self-renewal and multipotent differentiation (Ellis et al., 2004).

1.5.6.3 Nestin

Nestin is an intermediate filament protein that has been known as a protein marker for neural stem/progenitor cells (Gilyarov, 2008). Expression of nestin appears in many types of cells during development, especially in dividing cells of the central nervous system, peripheral nervous system and other tissues (Korzhevskii et al., 2008, Gilyarov, 2008). During the differentiation period,

nestin expression becomes gradually decreased and tissue-specific intermediate filament proteins start to be expressed. While nestin is therefore transiently expressed and is not found in fully differentiated cells its expression is more complex than being simply a marker of undifferentiated cells. To date, nestin has been detected in the two germinative zones of the brain: the SVZ of the lateral ventricle and the DG of the hippocampus (Gilyarov, 2008). There are two types of nestin-immunopositive cells in the SGZ of the dentate gyrus: early precursor and late precursor. The late precursor has been described as an immature neuron marker (Gilyarov, 2008).

1.5.6.4 c-Myc

c-Myc (also called Myc) is a member of the Myc family (so called protooncogene) including n-Myc and l-Myc. The crucial role of c-Myc is to induce cell proliferation by down-regulating p21, in stem cell self-renewal, angiogenesis and cell growth; however, c-Myc also inhibits terminal differentiation and apoptosis (Murphy et al., 2005, Nagao et al., 2008). In addition, c-Myc is expressed in neural progenitor cells and its expression promotes self-renewal in neurospheres of neural progenitor cells (Kerosuo et al., 2008). It has been found that inactivation of c-Myc in mice impairs granule cell precursor proliferation and attenuates self-renewal (Nagao et al., 2008). Interestingly, levels of c-Myc expression in adult mouse neural stem cells are higher than in embryonic stem cells (Kim et al., 2008).

1.6 Neurogenesis

The generation of neural cells in most mammalian brain regions occurs in the prenatal period. However, in 1962 Joseph Altman wrote an article entitled "Are new neurons formed in the brains of adult mammals". In 1963 the *Anatomical Record*, published the first image of newly generated granule cells identified in the dentate gyrus of the hippocampus (Kempermaan, 2006). This led Joseph Altman in 1965 to postulate postnatal hippocampal neurogenesis. The first report on neurogenesis in the adult olfactory bulb was published by Kaplan and Hinds in 1977 (Kempermaan, 2006). Results based on labelling dividing cells with radioactive thymidine demonstrating that newly born cells had a neuronal morphology. However many people remained unconvinced by this evidence. More recent work using BrdU labelling of dividing cells, retroviral lineage tracing and immunological identification of new neurons has now confirmed the correctness of Altman's observations in all mammalian species, including man, which have been investigated (Kempermaan, 2006).

At present it has been shown that there are only two main neurogenic regions within the adult mammalian brain; (i) the lateral walls of lateral ventricle which contribute cells to the rostral migratory stream and olfactory bulb and (ii) the subgranular zone of the dentate gyrus in hippocampus. In these regions neural stem cells continue to divide and generate new neurons and glia throughout life (Lledo et al., 2006, Mackowiak et al., 2004). A particularly striking demonstration of neurogenesis within the dentate gyrus has been shown using GFP encoding replication deficient retroviruses, see Fig 1.7 (Zhao et al., 2006).
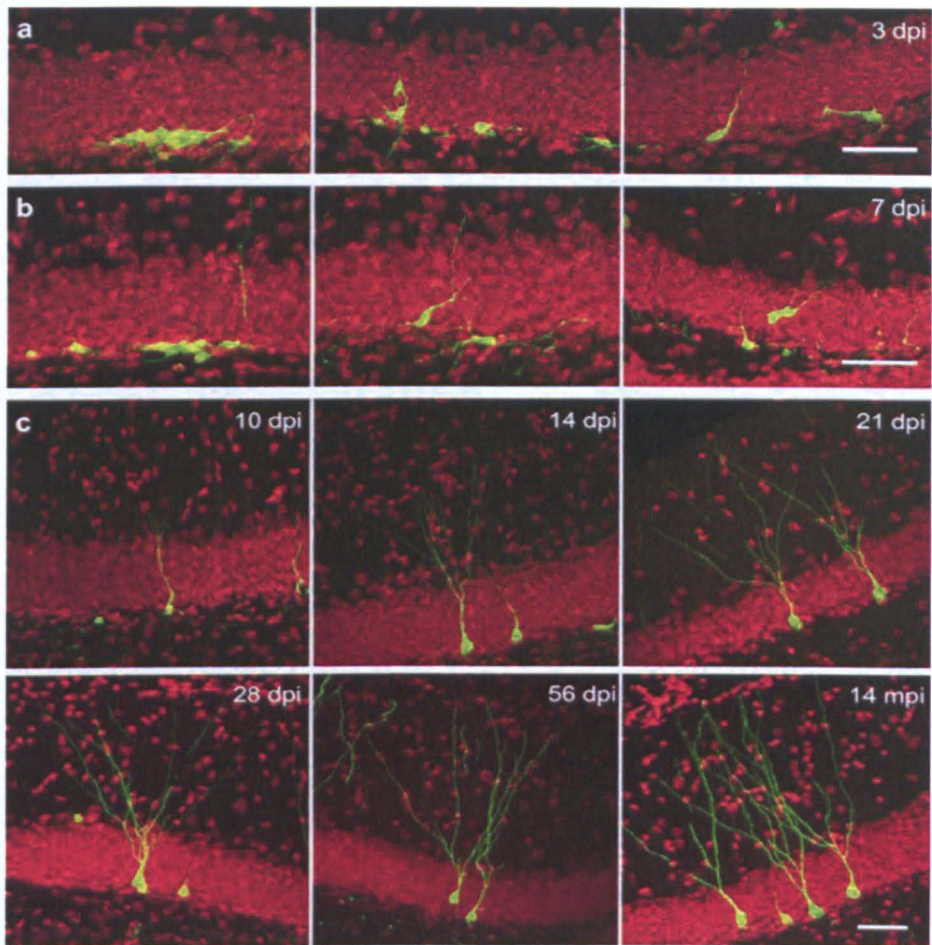
Figure 1.7 Taken from Zhao et al. 2006, shows mice put down at a series of time intervals (3 days to 14 months) after stereotaxic injection of GFP coding retrovirus. Only dividing cells are infected by the retrovirus which shows infected cells initially in the SGZ adjacent to the dentate gyrus (3 days after injection) followed by migration into the dentate gyrus and extension of dendrites into the molecular layer of the hippocampus in animals put down at progressively later ages.

Neurogenesis in the olfactory bulb has been observed in adult rodents and non-human primates. Neural progenitor cells in the SVZ of lateral ventricles produce new interneurons destined for the adult olfactory bulb. The SVZ is defined as a narrow area of one to two cells wide below the ependyma. The developing new neurons migrate from SVZ to the olfactory bulb via a particular chain migration called the "rostral migratory stream" (RMS) or "rostral migratory path" (RMP) (Kempermaan, 2006, Mackowiak et al., 2004). Recently the RMS has been demonstrated in humans (Curtis et al., 2007).

From observations in rodents, non-human primates and humans, the neuronal progenitor cells also divide in SGZ of dentate gyrus (Mackowiak et al., 2004). By definition, the SGZ is described as a layer three cell nuclei wide, adjacent to the basal cell band of the granular cell layer and a two nucleus-wide zone into the hilus (Kempermaan, 2006). The generated neuronal progenitor cells migrate from the SGZ into the granule cell layer of the dentate gyrus and then differentiate into neurons and glial cells (Mackowiak et al., 2004).

1.6.1 Postnatal hippocampal neurogenesis

During embryonic development, neural stem cells migrate from the walls of the ventricle (SVZ) to the developed hippocampus to form a secondary neurogenic area. During early postnatal development, the proliferation of these cells decreases rapidly from six hours to five days of age (Altman and Das, 1965). Then it builds up gradually to reach a peak at about 15 days tertiary neurogenesis and then substantially declines after this age although this decline is accompanied by an increase in size of hippocampus. The decrease of this

germinal pool, involving the number of undifferentiated cells is accompanied by an increase in the number of differentiated granule cells. In addition, the internal arm of granular layer and Ammon's horn are not completely developed at this age and showed only a diffuse cell mass of undifferentiated cells (Altman and Das, 1965). The development of the dentate gyrus takes place during gestation and substantially continues into the postnatal period until adulthood. Approximately 80% of granule cells in rats are produced after birth and neuronal production reaches a peak during the first postnatal weeks. In the guinea pig, in contrast, 80% of the granule cells of the dentate gyrus are generated before birth and 20% are developed postnatally, particularly during the first two weeks. This developmental pattern is similar to human and non-human primates (Guidi et al., 2005).

1.6.2 Adult hippocampal neurogenesis

Eriksson et al., (1998) showed the genesis and survival of new granule cell neurons in the dentate gyrus of the adult human brain. This indicates that some regions of the human brain are potentially self-renewable throughout life. However, neurogenesis declines gradually during adulthood and senescence which is associated with a reduction of granule cell progenitor proliferation (Kuhn et al., 1996). In addition to the human findings, the production of new hippocampal neurons has also been found in the dentate gyrus of two species of adult macaque monkeys (Gould et al., 1999). The approximate rate of generation of new granule neurons is 9,000 cells per day in the adult rat hippocampus with over 70% of new cells differentiating into neurons (Cameron and McKay, 2001, Zhao et al., 2006). The number of the newly

generated granule cells every month is about 6% of the granule cell population (Cameron and McKay, 2001). The large number of the new neuronal cells generated in dentate gyrus, suggests that they have an essential role in the function of hippocampus. From these studies, therefore, adult neurogenesis may be an important phenomenon in hippocampal function.
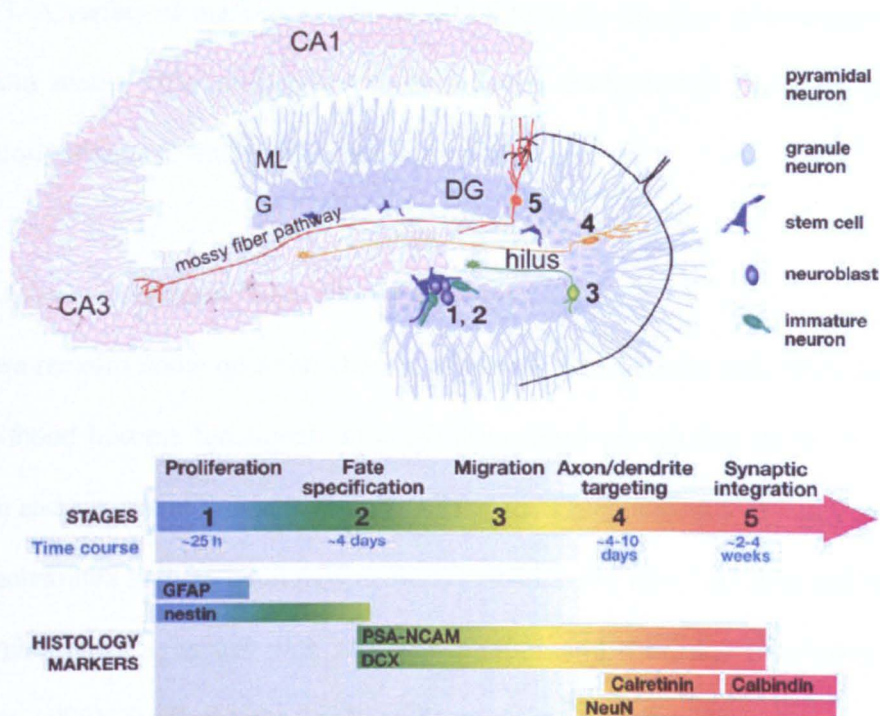
Figure 1.8 Diagram illustrates neurogenesis in the dentate gyrus of the hippocampus from neuronal stem cells in the SGZ. In adult generation of new granular neurons in the dentate gyrus of hippocampus proceed five developmental stages: proliferation, fate specification, migration, axon/dendrite targeting, and synaptic integration, respectively. DG, dentate gyrus; ML, molecular cell layer; GL, granular cell layer (Adapted from Ming and Song, 2005).

Neurogenesis is composed of three processes; cell proliferation, migration, and differentiation see Fig. 1.8 (Ming and Song, 2005). Neural stem cells in the SGZ of the hippocampal dentate gyrus give rise to transit amplifying cells which differentiate into immature neurons. Subsequently, these cells migrate into the granule cell layer and then mature into new granular neurons. These neurons receive inputs from the entorhinal cortex and extend projections into CA3. A variety of markers can be used to follow the progress of neurogenesis. Nestin marks early progenitors or stem cells, doublecortin marks immature neurons whereas NeuN marks mature neurons.

1.7 Neurogenesis and hippocampal function

There remains some question about whether the new granule cells born during adulthood become functional, although it has been proved that these cells are born and survive in the dentate gyrus of the adult hippocampus. A study in rats demonstrated that the adult new neurons extend axons into CA3 area and make morphological synapses with adjacent neurons and dendrites (Markakis and Gage, 1999). Similarly, the newly adult-generated granule cells in rats give off axonal projections into mossy fibre pathways. After mitosis, the immature granule cells also promptly send mossy fibres into CA3 field between days 4-10 (Hastings and Gould, 1999). Furthermore, it has also been found that mature new granule neurons display gene expression patterns, supporting the idea that these cells participate in functional hippocampal networks induced by learning experiences (Ramirez-Amaya et al., 2006). Another intriguing possibility is that the newly generated granule cells develop associations with existing hippocampal circuits and replace earlier generated cells which are removed by

apoptosis. In this way, postnatal cells may replace cells produced prenatally (Dayer et al., 2003).

As described above, the adult hippocampus has been recognized to have numerous cognitive functions, including learning, spatial memory, and long-term recognition memory (Suzuki and Clayton, 2000, Brown and Aggleton, 2001). Several lines of evidence suggest that the rate of adult neuronal proliferation in the subgranular zone of the dentate gyrus is both altered by emotional, pharmacological and behavioural factors and that the production of new neurones is related to learning and memory. Intriguingly, enhancement of the production of new neurons improves long-term recognition memory while antimitotic agents that diminish hippocampal neurogenesis, result in impaired long-term memory (Bruel-Jungerman et al., 2005, Duman et al., 2001b, Gould et al., 1999).

1.8 Modulation of hippocampal neurogenesis

The stem cell/progenitor activity and fate of the hippocampal SGZ are regulated by cellular and molecular interactions. Adult neurogenesis is a representative of extremely dynamic processes in the mature CNS that responds to numerous intrinsic and extrinsic influences (Table 1.2). Cell generation and cell death have to be balanced since elimination of surplus cells is one of the key mechanisms in controlling adult neurogenesis. New generated granule cells are preferentially integrated in spatial learning circuits in the dentate gyrus (Kee et al., 2007). Early phase of learning does not alter neurogenesis but increases the cell survival of adult born cells, which may be

involved in the encoding of memory. Learning itself requires death of the neurons born during the early phase of learning to suppressing old unnecessary neurons and/or the immature neurons that are too old or have not established learning-related synaptic connections. This cell death phenomenon may involve in memory trace consolidation. However, there is an increase in the number of cells generated contingently with the late phase training. This phenomenon indicates that learning-induced neurogenesis is not associated with learning performance but may be involved in a resetting process for a new memory (Abrous et al., 2005).

1.8.1 Vascular niche

The generation of new granule neurons in the dentate gyrus of the hippocampus is regulated by numerous local factors. This has led to the idea of a stem cell niche within the SGZ where adjacent cells, local growth factor production and the extracellular matrix can influence stem cell proliferation. The vascular system within the SGZ may provide a vascular stem cell niche which may be one of the most influential factors in either up or down regulating cell proliferation. It is a functional unit, consisting of the precursor cells, astrocytes, endothelial cells, microglia or macrophages, extracellular matrix, and close contact with the basal membrane (Fig. 1.9). The vascular niche is implicated in stem and progenitor cell generation, maintenance, repair, and also promotes neuronal differentiation. It is a place where precursor cells interact with the environment. In this unit, cell-to-cell interaction takes place and the precursor cells are capable of receiving local regulatory cues (Scadden, 2006, Kempermaan, 2006). Palmer et al. (2000) have revealed that precursor

cell proliferation in adult SGZ is paralleled with the vasculature. Adult hippocampal neurogenesis develops within an angiogenic niche where mesenchymal-derived cells in combination with circulating factors influence the continued development of the adult central nervous system.
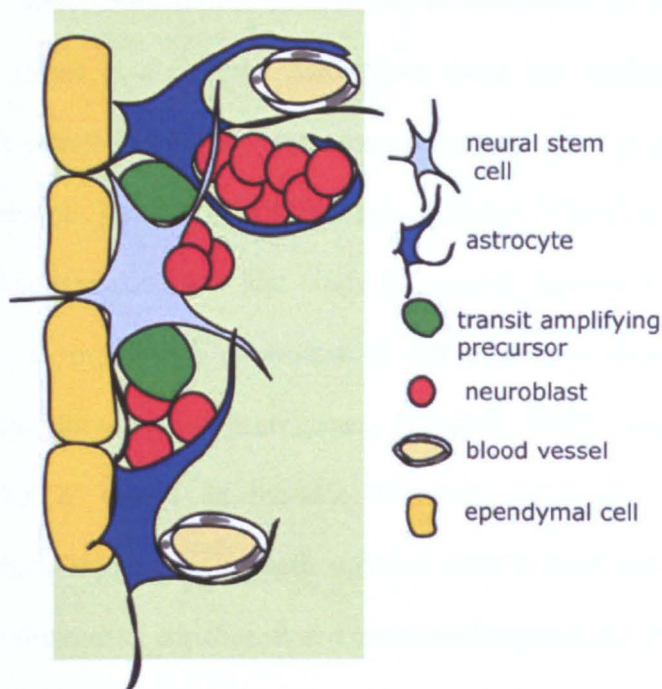
Figure 1.9 Vascular niche is also called stem cell niche which is defined as the environment that stem cells reside and interacts with ependymal cells, blood vessels, astrocytes and neuronal progenitor cells (Adapted from http://www.scitopics.com/Neurogenesis_in_the_adult_brain_the_subependyma l_zone).

Indeed, it now appears that microvascular endothelial cells support neuronal expansion and survival in the CNS by secreting soluble neurotrophin including brain-derived neurotrophic factor (BDNF) whereas neural stem cells can produce angiogenic factors (Leventhal et al., 1999). Endothelial cells function as a source of soluble factors that induce self-renewal, suppress differentiation of neural stem cells, and also enhance their neuron production. Thus endothelial cells were characterized as a powerful component of the neural stem cell niche (Shen et al., 2004). Aside from these few studies, growth factors which are secreted by blood vessels have been determined to participate with neural generation. Vascular endothelial growth factor (VEGF) is a protein secreted under hypoxic condition that binds to receptor tyrosine kinases on endothelial cells to promote angiogenesis. It has also been shown to be associated with the stimulation of neurogenesis (Jin et al., 2002). Hippocampal expression of VEGF causes an increase of neurogenesis and cognitive improvement which associate with both enriched environment and running. Furthermore, environmental enrichment and increased hippocampal activity are associated with endothelial cell production, and clusters of dividing cells occur around new blood vessels (Cao et al., 2004).

1.8.2 BDNF

The neurotrophin, BDNF, is a polypeptide growth factor that has been shown to have not only an influential role in regulating the proliferation and survival of neurons but also in prohibiting inordinate neuronal cell death (Linnarsson et al., 2000). BDNF also plays essential roles in controlling the survival and the formation of synaptic plasticity (Cheng et al., 2003). The BDNF protein is

generated by granule cells in the hippocampal dentate gyrus (Conner et al., 1997, Wetmore et al., 1990). Levels of the BDNF generally correlate with the levels of cell proliferation in the SGZ (Kempermaan, 2006). BDNF expression has been shown to be associated with the process of learning and adult neurogenesis. Additionally, BDNF is considered to be involved in mediating the effects of antidepressants (Nibuya et al., 1995, Shirayama et al., 2002). An enhancement of BDNF expression promotes an induction of granule precursor cell proliferation in the rat hippocampus (Katoh-Semba et al., 2002). Infusion of BDNF into the hippocampal dentate gyrus increases granule cell proliferation in normal adult rat (Scharfman et al., 2005). Conversely, adult hippocampal neurogenesis is decreased in heterozygous BDNF knockout mice in comparison with wild-type mice. The reduction of neurogenesis is correlated with a decrease in the size of the dentate gyrus (Rossi et al., 2006, Lee et al., 2002). BDNF has been found to be involved in producing long term potentiation (LTP) which is required for memory consolidation (Bekinschtein et al., 2008, Korte et al., 1995, Lu et al., 2008, Minichiello et al., 1999). BDNF expression is upregulated in enhanced learning and memory in an enriched environment (Rossi et al., 2006, Cheng et al., 2003). In the process of neurogenesis within the SGZ, BDNF is required for the survival, migration and maturation of newly formed neurons and promotes their dendritic growth and branching. Reducing BDNF levels or BDNF signalling in the brain is associated with impaired spatial memory (Minichiello et al., 1999, Mustafa et al., 2008).

1.8.3 Genetic factors

Genetics have been shown to impact on the rate of hippocampal neurogenesis. For instance, different strains of adult mice housed under the same environment, show striking differences in baseline levels of cell proliferation, survival and differentiation (Kempermann et al., 1997). In addition, the genetically determined baseline level of the adult neurogenesis is associated with acquisition of spatial memory in the water maze which is a hippocampal-dependent task (Kempermann and Gage, 2002).

1.8.4 Enriched environment and learning

Neuronal activity and environmental factors control adult neurogenesis in both a positive and negative manner. Animals living in an enriched environment have shown an increase in hippocampal neurogenesis in comparison to naïve animals, suggesting that enrichment enhances new neuronal cell generation. Environmental enrichment is produced by social interaction and physical aspects of the environment such as sets of tunnels and objects including toys to stimulate exploratory behaviour (Bruel-Jungerman et al., 2005). It has been demonstrated that hippocampal-dependent learning tasks such as eyeblink conditioning and spatial water-maze training induce adult hippocampal neurogenesis (Gould et al., 1999). Whereas, running and enriched environment stimulate cell survival in the dentate gyrus compared with water-maze learning, swimming and standard housed groups. Furthermore, voluntary activity using running wheel enhances the generation of newborn hippocampal neurons (van Praag et al., 1999b). Physical activity and running, can also improve learning (water maze performance) and increase adult generated neurons in the

hippocampus (van Praag et al., 1999a). A rat would not usually choose swimming activity; therefore, it may cause psychological stress which may neutralize neurogenic stimuli. There is a report showing that environmental enrichment increases hippocampal neurogenesis. Enriched and irradiated animals show no difference in the visible platform training. However in hidden platform training, environmental enrichment improves spatial learning whereas irradiation does not reduce this effect of enrichment. This result indicates that increased hippocampal neurogenesis is not always associated with changes of environmental enrichment (Meshi et al., 2006).

1.8.5 Physiological and pathological states

Several studies of adult neurogenesis under pathological conditions have shown that epilepsy enhances progenitor cell proliferation in dentate gyrus (Ming and Song, 2005, Parent, 2007). In contrast decreased neurogenesis of granule cells in adult hippocampus appears in animals experiencing stress and depression (Duman et al., 2001a, Grote and Hannan, 2007). Stress promotes an induction of circulating corticosteroids which play a regulatory role in adult hippocampal neurogenesis (Grote and Hannan, 2007). Stress increases glucocorticoids in blood circulation, causing down-regulation of adult neurogenesis. In adult rat hippocampus, administration of glucocorticoids reduces generation of granule precursor cells (Grote and Hannan, 2007). Moreover, a reduction of hippocampal volume appears in patients exposed to prolonged stress and elevated glucocorticoids. This effect has been investigated by quantifying cell number in the post-mortem hippocampi of mood disorder patients (Duman et al., 2001a). Attenuation of mitotic activity of neuronal

47

progenitor cells in the dentate gyrus is also related to senescence in adult rats (Kuhn et al., 1996). A study of old mice, living in an enriched environment has shown an increase neurogenesis in the dentate gyrus (Kempermann et al., 1998).

1.8.6 Growth factors, neurotransmitters, antidepressants

Changes in neurotransmitter signalling have been shown to regulate hippocampal granule cell proliferation in adult brain, e.g. a decrease of hippocampal neurogenesis is correlated with reduction of serotonin (5-HT). In contrast, increasing serotonin synthesis induces granule cell proliferation in adult hippocampus. Furthermore, proliferation of progenitor cells in the adult hippocampus is up-regulated by administration of a 5-HT_{1A} agonist and blocked by pre-treatment with a 5-HT_{1A} antagonist (Duman et al., 2001b, Duman et al., 2001a). Activation of N-Metyl-D-Aspartate (NMDA) receptors, a glutamate receptor subtype, sustains a usual rate of adult hippocampal neurogenesis. Administration of NMDA receptor antagonist increases generation of granule cells in adult hippocampus but this is reduced by systemic administration of NMDA (Duman et al., 2001a).

Moreover, neuronal generation in adult brains is influenced by antidepressants. Antidepressants up-regulate the phosphorylation of the cAMP-CREB (cAMP response element binding protein) which in turn increases the level of BDNF expression that leads to induction of adult hippocampal neurogenesis (Duman et al., 2001b). Besides up-regulation of CREB and BDNF, antidepressants also

increase the level of 5-HT which is involved in alterations of neurogenesis in adult hippocampus (Santarelli et al., 2003).

Growth factors are extracellular signalling molecules i.e. epidermal growth factor (EGF) and fibroblast growth factor (FGF) increase cell growth and maintenance. EGF and FGF also retain the capability of stem cells to generate neurons and glia (Caldwell et al., 2001). In culture, the combination of EGF and FGF have the ability to induce the long-term growth of the embryonic, neonatal and adult neurospheres taken from human and rodent central nervous system, (Caldwell et al., 2001, Kempermaan, 2006). However, it has been demonstrated that EGF has a stronger potential than FGF to enhance adult neurogenesis by increasing numbers of BrdU cells (Kempermaan, 2006).

Table 1.2 Regulators of neurogenesis in the adult dentate gyrus (Adapted from Rao, 2006)

	Effects	Context
Genetic	Changes in proliferation Number of new neurons	Unknown loci Unknown loci
Environmental enrichment	Increased number of new neurons in the hippocampus Absence of effect in SVZ and olfactory bulb	Population cage, food treats, toys, running wheel
Physical exercise	Increases in proliferation and number of new neurons in the hippocampus	Running wheel
Learning	Increased in number of new neurons	Water maze, eye-blink conditioning
Stress and other hormones	Increased proliferation Reduced proliferation	Adrenalectomy, estrogen, Glucocorticoids, predator/psychosocial stress
Neurotransmitters	Increased proliferation Reduced proliferation	NMDA antagonists, serotonin, epinephrine, norepinephrine and dopamine signalling NMDA agonists, serotonin receptor antagonists, depletion of 5-hydroxytryptamine, antidepressants
Growth factors/mitogens	Increased proliferation and number of neurons	FGF-2, EGF, VEGF

1.9 Markers of cell proliferation in neurogenesis

1.9.1 5-bromo-2-deoxyuridine (BrdU)

In all mammalian species including humans, neurogenesis in the adult dentate gyrus has been demonstrated. The rate of cell proliferation is altered by numerous factors. Until now 5-bromo-2-deoxyuridine (BrdU) has been the most commonly used mitotic marker in such studies. However, it has been demonstrated that high doses of BrdU 60-600 mg/kg, i.p. possibly cause mutations in cells and consequently severe abnormalities of the developing tissue, especially in the CNS (Taupin, 2007). Diffusion of BrdU is uncertain among various tissues of the body following intraperitoneal injection. In the brain, for instance, diffusion of BrdU could be difficult since it may be reduced by the blood brain barrier and other metabolic factors, particularly in older animals. It has been reported that the BrdU injection before or after a learning experiment is likely to be a stressful procedure which stress is recognized to be able to inhibit neurogenesis and the BrdU incorporation into the dividing cells (Kee et al., 2002).

1.9.2 Endogenous markers in neurogenesis

Ki67, a nuclear protein, is an alternative method of detecting cell proliferation. Ki67 is present in all dividing cells throughout the mitotic cycle but is absent from resting cells (G_0) (Gerdes et al., 1984). It is an effective marker for determining the so-called growth fraction of a given cell population. Like BrdU, Ki67 is detectable with immunohistochemistry. Unlike BrdU, Ki67 is an endogenous marker and so is also a non-invasive marker in living cells. Ki67 is

a reliable marker of mitosis because its expression is strictly correlated with cell proliferation and to active phases of the cell cycle and its half-life is very short, even though the role of Ki67 is not known. Furthermore, studies have shown that Ki67 is present in all neuronal tissue of mammalians from rodents to humans (Kee et al., 2002, Scholzen and Gerdes, 2000). A study of neurogenesis in the hippocampal dentate gyrus has compared BrdU and Ki67. This has clearly proved that Ki67 has most of the benefits of BrdU and none of costs (Kee et al., 2002). Thus it is an excellent indicator for a comparative study of neuronal proliferation among different species or among different brain regions. However, it is not suitable for an experiment that involves tracing of cells over long periods (Kee et al., 2002).

In addition to Ki67, doublecortin (DCX) has been postulated as an alternative indicator for adult neurogenesis since DCX expression is associated with neurogenic processes (Couillard-Despres et al., 2005, Brown et al., 2003). DCX, a microtubule binding protein, is transiently expressed in differentiating/migrating and immature neurons within the adult mammalian brain, the SGZ of the dentate gyrus and olfactory bulb (Couillard-Despres et al., 2005, Abrahams et al., 1997). In newly formed cells of adult hippocampal dentate gyrus, expression of DCX can be detected for nearly 30 days (Brown et al., 2003). In the adult dentate gyrus, DCX expression in newly generated granule cells is detected during migration, early differentiation and the maturation period (Rao and Shetty, 2004). Once new born neurons have completely matured, they will stop expressing DCX protein (Couillard-Despres

et al., 2005). As these reasons, both Ki67 and DCX were utilised as neurogenesis markers in the experimental study.

1.10 Notch signalling

The Notch signalling pathway has been shown to mediate cell-cell communication between adjacent cells. Notch is a family of four cell surface receptors activated by ligands on adjacent cells (Yoon and Gaiano, 2005). Notch receptors are single pass transmembrane proteins (~320KDa) composed of a ~200KDa functional extracellular (NECD) and a ~120KDa transmembrane (TM) domains (Conboy et al., 2007). The full length Notch receptor (NFL) presents at the cell surface as a heterodimer and the TM domain is cleaved by presenilins complex to yield a ~110KDa Notch intracellular domain (NICD) (Conboy et al., 2007). The NECD contains multiple epidermal growth factor (EGF) repeats, which regulate ligand binding and three Lin12 repeats. Whereas the INCD containes a RAM sequence, ankyrin repeats, a tranactivation domain (TAD) and PEST sequence is involved in signal transduction (Fig. 1.10) (Allenspach et al., 2002, Fiuza and Arias, 2007).

EGF-like repeats   Lin12-repeats   RAM   Ankylin repeats   TAD   PEST

Extracellular domain   Intracellular domain
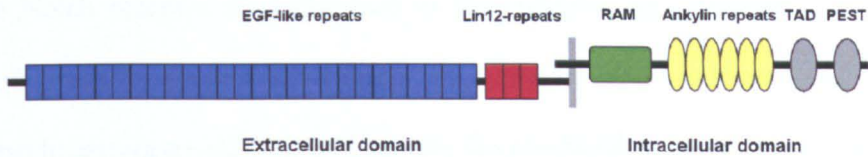
Figure 1.10 Structure of Notch which is composed of extracellular and intracellular domains. The extracellular domain is composed by up to 36 EDF-like repeats and Lin12 repeats. In the intracellular domain, Notch consists of a region called RAM, Ankylin repeats, TAD and PEST.
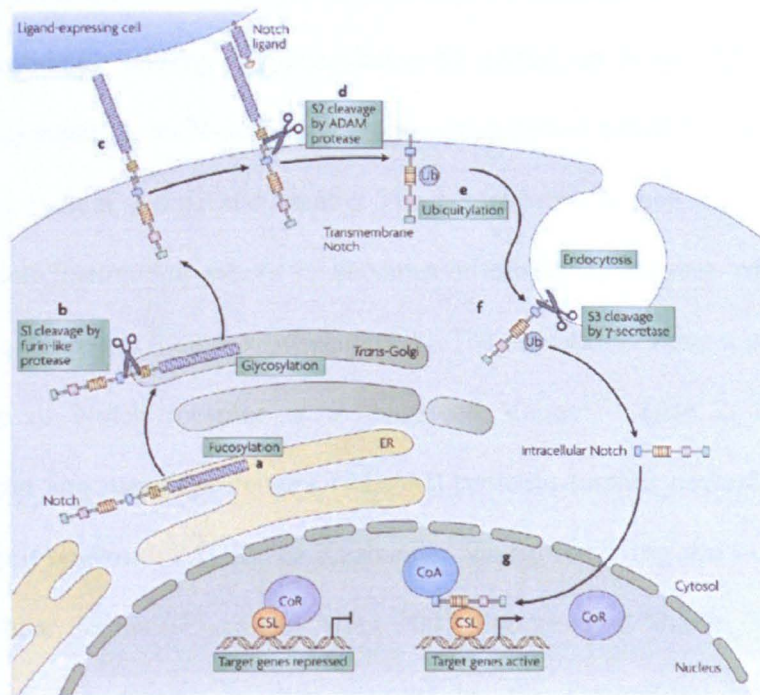
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Figure 1.11 Notch signalling pathway. Notch binding on one cell to ligand on another cell elicits two steps of proteolytic cleavages. Firstly, Notch is cleaved at the S2 site by ADAM protease and then creates a substrate for S3 cleavage by γ-secretase. This process causes release of Notch intracellular domain that translocates into the nucleus and associates with the DNA-binding CSL transcription factor while co-repressors are released (Adapted from Osborne and Minter, 2007).

The Notch receptor is synthesized in the endoplasmic reticulum (ER) as a single precursor protein (pre-Notch). Then, the pre-Notch is fucosylated by *O*-fucosyltransferase1 (O-Fut) to facilitate the pre-Notch transportation from ER to the Golgi apparatus. This process is also crucial for the production of a functional receptor (Bray, 2006, Fiuza and Arias, 2007). Within the Golgi apparatus, a furine-like protease cleaves the pre-Notch at the S1-cleavage site to produce a non-covalent heterodimer receptor comprised of the extracellular and intracellular domains (Fiuza and Arias, 2007, Osborne and Minter, 2007). The heterodimer undergoes glycosylation by adding an N-acetylglucosamine (GlcNAc) sugar by an N-acetylglucosaminyltransferase called Fringe proteins, including radical, manic and lunatic. The heterodimer is then translocated to the plasma membrane where it becomes available to interact with Notch ligands on adjacent ligand-expressing cells. The interaction induces proteolytic cleavage of Notch receptor at a conserved Valine[1711] (site 2, S2) by a disintegrin and metalloproteinase (ADAM) protease tumour necrosis factor-α converting enzyme (TACE), or Kuzbanian (Kuz), removing the bulk of the extracellular domain (Fiuza and Arias, 2007, Osborne and Minter, 2007). The transmembrane domain is then cleaved by the presenilins complex (γ-secretase) at site 3 (S3) between Glycine[1743] and Valine[1744], releasing the NICD from the transmembrane domain (Fiuza and Arias, 2007, Osborne and Minter, 2007). NICD then translocates into the nucleus and binds to the transcription factor CSL (CBF1-RBP-Jk). This interaction leads to transcriptional activation by displacement of corepressors (CoR) and simultaneous recruitment of coactivators (CoA), including mastermind-like proteins (MAML1) (Fig. 1.11) (Allenspach et al., 2002, Fiuza and Arias, 2007, Osborne and Minter, 2007).

1.10.1 Notch1 in neurogenesis, progenitor or stem cells

Notch signalling is used by neurons in the hippocampus of the adult mouse brain (Wang et al., 2004). In addition, Notch1 expression has been found in the adult human hippocampus (Nagarsheth et al., 2006, Berezovska et al., 1998). It has been known that Notch signalling is triggered by its ligands expressed on neighbouring cells in the SGZ and dentate gyrus (Breunig et al., 2007, Stump et al., 2002). This activation leads to the cleavage of the transmembrane domain of the Notch receptor and then the release of the intracellular domain ICD which translocates into the nucleus and associates with the transcription factor RBP-J. This complex activates Hes1 which Notch1 ICD functions effectively to upregulate Hes1 promoter activity (Kageyama and Ohtsuka, 1999) and subsequently regulates maintenance of neural stem phenotype and progenitor cell proliferation (Kageyama and Ohtsuka, 1999, Kageyama et al., 2005).

Recent evidence has suggested that impairment of the Notch signalling pathway by reducing Notch1 expression might decrease neurogenesis and progenitor survival (Oishi et al., 2004). Expression of Notch1 is correlated to the regions where neurogenesis takes place. Furthermore, activation of Notch1 expression occurs when neurogenesis is activated (Hatakeyama and Kageyama, 2006). During neural development and in the germinal zones of the adult brain, Notch1 functions to regulate neurogenesis and cell fate determination by preventing neural differentiation and maintaining neural precursor and the stem cell proliferation (Breunig et al., 2007, Wang et al., 2004, Hitoshi et al., 2002). Additionally, it has been found that Notch1 regulates NSC self-renewal (Potten

et al., 2006). However, Notch1 is not only involved in alterations of neurogenesis and survival but also plays a crucial role for the regulation of neural stem cell population *in vivo* and *in vitro* (Hitoshi et al., 2002).

Besides its known function in neurogenesis, Notch1 also controls the formation of dendritic morphology in the postnatal hippocampal dentate gyrus (Breunig et al., 2007, Wang et al., 2004, Hitoshi et al., 2002). In post mitotic neurons, Notch1 is important for the later stages of granule cell maturation and dendritic branching (Redmond et al., 2000, Breunig et al., 2007). A recent study has reported that heterozygous knock out adult mice demonstrate impairments of spatial learning and memory (Costa et al., 2003). In addition, transgenic mice with reduced expression of Notch1 show reduced LTP which can be rescued by addition of Notch signalling components (Wang et al., 2004). The Notch signalling pathway has been proposed to maintain the integration of synapses transiently produced during memory consolidation in adult rats (Conboy et al., 2007).

1.10.2 Notch1 in cancer

Notch1 has multifunctional roles in regulating cellular differentiation, development, proliferation and survival in the animal kingdom. Notch1 in human cancer cells can function as either a tumour suppressor or an oncogene (Fig. 1.12) (Kunnimalaiyaan and Chen, 2007, Allenspach et al., 2002). Firstly, the oncogenic function of Notch1 was investigated in human T-cell neoplasia. Subsequently, upregulation of Notch1 has been demonstrated in several types of cancer, for instance pancreatic cancer, colon cancer, non-small cell lung

cancer, cervical cancer, renal cell carcinoma and several lymphomas. In these types of cancer, Notch1 exhibits a powerful oncogenic function by inhibiting differentiation and apoptosis (Kunnimalaiyaan and Chen, 2007). In contrast, Notch1 signalling expression is very low or absent in prostate cancer, and in neuroendocrine tumours (NETs) such as small-cell lung cancer (SCLC), pancreatic carcinoid and medullary thyroid cancer (MTC). Expression of Notch1 in these cancers results in reduction of cell growth (Sriuranpong et al., 2001, Allenspach et al., 2002, Kunnimalaiyaan and Chen, 2007). Taken together, inactivation of the Notch1 oncogene leads to growth inhibition and induction of apoptosis whereas activation of Notch1 tumour suppressor results in growth suppression (Kunnimalaiyaan and Chen, 2007). These studies led to investigating a potential role of Notch1 in affecting cellular differentiation, proliferation and/or survival in cancers. Thus, understanding of Notch signalling in regulating normal development and malignant transformation may be essential for novel cancer therapeutics.
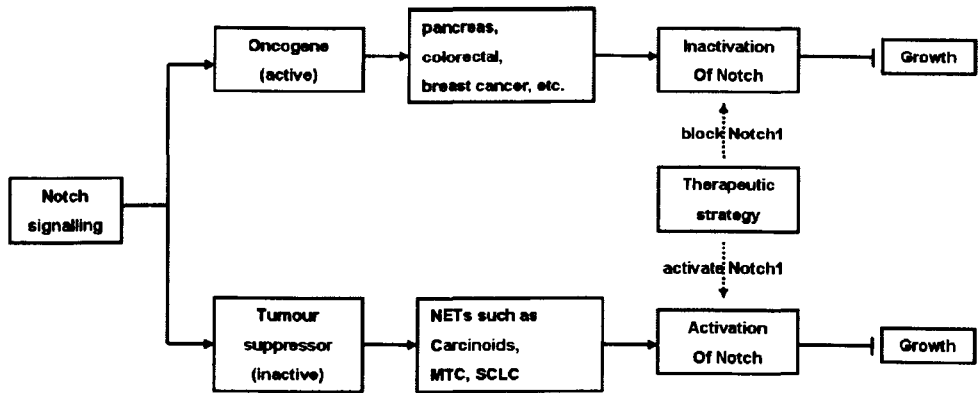
Figure 1.12 Diagram of the Notch1 signalling pathway in cancer. The functions of Notch1 are dependent on a certain cellular context. Abbreviations: MTC, medullary thyroid cancer; NETs, neuroendocrine tumours; SCLC, small-cell lung cancers (Adapted from Kunnimalaiyaan and Chen, 2007).

1.11 Behavioural tests in this study

1.11.1 Novel object location (NOL) task

Studies in human, patients were tested their ability in detecting movement of objects or dots (Smith et al., 1996). Patients with right hippocampal damaged showed deficits in their spatial memory for the location of the objects (Nunn et al., 1999). Similarly, in rodents, NOL has been used as a standard behavioural test to investigate spatial memory. A novel-preference paradigm is used in the NOL test to measure the memory for spatial locations within a familiar area. In this test, two identical objects are presented in the choice (test) trial, which one object is moved to a novel location (Dix and Aggleton, 1999, Ennaceur et al., 1997). Rodents naturally prefer to approach and explore an object in a novel location, a response which was not required training or reward. They normally also prefer to explore an object in a novel location more than an object in a

familiar location (Dere et al., 2007), indicating that rodents remember the features of an object and also the spatial location in which it was encountered. Rodents naturally approach and inspect objects by directing their noses to sniff the object and manipulating the object by their forepaws. The NOL test uses the natural tendency of rats to spontaneously explore an object placed in new position without primary reinforcement (i.e. food or electric shock delivery), spatial learning, the learning of rule and the learning of response-reward association which needed in other behavioural tests (Dix and Aggleton, 1999, Ennaceur et al., 2005). Therefore, it does not need lengthy pretraining and does not induce arousal and stress caused by using negative reinforcement as compared with the water maze and the inhibitory and active avoidance of fear conditioning (Ennaceur and Delacour, 1988). Several studies have reported that impairment in the performance of the NOL test occurs in rats who have been given a lesion in the hippocampus, indicating that the hippocampus, per se, is critically important for this task (Dere et al., 2007). Moreover, DG lesioned animals showed lack of reexploration the objects in novel locations, suggesting that the DG plays an important role to acquisition of the novel information (Lee et al., 2005). Since spatial information or spatial mapping is processed in the hippocampus, NOL should be an excellent behavioural model to quantify and utilized to study spatial memory in rodents.

1.11.2 Contextual fear conditioning

It has been known that the hippocampus has an important role in learning and memory. Declarative, relational, and spatial memory are hippocampal-dependent while others (e.g. procedure and implicit) are hippocampal-independent. Recently, contextual fear conditioning has been recognized as a popular behavioural model for learning and memory that is dependent on the hippocampus (Fanselow, 2000, Mei et al., 2005). Contextual fear conditioning is displayed by a rat after exploring a novel environment (a shock chamber) in which it has received an aversive electric footshock which is approximately 0.5-2 s, 0.3-1.5 mA and one to four shocks (Fanselow, 2000). Lesions of the hippocampus blocked freezing to contextual fear associated with shock (Fanselow, 2000, Fendt and Fanselow, 1999). Similarly, disruption of hippocampal neurogenesis with X-irradiation caused impairment of contextual fear conditioning (Saxe et al., 2006).

Fear is a powerful emotional experience and also a defensive behaviour that protect animals and humans from potentially dangerous environmental threats. Contextual fear conditioning experiments use an electric shock as an unconditioned stimulus (US) which is similar to Pavlov's meat powder. The contextual cue (the shock chamber) functions as a conditioned stimulus (CS) which predicts the happening of the shock and evokes a state of fear (e.g. freezing, called the conditional response (CR)). The contextual cue plays an important role like the bell in Pavlovian conditioning's experiment (Fanselow, 2000). Fear-potentiated startle, freezing, tachycardia, analgesia, conditioned defensive burying, ultrasonic vocalization and increased release of several

hormones can be quantified in rats that have experienced fear conditioning (Fendt and Fanselow, 1999). Freezing has been used as an index of conditioned fear and assessed by investigation of direct observation. Freezing is defined as a state of motionless, including immobility of vibrissae, except movement that is associated with respiration. Freezing reduces the impact of shock and generally occurs while the animal displays a crouching posture, piloerection and lowering of head and tail. Ordinarily, rats never lie down when they are freezing, for this reason; freezing is distinguishable from resting or sleeping (Fendt and Fanselow, 1999, Fanselow, 2000, Mei et al., 2005).

Studies have demonstrated that hippocampal lesioned rats showed contextual fear deficit, indicating that the hippocampus is required in the processes of the formation, storage and consolidate contextual memory. In particular a contextual fear memory deficit in animals with lesions made briefly after training is greater than animals with lesions made a month or more after training. Therefore, the hippocampus is not necessary for old contextual fear memories (Fanselow, 2000). The lesioned rats are hyperactive to explore new environment, but it is easily interrupted with freezing. This hyperactivity in hippocampal lesioned animals is possibly produced by the failure of the formation of a cognitive map of spatial location (Fanselow, 2000).

Besides the hippocampus, it has been found that the amygdala is also involved in freezing to a context associated with shock (Fendt and Fanselow, 1999). Neural circuitry of contextual fear conditioning is shown in Figure 1.12. The hippocampus functions importantly to provide contextual information to the

amygdala, while the lateral and basolateral nuclei of the amygdala are importantly required for acquisition of freezing. Particularly, the amygdala plays a pivotal role to converge the CS and US information. There is a report showing that an impairment of the amygdala block freezing to contextual cues (Maren, 2001). Together, an acquisition of contextual fear conditioning is dependent on the association of the hippocampus and amygdala.
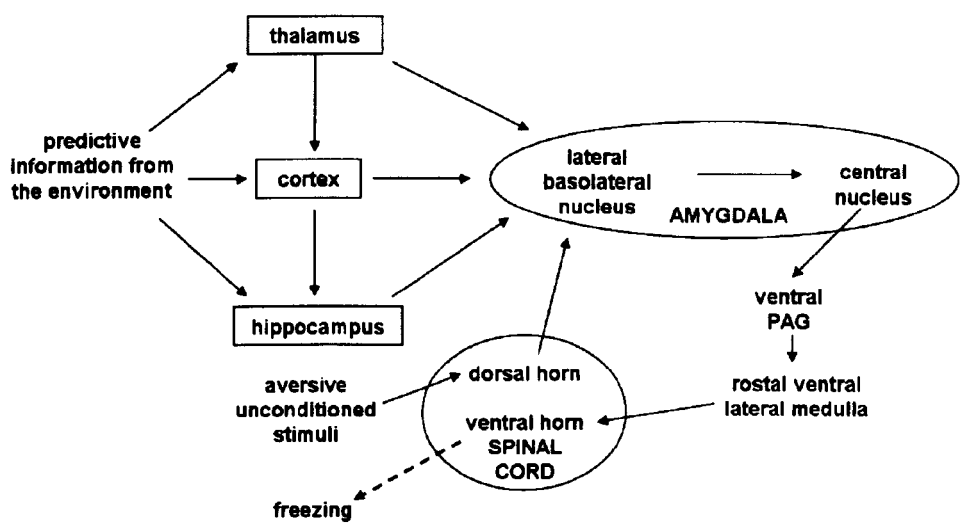
Figure 1.13 Hypothetical circuit mediating the different aspects of conditioned fear. Abbreviations: PAG, periaqueductal gray (Adapted from Fendt and Fanselow, 1999).

1.12 Aims and objectives

The studies presented in this thesis were carried out to examine whether chronic exposure to VPA produces changes in memory and neurobiology in the rat brain. The aim of this research was to determine and investigate the effects of VPA on an association of hippocampal memory and neurogenesis, on hippocampal neural stem cells and on cell proliferation of three cancer cell lines. The objectives of the present studies were as followed:

- Determine the chronic effects of VPA treatment on behavioural testing of recognition memory which were carried out by hippocampus-dependent tests: novel object location (NOL) task and contextual fear conditioning.

- Examine the effects of exposure to VPA on hippocampal neurogenesis by measuring proliferating cell numbers in the dentate gyrus and determining levels of neurtrophin BDNF, DCX and Notch1 expression.

- Assess a correlation between hippocampus-dependent memory tests and any changes of neurogenesis within the same animals.

- Determine the effects of VPA on hippocampal neural stem cells by examining cell proliferation and neural stem cells markers: Sox2, nestin, c-Myc and Notch1 *in vitro*.

- To evaluate the effects of VPA on the cell proliferation and on level of Notch1 expression of 3 cancer cell lines: Epn1 an ependymoma, Med1, a medulloblastoma and neuroblastoma (SHSY5Y).

Chapter 2

VPA produces deficits in hippocampus-dependent

cognition and inhibits cell proliferation in the SGZ

2.1 Introduction

Valproic acid (VPA) is used clinically as an anti-convulsant and mood stabilizer in both adults and children and has been proposed as a possible treatment for cancers and HIV infection (Henry, 2003, Lehrman et al., 2005, Buckley, 2008). Its exact mode of action is still not entirely clear but it appears to reduce neuronal activity by blocking sodium and calcium channels, elevating GABA and reducing aspartate levels within the brain (Kwan et al., 2001). VPA also functions as a therapeutically useful mood stabilizer by stimulating extracellular signal-regulation kinase (ERK) pathway (Hao et al., 2004). Despite its wide use, VPA has however long been known as a teratogen, causing neural tube defects and more recently has been associated with behavioural problems in children exposed to the drug during gestation (Meador et al., 2008, Nicolai et al., 2008). These effects are likely to be caused by its action as a histone deacetylase inhibitor (Gurvich et al., 2005, Phiel et al., 2001). Exposure of F9 teratocarcinoma or HeLa cells to 0.25 and 2 mM VPA causes hyperacetylation of DNA which leads to increased expression of growth arrest and pro-differentiation genes (Gottlicher et al., 2001, Kostrouchova et al., 2007). Both *in vitro* and *in vivo*, exposure to VPA leads to an inhibition of cell proliferation primarily by up regulation of p21/waf1, a cyclin-dependent kinase inhibitor, together with an increase in apoptosis (Das et al., 2007, Li et al., 2005).

Despite its low toxicity, a number of reports have documented a range of mild to moderate cognitive impairments, including memory deficits, in adult patients taking VPA (Senturk et al., 2007, Cysique et al., 2006, Carpay et al.,

2005, Gualtieri and Johnson, 2006). The association of these neuropsychological effects with the treatment rather than other factors coexisting with the patients condition is strengthened by reports of improvements in cognition when VPA is discontinued (Lossius et al., 2008, Ristic et al., 2006, Masmoudi et al., 2006, Hommet et al., 2007). The causes of these cognitive changes could be the generalized neuro-suppressant effect of this compound, but may also be due to more specific effects on those brain regions in which neurogenesis continues.

There are two regions in the adult mammalian brain which continue to generate new neurons throughout life, the sub granular zone (SGZ) of the dentate gyrus within the hippocampus and the sub ventricular zone (SVZ) in the lateral walls of the lateral ventricles (Abrous et al., 2005, Ehninger and Kempermann, 2008, Eriksson et al., 1998). Proliferation of neural stem cells within the SGZ continuously produces new granule cell neurons which are incorporated within the dentate gyrus (Ehninger and Kempermann, 2008, Abrous et al., 2005). It has been postulated that the newly generated neurons in this region contribute to hippocampal function and the rate of adult neurogenesis in the hippocampus is correlated with the degree of hippocampal dependent learning and memory (Kitabatake et al., 2007). Specifically factors which affect neurogenesis are also found to affect performance in hippocampal-dependent memory tasks (Bruel-Jungerman et al., 2005, Snyder et al., 2005, Mustafa et al., 2008, Duman et al., 2001).

Two recent papers have looked at the effect of VPA on hippocampal neurogenesis and come to opposite conclusions. Hsieh et al. treated rats with VPA (300 mg/kg) by i.p. injection twice daily for 14 days while also giving VPA in drinking water (12 g/litre). At the end of this period, cell proliferation in the SGZ was significantly reduced although neuronal differentiation of those cells which continued to proliferate had been enhanced (Hsieh et al., 2004). In contrast, Hao et al. (2004) treated mice with VPA (20 mg/kg) in their food for 6 weeks and found an increase in cell proliferation in the SGZ. It is difficult to reconcile these two results although the difference in the route of drug administration may be significant. In neither study were any behavioural tests carried out and so it is unknown whether the reported changes in neurogenesis caused changes to hippocampal function. Patients taking VPA may have other confounding variables that affect cognition. In particular epilepsy itself has been found to cause memory impairments making it difficult to separate the effects of VPA from other aspects of a patient condition (Hirsch et al., 2003, Loiseau et al., 1983, Kwan and Brodie, 2001). The present study uses an animal model to investigate the cognitive effects of VPA exposure in order to avoid these confounding variables.

2.1.1 Aims of this chapter

The present study was carried out to examine whether exposure to VPA in adult animals causes cognitive deficits. The effects of VPA on behavioural testing of recognition memory which were carried out by novel object location (NOL) and contextual fear conditioning tasks. The hippocampal tissue obtained from one half of the brain of the animals at the end of the behavioural testing

was used to investigate the effects of VPA on dividing cell numbers in the dentate gyrus. This was carried out by immunohistochemistry to detect the proliferation marker Ki67. Contradictive findings have been reported in the literature (Hao et al., 2004, Hsieh et al., 2004). Hippocampal tissue from the other cerebral hemisphere of the same brains was processed for Western blotting to detect BDNF, DCX and Notch1 proteins. Measurement of Ki67 was carried out to corroborate the effect of VPA on NOL and contextual fear conditioning tasks employed in this study. Moreover, measures of BDNF, DCX and Notch1 proteins aimed to corroborate changes in neurotrophic factor, survival of newborn neurons and stem cell marker with cognition and cell proliferation. Additionally, BDNF and DCX were measured in the frontal cortex of the same cerebral cortex tissue as hippocampus. This was done to determine the regional-specificity of the effect of VPA in differentiation observed.

2.1.2 Hypothesis

- Cell proliferation in the dentate gyrus will be reduced by treatment with VPA as found by Hsieh et al 2004

- Changes in cell proliferation will be associated with changes in Notch1 levels as it has been suggested that this receptor is involved in controlling neural stem cell proliferation

- Lower levels of cell proliferation will be correlated with reduced levels of DCX indicating that neural differentiation has been affected.

- VPA may also affect BDNF levels within the hippocampus and this may provide a mechanism to alter hippocampal function independent of cell proliferation in the SGZ

- This change in cell proliferation in the SGZ will be correlated with behavioural changes as measured by the novel object location (NOL) and contextual fear conditioning.

- The expression of BDNF and DCX in the frontal cortex tissue will not be significant different between control and VPA-treated animals. Effect of VPA will be specific to the hippocampus

2.2 Materials and Methods

2.2.1 Animals

Adult male Lister hooded rats (Charles River Laboratories, UK), weighting approximately 220-250 g were acclimatized to the Biomedical Service Unit (BMSU), University of Nottingham) for 11 days before experiments started. Prior to experiments, animals were randomly divided into two groups; a control group and a drug-treated group. In the control group, cages 1 and 2 held three animals while cage 3 held four animals. In the drug-treated group, similarly, cages 4 and 5 held three animals whereas cage 6 held four animals. Animals were housed in a temperature-controlled ($20 \pm 1°C$) and humidity-controlled (45-65%) an animal facility with a 12 h light/12 h dark cycle and had access to food and water. The lights were on at 07.00 GMT and the light intensity in cages was 100 - 300 lux. During experiments, animals were weighed everyday. All behavioural tests were recorded by video cameras mounted above the apparatus. All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Acts, 1986, under project licence 40/2715 and personal licence 40/8885.

2.2.1.1 Treatment

Drug-treated animals (n = 10) received two daily intraperitoneal injections approximately at 10.00 + 15.00 hr GMT of VPA (300 mg/kg dissolved in 0.9% saline; Sigma) at a volume of 1 ml/kg for 10 days while control animals (n = 10) were received equally numbers of saline injections (Fig.2.1). This protocol was chosen from a treatment regime which statistically significantly reduces

seizure frequency in spontaneously epileptic rats (Nissinen and Pitkanen, 2007) and is similar to that used by Hsieh et al. (2004), the difference being that VPA was not administered in drinking water as well as by injection.

```
  d1-d10   d16    d17           d23-24
 ─┤ saline ├┤ H ├┤ NOL ├┤ Contextual fear conditioning ├─

  d1-d10   d18    d19           d23-24
 ─┤  VPA   ├┤ H ├┤ NOL ├┤ Contextual fear conditioning ├─
```
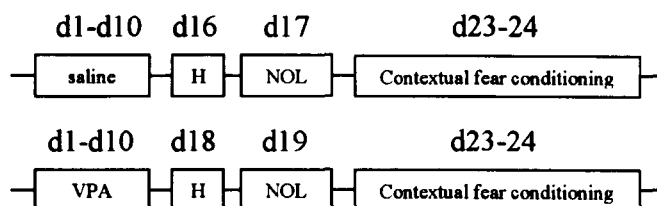
Figure 2.1 Schematic of injection and experimental paradigm. Saline or VPA was injected two daily for 10 days (d1- d10). After treatment, animals were habituated 1 h and then they were assessed for NOL and contextual fear conditioning. Abbreviation: H, habituation; NOL, novel object location.

2.2.2 Behavioural testing

2.2.2.1 Novel object location (NOL) task

The NOL was used to perform behavioural testing of hippocampal-based recognition memory. This test was modified from Dix and Aggleton (1999) and recorded with a video camera mounted directly above each test arena, which was connected to a computer using a program called EthoVision® (EthoVision®, Version 3.1, Noldus, Wageningen, The Netherlands) as described previously by Mustafa et al (2008). EthoVision® tracked a rat's position by comparing and contrasting its movement and colour to its surroundings. Task apparatus consisted of two open square translucent Perspex boxes (39x23.5x30 cm, Whatmore Creative Plastic, UK) which were called Arena 1 and Arena 2. Experiments were performed in constant light (80 Lux at

floor level in the arena) between 10.00 and 14.00 h. The objects to be explored were identical blue commercial plastic water bottles of height 7 cm which had cartoon faces on them. Each object was weighed down with metal bars inside them so that they could not be displaced by the rats. Before starting behavioural testing, each animal was habituated for one hour in the Perspex boxes to be familiar with the arena and environment. After habituation, animals were returned back to the same home cage. On the test day, the trial was divided into two trials; a familiarization trial and a choice trial. In the familiarization trial, animals were placed in the arenas for three minutes to explore two identical objects which were placed firmly in each arena at specific locations. The starting location of objects in the familiarization trial could be either in adjacent corners of the arena or in opposite corners. After three minutes, control and treated rats were removed back to their home cages for five minutes the inter-trial interval (ITI). Prior to the choice trial, the objects and the boxes were cleaned thoroughly with 20% ethanol to eliminate olfactory clues. One object was moved to the adjacent or opposite corner depending upon their location in the familiarization trial. Initial object locations (in adjacent or opposite corners) was randomized (Fig.2.2). In the choice trial, animals were placed back in the arenas to explore the objects for three minutes and then returned back to their home cages. The exploration time of a rat was recorded in seconds using stopwatches. The exploratory activity was defined as actively exploring by directing the nose towards the object at a distance of less than 2 cm (Dix and Aggleton, 1999), not including turning around or sitting on the object. Exploratory activity data in the choice trial was converted to a mean score of discrimination index. Discrimination index is defined as the difference

in exploration time between the two objects in the choice trial (Dix and Aggleton, 1999). Determination of discrimination index was to measure whether the mean scores differed from zero and this would also contribute to analyse discrimination of experimental groups individually by comparing them to zero.

Familiarization Trial   **Choice Trial**
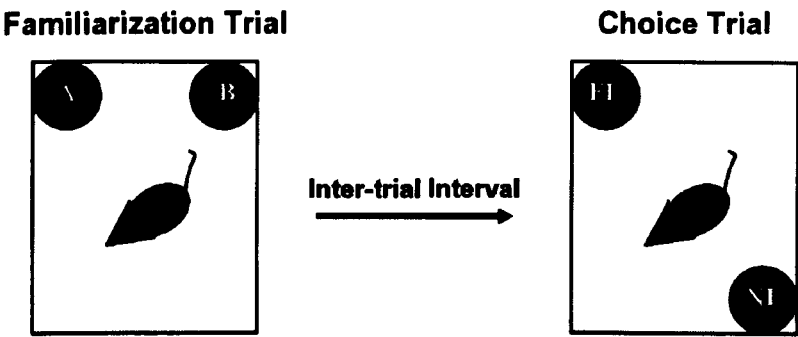
Figure 2.2. Schematic representation of novel object location protocol. This protocol was divided into two trials; a familiarization trial and a choice trial. In the first trial, a rat was placed in the familiarization trial to explore two identical objects, A and B for three minutes. Then the rat was returned back to its home cage for a five minute inter-trial interval. In the choice trial, the rat explored the duplicates of the objects which one object was placed in the same location (familiar location, FL) and the other was moved to a novel location (NL) in the corner opposite to its original location. The location of the objects was randomly located in both familiarization and choice trials.

2.2.2.2 Contextual fear conditioning

The procedure of contextual fear conditioning was modified from Resstel et al. (Resstel et al., 2006). This trial was carried out in two sessions of testing which comprised two days of trials. On day 1 or shock day, each rat was individually placed in the chamber (width x length x height = 24 cm x 25 cm x 26 cm) with a metal grid floor. The floor of the chamber was connected to a shock generator which produced an output of 0.4 mA and also connected to an oscillator in order to measure the electric impulse. Rats were allowed to explore the chamber for one minute before shock and the period of shock delivery was 10 minutes. During the shock period, each rat was received 10 shocks at one minute intervals and each shock was approximately one second. Subsequently, rats were returned to their home cages. The chamber was cleaned with 20% ethanol between each set of trials to ensure the absence of olfactory cues. On day 2, rats were placed into the same chamber for 10 minutes without any shocks. Simultaneously, freezing behaviour was scored continuously during the first three minutes. Freezing was defined as the complete immobility, including movement of vibrissae except for movement related to respiration, whereas animals were usually in crouching posture for a minimum of 0.5 s (Saxe et al., 2006). Scoring the first three minutes of behaviour was chosen as a previous investigation showed that differences between shocked and unshocked animals were most marked during this period.

2.2.3 Brain tissue analysis for cell proliferation by using Ki67

2.2.3.1 Brain tissue preparation

The animals were killed 15 days after behavioural analysis by cervical dislocation, the brains removed, one half was used for immunohistochemistry and the hippocampal formation and frontal cortex dissected from the other for Western blotting. For Immunohistochemistry, half brains were cryoprotected in 30% sucrose for 30 minutes at 4°C to prevent tissue damage due to freezing. Then each brain was placed in plastic moulds filled with OCT compound (VWR International Ltd., UK). After that, the moulds were snap-frozen by submerging in iso-pentane (used to prevent damage of blocks of tissue) which was cooled in liquid nitrogen. Frozen brains were stored at -80°C prior to sectioning and examination. For sectioning, frozen brain blocks from each group were sectioned through the whole dentate gyrus at a thickness of 20 μm (between Bregma -2.3mm to -6.3mm) in the coronal plane using a cryostat (Leica CM 1900). Sections were mounted on APES (3-aminopropylmethoxysaline) coated slices (See Appendix A1.1), with three sections per slide. Slides were kept at -20°C until further analysis.

2.2.3.2 Immunofluorescent staining

For quantification of cell proliferation in the SGZ of the dentate gyrus every 20th section was selected for staining throughout the entire length of the dentate gyrus (in a total of 8 sections) using a systemic random sampling method to provide an efficient and unbiased sample (Mayhew and Burton, 1988). The sections from each brain were quantified for the number of Ki-67

positive cells in the SGZ of the hippocampal dentate gyrus. Only dividing cells within 3 cell diameters of the inner edge of the dentate gyrus were scored as this is normally taken as the location of the SGZ (Kempermaan, 2006). Previous studies have shown that over 70% of dividing cells in this region contribute neurons in the dentate gyrus (Zhao et al., 2006).

For the staining protocol, the selected slices were defrosted for 10 minutes. A PAP marker pen (Vector Labs, UK), which is a hydrophobic barrier, was used to circle around each individual section. All washing was done with phosphate buffered saline (PBS) and all procedures were done at room temperature (RT) in a covered humidity chamber. After the PAP markings were dry, the sections were washed three times to remove the OCT compound. The sections were fixed with 0.5% paraformaldehyde (PFA) (See Appendix A1.1) for three minutes and washed three times. The sections were then treated with the primary antibody monoclonal Mouse Ki67 (Novocastra NCL-KI-67-MM1) at a concentration of 1:150 in PBS for 60 minutes. Following this, the sections were washed gently three times and then treated with the secondary antibody Alexa 488 Rabbit Anti-mouse IgG (Molecular Probes) at a 1:300 working dilution in PBS for 40 minutes. Then, the sections were once again washed three times. Before investigation, all the sections were counter stained with Propidium iodide (Sigma-Aldrich, Inc, St.Louis, USA), a nuclear stain which was diluted with PBS to a 1:3,000 working solution, for 30 seconds. After staining was complete, the sections were washed three times and mounted in glycerol and cover-slipped for observation by fluorescence microscopy.

2.2.3.3 Fluorescence microscope

The fluorescein isothiocyanate (FITC) fluorescence filter was used to quantify Ki67 stained sections using a X40 objective on a Nikon EDF-3 Fluorescence Microscope. Nuclei stained with propidium iodide (PI) on the same sections were observed using the red (TRITC) filter. Images of the dentate gyrus regions were viewed by a SPOT QE version 4.2 digital camera run by the SPOT advanced software run on a PC using Microsoft XP operating system.

2.2.3.4 Microscopic quantification

To avoid scoring artefacts only Ki67 positive cells in the SGZ which also showed a PI positive nucleus were counted. The dividing cells were counted along the entire length of the upper and lower blades of the dentate gyrus, starting from where the upper and lower blades meet. The number of dividing cells in 2-3 adjacent sections on the same slide was averaged and then the averaged counts of eight slides from each brain were compiled to get a measure of the sum of proliferating cells in one dentate gyrus in each animal. The mean number of proliferating cells from both control and drug-treated groups were calculated and statistically analysed. The sum of proliferating cells from both control and drug-treated groups were multiplied 22 to produce estimates of proliferating cell number (Huang and Herbert, 2006) and statistical analysis was performed to compare the number of the cell counts.

2.2.4 Western blot analysis of BDNF, DCX and Notch signalling expression in hippocampus and frontal cortex

2.2.4.1 Sample preparation

Hippocampus and frontal cortex from half of each brain was lysed in lysis buffer (20mM Tris-HCl, pH 7.6, 1mM EGTA, 320 mM Sucrose, 0.1% Triton X100, 1mM NaF, 10mM Beta glyceronphosphaste) to produce a 100 mg/ml solution. The samples were homogenized with a pestle to ensure all protein was released and then rotated on a wheel for 10 minutes at 4°C. Then, the samples were centrifuged at 13000rpm for 10 minutes at 4°C. The supernatant was removed and from each sample was pipetted 20 µl into an eppendorf to quantify protein concentration by Lowry assay. The remaining sample was added 6X solubilisation buffer to solubilise the protein and stored at -20°C.

2.2.4.2 Lowry test for protein concentration

A standard curve was set up using 1 mg/ml stock solution of bovine serum albumin (BSA) diluted in distilled water to prepare the concentration range of 0 to 0.5 mg/ml (See Appendix A1.2). 20 µl of the homogenized sample was diluted (1:10) in distilled water to a volume of 200 µl. All diluted BSA standards and homogenized samples were added to 1ml of Lowry AB solution (See Appendix A1.2) and incubated at room temperature for 10 minutes. Folin reagent was diluted 1:1 with distilled water and added at 100 µl per sample and incubated at room temperature for 45 minutes. After Folin reagent had reacted with the Lowry AB for 45 minutes, each standard and sample was placed, in triplicate, into a 96 well plate and the optical density (OD) was read at 750nm.,

using a Dynex MRX Model 96 Well Plate Reader (MTX Lab Systems Inc., USA). The colourimetric reading and the protein concentration value within each sample were analysed and presented by Revelation software (MTX Lab Systems Inc., USA). All the samples were then normalized with 2X solubilisation buffer diluted 1:1 with lysis buffer.

2.2.4.3 Casting the gel

During this study, 0.75 mm, 1.0 mm or 1.5 mm gel were used for electrophoresis depending upon the volume of sample to be loaded. The components and the volumes for 8%, 10% and 12% gel preparation are shown in Appendix A1.2. The glass plates were cleaned with IMS and distilled water before casting the gel and then placed them into a cast. The resolving gel was pipetted into the cast and pipetted 100 µl of saturated butanol onto the top of the gel to remove any bubbles. The gel was allowed to set for 20 minutes. After the gel had set, the butanol was poured off and the gel was washed three times with distilled water. Blotting paper was used to remove excess water to make sure the gel was dry. The stacking solution was pipetted on top of the resolving gel and a comb was placed into the gel to create wells. Once set, the comb was removed and the gel was washed thoroughly with distilled water, especially all the wells.

2.2.4.4 Running and transferring the gel

Prior to protein separation, the solubilised samples were denatured by warming them on a heat block at 95°C for 5 minutes and then vortexed and centrifuged at 1300rpm for 1 minute. The gel was placed into an electrophoresis tank (BIO-

81

RAD Laboratories, Hertfordshire, UK) and filled with 1X running buffer in distilled water. 30 µg proteins per lane were subjected to 12% SDS-PAGE to assess BDNF and DCX while 50 µg proteins per lane were subjected to 8% SDS-PAGE to assess Notch1. Additionally, 2 µl of a molecular weight marker (PageRuler plus Prestained protein ladder, Fermentas UAB, Lithuania) was run parallel to the samples to determine the size of the protein of interest. The gel was run for approximate 45 minutes at 200V.

Subsequently, the proteins from the gel were transferred onto a nitrocellulose membrane (Hybond™, Amersham Biosciences, Buckinghamshire, UK). In transferring process, the gel was placed into a cassette, placed onto a piece of blotting paper and then a piece of nitrocellulose membrane was placed on top of the gel which followed by another piece of blotting paper. The cassette was closed and placed into a transfer tank filled with transfer buffer and an ice pack. This process was run at 4°C for 60 minutes at 100V. After transferring, the nitrocellulose membrane was stained with Ponceau solution (Sigma-Aldrich, Inc, St,Louis, USA) to indicate the amount of protein successfully transferred. The membrane was washed in Tris-buffered saline tween-20 (TBST) solution (See Appendix A1.2) to remove the Ponceau solution.

2.2.4.5 Immunodetection

Prior to immunodetection, the membrane for detecting BDNF and DCX was blocked with 5% milk solution (dried milk powder dissolved in TBST) whereas the membrane for detecting Notch1 was blocked with Odyssey buffer (LI-COR Biosciences) 1:1 diluted in PBS, shaking platform for 1 hour at room

temperature. The membrane was incubated overnight with primary antibodies diluted in 5% milk solution and placed on a constant shaking platform. The primary antibodies used in this study are as follow: anti-BDNF (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:500, polyclonal anti-doublecortin (DCX) antibody diluted 1:1000 (Cell Signaling Technology; Danvers, MA, USA), polyclonal anti-Notch1 (C20) diluted 1:200 (Sc-6014, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or monoclonal Mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody diluted 1:20000 (Abcam, Cambridge, UK). Afterwards, the membrane was rinsed 3 times, and then washed for 5 minutes 3 times and 15 minutes 3 times with TBST at room temperature. Subsequently, the membrane was incubated with secondary antibodies: IRDye 800CW goat anti-mouse (1:20000 diluted in 5% milk solution, LI-COR Biosciences), IRDye 680CW goat anti-rabbit (1:20000 diluted in 5% milk solution, LI-COR Biosciences) or IRDye 800CW donkey anti-goat (LI-COR Biosciences) 1:20000 diluted in Odyssey buffer (1:1 diluted in PBS). The membrane was analyzed in accordance with the manufactures instructions using on an Odyssey scanner (Licor) at both wavelengths, 700nm (red emission) and 800nm (green emission), and Odyssey software (Application software version 3.0).

2.2.4.6 Quantification for protein and data analysis

Hippocampal and frontal cortex tissue samples from both control and VPA-treated groups were run and repeated three times. Quantitative measurement of protein level was performed using Odyssey software (Application software version 3.0) by measuring the intensity of the protein band. Intensity levels of

BDNF and DCX expression were measured at 700nm while intensities of Nocth1 and GAPDH expression were measured at 800nm. The intensity values of BDNF, DCX and Notch1 for each sample were normalized by their corresponding GAPDH intensity values run on the same gel. The intensity values of each experimental group were pooled together and presented as the mean of BDNF, DCX and Notch1 protein expression. All pictures of the blots showed in this chapter were visualised using Adobe Photoshop Version 7.0 (Adobe Photoshop, USA) to increase contrast only to allow visible hardcopy prints for figures especially red and green images.

2.2.5 Statistical analysis

All statistical parameters were calculated using GraphPad Prism Version 4.03 (GraphPad Software Inc., USA). One- and two-way repeated measures ANOVA were used to analyze weight data. For the behavioural testing, unpaired Student's t-test was used to analyze total exploratory time between control and VPA-treated groups in sample and choice trials. The data of NOL within each group was analyzed using paired Student's t-test to compare means of exploratory time in the sample and choice trials. Comparisons of mean exploratory times between groups were performed using two-way repeated measures ANOVA. Analysis of the discrimination index and contextual fear conditioning were calculated using one sample t-test and unpaired Student's t-test. Proliferating cell count and Western immunoblotting data were analyzed by unpaired Student's t-test. Normality distribution of all data was analyzed using Column statistics (KS normality test, D'Agostino & Pearson omnibus

normality test or Shapiro-Wilk normality test). A probability level of $p < 0.05$

was considered statistically significant.

2.3 Results

2.3.1 Weight

To investigate the effect of VPA on weight gain, the weight of animals was monitored daily throughout the treatment. During the administration, the VPA-treatment rats showed a significant weight loss, approximately 16 grams ($p <$ 0.0001; one-way repeated measures ANOVA, Fig. 2.3), while animals received saline showed a slightly increase of weight gain, approximately 21 grams ($p <$ 0.0001; one-way repeated measures ANOVA, Fig. 2.3). Further analysis showed that the rats administered VPA showed a significant weight loss compared to animals received saline treatment ($F(1,162) = 8.958$, $p = 0.0078$, treatment variable, two-way repeated measures ANOVA). There was a significant change in the amount of weight gained in both control and VPA-treated animals over the time period observed ($F(9,162) = 5.918$, $p < 0.0001$, main effect of time, two-way repeated measures ANOVA, Fig. 2.3). Furthermore, a significant interaction between treatment and time was observed, indicating that treatment affected the rate of weight gain over the monitored period ($F(9,162) = 44.92$, $p < 0.0001$, interaction, two-way repeated measures ANOVA, Fig. 2.3).
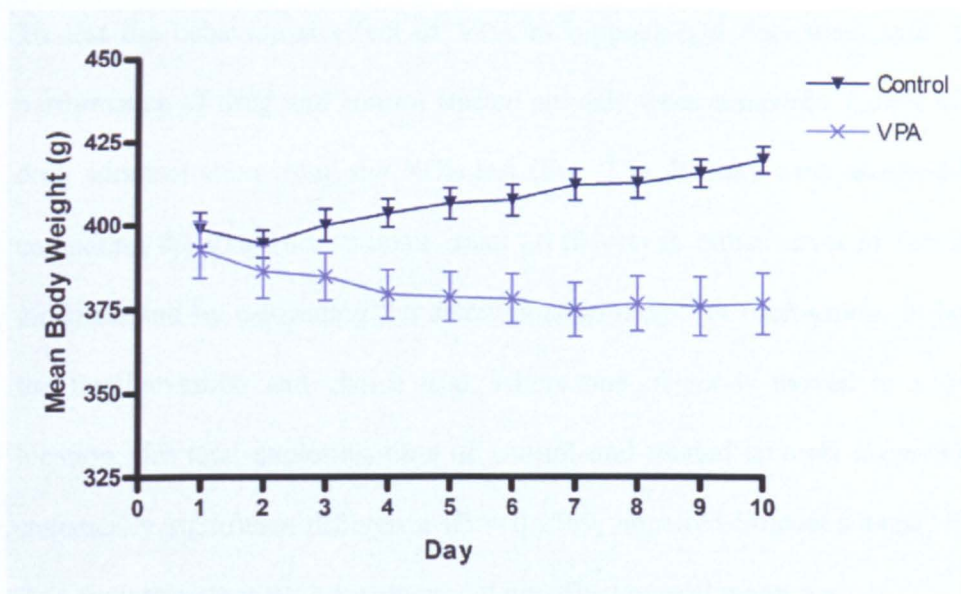
Figure *2.3* Mean body weight (± S.E.M) of the rats in each group was monitored during drug treatment (Days 1-10). This experiment compared between control and drug-treated groups which were received normal saline and 300 mg/kg VPA. Both control and VPA-treated animals showed a significant change in the rate of weight gain, $p = 0.0001$. Mean of weight gain in the drug-treated group (n = 10) was significantly lower than those in the control group (n = 10) ($p < 0.05$, two-way repeated measures ANOVA). There was a significant interaction effect between treatment and time ($p < 0.0001$, interaction). All comparisons were calculated using one- and two-way repeated measures ANOVA.

2.3.2 Novel object location (NOL)

To test the behavioural effect of VPA in hippocampal dependent task, the performance of drug and control treated animals were compared 7 days after drug administration using the NOL test (Fig. 2.1). Results were assessed by comparing the total time animals spent on objects in either novel or familiar locations and by calculating the discrimination index for each group. In both the familiarization and choice trial, where one object is moved to a new location, the total exploring time of control and treated animals showed no statistically significant difference ($P = 0.2569$, unpaired Student's t-test, Fig. 2.4), indicating that VPA-treatment did not affect overall motor activity.

Total exploratory time
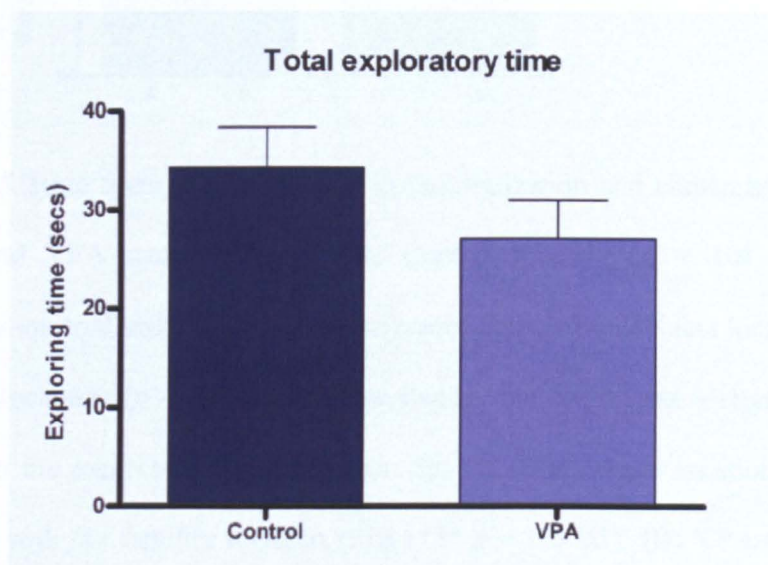
Figure 2.4 Total exploratory time during the NOL testing in controls and VPA treated animals. There was no significant difference in the amount of time exploring the objects between the control and VPA-treated animals ($p = 0.2569$). All statistic parameters were analyzed using unpaired Student's t-test.
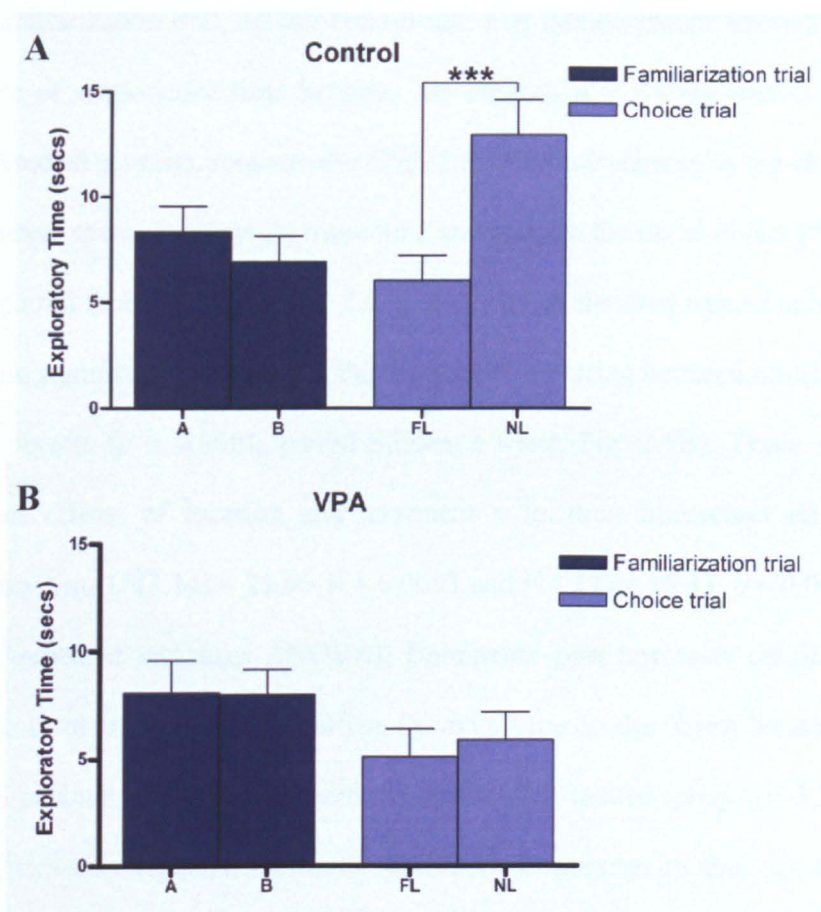
Figure 2.5 Time spent on each object in familiarization and choice trials by control and VPA-treated animals. (A) Control animals (n = 10) in the familiarization trial did not display a preference for the two objects located in A and B locations (p = 0.0518). In the choice trial, there was a significant increase in the exploratory time spent on objects in the novel location (NL) compared with the familiar location (FL) (*** p = 0.0002). (B) VPA-treated animals (n = 6) (7 days post treatment) spent the same amount of time exploring the objects in A and B locations in the familiarization trial (p = 0.1291). In the choice trial there was no significant difference in the time spent between objects in familiar and novel location (p = 0.9601). All comparisons assessed using paired Student's t-test.

In the familiarization trial, neither control nor drug treated groups showed any difference of exploratory time between the objects, p = 0.0518 and 0.1291 (paired Student's t-test), respectively (Fig. 2.5). Control animals in the choice trial however spent significantly more time attending to the novel object (* p = 0.0002, paired Student's t-test, Fig. 2.5A). In contrast, the drug treated animals showed no significant difference in the time spent exploring between novel and familiar objects (p = 0.9601, paired Student's t-test, Fig. 2.5B). There were significant effects of location and treatment x location interaction on the exploratory time ($F(1,14) = 23.66, p = 0.0003$ and $F(1,14) = 15.33, p = 0.0016$, two-way repeated measures ANOVA). Bonferroni post hoc tests confirmed that the control animals spent significantly more time on the object located in the novel position in comparison with the rats in VPA-treated group (t = 3.275, p <0.01, two-way repeated measures ANOVA). In contrast to this, the time spent exploring the object located in the familiar position exhibited no difference between the two groups (t = 0.4107, p >0.05, two-way repeated measures ANOVA, Fig. 2.5). This indicates that VPA-treated animals showed a significant deficit is a spatial working memory task.

In order to further analyse these results, the exploratory times in the choice trial were converted into a discrimination index to compare the ability of the animals to discriminate the familiar and novel objects between control and VPA-treated groups. The discrimination index between the novel and the familiar location after treatment were significantly above zero in control group (p = 0.0002, one sample t-test, Fig. 2.6) while the discrimination index in the VPA-treated group were not significantly different from zero (p = 0.1563, one

sample t-test, Fig. 2.6). Comparisons of the discrimination index between control and VPA-treated groups demonstrated a significant difference after treatment (p = 0.0016, unpaired Student's t-test, Fig. 2.6). These findings indicate that animals which received VPA did not discriminate the object remained in the familiar and the novel objects in this test.
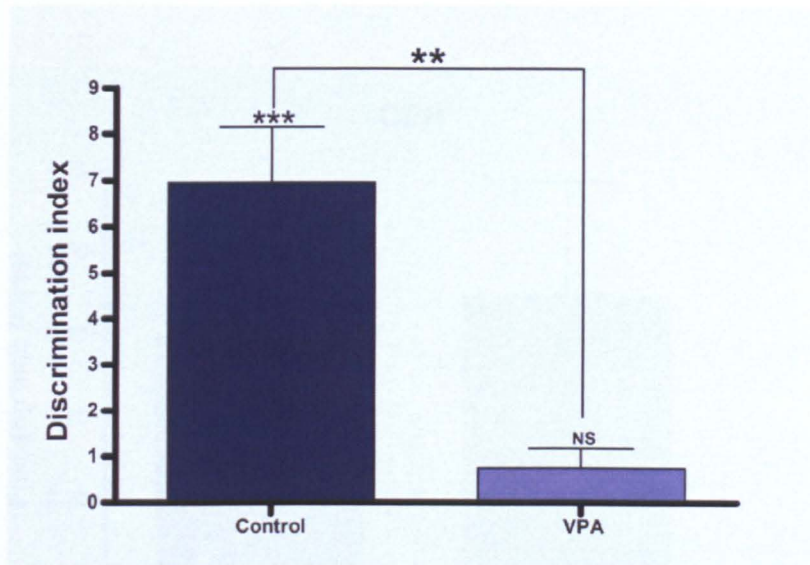
Figure 2.6 The discrimination index of control and VPA-treated rats in the novel location recognition task after treatment. The discrimination index is defined as the difference in exploration time between the two objects in the choice trial. The mean discrimination index of each group was compared against 0. The discrimination index of control group was significant greater than 0 (*** p = 0.0002). In contrast, VPA-treated animals had a discrimination index value that was not significant different from 0 (NS, p = 0.1563). The discrimination index showed a significant difference in comparison between groups (** p = 0.0016,). Statistic analysis was carried out using one sample t-test and unpaired Student's t-test.

2.3.3 Contextual fear conditioning test

Freezing behaviour was assessed by scoring the freezing time during the first three minutes and means of the freezing time were then calculated for each group. The results showed no significant difference between the freezing behaviour of control and drug-treated animals ($p = 0.8075$; unpaired t-test, Fig. 2.7).
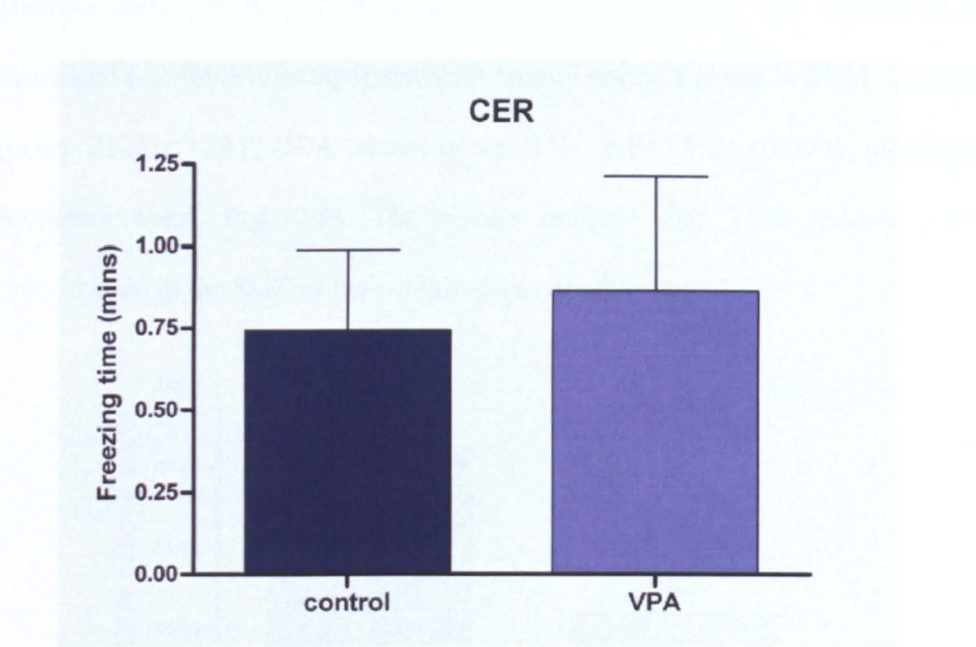
Figure 2.7 Effect of shock on freezing behaviour after treatment in control (n = 10) and VPA-treated (n = 6) animals. Data represents mean ± S.E.M of freezing time in minutes. Analysis of freezing behaviour between control and drug-treated animals was not found significantly different either before or after treatment, p-value = 0.7797 (unpaired Student's t-test).

2.3.4 Quantification of proliferating cells in the SGZ

To investigate the possible causes of the deterioration in the hippocampal spatial memory, the level of cell proliferation in the SGZ of the dentate gyrus was determined by staining for the cell proliferation maker Ki67 (Fig. 2.9). Ki67 positive cells were found in the SGZ adjacent to the inner edge of the dentate gyrus as described by previous investigators. The number of Ki67 positive cells per hippocampus in VPA-treated animals was significantly decreased (~2-fold) in comparison with control animals (mean ± SEM; control group: 2106 ± 129.9; VPA treated group: 1100 ± 84.55; P <0.0001, unpaired Student's t-test, Fig. 2.8). The results indicate that VPA reduced cell proliferation in the SGZ of the dentate gyrus of adult rats.
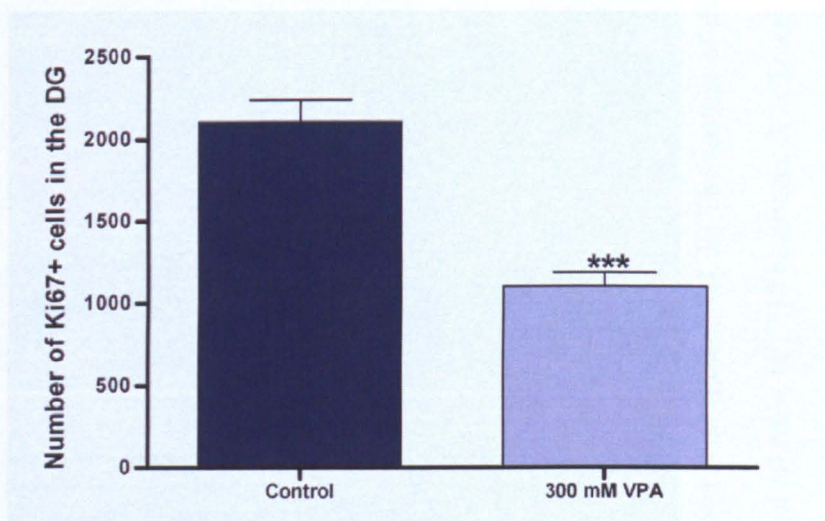
Figure 2.8 VPA reduced cell proliferation in the hippocampus. The number of Ki67 positive cells per hippocampus in VPA-treated animals (n = 10) was significantly decreased in comparison with control animals (n = 10, *** $p <$ 0.0001, unpaired Student's t-test).
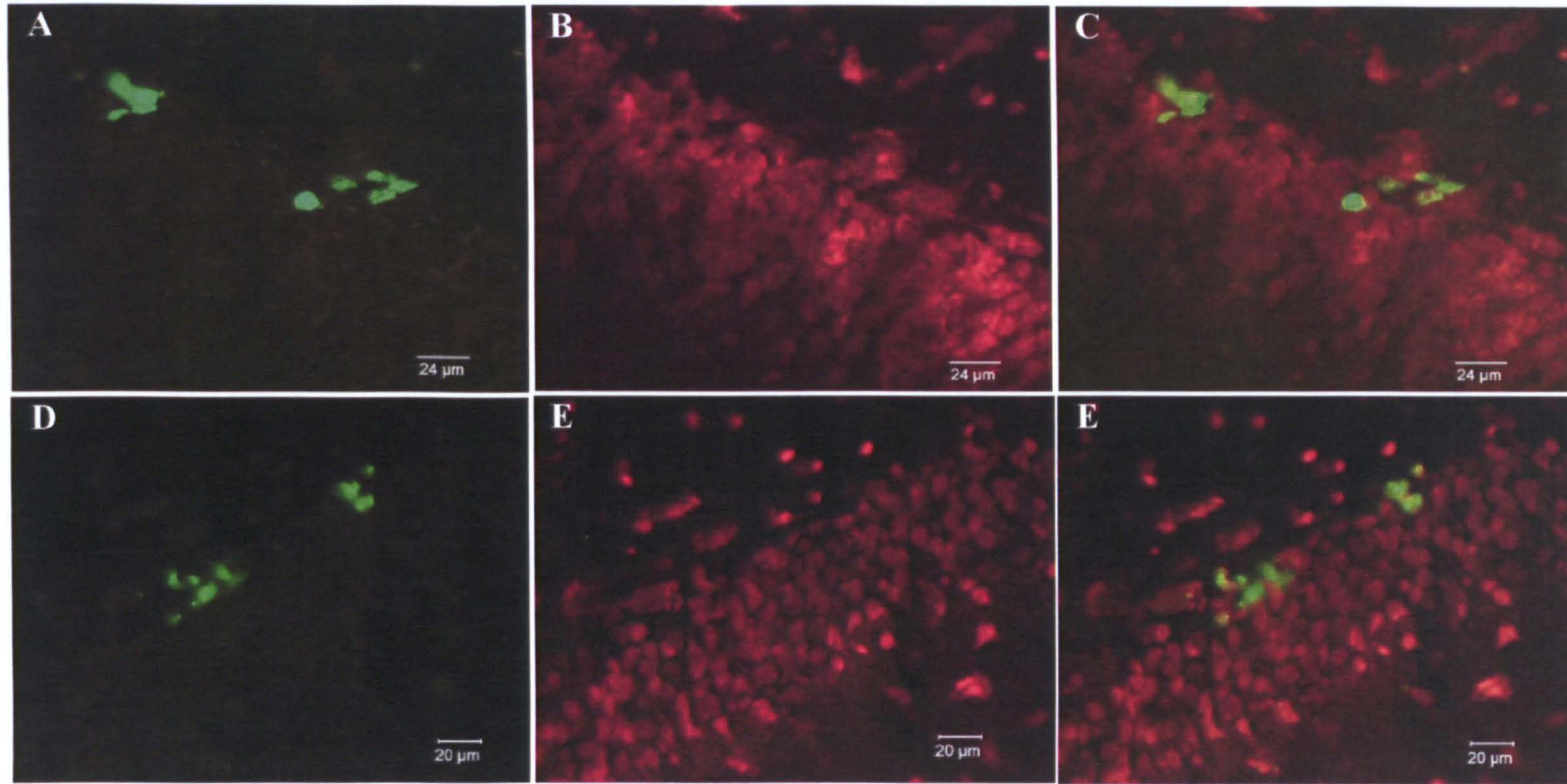
Figure 2.9 (A, D) Clusters of Ki67 positive cells (green). (B, E) The section of the dentate gyrus seen in (A, D) counterstained with Propidium iodide (PI), showing red nuclear staining of all cells. (C, F) The images A or D and B or E merged to show Ki67 positive cells located in the SGZ adjacent to the dentate gyrus. Upper panels control (saline-treated) rat, lower panels VPA-treated rat.

2.3.5 BDNF, DCX and Notch1 expression

Western blotting analysis was performed to investigate the effects of VPA on levels of BDNF and DCX expression in the hippocampus and frontal cortex. Whole brain and recombinant BDNF were used as positive control for DCX and BDNF respectively.

Immunoblots probed for BDNF expression showed a band at approximately 15 KDa (Fig. 2.10). The present study showed a significant reduction in BDNF levels within the hippocampal formation in VPA-treated animals compared to control animals ($p = 0.0053$, unpaired Student's t-rest, Fig. 2.11A). Conversely, BDNF levels in frontal cortex did not differ between control and VPA-treated groups ($p = 0.5352$, unpaired Student's t-test, Fig. 2.11A).

Probing for the cytoskeletal associated protein DCX which is transiently expressed in immature neurons, showed a band at approximately 45 KDa (Fig. 2.10). Protein levels of DCX did not show a significant reduction compared between control and VPA-treated groups in the hippocampus and frontal cortex, $p = 0.5736$ and 0.8769, respectively (unpaired Student's t-test, Fig. 2.11B).

The receptor Notch1 is expressed on undifferentiated neural stem cells where it is involved in controlling proliferation and in postmitotic neurons where promotes dendritic branching. Western blot analysis was used to detect a band of Notch1 at approximately 120 KDa (Fig. 2.12A). VPA-treated animals

showed a significant decrease of Notch1 expression in the hippocampus compared with controls ($p = 0.01$, unpaired Student's t-test, Fig. 2.12B).

These results indicate that VPA treatment led to a significant decrease of BDNF and Notch1 but not DCX in the hippocampus.
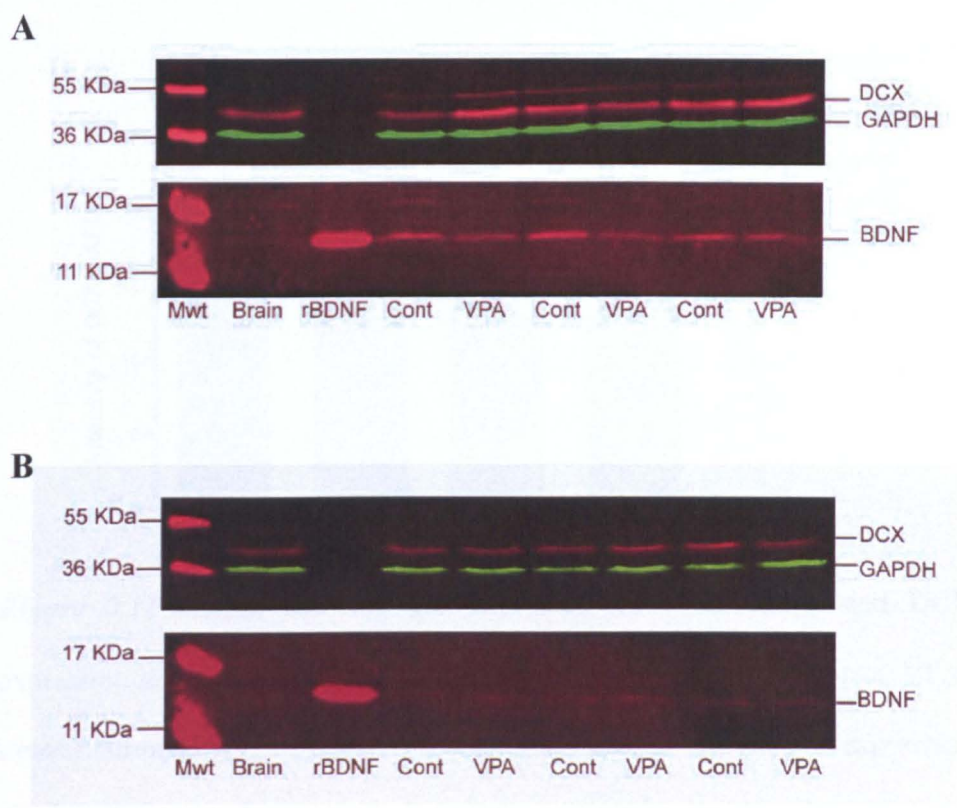
Figure 2.10 Photomicrographs of representative immunoblots showing detection of BDNF at 15 KDa and doublecortin at 45 KDa in the hippocampus (A) and frontal cortex (B). The positive control consisted of recombinant BDNF (lane 3) and whole brain for doublecortin (lane 2). GAPDH was observed at 36 KDa and was used as a loading control.

Cont = control, VPA = valproic acid and Mwt = Molecular weight marker.
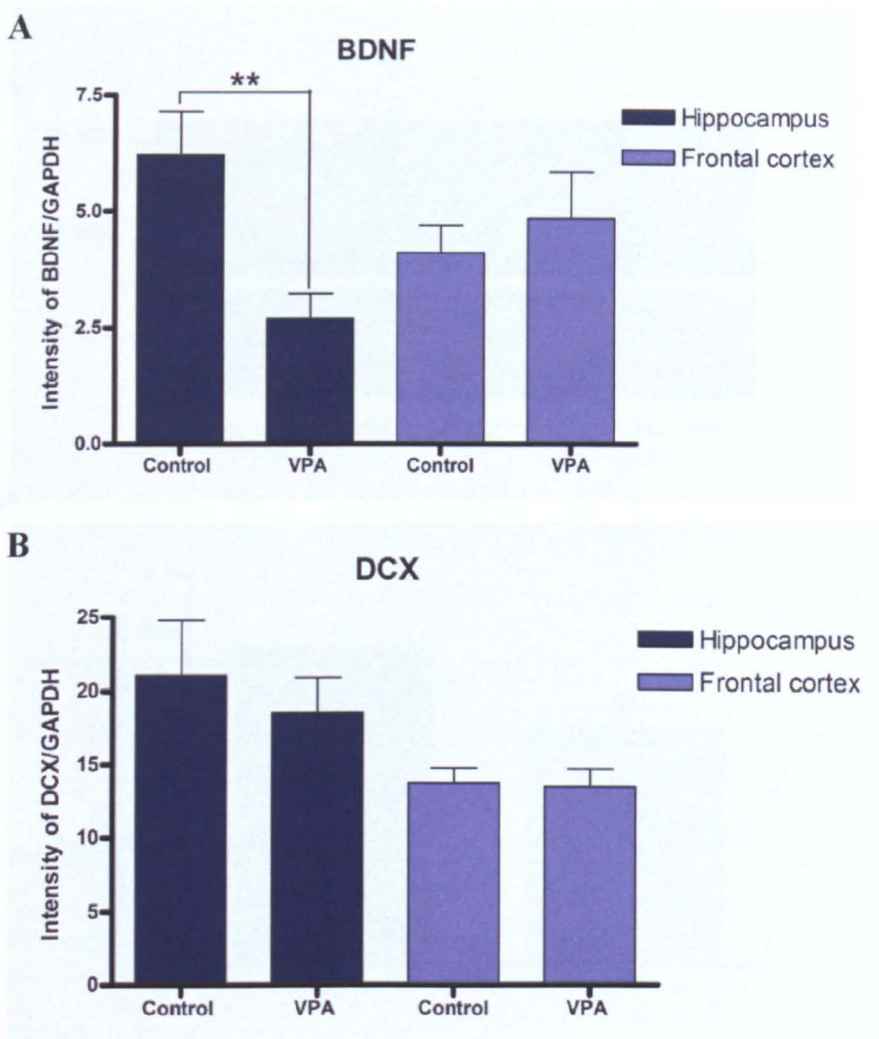
Figure 2.11 Graphs showing the effects of VPA on BDNF and DCX expression in hippocampus and frontal cortex. (A) In the hippocampus, VPA-treated animals (n = 8) showed a significant decrease of BDNF expression compared to control animals (n = 8) (** p = 0.0053). On the other hand, the levels of BDNF in VPA-treated animals was not significant different in comparison with control animals in frontal cortex (n = 9) (p = 0.5352). (B) DCX expression in both hippocampus (n = 8) and frontal cortex (n = 9) did not displayed a significant difference between control and VPA-treated groups, p = 0.5736 and 0.8769, respectively. Statistical significant was determined using unpaired Student's t-test.

97

A

130 KDa — | | Notch1

95 KDa —

36 KDa — | | GAPDH

Mwt   Control   VPA   Control   VPA   Control   VPA

B
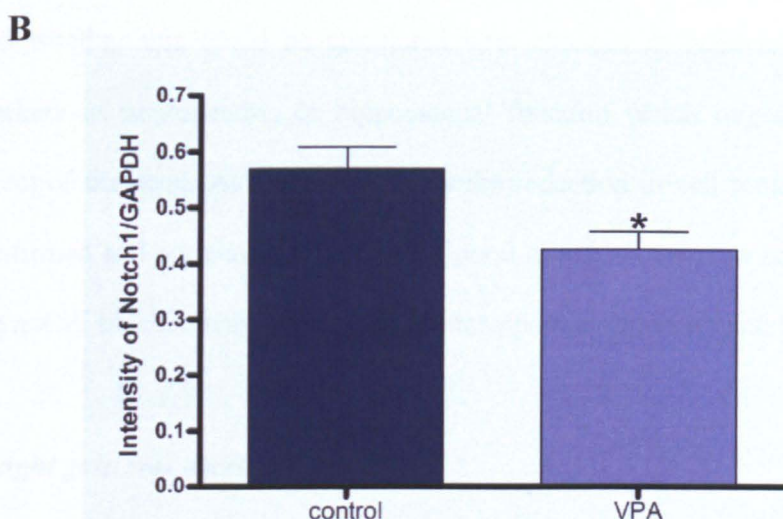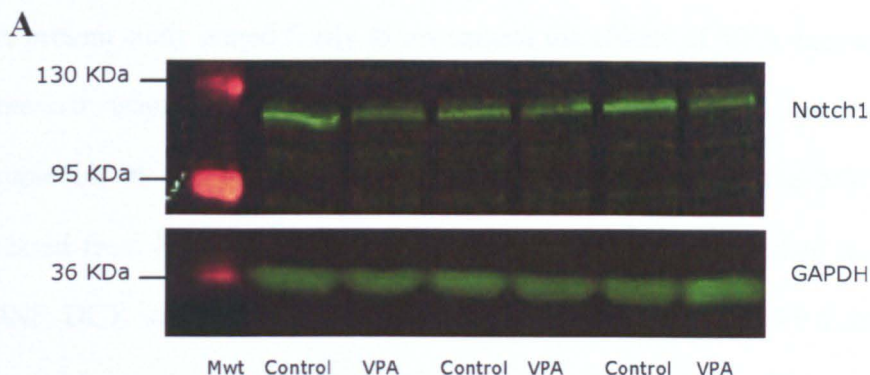
Figure 2.12 Western blot analysis for levels of Notch1 in animals administered VPA in hippocampus. (A) Photomicrographs illustrating immunoblot bands detected at 120 KDa. The expression of Notch1 was reduced in VPA-treated animals as indicated by the presence of fainter bands compared with control animals. GAPDH was used as a loading control. (B) Normalized wavelength at 800nm revealed that there was a significant decrease of Notch1 expression in VPA-treated animals (n = 9) in comparison with controls animals (n = 9) (* $p = 0.01$, unpaired Student's t-test). Cont = control, VPA = valproic acid and Mwt = Molecular weight marker.

2.4 Discussion

The present study aimed firstly to investigate the effects of VPA exposure on behaviour using the NOL and contextual fear conditioning tests. After completion of the behavioural testing, cell proliferation in the SGZ was assessed from half of each brain while the other half was used to quantify BDNF, DCX and Notch1 protein levels. This was done to see if VPA affected cell proliferation in the hippocampal neurogenic region, as reported previously, and whether this could be correlated with changes in behaviour and other markers of neurogenesis or hippocampal function which might explain the effect of the drug. As discussed below the reduction in cell proliferation was confirmed and correlated with a behavioural change in one test and with some but not all biochemical markers of hippocampal neurogenesis and function.

Weight gain was decreased by VPA

The present study of the adult rats exposed to VPA, showed that the weight gain of VPA-treated animals was significantly decreased compared to control animals which gained in weight throughout the experiment. There are conflicting reports on the effects of VPA on weight gain in patients. Exposure of women to anticonvulsant drugs during pregnancy results in decreased birth weights (Fonager et al., 2000) in contrast to a report comparing monozygotic twins or children treated with VPA which found a weight gain (Klein et al., 2005, Demir and Aysun, 2000). Weight change is not thought to be a normal side effect of VPA and the mechanism which could bring this about is not well understood.

VPA produced cognitive deficits in NOL but not contextual fear conditioning

VPA treatment did not affect locomotor activity or total exploration time in the NOL test compared to control animals. The NOL test which requires animals to recall the spatial layout of objects in an arena is regarded as a test of spatial working memory (Dix and Aggleton, 1999). The test uses the natural tendency of rats to spontaneously explore an object placed in new position without primary reinforcement, spatial learning or the learning of response-reward association which is needed in other behavioural tests. Therefore, it needs very little pretraining and does not cause arousal or stress (Dere et al., 2007). This test using the novelty-preference paradigm is closely related to human recognition memory observed in everyday life (Ennaceur and Delacour, 1988). Results from the choice trial, where control animals showed significantly more interest in the object moved to a novel location, showed that drug treated animals failed to show this behaviour. Animals treated with VPA showed an inability to discriminate between an object in a novel location and one in a familiar location. This result indicates that VPA can cause spatial memory deficits after chronic exposure.

In terms of the hippocampal formation the NOL test requires an intact dentate gyrus for animals to be able to carry it out (Mumby et al., 2002, Lee et al., 2005). The inability of VPA animals to discriminate between novel and familiar object locations suggests a deficit in the function of this particular brain region. As discussed later since the dentate gyrus is one of the few brain areas to continuously incorporate new neurons a decline in neurogenesis may be one reason for the cognitive impairment shown in this behavioural test.

In addition to the NOL test, the present study also scored freezing behaviour after exposure to an aversive stimulus in a particular location (contextual fear conditioning). This test also used the hippocampus but is thought to also involve other brain areas including the amygdala and brain stem (periaqueductal grey matter) (Fanselow, 2000). In contrast to the NOL test, the behaviour of control and drug-treated animals was not found to be significantly different.

Although the contextual fear test is not as specific a test of hippocampal function as the NOL, a reduction of neurogenesis in the hippocampus by focal X irradiation or genetic ablation of GFAP containing neural progenitors has previously been reported as causing an impairment in contextual fear conditioning (Saxe et al., 2006). This would suggest an association between neurogenesis and the ability to perform the test.

The results predicted here however do not suggest this connection. There are a several possible reasons for this. Firstly, the number of electric shocks used in this study (10 shocks) is greater than that used in some studies (3 shocks) (Saxe et al., 2006). Increasing the number shocks may not lead to an improvement in memory and in fact may cause difficulties for the brain in interpreting the association between stimulus and context and so reduce the ability to learn. In line with a human study, it has been reported that patients who are under conditions of high fear or hunger can not usually be assessed for learning and memory capacities (Ennaceur and Delacour, 1988). Additionally, the rats in this study were administered VPA before the contextual fear conditioning

testing. This time scale is different from post-training hippocampal lesions where animals were injected with VPA 120 and 30 minutes before the passive avoidance test which showed cognitive deficits (Balakrishnan and Pandhi, 1997; Pandhi and Balakrishnan, 1999). Support this view comes from the finding that post-training hippocampal lesions cause severe impairments in contextual fear conditioning. In post-training hippocampal lesions, the hippocampus is required for assembling the contextual stimuli into a context representation. Then, the context representation comes into association with footshock in the amygdala to acquiring contextual fear conditioning. On the other hand, contextual stimuli can come into association with footshock in the amygdala without using a configural strategy in pre-training hippocampal lesions (Maren, 2001). Thus they may be two of the reasons that made freezing behaviour show no significant difference between drug and control groups in this study.

VPA reduced proliferating cells in the SGZ of the hippocampal dentate gyrus
Adult neurogenesis in the hippocampus is confined to producing new granule cells in the dentate gyrus. These cells are derived from a proliferating pool of neural stem cells in the SGZ adjacent to the dentate gyrus. Recent work has shown that it is newly formed granule cells which are preferentially used in spatial learning tasks (Dupret et al., 2008, Kee et al., 2007). This observation explains why levels of neurogenesis in the hippocampus generally strongly correlate with hippocampal-dependent cognition (Zhao et al., 2008). Treatment with VPA significantly inhibited cell proliferation in the SGZ by the end of the experiment, a result in agreement with a previous study (Hsieh et al., 2004).

The route of administration of VPA is similar to that used by Hsieh (twice daily injections for 14 days) but without the additional VPA in drinking water. In contrast, administration of VPA only with food for 6 weeks has been reported as increasing hippocampal neurogenesis (Hao et al., 2004). It is unknown what the relative serum concentrations of VPA are by these two methods of drug administration but it is likely that i.p. injections lead to a higher peak serum concentration than that obtained through diet. It is possible that VPA has different effects at different concentrations, possibly stimulating proliferation at low concentrations while inhibiting it at higher concentrations.

The correlation between reduced proliferation in the SGZ and cognitive deficits is in line with recent work by Mustafa et al (2008) and other studies stating that pharmacological, environmental or physical influences on cell proliferation in this region correlate with changes in cognition (Drapeau et al., 2003, Kempermann, 2002, Madsen et al., 2003, Raber et al., 2004a, Shors et al., 2001). In particular the results presented here parallel those studies in which anti-mitotic drugs have been shown to reduce proliferation in the SGZ and impair cognition (Seigers et al., 2008, Shors et al., 2001). There have been some disagreements in what role hippocampal neurogenesis plays in spatial learning. Some authors have found a good correlation between neurogenesis and learning and have shown that reducing neurogenesis leads to deficits in this modality (Kee et al., 2007, Raber et al., 2004b, Kempermann et al., 1998). Others however have not found this association or have demonstrated improvements in cognition independent of neurogenesis and have queried the link between neurogenesis and spatial learning (Saxe et al., 2006, Shors et al.,

2002, Meshi et al., 2006). Recently a genetic approach to blocking neurogenesis has shown that a reduction in neurogenesis is associated with a significant decrease in spatial memory (Imayoshi et al., 2008). As discussed in this reference the differences in results may be due to differences in the extent neurogenesis is inhibited or to non specific effects on brain function.

VPA reduced hippocampal BDNF but not DCX

In addition to effects on behaviour and cell proliferation in adult rats, VPA treatment caused a significant decrease in BDNF expression in the hippocampus but not frontal cortex. The neurotrophin, BDNF, is expressed by dentate gyrus granule cells (Conner et al., 1997, Wetmore et al., 1990). BDNF has been shown to be important both in the process of learning and in adult neurogenesis as well as mediating the effects of antidepressants (Nibuya et al., 1995, Shirayama et al., 2002). BDNF is required for long term potentiation (LTP), a process required for memory consolidation (Bekinschtein et al., 2008, Korte et al., 1995, Lu et al., 2008, Minichiello et al., 1999). In the process of neurogenesis within the SGZ, BDNF is required for the survival, migration and maturation of newly formed neurons and promotes their dendritic growth and branching. Reducing BDNF levels or BDNF signalling in the brain is associated with impaired spatial memory (Minichiello et al., 1999, Mustafa et al., 2008). Two reports have found that chronic *in vivo* VPA treatment can elevate BDNF levels in the hippocampus (Fukumoto et al., 2001) while a further study showed a non significant reduction or no effect. In both cases where BDNF levels were increased, BDNF levels were measured immediately after the end of drug treatment. Within 24 hours however BDNF levels had

fallen to control levels (Fukumoto et al., 2001). In the present study, BDNF levels were measured after the behavioural testing, 2 weeks after the end of drug dosing. BDNF is required to maintain the synaptic plasticity underlying memory but is also produced and secreted in response to learning reviewed in (Lu et al., 2008). The lower BDNF levels found in VPA treated animals are consistent with their impairment in learning during the behavioural tests but could be due to the action of VPA on BDNF production or to reduced hippocampal activity which may be occurring in animals which fail to develop spatial memories.

After VPA treatment, animals showed a non significant reduction in DCX levels within the hippocampus. DCX is a microtubule associated protein which is required for neuronal cell migration and is transiently expressed maturing neurons in the SGZ and dentate gyrus (Couillard-Despres et al., 2005). During neurogenesis in the dentate gyrus, newly differentiating neurons appear to retain expression of DCX for several weeks during which time they complete their migration into the dentate gyrus and extend dendrites and axons (Rao and Shetty, 2004). DCX levels may have been increased in these animals by the differentiation promoting effects of VPA which as well as inhibiting cell proliferation can accelerate neuronal progenitor differentiation (Gottlicher et al., 2001, Hsieh et al., 2004).

VPA did not decrease BDNF and DCX in frontal cortex

BDNF and DCX levels in frontal cortex were also determined to investigate that not only newborn neurons in the hippocampus was affected by VPA

treatment but also the frontal cortex could be affected. BDNF and DCX expression were detected within the frontal cortex; however, there were no significant changes of BDNF and DCX expressions in VPA-treated animals compared to the control animals in this brain region. BDNF is considered to have an important role to contributing generation and/or survival of newly generated neurons in the adult olfactory bulb (Zigova et al., 1998). It has been revealed that level of DCX expression is substantially observed in newborn neurons of the olfactory bulb (Nacher et al., 2001). The expression of DCX in the dissected frontal cortex included the SVZ, RMS and main and accessory olfactory bulbs suggests that this portion comprises the second neurogenic zone in the adult brain (Abrous et al., 2005). This lack of effect on neurogenesis in the frontal cortex indicates that the VPA administration in this study did not affect the generation and/or survival of new neurons in the frontal cortex of the adult brain.

VPA caused a reduction of receptor Notch1 expression in the hippocampus

VPA treated animals in the present study showed a significant reduction in Notch1 protein levels within the hippocampus which correlated with their reduced performance in a spatial learning task. Although the contribution of reduced Notch1 levels to cognitive impairment is unclear, it is likely that it is affecting the generation, maturation and synaptic plasticity of newly formed neurons within the dentate gyrus. Notch comprises a family of four cell surface receptors activated by ligands on adjacent cells (Yoon and Gaiano, 2005). Notch receptors and their ligands are expressed throughout neural development and expression is retained in discrete regions of the adult brain including strong

expression in the SGZ and dentate gyrus (Breunig et al., 2007, Stump et al., 2002). Signalling through Notch appears to have several effects on the receptor expressing cells. During neural development and probably in the germinal zones of the adult brain, activation of the Notch1 receptor prevents neural differentiation and maintains neural precursor and the stem cell proliferation (Breunig et al., 2007, Hitoshi et al., 2002). In post mitotic neurons, Notch1 is important for the later stages of granule cell maturation and dendritic branching (Redmond et al., 2000, Breunig et al., 2007). Given these functions it is not surprising that alterations in the level of Notch1 expression have been found to influence learning and memory. Transgenic mice with reduced expression of Notch1 show reduced LTP which can be rescued by addition of Notch signalling components (Wang et al., 2004). Homozygous Notch1 knock out mice die before birth while heterozygous knock out animals survive but show impaired spatial learning (Costa et al., 2003).

Untreated epilepsy itself causes cognitive impairments (Aldenkamp and Bodde, 2005, Hirsch et al., 2003) and this is associated with increased but aberrant hippocampal neurogenesis in animal models (Crespel et al., 2005, Jessberger et al., 2007). VPA is able to normalize neurogenesis after induction of seizures in animal models and this may be one of the mechanisms by which it helps epileptic patients (Hsieh et al., 2004). However, it is worth nothing that treatment with VPA when neurogenesis is normal can produce cognitive impairments associated with reduced neurogenesis. This may be significant in the use of VPA as a mood stabiliser in conditions where neurogenesis is not elevated or aberrant.

Chapter 3

The effect of Valproic acid on cell proliferation and Notch signalling expression in hippocampal neural stem cells

3.1 Introduction

Valproic acid (VPA) has been clinically employed as an antiepileptic drug and a mood stabilizer for several decades. Although, VPA has been known to increase γ-aminobutyric acid (GABA) ergic function and block sodium and calcium channels, the mechanisms of action are currently not entirely clear (Eyal et al., 2004). In addition to its effects on neurotransmitters and ion channels, VPA has the potential to affect chromatin structure, which is important in determining whether stem cells either self-renew or differentiate (Hsieh et al., 2004). VPA has been shown to decrease cell proliferation and enhance neuronal differentiation which might be via the inhibition of HDACs. Suppression of cell proliferation and induction of apoptosis by VPA has been known to arrest the cell cycle at a G0/1 phase in multiple myeloma cells *in vitro* (Murabe et al., 2007). *In vivo* studies have shown that VPA also regulates differentiation and inhibits the proliferation of neuronal progenitor cells suggesting that the actions of VPA in controlling neural production could be modified for therapeutic use in patients (Jung et al., 2008). Exposure of embryonic rat cerebral cortex neural progenitor cells to 1 mM VPA induces a marked up-regulation of p21 expression, increasing differentiation and inhibiting cell proliferation (Jung et al., 2008).

Adult neural stem cells (NSC) residing in the SGZ of the hippocampal dentate gyrus give rise new granule cells for the entire life of the organism. New born neurons functionally contribute to existing circuits within the dentate gyrus (Ehninger and Kempermann, 2008, Abrous et al., 2005, Farioli-Vecchioli et al., 2008, Hsieh and Gage, 2004). Moreover, newly generated neurons in the SGZ

are used preferentially in aspects of hippocampal function such as learning and memory (Kitabatake et al., 2007) suggesting that adult hippocampal neurogenesis may be essential for these behaviours. NSCs in the mammalian CNS have been extensively used to investigate a model of the molecular mechanisms that control cell-fate specification (Hsieh and Gage, 2004). The undifferentiated NSCs maintain the potential to proliferate and self-renew which is the most important intrinsic capacity of stem cells (Shi et al., 2008, Galli et al., 2003). Stem cell self-renewal and differentiation are regulated by transcription factor, chromatin remodelling and epigenetic factors (Shi et al., 2008). In monolayer culture, proliferation of hippocampal progenitor cells treated with 1 mM VPA for 24 hours was dramatically decreased compared to untreated cells using BrdU staining (Hsieh et al., 2004).

In the previous chapter (Chapter 2), adult rats were administered two daily intraperitoneal injections of VPA at a dose of 300 mg/kg. This resulted in significant cognitive impairment as shown by the novel object location (NOL) test and a significant reduction of cell proliferation in the SGZ of the hippocampal dentate gyrus. VPA also produced a decrease in the levels of BDNF and Notch1 expression in hippocampal tissues. The cognitive deficit produced by VPA treatment might be due directly to the action of the drug on cell proliferation leading to a reduction in neurogenesis but could also be indirect, through changing levels of neurotrophic factors (e.g. BDNF); acting on the stem cell niche (e.g. Notch1) or affecting other brain regions (e.g. frontal cortex) which then influence hippocampal function. To further understand the direct effect of VPA on the neural stem cells in the SGZ of the

hippocampus investigations were carried out *in vitro* to see what changes in proliferation and gene expression occurred with VPA treatment. This approach has become a widely used as an important new tool for predicting drug effects and estimating their toxicity.

Treatment of a range of cell lines with a therapeutic dose (0.3 -0.7 mM) of VPA has been reported to inhibit histone deacetylases (HDACs) and enhance the acetylation of core histones in HeLa, F9 teratocarcinoma and Neuro2A neuroblastoma cells (Murabe et al., 2007). Thus, VPA can potentially directly affect transcription, which might cause stem cells to lose control over their ability to proliferate, differentiate, apoptosis or senesce. Taking as a starting point the results from the previous *in vivo* study, it was decided to look at the effects of VPA on cell proliferation and the expression of Notch1, Sox2, nestin and c-Myc genes in hippocampal NSCs *in vitro*. Both proliferation and Notch 1 levels were reduced by VPA treatment *in vivo* while nestin, Sox2 and c-Myc are genes known to be expressed by neural stem cells and changes in their level of expression may indicate changes in the ability of NSCs to   self-renew. Alteration of gene expression and proliferation in adult hippocampal NSCs could have direct effects on neurogenesis leading to subsequent changes in hippocampal function.

3.1.1 Aims of this chapter

To determine the direct effects of VPA on hippocampal neural stem cells by examining their proliferation and expression of the neural stem cells markers: Sox2, nestin, c-Myc and Notch1, *in vitro*.

3.1.2 Hypothesis

- Neurosphere and cell proliferation of hippocampal NSCs in culture will be significantly reduced by treatment with VPA.

- Levels of Notch1, Sox2, nestin and c-Myc gene expressions in hippocampal NSCs will be significantly decreased by treatment with VPA.

3.2 Materials and Methods

3.2.1 Analysis of effects of VPA on adult hippocampal NSCs

3.2.1.1 Isolation and propagation of adult rat hippocampal NSCs

Adult Lister hooded rats (Charles River Laboratories, UK), weighing approximately 160-200 g were killed by decapitation, the brains were removed from the skulls and then immersed into ice cold PBS. The hippocampus was dissected and diced into small pieces ($<1mm^3$). Following the dissection, all dissected tissue was pooled into a 30 ml universal tube. PBS was removed prior to tissue digestion. In order to digest the tissue, 1 ml of accutase (Millipore, California, USA) was added to the tube, followed by incubation for 45 minutes at 37°C to facilitate the dissociation of the tissue. During digestion, the tissue dissociation was aided by gentle pipetting using blue tips and P1000 until only very small clumps were present or the suspension looked cloudy. 10 ml of PBS was added to dilute the accutase and the tube was shaken to ensure the tissue was completely dissociated. The tissue was then centrifuged at 200g for 5 minutes and PBS was removed from the cell pellet. The tissue was resuspended in 4 ml of neural stem cell (NSC) medium [Dulbecco's Modified Eagles Medium/Ham's F12 (DMEM/F12 1:1, Invitrogen, CA, USA), Neurobasal media (Invitrogen, CA, USA), N2 supplement (Invitrogen, CA, USA), B27 (Invitrogen, CA, USA), 0.5% Pen/Strep (Sigma-Aldrich, Inc, St.Louis, USA), EGF (Sigma-Aldrich, Inc, St.Louis, USA) and FGF-2 (Invitrogen, CA, USA)] (see Appendix A1.4). 2 ml of the cell suspension was placed in a 6-well plate (Corning) and cultured at 37°C with 5% CO_2. The next

day, the medium is replaced with new medium ensure a fresh supply of nutrients. Every 3-4 days, the medium was replaced with new medium for actively growing culture.
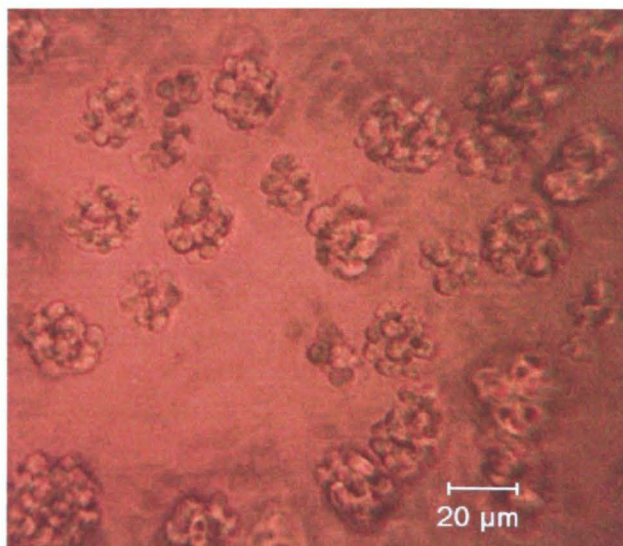
Figure 3.1 Phase contrast photograph of neurospheres of adult rat hippocampal NSCs.

3.2.1.2 Cell proliferation of adult rat hippocampal NSCs

Cultures of hippocampal NSC were assigned in one of three groups: control, 0.3 mM VPA or 1 mM VPA. Hippocampal neurospheres were transferred from a T75 tissue culture flask (Corning, Sigma-Aldrich, Inc, St.Louis, USA) to a 30 ml universal tube and centrifuged at 200rpm for 5 minutes. The medium was removed using an aspirator being careful not to disturb the cell pellet. 0.5-1 ml of accutase was added and the pellet resuspended. The cells were incubated for 5-10 minutes at 37°C to facilitate the dissociation of the cells. To dissociate the cells into single cells, the solution was gently pipetted using 1000P and then PBS was added to dilute the accutase. The density of the cells was counted

using an Improved Neubauer Hemocytometer (See Appendix A1.3). The cells were cultured at 2×10^5 cells in a T75 flask in 15 ml of NSC medium containing 20 ng/ml of EGF and FGF-2, maintained at 37°C with 5% CO_2. The following day, the NSCs were treated with 0, 0.3 or 1 mM VPA. After 72 hours in vitro, floating sphere colonies (neurospheres) which were bigger than a few cells were counted. Following neurosphere count, neurospheres were triturated with 0.5-1 ml of accutase, incubated at 37°C for 5-10 minutes. The neurospheres were dissociated into single cells and the density of neural stem cells was then assessed in control and VPA treated cultures by staining cells with Trypan blue. Quantification of numbers of neurospheres and single cells was performed in triplicate on separate cultures (Appendix A1.3). Means of the total number of neurosphere-forming NSCs and the density of neural stem cells were calculated and subjected to the statistical analysis.

3.2.1.3 Isolation and propagation of adult mouse hippocampal NSCs

Three brains of mice (male and female adult 129 and ME1 mice, Biomedical Service Unit (BMSU), University of Nottingham) were dissected and the hippocampi isolated. The hippocampi were transferred into ice cold PBS and then proceeded as detailed previously in section 3.2.1.1, chapter 3.
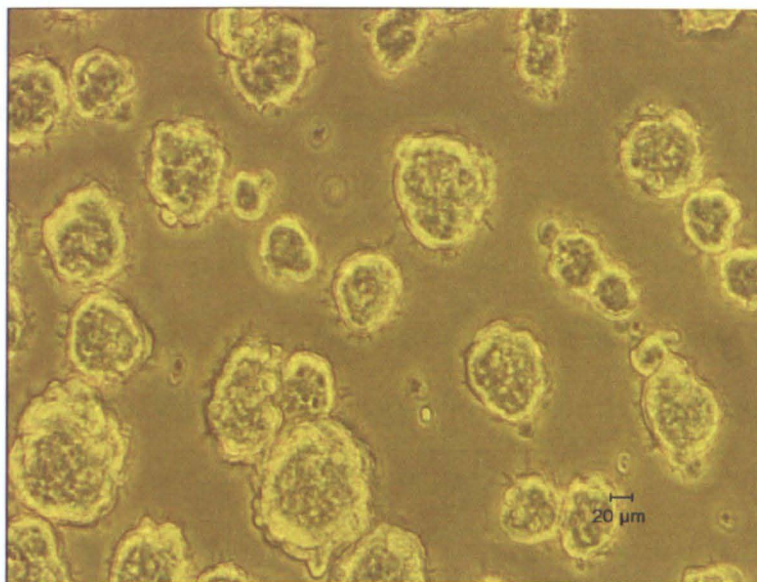
Figure 3.2 Phase contrast photograph of neurospheres of adult mouse hippocampal NSCs.

3.2.1.4 Cell proliferation of adult mouse hippocampal NSCs

Adult hippocampal neurospheres were dissociated with accutase as described previously (section 3.2.1.1, chapter 3) and then seeded at 12.5×10^6 cells in 5 ml of NSC medium in a T25 flask. Hippocampal NSCs were incubated for 2 hours at 37°C and then treated with 0, 0.3 and 1 mM VPA. Following 48 hours incubation, numbers of hippocampal NSCs were counted by staining cells with Trypan blue as detailed previously (section 3.2.1.2, chapter 3). Analysis of density of adult mouse hippocampal NSCs was performed a total of three times on different samples per condition (n = 3) (Appendix A1.3).

3.2.2 Analysis of quantitative real-time reverse transcription polymerase chain reaction (qPCR) of Sox2, c-Myc, nestin and Notch1 gene expressions in VPA treated adult mouse hippocampal NSCs

3.2.2.1 VPA treatment

Cultures of mouse hippocampal NSCs were assigned in one of three groups: 0, 0.3 or 1 mM VPA. Hippocampal neurospheres were transferred from a T25 flask (Corning, Sigma-Aldrich, Inc, St.Louis, USA) to a 30 ml universal tube and centrifuged at 200g for 5 minutes. The medium was removed using an aspirator being careful not to disturb the cell pellet. 0.5-1 ml of accutase was added and the pellet resuspended. The cells were incubated for 5-10 minutes at 37°C to facilitate the dissociation of the cells. To dissociate the cells into single cells, the solution was gently pipetted using 1000P and then the accutase was diluted by adding PBS. The density of the cells was counted using Trypan blue assay. The cells were cultured at 1.25×10^6 cells in a T25 flask in 5 ml of NSC medium containing 20 ng/ml of EGF and FGF-2, maintained at 37°C with 5% CO_2. After 2 hours, the NSCs were treated with 0, 0.3 or 1 mM VPA. After 48 hours in vitro, the hippocampal neurospheres were removed from the T25 flask to a 30 ml universal tube and centrifuged at 200g for 5 minutes. Then the hippocampal neurosphere-forming NSCs were washed with cold PBS and centrifuged at 200g for 5 minutes. The pellet of the hippocampal neurosphere-forming NSCs was collected for cDNA preparation. The treatment was performed in triplicate.

3.2.2.2 RNA extraction

The hippocampal neurosphere-forming NSCs were lysed in TRI REAGENT (Sigma-Aldrich, Inc, St,Louis, USA) at $5\text{-}10 \times 10^6$ cells per 1 ml of the reagent by repeated pipetting. The sample was incubated for 5 minutes at room temperature to ensure complete dissociation of nucleoprotein. Then, chloroform (Fisher Scientific UK Ltd, Leicestershire, UK) was added to the sample at 0.2 ml per ml of TRI REAGENT used. The sample was shaken vigorously for 15 seconds and incubated for 2-15 minutes at room temperature. The sample was centrifuged at 12,000g for 5 minutes at 4°C. This resulted in the sample being separated into 3 phases: a red organic phase (containing protein), an interphase (containing DNA) and a colourless upper aqueous phase (containing RNA). The aqueous phase was transferred to a new tube and 0.5 ml of isopropanol (Fisher Scientific UK Ltd, Leicestershire, UK) per ml of TRI REAGENT added. Then the sample was mixed very well and incubated for 5-10 minutes at room temperature. After incubation, the sample was centrifuged at 12,000g for 10 minutes at 4°C. Once a pellet of the RNA precipitate was form on the side and bottom of the tube, the supernatant was removed and the RNA pellet washed with at least 1 ml of 75% ethanol per 1 ml of TRI REAGENT used. The sample was vortexed and then centrifuged at 13,000g for 5 minutes at 4°C. After washing with 75% ethanol, the supernatant was removed without disturbing the pellet by using a pipette. The RNA pellet was dried briefly for about 5 minutes by turning the tube up side down and put on a paper towel. Once the RNA pellet dried but was not completely dry, 10-30 µl of DEPC-treated water (Invitrogen, CA, USA) was added to the sample. Then the sample was mixed and centrifuged for a few seconds. 1.2 µl of RNA

sample was analysed for RNA concentration using a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, DE, USA) and recorded 260/280 values were used to confirm the purity of samples. The RNA sample could be stored at -80°C.

3.2.2.3 First-strand complementary deoxyriboneuclease (cDNA) synthesis

RNA purification was preceded by elimination of DNA using Deoxyribonuclease I, Amplification Grade (DNase I, Amp Grade) (Invitrogen, CA, USA) prior to RNA-PCR amplification. 3 μg RNA sample, 2 μl DNase I (Amp Grade, 1 U/μl) and 1.5 μl 10X DNase I reaction buffer were added into a 1.5 – 2 ml eppendorf and then DEPC-treated water (Invitrogen, CA, USA) was added to make 15 μl. The sample was thoroughly mixed and centrifuged for a few seconds and then was incubated for 40 minutes at room temperature to deactivate DNA. The sample was heated for 10 minutes at 65°C to reactivate RNA and the sample was ready to use in reverse transcription, prior to amplification. After DNA purification, the concentration of the sample would be 200 ng/μl.

For cDNA synthesis, duplicate 0.5 ml eppendorfs are prepared for positive and negative reverse transcriptase samples which are used in the amplification reaction. For 20 μl reaction volume, 1 μl random primer (Promega, WI, USA), 1 μl 10 mM deoxyribonucleoside triphosphate (dNTP) mix (Promega, WI, USA) and 2 μg RNA for the positive reverse transcriptase sample or 1 μg RNA for the negative reverse transcriptase sample were added into the eppendorf

and then added DEPC-treated water (Invitrogen, CA, USA) to 13 µl. Before

incubation, the sample was mixed thoroughly and then centrifuged for a few

seconds. The sample was heated for 5 minutes at 65°C and incubated on ice for

at least 1 minute. The sample was centrifuged for a few seconds. The sample

was mixed with 4 µl 5X first-strand buffer (Invitrogen, CA, USA), 1 µl 0.1 M

dithiothreitol (DTT) (Invitrogen, CA, USA) and 1 µl superscript III reverse

transcriptase (Invitrogen, CA, USA) was added to the positive reverse

transcriptase sample (omitted from the negative reverse transcriptase sample).

The sample was mixed by pipetting gently up and down and centrifuged for a

few seconds. After that, the sample was incubated for 5 and 45 minutes at 25°C

and 50°C respectively. For inactivating the reaction, the sample was heated for

15 minutes at 70°C. The sample was centrifuged for a few seconds and then

could be used as a template for amplification or stored at -20°C. The

concentration of the sample would be 100 mg/µl.

3.2.2.4 Reverse transcriptase polymerase chain reaction (RT-PCR)

For a 10 µl reaction, 1 µl 10X High yield reaction buffer with 25 mM $MgCl_2$

(Buffer A) (Kapabiosystems, MA, USA), 0.8 µl 10 mM dNTP

(Deoxyribonucleotide triphosphate) mix (Promega, WI, USA), 0.1 5U/µl

KapaTaq DNA polymerase (Kapabiosystems, MA, USA), 0.2 µl 10 µM β-actin

forward primer (Eurofins MWG Operon, Ebersberg, Germany) or mouse Sox2

forward primer (Eurofins MWG Operon, Ebersberg, Germany), 0.2 µl 10 µM

β-actin reverse primer (Eurofins MWG Operon, Ebersberg, Germany) or

mouse Sox2 reverse primer (Eurofins MWG Operon, Ebersberg, Germany),

6.7 µl HPLC water (Invitrogen, CA, USA) and 1 µl cDNA (100 µg/µl for

positive reverse transcriptase sample and 50 µg/µl for negative reverse transcriptase sample) were added into an RNase-free, 0.5 ml microcentrifuge tube. The sample was heated for 10 minutes at 95°C to denature the double strand melts open to single stranded DNA. For the amplification, the sample was firstly denatured at 95°C for 30 seconds. In the annealing step, the temperature was lowered to 60°C for 30 seconds allowing the primer to bind to the single-stranded DNA. After annealing, Tag polymerase synthesized a new DNA strand complementary to the DNA template strand by adding dNTPs at 72°C for 45 seconds. These reactions were performed for 35 cycles. Then the PCR products were ready for agarose gel electrophoresis.

Table 3.1 **Summary of final concentration of 10 µl RT-PCR reaction**

Components	Volume (µl)	Final concentration
HPLC water	6.7	-
10 mM dNTP	0.8	0.8 mM
10 µM Forward primer	0.2	200 nM
10 µM Reverse primer	0.2	200 nM
5 U/µl KapaTaq polymerase	0.1	0.05 U/µl
10X Buffer (A) 25 mM MgCl$_2$	1 -	1X 2.5 mM
100 ng/µl cDNA	1	100 ng/reaction

Table 3.2 **Nucleotide sequence of the forward and reverse primers for qPCR in mouse hippocampal NSCs**

Gene name	Gene identity primers (5'→3')	Total
mNestinRTF mNestinRTR	CGC TGG AAC AGA GAT TGG AAG G (22 bp) GTC TCA AGG GTA TTA GGC AAG (21 bp)	376 bp
mSox2qF mSox2qR	AGG GCT GGG AGA AAG AAG AG (20 bp) CCG CGA TTG TTG TGA TTA GT (20 bp)	94 bp
mMycQF mMycQR	AGC CCC TAG TGC TGC ATG A (19 bp) TCC ACA GAC ACC ACA TCA ATT TC (23 bp)	110 bp
clath up clath dn	GAC AGT GCC ATC ATG AAT CC (20 bp) TTT GTG CTT CTG GAG GAA AGA A (22 bp)	600 bp
GAPDH	f-GGG TGG AGC CAA ACG GGT C (19 bp) r-GGA GTT GCT GTT GAA GTC GCA (21 bp)	500bp
18s rRNA F 18s rRNA R	GGT CCG ACC ATA AAC GAT GCC (21 bp) TGG TGG TGC CCT TCC GTC AAT (21 bp)	130bp
Notch1 F Notch1 R	TCAATGCCGTGGATGACCTA CCTTGTTGGCTCCGTTCTTC	100bp

3.2.2.5 Agarose gel electrophoresis

The PCR products were analyzed on 1.4% (w/v) an agarose gel, dissolved 0.7 g of agrose (Invitrogen, CA, USA) in 50 ml of 1X Tris-Acetate-EDTA (TAE) buffer (Appendix A1.5) by heating to boiling in a microwave. After that, 5 µl of ethidium bromide (10 mg/ml, Sigma-Aldrich, Inc, St.Louis, USA) was added into the gel solution to get a final concentration of 0.1 µg/ml. The gel solution was poured into a casting tray and a comb was placed into the gel to

create wells. The gel was allowed to solidify before removing the comb. After gel casting, the gel was submerged into 1x TAE buffer. Before loading, 1 µl of 5x gel-loading buffer (New England Biolabs) was added into the PCR products. 1 kb or 100 bp (New England Biolabs) (200 ng) were run alongside the PCR products to identify sizes of PCR products of interest. 8 µl of the PCR products were loaded into the wells. The gel was run in 1X TAE buffer in an electrophoresis tank (BIO-RAD Laboratories, Hertfordshire, UK). The gel was run in 1X TAE buffer at 120 V for 10 minutes and then visualized in a MultiMage light cabinet and photographed using an AlphaImager 1220 Documentation & Analysis System (Alpha Innotech Corporation).

3.2.2.6 Selection of suitable reference genes

Quantitative PCR was performed in a volume of 25 µl/reaction. 12.5 µl Brilliant SYBR Green QPCR Master Mix (Stratagene, Aligent Technologies Company, CA, USA), 0.375 µl 10 µM GAPDH forward primer (Eurofins MWG Operon, Ebersberg, Germany) or 0.375 µl 10 µM 18s forward primer (Eurofins MWG Operon, Ebersberg, Germany) or clathrin forward primer (Eurofins MWG Operon, Ebersberg, Germany), 0.375 µl 10 µM GAPDH reverse primer (Eurofins MWG Operon, Ebersberg, Germany) or 18s reverse primer (Eurofins MWG Operon, Ebersberg, Germany) or clathrin reverse primer (Eurofins MWG Operon, Ebersberg, Germany), 0.375 µl 20 µM Rox reference dye (Stratagene, Aligent Technologies Company, CA, USA), 10.9 µl HPLC water (Invitrogen, CA, USA) and 0.5 µl 100 µg/µl cDNA sample were added into an RNase-free, 0.5 ml microcentrifuge tube. The reaction was divided into three segments. Firstly, the reaction mixture was heated to 95°C

for 10 minutes. Secondly, the reaction mixture was performed in 40 amplification cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 45 seconds. Finally, the mixture was incubated at 95°C, 55°C and 95°C for 1 minute, 30 seconds and 30 seconds, respectively. All qPCRs were performed in triplicate using the Mx3005p qPCR system (Stratagene, Aligent Technologies Company, CA, USA).

Table 3.3 **Summary of final concentration of 25 µl qPCR reaction**

Components	Volume (µl)	Final concentration
HPLC water	10.9	-
2X Master mix	12.5	1X
10 µM Forward primer	0.375	150 nM
10 µM Reverse primer	0.375	150 nM
20 µM Rox reference dye	0.375	300 nM
100 ng/µl cDNA	0.5	50 ng/reaction

3.2.2.7 *Effect of VPA on Notch1, Sox2, nestin and c-Myc gene expressions using qPCR*

The 25 µl of PCR mixture included 12.5 µl Brilliant SYBR Green QPCR Master Mix (Stratagene, Aligent Technologies Company, CA, USA), 0.375 µl 10 µM 18s forward primer (Eurofins MWG Operon, Ebersberg, Germany) or mouse Notch1 forward primer (Eurofins MWG Operon, Ebersberg, Germany) or mouse Sox2 forward primer (Eurofins MWG Operon, Ebersberg, Germany) or mouse Nestin forward primer (Eurofins MWG Operon, Ebersberg, Germany) or mouse c-Myc forward primer (Eurofins MWG Operon,

Ebersberg, Germany), or 18s reverse primer (Eurofins MWG Operon, Ebersberg, Germany) or mouse Notch1 reverse primer (Eurofins MWG Operon, Ebersberg, Germany) or mouse Sox2 reverse primer (Eurofins MWG Operon, Ebersberg, Germany) or mouse Nestin reverse primer (Eurofins MWG Operon, Ebersberg, Germany) or mouse c-Myc reverse primer (Eurofins MWG Operon, Ebersberg, Germany), 0.375 µl 20 µM Rox reference dye (Stratagene, Aligent Technologies Company, CA, USA), 10.9 µl HPLC water (Invitrogen, CA, USA) and 0.5 µl 100 µg/µl cDNA sample. All qPCRs were performed in triplicate. The reaction was performed as described earlier (section 3.2.3.6, chapter 3). The expression of each gene was normalized to 18s in order to calculate relative levels of transcript.

3.2.3 Statistical analysis

The data were expressed as mean ± SEM and analyzed by GraphPad Prism Version 4.03 (GraphPad Software Inc., USA). Statistical analysis was performed using one-way ANOVA followed by the Bonferrini's multiple comparison test. The significant level was set at $p < 0.05$.

Prior to qPCR analysis, all the samples were assessed cDNA quality using gel electrophoresis. Discrete intact RNA bands should be observed in the RT-PCR products but not the no RT-PCR products. This would verify that all the samples were not contaminated with genomic DNA.

In qPCR experiments, it was necessary to quantify reference gene as normaliser gene side-by-side with the gene of interest. The results were

calculated as relative quantity to the control, where the control sample was assigned an arbitrary quantity of "1" and all the other samples were examined in terms of their difference to this sample. This was done in accordance with previous publications and the manufacturer instructions (Stratagene, Aligent Technologies Company, CA, USA).

For accurate data analysis and meaningful statistics, negative controls known as no template control (NTC) were determined in every run. Ideally, signal amplification should not be detected in the NTC sample wells. NTCs were provided to control for external contamination or other factors that could result in a non-specific increase in the fluorescence signal.

At the end of the amplification reaction, qPCR products from each sample were run as a melting curve, also known as dissociation curve, to determine whether anything other than the gene of interest was amplified in the qPCR reaction. Most qPCR products would melt somewhere in the range of 80 - 90°C. Ideally, the melting temperature should be the same in all the reactions where the samples were amplified. If any secondary peaks or shoulders were seen on the peak of interest, it indicated that something other than the gene of interest was present among the reaction products. If these secondary peaks were present in the NTC wells, it might indicate primer dimer formation or the present of contamination by a sequence that was also amplified during the reaction. Since primer dimers would typically have a lower melting temperature and smaller peak. All melting curves showed a single peak.

3.3 Results

3.3.1 VPA reduced cell proliferation of adult rat hippocampal NSCs

In the present study, hippocampal neurospheres and NSCs were quantified in order to investigate the effect of VPA treatment on cell proliferation of adult rat hippocampal NSCs. Rat hippocampal NSCs were cultured and treated with either 0.3 or 1 mM VPA for 72 hours. Following the treatment, the numbers of floating sphere colonies (neurospheres) were counted (Fig. 3.5). Neurosphere formation of adult rat hippocampal NSCs was reduced by VPA (0.3 and 1 mM) to 44% and 10%, respectively. The results showed that there was a significant difference among the experimental groups ($p < 0.001$, one-way ANOVA, Fig. 3.4 A). Comparison of treatment groups with control group, VPA treatment significantly decreased the rate of neurosphere-forming NSCs in a dose responsive manner compared to control culture (*** $p < 0.001$, one-way ANOVA, Fig. 3.4 A).

Following quantification of hippocampal neurospheres, the neurospheres were triturated into single cells and then counted to investigate cell proliferation of adult hippocampal NSCs. Total number of single cells was 27% and 3%, respectively, when 0.3 and 1 mM VPA were added for 72 hours compared to untreated culture. The hippocampal NSC numbers in 0.3 and 1 mM VPA treatment groups showed a significant decrease in comparison to untreated culture (*** $p < 0.001$, one-way ANOVA, Fig. 3.4 B). The results also revealed that comparison of total numbers of NSCs was significantly different

in a dose-dependent manner among the experimental groups ($p < 0.001$, one-way ANOVA, Fig. 3.4 B).

In addition, the average of NSC number per neurosphere showed a significant decrease among experimental groups ($p < 0.0001$, one-way ANOVA, Fig. 3.3) Moreover, the number of NSCs per neurosphere significantly decreased in a concentration dependent fashion between 0.3 and 1 mM VPA in comparison to control group (*$p < 0.01$ and ** $p < 0.001$ respectively, one-way ANOVA, Fig. 3.3).

These results indicated that VPA reduced neurosphere formation, total number of single cells and number of NSCs per neurosphere in a concentration manner.
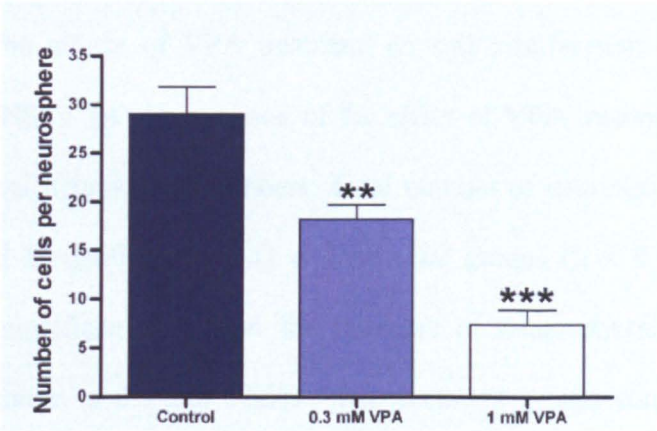
Figure 3.3 The effects of VPA on number of NSCs per neurosphere. Comparing the number of NSCs per neurosphere among experimental groups, there was a significant decrease ($p < 0.0001$). The number of NSCs per neurosphere showed a significant reduction in dose-dependent manner in 0.3 and 1 mM VPA treatment groups compared with untreated culture (**$p < 0.01$ and *** $p < 0.001$, respectively). All statistic analysis was assessed using one-way ANOVA.
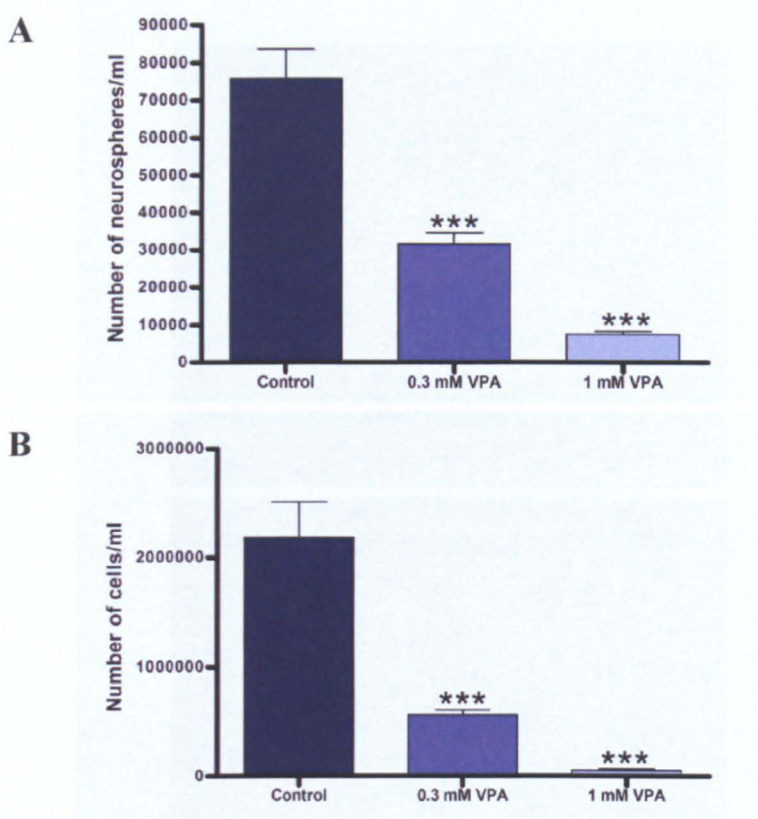
Figure 3.4 The effects of VPA treatment on cell proliferation of adult rat hippocampal NSCs. (A) Comparison of the effect of VPA treatment on adult rat hippocampal neurosphere numbers. Total number of neurosphere-forming NSCs showed a significant among experimental groups ($p < 0.001$). There were also a significant reduction the numbers of neurospheres in a dose responsive manner in 0.3 and 1 mM VPA treatment groups compared with untreated culture (*** $p < 0.001$). (B) The effect of VPA on hippocampal NSC proliferation was concentration dependent. 0.3 and 1 mM VPA-treated cultures revealed a significant decrease in number of hippocampal NSCs in comparison to untreated culture (*** $p < 0.001$). Comparing total hippocampal NSC number of all experimental groups showed that there was a significant difference ($p < 0.001$). All statistic analysis was assessed using one-way ANOVA.
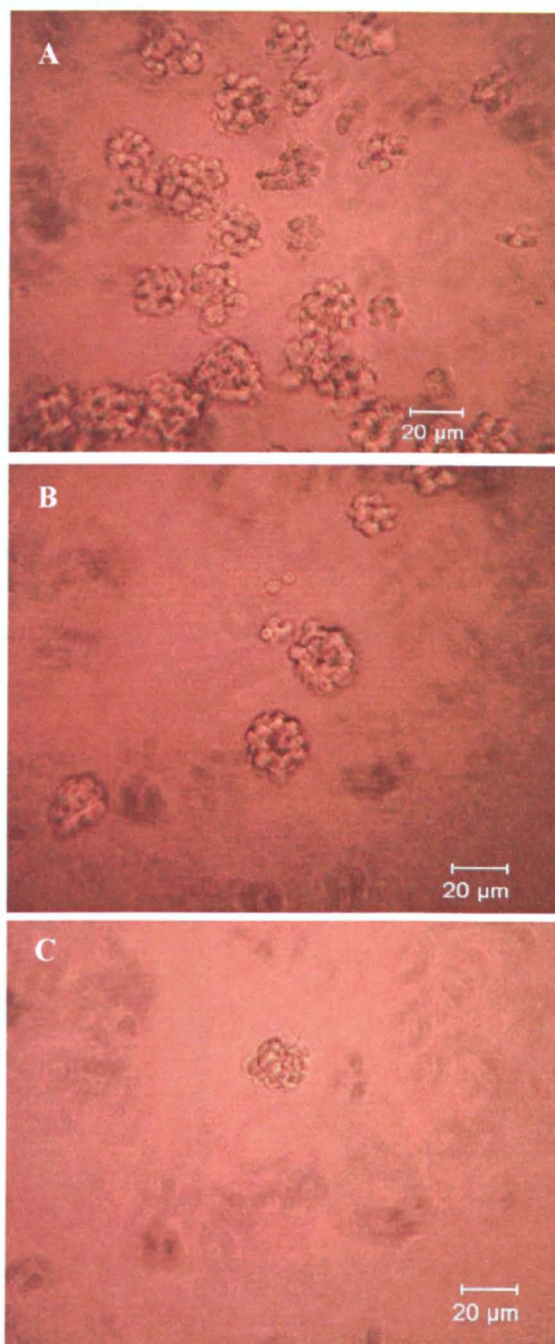
Figure 3.5 Phase contrast photomicrographs illustrating the effect of VPA on neurosphere formation of adult rat hippocampal NSCs. Photographs of neurosphere-forming NSCs treated with 0 mM VPA or untreated culture (A), neurosphere-forming NSCs treated with 0.3 mM VPA (B) and neurosphere-forming NSCs treated with 1 mM VPA (C).

3.3.2 VPA reduced cell proliferation of adult mouse hippocampal NSCs

Following 48 hours VPA treatment, the neurospheres (Fig. 3.7) were triturated into single cells and then counted to investigate cell proliferation of adult hippocampal NSCs. At 0.3 and 1 mM adult mouse hippocampal NSC viability was reduced to approximately 29% and 19%, respectively. The hippocampal NSC numbers in 0.3 and 1 mM VPA treatment groups showed a significant decrease in comparison to untreated culture (## $p < 0.01$ and ** $p < 0.01$, respectively, one-way ANOVA, Fig. 3.6). The results also revealed that comparison of total numbers of mouse hippocampal NSCs was significantly different in a dose-dependent manner among the experimental groups ($p <$ 0.0008, one-way ANOVA, Fig. 3.6).
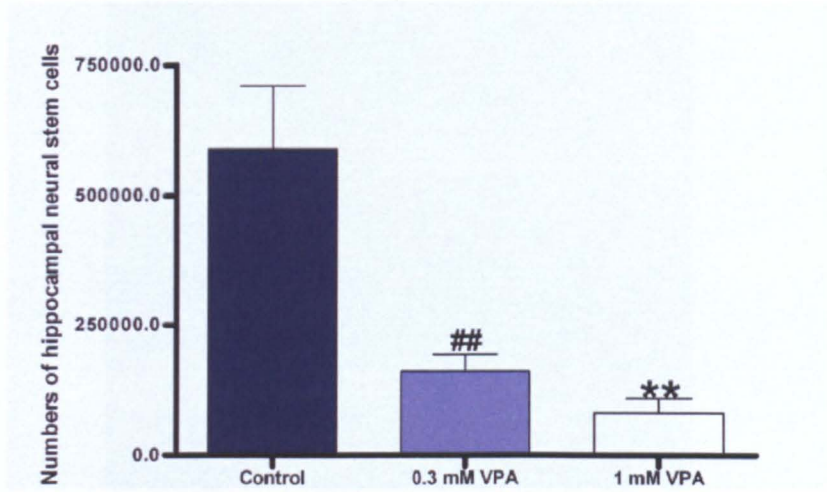
Figure 3.6 The effect of VPA on adult mouse hippocampal NSC proliferation was concentration dependent. Comparing total hippocampal NSC number of all experimental groups revealed that VPA treatment statistically significant decreased cell proliferation ($p = 0.0008$). There was a statistically significant reduction in number of hippocampal NSCs in 0.3 and 1 mM VPA-treated cultures in comparison to untreated culture (## $p < 0.01$ and ** $p < 0.01$, respectively). All statistic analysis was assessed using one-way ANOVA.
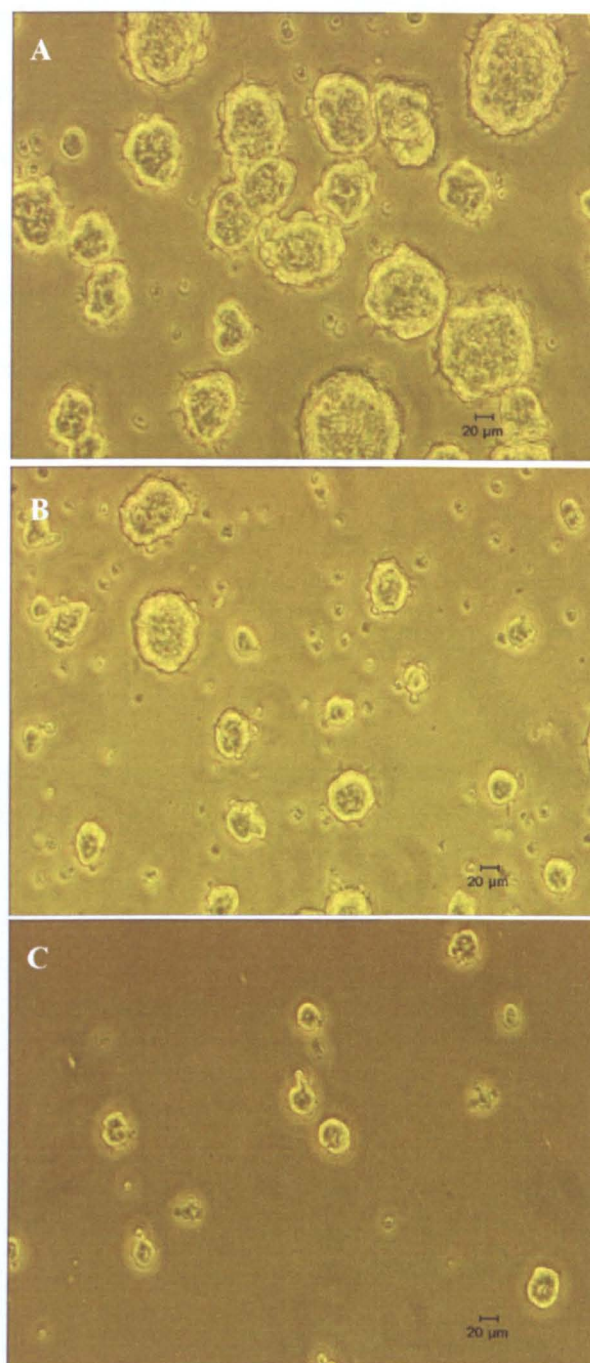
Figure 3.7 Phase contrast photomicrographs illustrating the effect of VPA on neurosphere formation of adult mouse hippocampal NSCs. Photographs of neurosphere-forming NSCs treated with 0 mM VPA or control culture (A), neurosphere-forming NSCs treated with 0.3 mM VPA (B) and neurosphere-forming NSCs treated with 1 mM VPA (C).

3.3.3 β-actin and Sox2 gene expression using RT-PCR

All cDNA samples were checked to investigate the quality of the cDNA preparation. This was achieved by comparing the RT-PCR with a no RT, PCR reaction to determine mRNA expressions of β-actin and Sox2 in each sample. The results showed that the mRNA expressions of β-actin and Sox2 in all positive reverse transcriptase samples were detected at 500 and 94 bp respectively but not the negative reverse transcriptase samples (Fig. 3.8 and 3.9). These results indicate that all samples were not contaminated with genomic DNA.
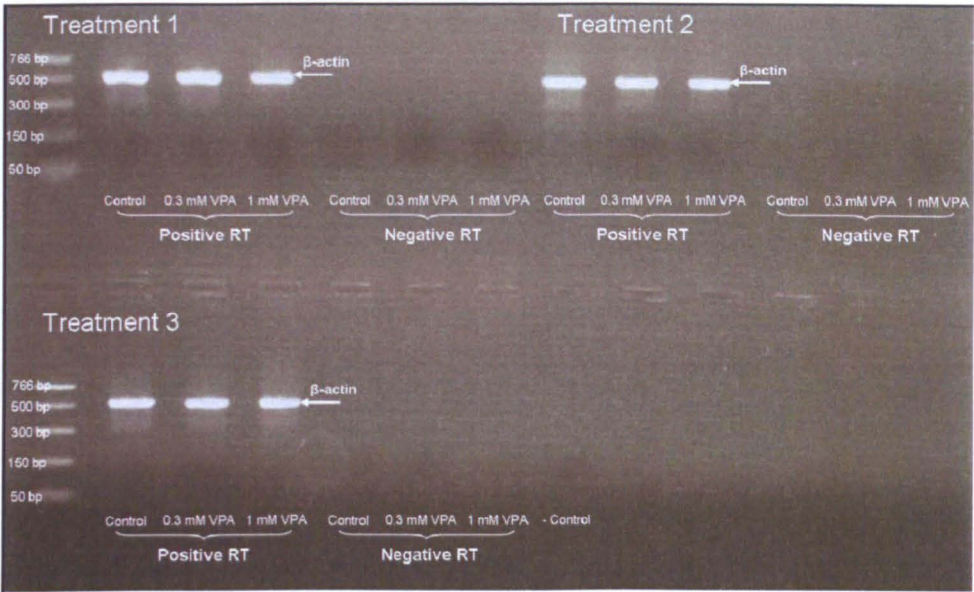
Figure 3.8 Expressions of β-actin in adult mouse hippocampal NSC samples from three treatments. The positive reverse transcriptase samples were detected at 500 bp. By contrast, no β-actin was detected in the negative reverse transcriptase samples.
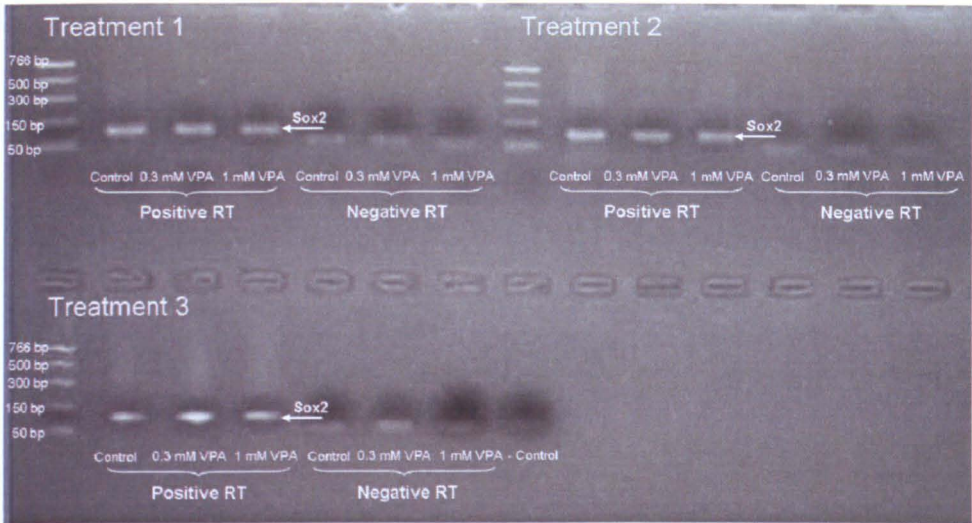
Figure 3.9 Expressions of Sox2 in adult mouse hippocampal NSC samples
from three treatments. The positive reverse transcriptase samples were detected
at 94 bp whereas the negative reverse transcriptase samples were not detected.

3.3.4 Expression of reference genes following VPA treatment in adult mouse hippocampal NSCs using qPCR

The three reference genes displayed a different range of threshold cycle (Ct) values (Fig. 3.10A) and dissociation curve (Fig. 3.10B). The dissociation curve plot of these genes showed three fluorescence peaks: 18s rRNA, centred approximately 84°C; GAPDH, centred approximately 86°C and clathrin, centred approximately 80°C (Fig. 3.10B). Comparative quantitation of levels of Ct value of 18s rRNA, GAPDH and clathrin between untreated or control samples and VPA-treated samples were performed using comparative quantitation module in the MxPro^{TM} software (Stratagene, Aligent Technologies Company, CA, USA). Then, the data was shown as Ct value of each reference gene (Figure 3.10). There was no statistically significant difference of Ct values of GAPDH and clathrin genes between control and VPA-treated samples ($p > 0.05$, one-way ANOVA, Fig. 3.11). Similarly, Ct values of 18s rRNA remained unaffected in both the control (untreated) as well as VPA-treated samples ($p > 0.05$, one-way ANOVA, Fig. 3.11). These genes were not affected by treatment with VPA, indicating tolerance to cellular activation and experimental conditions. However, expression of 18s rRNA showed the greatest consistency in different samples as compared to GAPDH and clathrin (Fig. 3.11). Therefore, 18s rRNA was chosen as the most suitable reference gene for gene expression profile in this study. This is in line with a report demonstrated that 18s rRNA is suitable reference gene for use as internal control for relative gene expression quantification (Saviozzi et al., 2006).
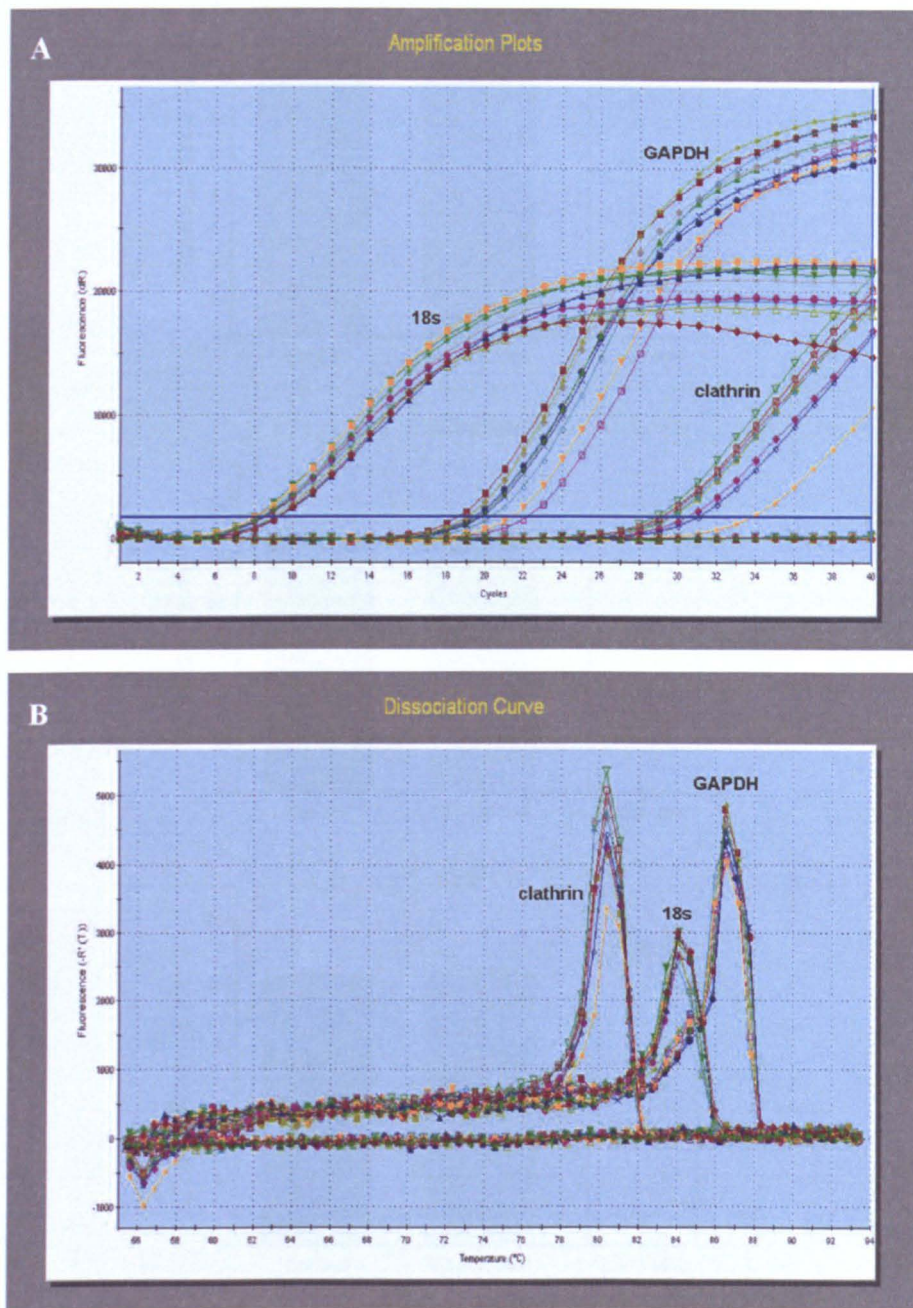
Figure 3.10 Amplification plots and dissociation curve of the three reference genes following VPA treatment in adult mouse hippocampal NSCs. (A) Ct values for each reference gene were shown in a different range. (B) Dissociation temperature of each gene was displayed without any primer dimer contamination and unintended amplification products.
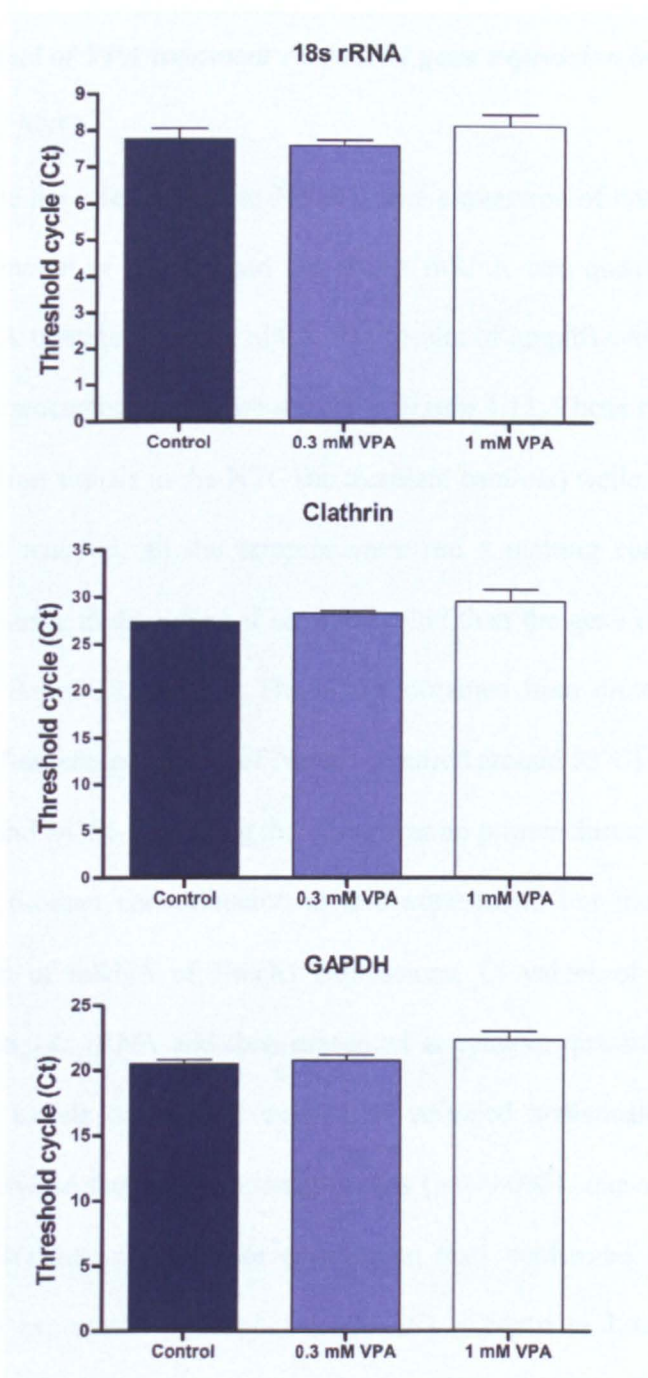
Figure 3.11 Quantitative PCR analysis of reference genes following VPA treatment in NSCs. Levels of Ct values of 18s rRNA ($p > 0.05$), clathrin ($p > 0.05$) and GAPDH ($p > 0.05$) show no significant differences between control (untreated) and treated samples. The data shown here represents the average of three different experiments performed each sample in triplicate using the same conditions. All statistic analysis was assessed using a one-way ANOVA.

3.3.5 The effect of VPA treatment on Notch1 gene expression in adult mouse hippocampal NSCs

To investigate the role of VPA in Notch1 gene expression of neurosphere-like NSCs, the amount of Notch1 and 18s rRNA mRNA was quantified after 48 hours of VPA treatment. Using qPCR, the results of amplification plots of Ct values and dissociation curves are shown in Figure 3.13. These results showed no amplification signals in the NTC (no template controls) wells. At the end of amplification reaction, all the samples were run a melting curve known as dissociation curve to determine if anything other than the gene of interest was amplified in the qPCR reaction. The results obtained from dissociation curve showed two fluorescence peaks of Notch1 (centred around 83°C) and 18s RNA (centred around 84°C), indicating that there was no primer-dimer or some other non-specific product contamination in this experiment. For the comparative quantification of mRNA of Notch1 expressions, Ct values of Notch1 were normalized to 18s rRNA and then presented as relative quantity expressions (Fig. 3.12). Levels of Notch1 expression revealed statistically significant difference between these experimental groups ($p < 0.0001$, one-way ANOVA, Fig. 3.12). Bonferroni's multiple comparison tests confirmed that levels of Notch1 gene expression in hippocampal NSCs exposed to 1 mM VPA was statistically significant lower than the untreated cells ($p < 0.001$, one-way ANOVA, Fig. 3.12). In contrast, Notch1 gene expression in 0.3 mM VPA-treated cells was not different from the untreated cells ($p > 0.05$, one-way ANOVA, Fig. 3.12).
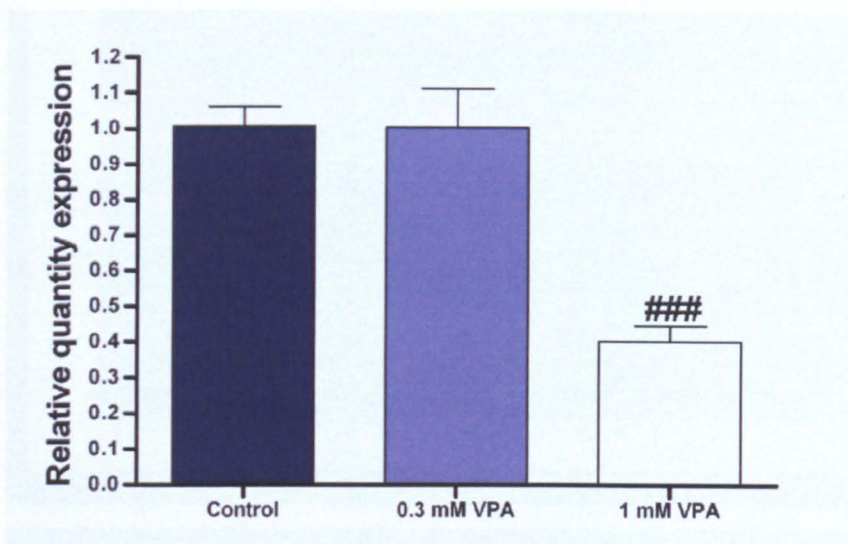
Figure 3.12 Effects of VPA on Notch1 gene expression in adult mouse hippocampal NSCs. Levels of Notch1 expression revealed statistically difference between these experimental groups (### $p < 0.0001$). The effect of VPA on Notch1 mRNA levels differed significantly between 1 mM VPA-treated and control samples ($p < 0.001$). In contrast, there was no significant difference between 0.3 mM VPA and untreated samples ($p > 0.05$). The data showed the average of relative quantity expression from 3 experiments performed each sample in triplicate using the same conditions. Statistical significance was determined using one-way ANOVA.
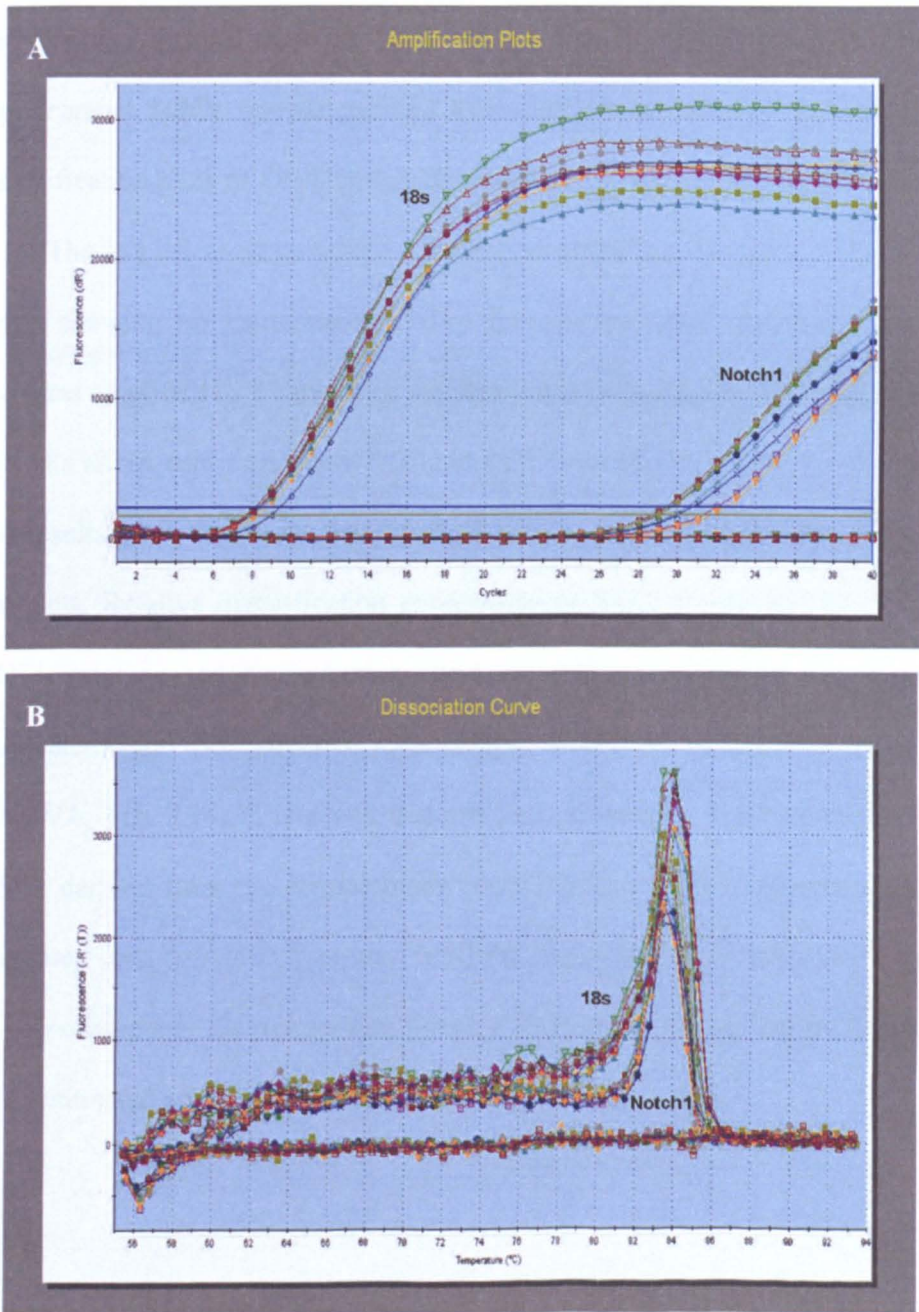
Figure 3.13 Amplification plots (A) and dissociation curve (B) of Notch1 and 18s rRNA genes following VPA treatment in adult mouse hippocampal NSCs. When the amplified products of Notch1 and 18s rRNA were subjected to dissociation curve analysis, the fluorescence peaks were detected approximately at 83°C and 84°C respectively.

3.3.6 Effects of VPA Sox2 gene expression in mouse hippocampal NSCs

Comparative quantification of Sox2 mRNA and 18s rRNA in adult mouse hippocampal NSCs was determined following 48 hours of VPA treatment. Amplification plots of Ct value and dissociation curves are displayed in Figure 3.15. The amplification curve did not show an amplification signal in the NTC wells showing no contamination. The dissociation curve analysis of PCR products amplified in the presence test showed two fluorescence peaks of Sox2 and 18s rRNA centred around 77°C and 84°C respectively. These verified that the results were free from the signal of primer-dimers or other non-specific products. Relative quantification expressions of Sox2 normalized to the 18s rRNA reference gene, showed a statistical difference between the control (untreated) and VPA-treated hippocampal NSCs ($p = 0.0002$, one-way ANOVA, Fig. 3.14). There was a significant decrease in Sox2 expression in NSCs derived from the hippocampus treated with 1 mM VPA compared to untreated cells ($p < 0.05$, one-way ANOVA, Fig. 3.14) while Sox2 expression in hippocampal NSCs treated with 0.3 mM VPA was not significantly different from untreated cells ($p > 0.05$, one-way ANOVA, Fig. 3.14).
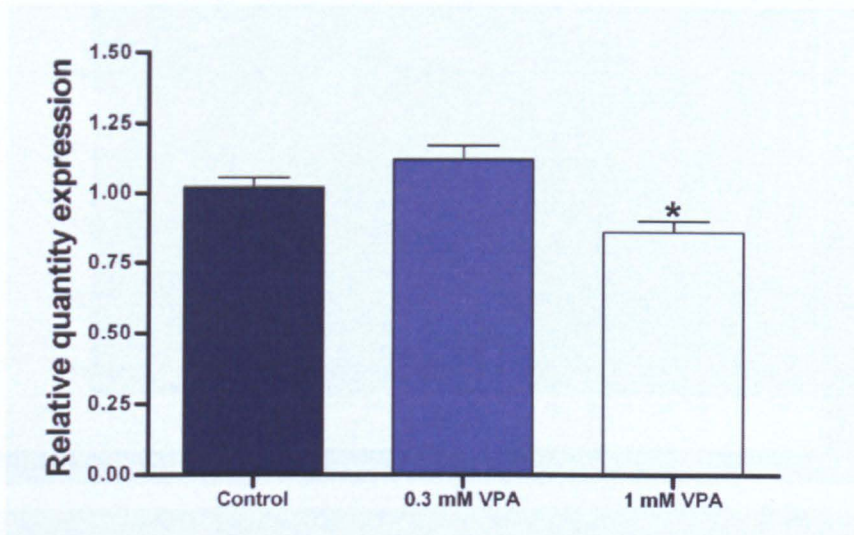
Figure 3.14 Effects of VPA on Sox2 gene expression determined by qPCR. Levels of Sox2 expression revealed a statistical difference between these experimental groups ($p = 0.0002$). 1 mM VPA produced a significant reduction of Sox2 mRNA levels in hippocampal NSCs compared to control cells (* $p <$ 0.05). Conversely, Sox2 gene expression was not affected in 0.3 VPA-treated samples as compared to the control samples ($p > 0.05$). The data showed the average of relative quantity expression from 3 experiments performed in triplicate using the same conditions. All statistic analysis was assessed using one-way ANOVA.
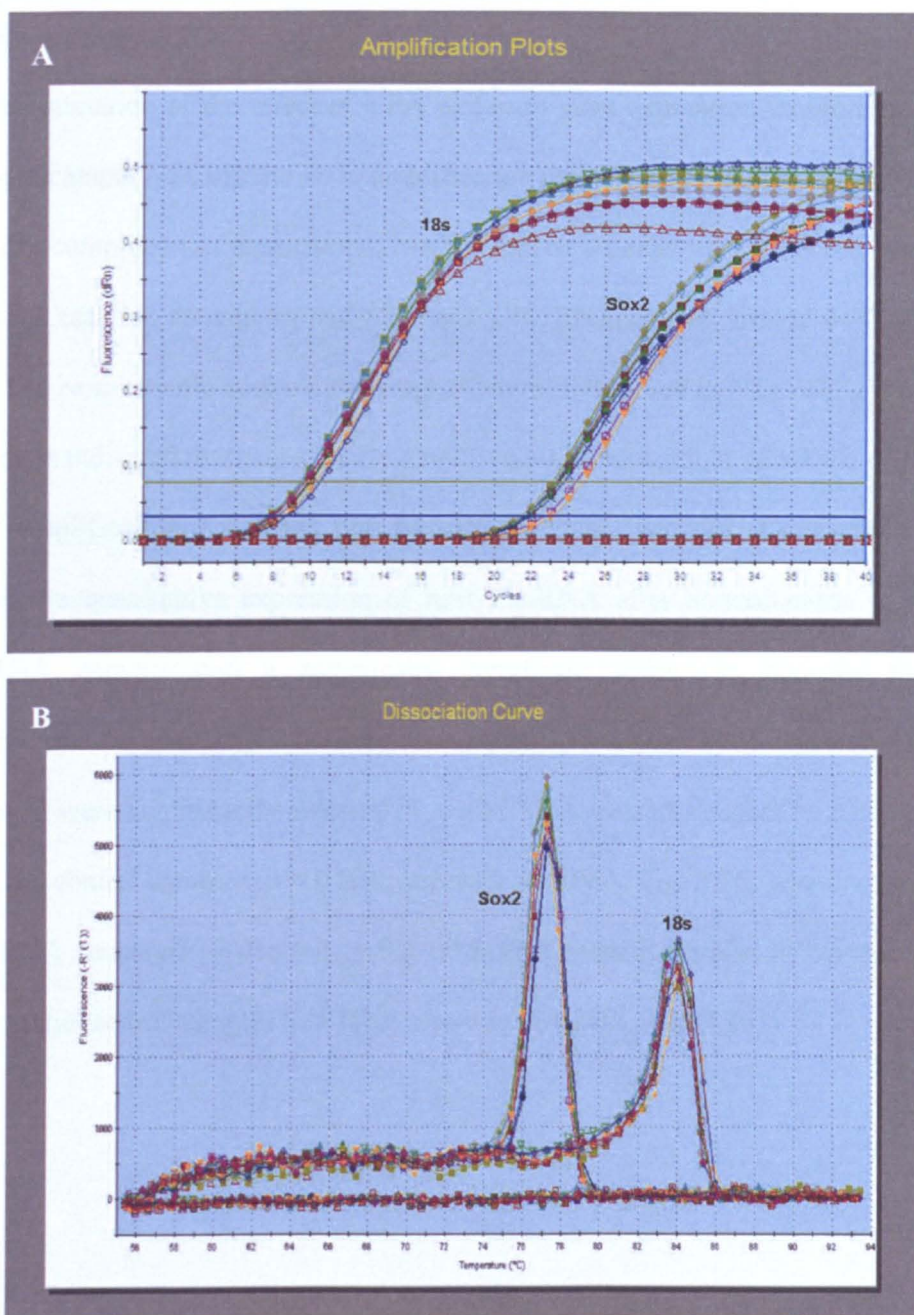
Figure 3.15 Quantitative PCR amplification plots (A) and dissociation curve (B) of a reaction with Sox2 and 18s rRNA genes following VPA treatment in adult mouse hippocampal NSCs. The amplified products of Sox2 and 18s rRNA were melted approximately at 77°C and 84°C respectively.

3.3.7 VPA down-regulates nestin gene expression in adult mouse hippocampal NSCs

Determination of the effect of VPA on nestin gene expression in adult mouse hippocampal NSCs is shown in amplification and dissociation plots (Fig. 3.17). After completion of dissociation, two fluorescence peaks were observed: nestin in the reaction centred around 85°C and 18s rRNA centred around 84°C (Fig. 3.17). Non-specific amplification signal was not observed in NTC wells. These results indicated that non-specific amplification, which might affect the quality of amplification data, was not detected in these reactions. Comparison of relative quantitative expression of nestin mRNA after normalization to 18s rRNA demonstrated a statistically significant difference between these experimental groups ($p < 0.0001$, one-way ANOVA, Fig. 3.16). Nestin mRNA levels were significantly reduced in 1 mM VPA-treated samples as compared to the control samples ($p < 0.001$, one-way ANOVA, Fig. 3.16) whereas nestin mRNA remained unaffected in 0.3 mM VPA-treated samples in comparison with the control samples ($p > 0.05$, one-way ANOVA, Fig. 3.16).
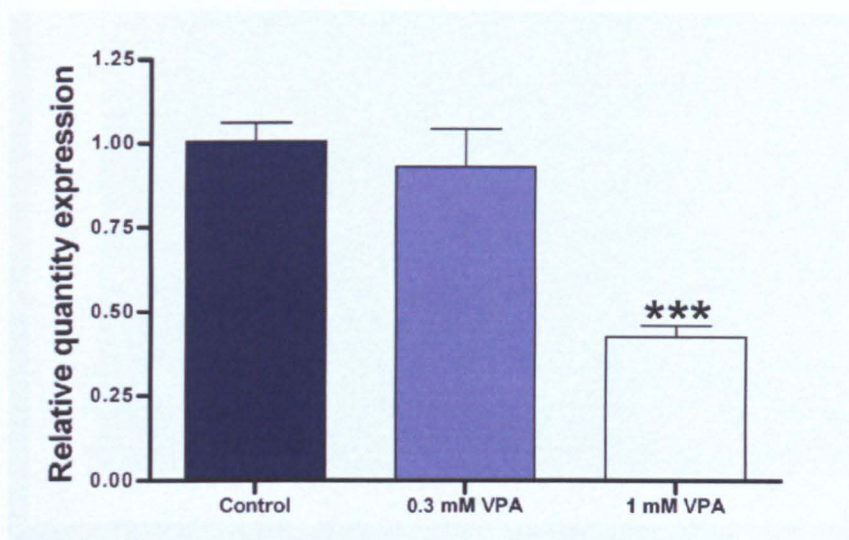
Figure 3.16 Effects of VPA on nestin gene expression in adult mouse hippocampal NSCs. Significant difference in nestin gene expression was detected in these experimental groups ($p < 0.0001$). The effect of VPA on nestin mRNA levels differed significantly between 1 mM VPA-treated and control samples (*** $p < 0.001$). In contrast, there was no significant difference between 0.3 mM VPA and control samples ($p > 0.05$). The data showed the average of relative quantity expression from 3 experiments performed in triplicate using the same conditions. Statistic significance was determined using one-way ANOVA.
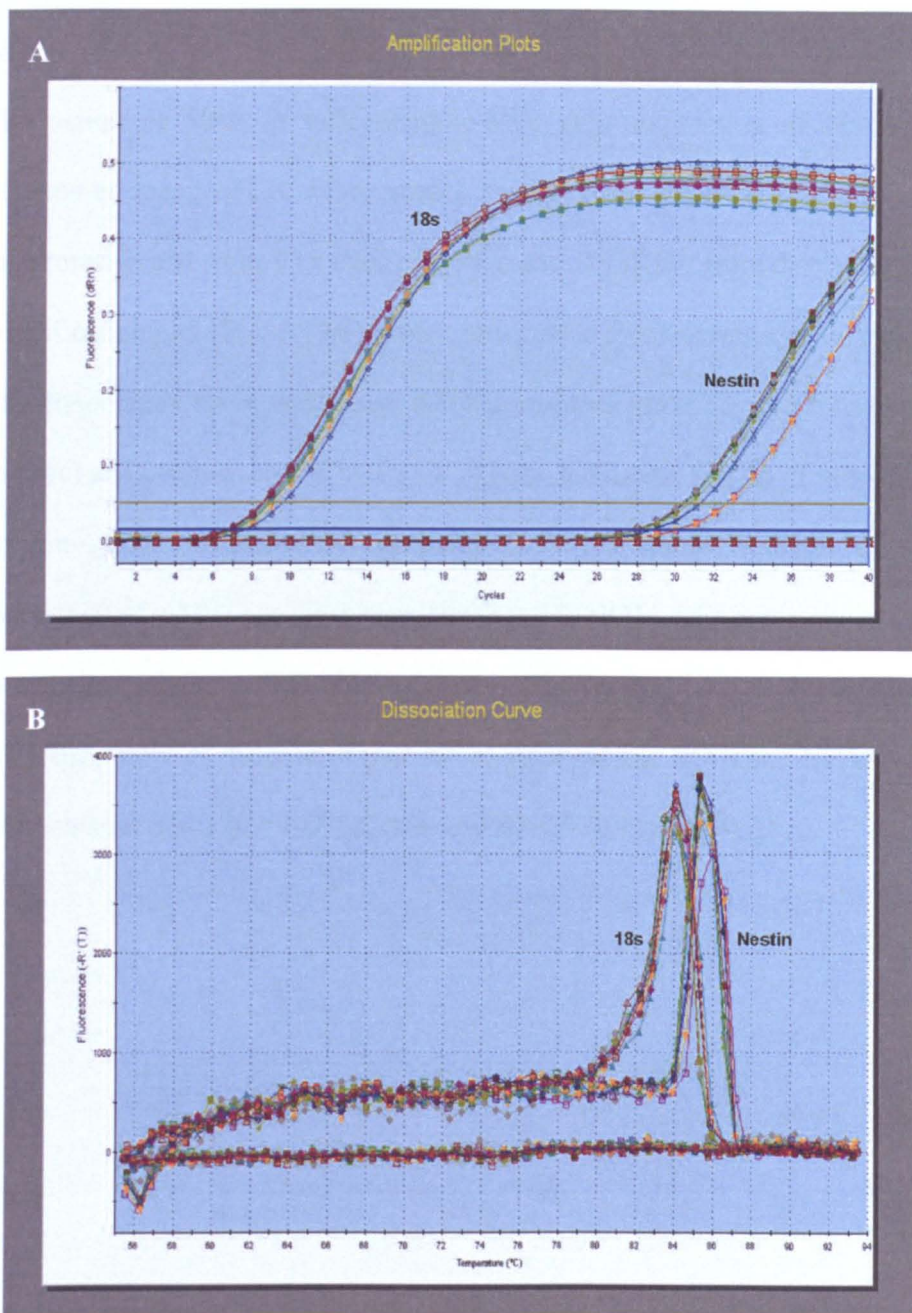
Figure 3.17 Amplification plots (A) and dissociation curve (B) of detection of nestin and 18s rRNA gene expression following VPA treatment in adult mouse hippoacampal NSCs using qPCR. The fluorescence peaks of nestin and 18s rRNA were detected approximately at 85°C and 84°C respectively.

3.3.8 Effects of VPA on c-Myc gene expression in adult mouse hippocampal NSCs

The action of VPA in influencing c-Myc gene expression of NSCs was determined using qPCR. Fluorescence signals from the PCR reactions were monitored in real-time, Ct values of c-Myc and 18s rRNA were displayed as an amplification plot (Fig. 3.19A), which reflected in fluorescence during cycling. The dissociation curve was shown two fluorescence peaks centred around 81°C (c-Myc) and centred around 84°C (18s rRNA), indicating that no primer-dimers or non-specific amplification would artificially increase fluorescence. The expression of c-Myc gene was normalized to 18s rRNA reference gene in order to calculate relative levels of transcription. The results showed no statistically difference between the control (untreated) hippocampal NSCs and VPA-treated hippocampal NSCs ($p = 0.0522$, one-way ANOVA, Fig. 3.18).
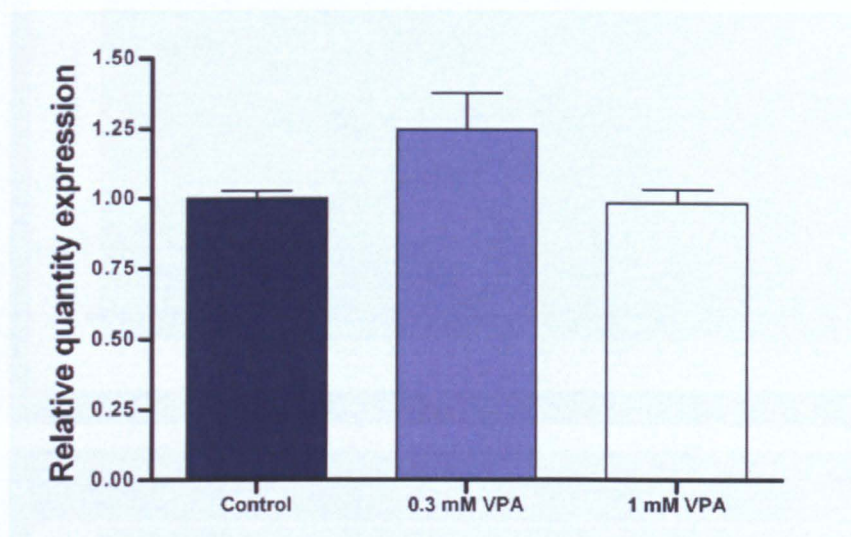
Figure 3.18 Effects of VPA on c-Myc gene expression in adult mouse
hippocampal NSCs. c-Myc gene expression was not affected in 0.3 and 1 mM
VPA-treated samples as compared to the control samples ($p = 0.0522$). The
data showed the average of relative quantity expression from 3 experiments
performed in triplicate using the same conditions. Statistical significance was
determined using one-way ANOVA.
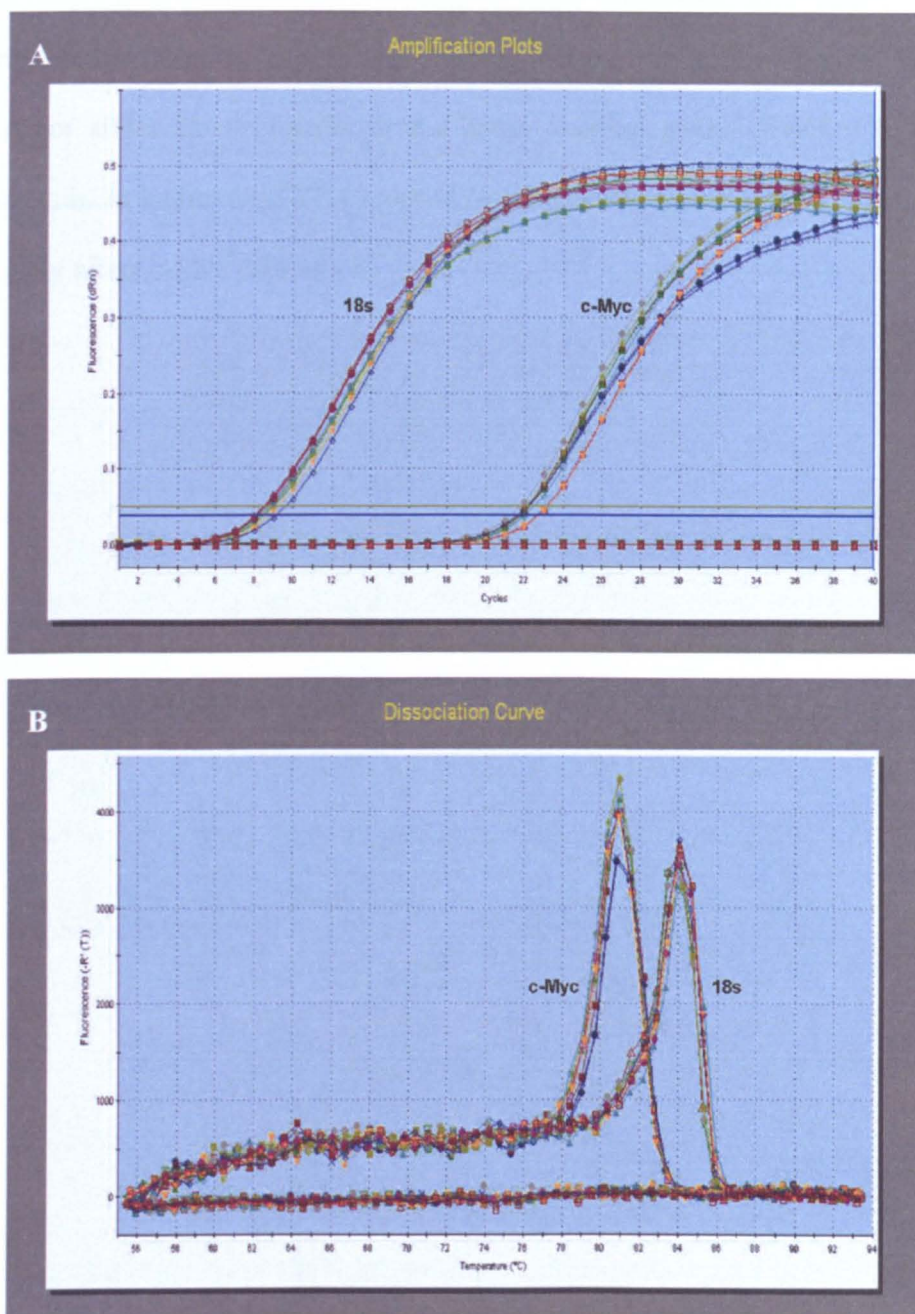
Figure 3.19 Amplification plots (A) and dissociation curve (B) of c-Myc and
18s RNA genes were detected in VPA-treated hippocampal NSCs by qPCR.
The amplified products of c-Myc and 18s rRNA were melted approximately at
81°C and 84°C respectively.

The results show that VPA produced a dose dependent reduction in cell proliferation in mouse and rat hippocampal derived NSCs. Furthermore, VPA did not affect the expression of the house keeping genes GPDH, 18s and clatherin. At higher dose VPA reduced Notch1, Sox2 and nestin expression but did not affect c-Myc expression.

3.4 Discussion

The chronic VPA administration in the previous chapter showed a significant decrease of cell proliferation in the SGZ of the dentate gyrus together with decreases in BDNF and Notch1 levels which were correlated with poorer performance in a spatial memory test. The present chapter sought to investigate whether VPA was having a direct effect on hippocampal neural stem cells isolated from the influences that might be coming from surrounding tissues. Two concentrations (0.3 and 1 mM) of VPA were used to treat cells. Both concentrations are within the human therapeutic range (50 – 150 mg/l or 0.35 – 1.04 mM) (Hao et al., 2004). Even the higher concentration has been reported as being non toxic to progenitor cells in the hippocampus (Jung et al., 2008). The results show that VPA (0.3 and 1 mM) inhibited hippocampal neurosphere formation and cell proliferation in a concentration-dependent manner (Fig. 3.5 and 3.7). Additionally, 1 mM VPA reduced levels of Notch1, Sox2 and nestin mRNA expression but not 0.3 mM VPA. In contrast, both 0.3 and 1 mM VPA had no effect on levels of c-Myc mRNA expression.

VPA inhibited adult hippocampal neural stem cell (NSC) proliferation

The results show that VPA inhibited hippocampal neurosphere formation and cell proliferation in a concentration-dependent manner (Fig. 3.5 and 3.7). This result was obtained with both mouse and rat hippocampal NCSs. VPA is well known to inhibit cell proliferation *in vitro* (Takai et al., 2004). Other investigators have shown an effect in both cerebral cortex derived cells and hippocampal NCSs at 0.3 and 1 mM (Jung et al., 2008, Hsieh et al., 2004). In a non-toxic concentration (1 mM) to the hippocampal progenitor cells of VPA, it

has been recently shown that VPA treatment for 48 hours decreases cerebral cortex progenitor cell proliferation (Jung et al., 2008). Moreover, there is a dramatic reduction of hippocampal neuronal progenitor cells treated with 1 mM VPA for 24 hours in comparison with untreated cells (Hsieh et al., 2004). These data fit well with the results of the present study which shows an inhibition of neurosphere formation (Fig. 3.5 and 3.7) and also a reduction of cell proliferation of hippocampal NSCs after exposure to VPA in a dose dependent manner. The previous study revealed that VPA caused a significant decrease of dividing cells in the hippocampal dentate gyrus (section 2.2.4, Chapter 2), which is in line with a report showing that VPA (300 mg/kg) induces cell proliferation inhibition of adult hippocampal neural progenitor cells *in vivo* (Hsieh et al., 2004). In culture, VPA has been reported to gradually decrease embryonic stem cell proliferation in a dose-dependent manner by inhibiting HDACs (Murabe et al., 2007). Inhibition of histone deacetylation *in vitro* and *in vivo* has been found to suppress cell proliferation and that this effect is associated with hyperacetylation of histones. This causes chromatin relaxation and compaction, leading access to promoter DNA for transcriptional activators and consequently, activation of gene expression (Murabe et al., 2007, Mai et al., 2005, Hsieh et al., 2004). In addition, VPA regulates cell proliferation in neuronal progenitor cells via p21 pathway which is a common target of transcription protein expression for HDAC inhibitor (Jung et al., 2008, Hsieh et al., 2004, Yu et al., 2009). NSCs are precursor cells that generate granule cells in the adult dentate gyrus. Adult newly generated granule neurons are not only functionally incorporated with hippocampal circuits and existing granule neurons but also express gene and protein known

to play a crucial role in synaptic plasticity related to memory formation (Emsley et al., 2005, Bruel-Jungerman et al., 2007, Gould et al., 1999). The present study shows that exposure of adult hippocampal progenitors to VPA directly reduces their proliferation, independent of effects on surrounding tissues. This reduction in one of the stages of neurogenesis may therefore explain the cognitive impairment found after *in vivo* treatment. *In vitro* treatment reduced the number of neurospheres forming and also reduced their size. This may indicate that VPA is toxic to progenitor cells and inhibits their proliferation.

Levels of Notch1 gene expression in adult mouse hippocampal NSCs was decreased by higher concentrations of VPA

The present study demonstrates that 1 mM VPA treatment significantly decreased levels of receptor Notch1 gene expression in hippocampal NSCs while 0.3 mM VPA did not show a reduction of Notch1 gene expression compared to untreated cells (Fig. 3.12 and 3.13). This result does not seem to be due to a non specific reduction in genome transcription as both house keeping and Sox2 and Nestin expression is unaffected by this concentration of VPA. The reduction in Notch1 expression in NSCs parallels the reduction in Notch1 protein found *in vivo* after VPA treatment (Chapter 2). The *in vivo* study looked at Notch1 levels in the whole hippocampal formation. However Notch1 is expressed both by proliferating NSCs in the SGZ and on the molecular layer dendrites of postmitotic granule cells (Breunig et al., 2007) making it difficult to know whether VPA is affecting Notch1 on dividing or post-mitotic cells. The Notch signal is important in proliferation, apoptosis and

differentiation and is transmitted through the Notch cell surface receptor in combination with other cellular factors (Artavanis-Tsakonas et al., 1999). According to some reports, Notch1 activation is essential for the maintenance and generation mammalian NSCs (Oishi et al., 2004, Chojnacki et al., 2003, Nyfeler et al., 2005). During neural development, Notch pathway is importantly involved in maintaining multipotency (Tokunaga et al., 2004, Kohyama et al., 2005). Moreover, it has been revealed that Notch1 prevents neural differentiation and maintains neural precursor and stem cell proliferation in order to regulate neurogenesis and cell fate determination (Breunig et al., 2007, Wang et al., 2004, Hitoshi et al., 2002). In addition, it has been found that Notch1 signalling regulates NSC self-renewal and promotes survival of neural progenitor cells (Hitoshi et al., 2002, Potten et al., 2006). Notch signalling has been found to be involved in regulating of maintenance and proliferation of NSCs by their density in culture (Mori et al., 2006). Notch is known to inhibit neuronal differentiation and maintain neural stem cells by forming complex with RBP-J and then this complex becomes a transcriptional activator and induces Hes1 and Hes5 expression (Kageyama et al., 2005). Notch signalling is mediated through expression of effector genes from the HES family. Hes genes control NSC maintenance and self-renewal by repressing proneuronal genes, such as Mash1, Math and Neurogenin (Ngn) (Qu and Shi, 2009). Notch signalling also controls NSC maintenance, self-renewal and differentiation by mediating the membrane-associated protein Numb (Qu and Shi, 2009). In NSCs, however, induction of Hes genes by RBP-Jk mediated Notch1 activation maintains stem cell character and inhibits neurogenesis (Shimizu et al., 2008). Recent evidence indicates that the absence

of Notch signalling pathway promotes a decrease of dividing cells and premature differentiation of the early-born cell types (Kageyama and Ohtsuka, 1999). Disruption of Notch1 expression inhibits neurogenesis by impairing survival of neural progenitor cells, causing apoptosis (Crews et al., 2008). Notch, RBP-Jk and Hes genes function to maintain dividing precursor cells by inhibiting cell differentiation (Kageyama and Ohtsuka, 1999). Unlike a reduction of Notch1 expression, induction of Notch1 gene expression enhances the rate cell proliferation of NSCs (Zhang et al., 2008). In cultures, activations of Notch1 are essential for promoting self-renewal and cell proliferation to form neurospheres in rodents (Kohyama et al., 2005, Chen et al., 2008). In contrast, ablation of Notch1 signalling by a γ-secretase inhibitor or use of deficient in PS1 mice impairs neurosphere formation (Chen et al., 2008). Taken together, a reduction of Notch1 gene expression in the hippocampal NSCs caused by 1 mM VPA in the present study might impair survival and self-renewal of hippocampal NSCs. Furthermore, it might inhibit neurogenesis and induce cell differentiation. Notch1 expression in neuroendocrine cells was also induced by VPA as discussed in Chapter 4.

Levels of nestin gene expression in adult mouse hippocampal NSCs were reduced by higher concentrations of VPA

This study shows that VPA (1 mM) significantly inhibited expression of nestin in hippocampal derived NSCs. Nestin, an intermediate filament protein, is known as a protein marker for neural stem/progenitor cells (Gilyarov, 2008). Nestin is expressed in dividing cells of SVZ of the lateral ventricle and the hippocampal dentate gyrus (Korzhevskii et al., 2008, Gilyarov, 2008). There is

a report revealing that 0.30 and 1 mM VPA enhances neuronal differentiation of adult progenitor cells (Hsieh et al., 2004) but nestin was not looked at. During the differentiation period, it has been found down-regulation of nestin while the progenitor cells exit the cell cycle and start to differentiate. Also, loss of nestin expression has been detected in an early stage of NSC differentiation both *in vivo* and *in vitro* (Mellodew et al., 2004). Nestin is not found in fully differentiated cells, therefore, loss of nestin can be considered as the earliest signification of the progression from precursor to differentiated phenotypes (Mellodew et al., 2004, Gilyarov, 2008). In the SGZ of the hippocampal dentate gyrus, nestin is expressed in NSCs and transiently amplifying progenitor cells but not neuroblasts (Kempermann et al., 2004). Thus, a decrease of nestin in NSCs indicates a reduction of NSCs and transiently amplifying progenitor cells.

Loss of nestin in an early event of NSC differentiation has been discovered to be regulated by Notch signalling. Moreover, Notch signalling pathway controls the alteration of nestin degradation to maintain the undifferentiated state of stem cells (Mellodew et al., 2004). Notch1 mRNA has been detected in nestin-containing cells rather than post-mitotic neurons in neurosphere assay (Irvin et al., 2001). Similarly, nestin which is a progenitor marker has been found on Notch1-expressing cells on the surface of neurospheres. It is interesting to note that, *in vitro*, down-regulation of Notch1 expression results in a decrease of nestin-expressing cells. This suggests that Notch1 is involved in nestin expression. Therefore, differentiation of both NSCs and transiently amplifying cells is possibly modulated by alteration of Notch1 signalling (Nyfeler et al.,

2005). Additionally, it has been revealed that down-regulation of nestin activates apoptosis of neuronal progenitor cells, suggesting that nestin plays an important role in survival determinant (Sahlgren et al., 2006). The hippocampal NSCs were treated with 0.3 and 1 mM VPA in the present analysis to investigate the effect of VPA on nestin gene expression. The results show that 1 mM VPA decreased level of nestin expression but not 0.3 mM VPA (Fig. 3.16 and 3.17). These findings indicate that loss of nestin may be due to a reduction of receptor Notch1 which functions to maintain progenitors. It is possible that a decrease of nestin expression in hippocampal NSCs may lead to differentiating of both NSCs and transiently amplifying cells and apoptosis.

VPA reduced levels of Sox2 gene expression in adult mouse hippocampal NSCs

Sox2, a transcription factor, is known to have the potential to regulate stemness, neural stem cell proliferation and neurogenesis (Cauffman et al., 2009, Episkopou, 2005). Interestingly, cell proliferation of precursor cells in neurogenic regions is decreased in Sox2 heterozygous knockout mice. Thus it is likely that Sox2 is essential for precursor cell proliferation and/or for maintaining NSCs (Episkopou, 2005). Similarly, it has been found that Sox2 is needed to maintain NSCs in the hippocampus *in vivo* and in neurosphere cultures (Favaro et al., 2009). Premature differentiation of precursors, which is one of the three processes of neurogenesis, is induced when Sox2 is inhibited by dominant-negative construct. This was caused by promoting the exit of neural progenitors from the cell cycle and mediating the expression of early neuronal markers (Episkopou, 2005). Furthermore, inefficient neurogenesis has

been found in Sox2 mutant adult mice by showing a reduction of stem/precursor cell number and the ability of neuronal differentiation of their progeny in the dentate gyrus (Ferri et al., 2004).

In an embryonic stem cell differentiation assay, expression of Sox2 levels have been shown to be higher under the condition of 1.5 mM VPA compare to 0, 0.3 and 0.7 mM VPA (Murabe et al., 2007). This appears to contradict the previous literature and the findings in the present study. I have demonstrated no alteration of Sox2 mRNA expression in hippocampal NSCs exposed to 0.3 mM VPA whereas 1 mM VPA produced a reduction of Sox2 mRNA expression. Our results are consistent with the reduction in cell proliferation found.

Regulation of Notch1, Sox2 and Nestin

Sox2 and Notch1 appear to be able to regulate each others expression. Sox2 has been reported to have the ability to control neural stem cell maintenance and self renewal in embryonic brains and the adult nervous system by activating the Notch downstream effector gene Hes5 (Qu and Shi, 2009). Some studies demonstrate that Notch is essential for up-regulating Sox2 in the neural tube formation. Furthermore, Notch signalling and Sox2 are equally required to repress neuronal differentiation (Yan et al., 2009). However, some studies have revealed that Notch1 expression is increased by over-expression of Sox2 to upstream of Notch signalling. Notch signalling pathway is greatly inhibited in retina-specific Sox2 ablation while the Notch signalling downstream effectors Hes5 is moderately reduced in the hippocampus of Sox2 mutants (Favaro et al., 2009). There also is evidence that Notch1 is directly affected By Sox2 (Pevny

and Nicolis, 2009). These might reveal an association regarding how Sox2 and Notch signalling interface which is in line with the simultaneous reduction of Sox2 and Notch1 mRNA expressions in hippocampal derived NSCs in the present study.
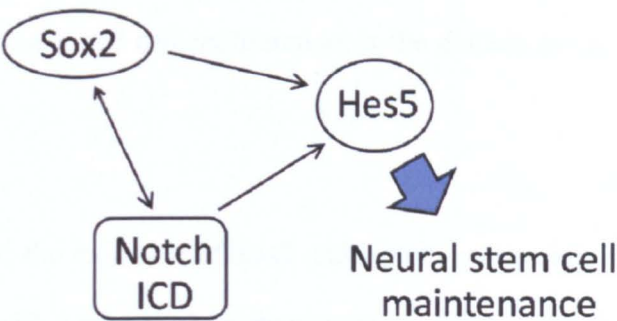
Figure 3.20 Cell intrinsic regulators in neural stem cell maintenance.

The reduction of Sox2 gene could be due to the action of 1 mM VPA might affect a powerful function of Sox2 to maintain stemness by self-renewing to be stem cells rather than transit amplifying progenitor cells. In stem cell biology, type-1 cells and type-2 cells correspond to stem cells and transit progenitor amplifying cells, respectively. Transit progenitor amplifying cells have less potentially ability than stem cells for self-renewal but have higher probability of undergoing terminal differentiation (Jones and Watt, 1993). Sox2 can be detected in type-1 and type-2a cells but not type-2b (Kempermaan, 2006). Likely to Sox2, type-2a cells are also expressed nestin whereas type-2b cells are not positive for nestin. Moreover, type-3 cells or neuroblasts are not expressed Sox2 and nestin (Kronenberg et al., 2003, Ehninger and Kempermann, 2008). Additionally, the reduction of Sox2 and nestin expression in hippocampal NSCs treated with 1 mM VPA might be caused by an

induction of cell differentiation. VPA has been reported that 1 mM VPA produced an increase of neuronal differentiation (Yu et al., 2009, Jung et al., 2008). Interestingly, nestin positive cells are extremely reduced in the dentate gyrus of Sox2 mutant adult mice (Ferri et al., 2004). In adult mice, ablation of Sox2 in Sox2-expressing cells produces a dramatic decrease in nestin-positive stem/precursor cells and cell proliferation in the dentate gyrus (Favaro et al., 2009).

Taken together, the reduction of Sox2 and Notch1 expression in hippocampal NSCs treated with 1 mM VPA might change the balance between maintaining the self renewal of stem cells and allowing cells to differentiate. Moreover, Sox2 and nestin gene expression in hippocampal NSCs exposed to 1 mM VPA in this research was lower than untreated cells, suggesting that these cells had been driven to become type-2b and type-3 rather than type-1 and type-2a.

VPA did not alter levels of c-Myc gene expression in adult mouse hippocampal NSCs

Evaluation of alteration of c-Myc gene expression is essential for determining the effect of VPA on cell proliferation via molecular mechanisms that regulates cell-fate. The present study reveals that 0.3 mM and 1 mM VPA did not alter levels of c-Myc gene expression in hippocampal NSCs in comparison with untreated cells (Fig. 3.18 and 3.19). In contrast, both rat and mouse adult hippocampal NSC proliferation in the previous study was significantly inhibited by VPA in a dose-dependent manner. These are not in line with a study which shows gradually down-regulation of c-Myc protein levels of acute

myeloid leukemia (AML) caused by 0.5, 1 and 3 mM VPA. Besides the levels of c-Myc protein reduction, level of c-Myc mRNA expression is down-regulated in AML cells treated with 1 mM VPA for 24 hours (Cheng et al., 2007). Moreover, inactivation of c-Myc causes an inhibition of granule cell precursor proliferation and self-renewal (Nagao et al., 2008). Corresponding to the down-regulation of c-Myc expression, ectopic expression of c-Myc enhances neural progenitor cell proliferation *in vitro* and *in vivo* (Fults et al., 2002). VPA also has a potential to decrease c-Myc expression and enhance apoptosis and differentiation of AML cells (Cheng et al., 2007). Similarly to the stem cell investigation, c-Myc is involved in regulation of cell proliferation, self-renewal and cell differentiation (Murphy et al., 2005, Nagao et al., 2008). Expression of c-Myc also induces self-renewal of neural progenitor cells in neurosphere culture (Kerosuo et al., 2008). However, this lack of effect on c-Myc gene expression in the hippocampal NSCs suggests that c-Myc might not be the major target responsible for the observed effects of VPA treatment in the present study.

In conclusion, the research in this chapter provides evidence for direct effects of 0.3 and 1 mM VPA treatment on hippocampal NSCs. The result shows a dose-dependent reduction of neurosphere formation and hippocampal NSC proliferation. This is in agreement with the animal model in the previous study was used to demonstrate the side effects of VPA which shows a decrease of cell proliferation in the hippocampal DG. However, VPA inhibited the expression of three markers of stem cells, Notch1, Sox2and nestin, but not c-Myc. This stem cell study indicates that VPA might cause a reduction of

hippocampal NSC proliferation via Notch1, Sox2 and nestin mechanisms rather than c-Myc.

Chapter 4

The effect of Valproic acid on cell proliferation

and Notch1 expression in Epn1, Med1 and

SHSY5Y cancer cell lines

4.1 Introduction

As described previously, VPA is a potent inhibitor of cell proliferation both *in vivo* and *in vitro*. This effect being linked to its action as a histone deacetylase inhibitor (Eyal et al., 2004, Chen et al., 2007, Gurvich et al., 2004). I have shown in Chapter 2 that VPA treatment *in vivo* reduced the expression of the receptor Notch1 and this was associated with a reduction in proliferation in the SGZ of the hippocampus.

The role of Notch1 is "context dependent", that is it can have different effects in different tissues and cell types. Activation of Notch1 during neural development is associated with increased cell proliferation, an inhibition of differentiation and the maintenance of a stem cell phenotype (Guentchev and McKay, 2006, Shimizu et al., 2008). However recent papers indicate that, in some cancers, VPA reduces cell proliferation but also induces the expression of Notch1. To further investigate the effects of VPA on Notch1 expression, a series of cancer cell lines were treated with VPA to test what the effects of this drug are on Notch1 levels. In cancer Notch1 can act as an oncogene promoting cell proliferation and suppressing differentiation or can induce cell cycle arrest and apoptosis (Kunnimalaiyaan and Chen, 2007).

VPA appears to always inhibit cell proliferation, induce cell cycle arrest and apoptosis in many types of cancer both *in vitro* and *in vivo*. For instance, VPA induces cell cycle arrest and apoptosis in small cell lung cancer, human thyroid cancer and endometrial tumour derived cells (Platta et al., 2008, Shen et al.,

2005, Blaheta et al., 2005). Similarly in neuronal tumours, VPA suppresses cell proliferation in neuroendocrine tumour derived cells (Greenblatt et al., 2007).

The Notch1 signalling pathway, is not active in all tissues, but is expressed at several stages of neural development where it has been reported to have a powerful influence. Notch1 is involved in regulating cell fates, cell proliferation and cell death (Bray, 2006). In some cancers, Notch1 per se can function as an oncogene that induces proliferation and suppresses apoptosis and cell differentiation, promoting malignancy (Kunnimalaiyaan and Chen, 2007). In these cases increased expression of Notch1 is associated with increased growth of the tumour (Wang et al., 2006c, Wang et al., 2006b, Wang et al., 2006a). In contrast, in some cancers, induction of expression of Notch1 signalling by VPA is associated with a reduction in tumour growth, cell cycle arrest and apoptosis. These cancers include small cell lung cancer and medullary thyroid cancer cells (Platta et al., 2008, Greenblatt et al., 2008). In the nervous system, VPA produces growth arrest in neuroendocrine cancer cells, *in vitro* and *in vivo*, and this is associated with an activation of Notch1 signalling (Greenblatt et al., 2007).

As Notch1 expression appears to have significant but sometimes contradictory effects on cell proliferation or cell cycle arrest it was decided to test the effects of VPA on cell proliferation and Notch1 levels in a variety of cancer cell lines. These provide a comparison to its effects on primary hippocampal neuronal stem cells.

Cell lines

Epn1 is a human cancer cell line which was cloned from an ependymoma (obtained from the Children Brain Tumour Research Centre, Nottingham, UK). This type of tumour is a form of glioma that arises from the ependymal cells lining the ventricular system (Puget et al., 2009, Agaoglu et al., 2005). Ependymomas are relatively rare tumours, accounting for 3% of all primary brain tumours and 50% of these tumours are found in children younger than 6 years old (Reni et al., 2004, Puget et al., 2009). The etiology of ependymomas is not well understood, current treatment includes surgery, radiotherapy and chemotherapy, but survival rates remain poor (Bouffet and Foreman, 1999).

The cell line Med1 was isolated from human medulloblastomas (obtained from the Children Brain Tumour Research Centre, Nottingham, UK). These tumours originate in the cerebellum or posterior fossa and are described as primitive neuroectodermal tumours (PNET) (Li et al., 2005). Unlike other brain tumours, this tumour type is invasive and spreads via the cerebrospinal fluid to various locations in the brain and spine. Approximately 15% to 40% of all childhood brain tumours and 30% to 40% of all posterior fossa tumours are medulloblastomas (Greenberg et al., 2001). Medulloblastomas in children tend to occur near the vermis that connects the two cerebellar hemispheres, whereas in adults this tumour arises in the cerebellar hemispheres (Greenberg et al., 2001). Prognosis of medulloblastomas remains poor, especially in infants and young children (Li et al., 2005) and the cause of medulloblastomas is unknown.

The SHSY5Y cell line is derived from human neuroblastoma (Biedler et al., 1973). This type of tumour occurs mostly in infancy and children younger than 5 years old, with an annual incidence of a hundred new cases diagnosed in the UK each year. It is the most common solid cancer in childhood and approximately 15% of all children with cancer will die from neuroblastoma (de Ruijter et al., 2005). The average survival period of children with intracranial neuroblastoma is normally not more than 1 year from time of diagnosis (Gao et al., 2007). Although the cause of neuroblastomas is unknown, they arise from neural crest cells of the sympathetic nervous system, and are also known as neuroendocrine tumours (de Ruijter et al., 2005, Stockhausen et al., 2005).

VPA, a well-tolerated anticonvulsant, has demonstrated a potential as a novel therapeutic agent due to its HDAC inhibitor effects on transcriptional regulation within cancer cells (Gottlicher et al., 2001). Transcriptional control by acetylation is one of the most important mechanisms by which the N-terminal tails of histones determine cell fate. Therefore, affecting acetylation by VPA affects transcription and is a possible potential approach in cancer therapeutics (Chen et al., 2006, Sakajiri et al., 2005).

This study was aimed to investigate the ability of VPA to reduce proliferation in Epn1, Med1 and SHSY5Y cells and to see if this effect was associated with changes in Notch1 levels. It is important to understand whether Notch1 signalling plays a role in controlling proliferation in these cells and whether it is acting as a tumour suppressor or an oncogene. Thus, alteration of Notch1 signalling by VPA may be an effective new therapy for these types of cancer

167

and may produce milder side effects compared to the currently used chemotherapeutic treatment with or without radiotherapy.

4.1.1 Aims of this chapter

Determine effect of VPA on Epn1, Med1 and SHSY5Y cell proliferation. Determine whether these cell lines express Notch 1 and whether this is affected by VPA treatment.

4.1.2 Hypothesis

- Cell proliferation of Epn1, Med1 and SHSY5Y cells in culture will be significantly reduced by treatment with VPA.

- Levels of Notch1 expressions, if present, in Epn1, Med1 and SHSY5Y cells will be significantly altered by treatment with VPA.

4.2 Materials and Methods

4.2.1 Effects of VPA treatment on Epn1 and Med1 cell lines

4.2.1.1 Growing and culturing of Epn1 and Med1

Human ependymoma cell line (Epn1) and human medulloblastoma cell line (Med1), were kindly provided from the Children Brain Tumour Research Centre, Nottingham, UK. These cell lines were derived by passaging primary tumour samples and selecting lines that continued to grow. The cell lines were grown in Dulbecco's Modified Eagles Medium (DMEM) (GibcoBRL, Paisley, UK), 10% Fetal Bovine Serum (FBS; GibcoBRL, Paisley, UK), 1% L-Glutamine (Sigma-Aldrich, Inc, St.Louis, USA) and 0.5% Penicillin/Streptomycin (Sigma-Aldrich, Inc, St.Louis, USA). Cells were grown at 37°C and the media was changed every 2-3 days.

4.2.1.2 Cell proliferation assay

Epn1 and Med1 were seeded into 6-well plates at a density of 7500 cells per well. The next day, the medium was replaced with cell culture medium containing 0, 1, 2 and 3 mM VPA (Sigma-Aldrich, Inc, St.Louis, USA) and then incubated for 72 hours. Before the cells reached 80% confluence, the cells were washed with pre-warmed 1X PBS to remove any residual growth media. Trypsin (0.5% trypsin, 0.53mM EDTA; Sigma-Aldrich, Inc, St. Louis, USA) was then applied to the side of each well to completely cover the monolayer. The 6-well plates were incubated at 37°C for a couple of minutes or until the cells had detached. After two minutes, the medium was added to the well and

then the cells were removed to a universal tube. The density of cells in control and VPA treated groups was quantified by staining with Trypan blue. Quantification of numbers of *Epn1* and *Med1* cells were carried out in triplicate. The mean ± standard error of the mean (SEM) of triplicate samples was calculated for each condition.

4.2.1.3 Immunostaining for cell proliferation by using Ki67

Cultures of Epn1 and Med1 cell lines were assigned to one of two groups: control group or VPA-treated group. The cell lines were seeded 200 cells per well. After incubating the cells for 24 hours at 37°C, the control cells received new fresh culture medium while the treated culture received culture medium contained 2.5 mM VPA. The cells were incubated for 3 days at 37°C. Next, immunofluorescence analysis was performed. The cells were fixed with 1% paraformaldehyde (PFA) in PBS for 3 minutes at room temperature and followed by washing three times with PBS. The cells were then incubated for 60 minutes at room temperature with Mouse anti-rat monoclonal anti-Ki67 (Novocastra NCL-KI-67-MM1), diluted 1:150 in PBS. Following washes with PBS 3 times, the binding of primary antibodies were detected by incubating with Alexa 488 Rabbit anti-Mouse IgG (1:300, Molecular Probes, Invitrogen, Paisley, UK) for 40 minutes at room temperature. The secondary antibody was removed by three washes in PBS. Subsequently, all cell cultures were subject to nuclear counterstaining with Propidium iodide (PI) (Molecular Probes, Invitrogen, Paisley, UK), diluted 1:3,000 in PBS. Then, the cultures were incubated for 30 seconds at room temperature and washed 3 times with PBS.

Positive staining was quantified using the Nikon Optiphot-2 microscope (Nikon, Surrey, UK) and viewed using the SPOT advanced software, version 3.5 (Diagnostic Instruments Inc, Michigan, USA). Systemic random sampling was used to observe 5 different fields of view per sample (n = 3). Ki67 positive cells were quantified using the fluorescein isothiocyanate (FITC) fluorescence filter and all nuclei were observed using the red (TRITC) filter. Two pictures taken per field were merged using the filters as described previously to show the image of Ki67 positive cells associated with their nuclei. Cell proliferation was determined and presented as means of Ki67 positive cell number.

4.2.1.4 Optimisation of detection of Notch1 using the C2C12 myoblast cell line for Western blot analysis

The detection of Notch1 was optimized using the C2C12 mouse embryonic myoblast cell line obtained from the American Type Culture Collection (ATCC) as a positive control for Western blot assay. C2C12 cells were harvested when they reached approximately 80% confluence. The cells were washed with 5 ml of ice cold PBS and then added 5 ml of ice cold cell dissociation buffer (Sigma-Aldrich, Inc, St.Louis, USA) to the cultured flask. After incubating the flask on ice for 5minutes, the cells were removed using a cell scraper and thoroughly transferred into a 30 ml universal tube. The flask were added a further of 5 ml of ice cold cell dissociation buffer to ensure all cells were removed from the flask and removed the buffer into the tube. The sample was kept on ice at all times. The universal tube was centrifuged at 1000rpm for 5 minutes at 4°C. The supernatant was removed using a suction pump being careful not to disturb the cell pellet. Lysis buffer (20mM Tris-HCl,

pH 7.6, 1mM EGTA, 320 mM Sucrose, 0.1% Triton X100, 1mM NaF, 10mM

Beta glyceronphosphaste) was added into the tube to resuspend the sample.

The sample was transferred into a new eppendorf and homogenized using a

pellet pestle to ensure all protein was released and then rotated on a wheel for

30 minutes at 4°C. After rotating 30 minutes, the sample was centrifuged at

14000rpm for 5 minutes at 4°C. Following the homogenization, the supernatant

was removed and placed 5 µl of each sample into an eppendorf for the Lowry

test to determine protein concentration as described in Chapter 2 (section

2.2.4.2). The remaining sample was resuspended in 6X of Sodium

dodecylsulphate polyacrylamine gel electrophoresis (SDS-PAGE) loading

buffer. After the Lowry assay, the sample was normalized to the same

concentration with 2X SDS-PAGE loading buffer. 50 µg proteins per lane were

loaded onto 10% SDS-polyacrilamide gel, electrophoresed for 45 minutes at

200v and blotted onto nitrocellulose membrane for 90 minutes at 100v, 4°C.

The membrane was blocked with Odyssey buffer (1:1 diluted in PBS, LI-COR

Biosciences) on the shaking platform for 1 hour at room temperature.

Following the blocking process, the membrane was incubated overnight at 4°C

with either a Goat anti-Notch1 (1:200, Sc-6014, Santa Cruz Biotechnology,

Santa Cruz, CA, USA) or a Mouse anti-GAPDH antibody (1:20000, Abcam,

Cambridge, UK), diluted in 5% dried milk solution. Then, binding of the

primary antibody was detected by incubating the membrane with either IRDye

800CW donkey anti-goat (LI-COR Biosciences) or IRDye 800CW goat anti-

mouse (LI-COR Biosciences), 1:20000 diluted in Odyssey buffer (1:1 diluted

in PBS). Immunoreactivity was determined using Odyssey scanner (Licor) at

wavelengths 800nm (green emission), and Odyssey software (Application software version 3.0).
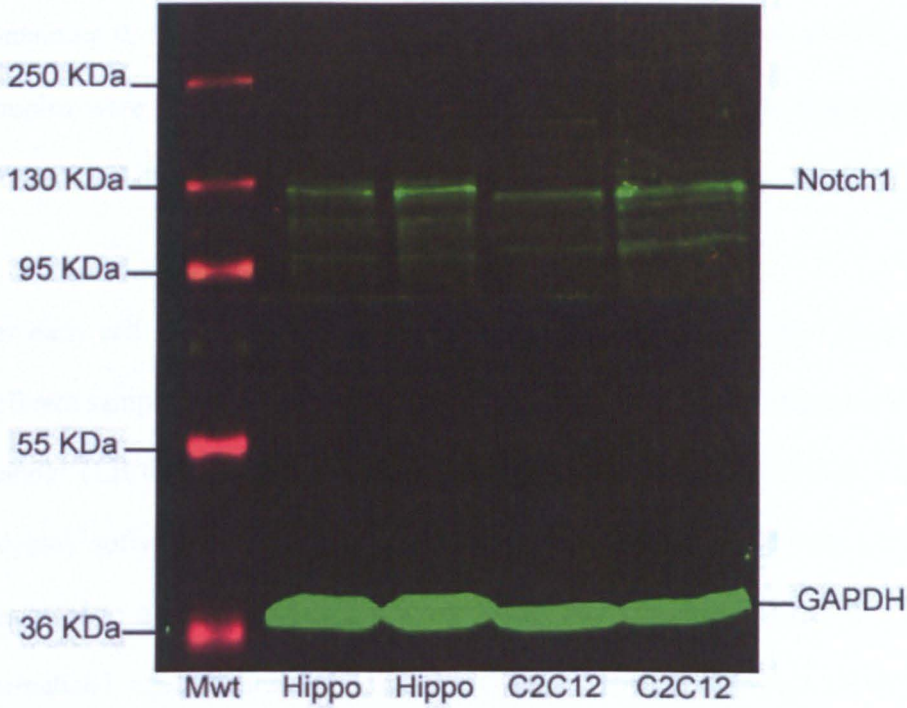
Figure 4.1 The expression of receptor Notch1 in C2C12 cell line and hippocampal brain tissue. The nitrocellulose membrane was scanned using Odyssey scanner (LICOR) at wavelength 800nm. Immunoblot bands were detected at 120 and 36 KDa for Notch1 and GAPDH respectively. Mwt = Molecular weight marker, C2C12 = C2C12 cell line and Hippo = hippocampal brain tissue.

4.2.1.5 Effects of VPA on Notch1 expression in Epn1 and Med1 cell lines using Western blot analysis

Epn1 and Med1 cell lines were seeded at a density of 10×10^4 cells/ml. The following day, the medium was replaced with new cell culture medium containing 0, 1, 2 and 3 mM VPA. Following 72 hours incubation at 37°C, the samples were harvested and prepared for Western blot assay as described in section 4.2.1.4.

For each cell line, western blotting was performed a total of three times on different samples per condition (n = 3). After scanning each blot using Odyssey scanner (LICOR), intensity of the immunoblot bands were measured using Odyssey software (Application software version 3.0). Intensity measurement was used to quantify protein levels of Notch1 expression at 800nm and then normalized against intensity levels of GAPDH. The normalized intensities were calculated into means of Notch1 expression levels. Adobe Photoshop Version 7.0 (Adobe Photoshop, USA) was used to increase contrast only in order to allow visible hardcopy prints for figures especially red and green images in this chapter.

4.2.2 Effects of VPA treatment on SHSY5Y cell line

4.2.2.1 Growing and culturing of SHSY5Y

The SHSY5Y cell line was obtained from the ATCC. The cell line was cultured in Dulbecco's Modified Eagles Medium (DMEM) (GibcoBRL, Paisley, UK), 10% Fetal Bovine Serum (FBS; GibcoBRL, Paisley, UK), 1% L-

Glutamine (Sigma-Aldrich, Inc, St.Louis, USA) and 5% Penicillin/Streptomycin (Sigma-Aldrich, Inc, St.Louis, USA). Cells were cultured at 37°C 5% CO_2 and the media was changed every 2-3 days.

4.2.2.2 Cell proliferation assay

SHSY5Y cells were seeded at a density of 6.69 X 10^4 cells into a T25 flask. Following overnight incubation, SHSY5Y cells was treated with media containing VPA at a concentration of 0, 1, 2 or 3 mM and maintained at 37°C for 72 hours. Following the incubation, the cell reached approximately 80% confluence, the cells were washed with pre-warmed 1X PBS to remove any residual growth media. Trypsin (0.5% trypsin, 0.53mM EDTA; Sigma-Aldrich, Inc, St. Louis, USA) was then applied to the side of the flask to completely cover the monolayer. The flask was incubated at 37°C for a couple of minutes or until the cells had detached. After two minutes, the medium was added to the flask and then the cells were removed to a universal tube. The density of SHSY5Y cells in non-treated and treated groups was determined using Trypan blue staining. Quantification of numbers of SHSY5Y cells were carried out in triplicate. The mean ± the standard error of the mean (SEM) of triplicate samples was calculated for each condition.

4.2.2.3 Quantification of Notch1 expression in SHSY5Y by SDS-PAGE and immunoblotting

SHSY5Y cell line was seeded at a density of 10 X 10^4 cells/ml. The following day, the medium was replaced with new cell culture medium containing 0, 1, 2 and 3 mM VPA, maintained at 37°C for 72 hours. After the cells reached

approximately 80% confluence, they were harvested as described earlier (section 4.2.1.4, chapter 4) to prepare samples for Western blot assay. Binding of primary antibody was detected by incubating the membrane with either IRDye 680CW donkey anti-goat (LI-COR Biosciences) or IRDye 800CW goat anti-mouse (LI-COR Biosciences).

Western blotting was performed a total of three times on different samples per condition (n = 3). Each blot was scanned as described in section 4.2.1.5 in this chapter. Intensity measurement was used to quantify protein levels of Notch1 expression at 700nm and then normalized against intensity levels of GAPDH expression at 800nm. The normalized intensities were calculated into means of Notch1 expression levels. Adobe Photoshop Version 7.0 (Adobe Photoshop, USA) was used to increase contrast only in order to allow visible hardcopy prints for figures especially red and green images in this chapter.

4.2.3 Statistical analysis

The data of cell proliferation and Notch1 expression were expressed as mean ± SEM values and analyzed by GraphPad Prism Version 4.03 (GraphPad Software Inc., USA). One-way analysis of variance (ANOVA) was used to determine significant difference among the experimental groups followed by Bonferrini's multiple comparison test to compare between groups. The criterion for statistical significance was $p < 0.05$.

4.3.1 VPA inhibited cell proliferation of Epn1 and Med1 cell lines in culture

Following 72 hours treatment, a comparison of the number of Epn1 cells in all experimental groups revealed that there was a significant difference among the experimental groups ($p = 0.0019$, one-way ANOVA, Figure 4.2). VPA (1, 2 and 3 mM) reduced Epn1 cell proliferation to 98%, 83% and 60%, of control culture respectively. Cell proliferation in Epn1 cells treated with 1 and 2 mM VPA groups was not significantly reduced as compared to untreated cells (both; $p > 0.05$, one-way ANOVA, Figure 4.2). In contrast, there was a statistically significant reduction of cell proliferation in 3 mM VPA-treated group compared to untreated group ($p < 0.01$, one-way ANOVA, Figure 4.2).

Similarly treated cultures of Med1 cells showed a statistically significant reduction in proliferation ($p = 0.0046$, one-way ANOVA, Figure 4.3). Cell proliferation of Med1 exposed to 1, 2 and 3 mM VPA was reduced to 91%, 85% and 63% of control cultures, respectively. The degree of cell proliferation in 3 mM VPA-treated Med1 cells was significant lower than untreated cells ($p < 0.01$, one-way ANOVA, Figure 4.3). However, both 1 and 2 mM VPA-treated groups did not show a statistically significant reduction in comparison to untreated group ($p > 0.05$, one-way ANOVA, Figure 4.3).

These results indicate that VPA appeared to produce a dose dependent reduction in cell proliferation in Epn1 and Med1 cells. 3 mM VPA treatment caused significant reductions of cell proliferation in both Epn1 and Med1 cell lines but not in 1 and 2 mM VPA-treated groups.
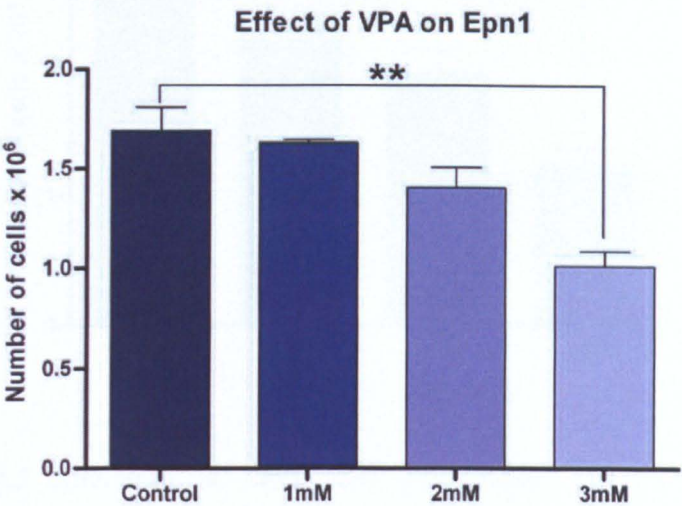
Figure 4.2 Analysis of the effect of VPA treatment on cell proliferation of Epn1 cell line. Graphs represent the mean (± SEM) of numbers of cells. Overall, cell proliferation showed a significant difference among the experimental groups ($p = 0.0019$). Bonferrini's multiple comparison analysis revealed unaltered cell proliferation in 1 and 2 mM VPA groups in comparison to control group ($p > 0.05$). Numbers of Epn1 cells exposed to 3 mM VPA were significantly reduced in comparison to control culture (** $p < 0.01$). Statistic analysis was carried out using one-way ANOVA.

Effect of VPA on Med1
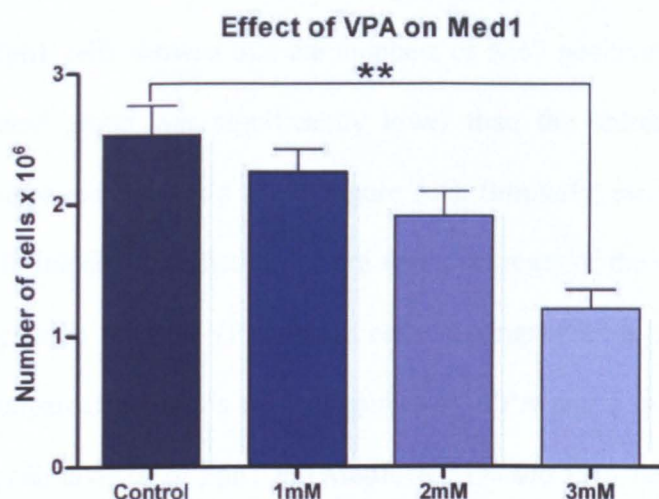
Figure 4.3 Alteration of Med1 cell proliferation after VPA treatment. Following 72 hours treatment, there was a statistically significant change in the number of cell proliferation amongst these experimental groups ($p = 0.0046$). Bonferrini's multiple comparison analysis confirmed that 3 mM VPA treatment caused a reduction of Med1 cell proliferation compared to control culture ($p < 0.01$) but Med1 cells in 1 and 2 mM VPA-treated groups remained unaffected as compared to control group ($p > 0.05$). All comparisons assessed using one-way ANOVA.

4.3.2 Numbers of Ki67 positive cells of Epn1 and Med1 cell lines were reduced by VPA

To confirm the previous results, Epn1 and Med1 cultures were stained for the proliferation marker Ki67 (Figure 4.5 and 4.6). Comparing untreated and treated Epn1 cells showed that the numbers of Ki67 positive cells in 2.5 mM VPA-treated group was significantly lower than the untreated group ($p <$ 0.0428, unpaired Student's t-test, Figure 4.4). Similarly, staining for Ki67 in Med1 cell line demonstrated that there was a decrease of the numbers of Ki67 positive cells in 2.5 mM VPA-treated cells in comparison to control cells ($p <$ 0.0475, unpaired Student's t-test, Figure 4.4). VPA at 2.5 mM was potent in reducing cell division of Epn1 and Med1, by 21% and 19%, respectively.
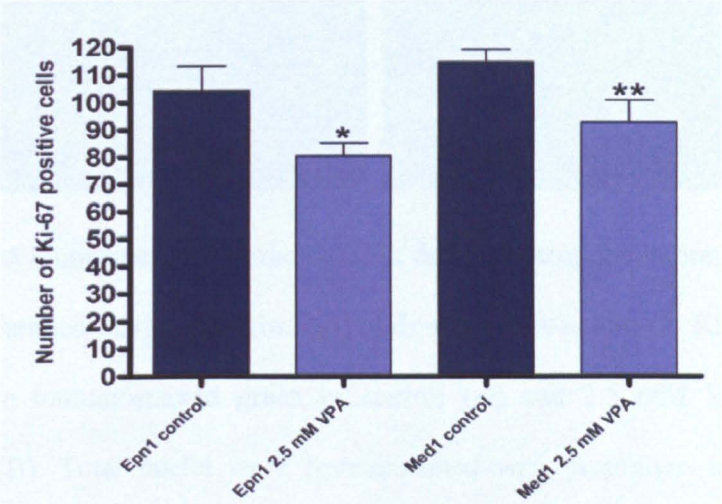
Figure 4.4 Quantification of Ki67 positive cells of Epn1 and Med1 in the presence or absence of 2.5 mM VPA. All data shown are from three experiments in parallel cultures. This analysis revealed that the numbers of Ki67 positive cells in control culture was significant greater than 2.5 mM VPA culture in both Epn1 and Med1 cell lines (*$p <$ 0.0428 and ** $p <$ 0.0475). All comparisons were calculated using unpaired Student's t-test.

Control culture   **2.5 mM VPA culture**
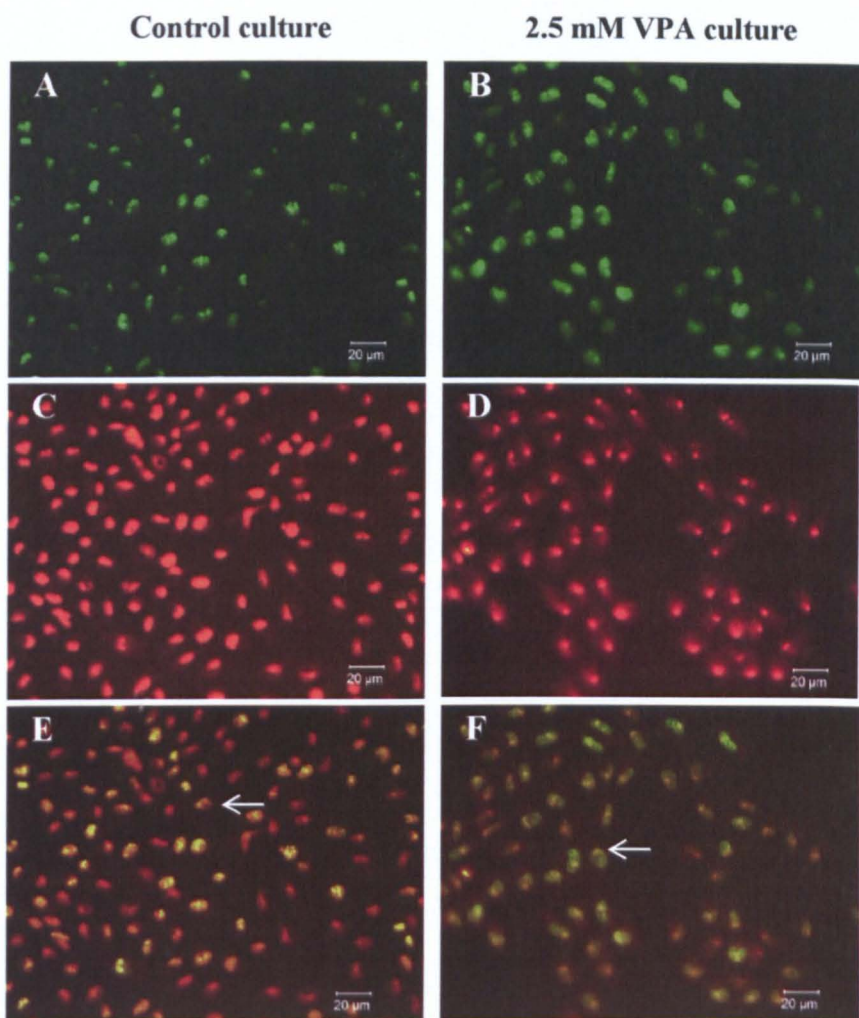
Figure 4.5 Fluorescent photomicrographs demonstrating the expression of the cell proliferation marker Ki67 in Epn1 cells after VPA treatment. Ki67 positive cells were immunostained green in control (A) and 2.5 mM VPA-treated cultures (B). Total nuclei were counterstained with propidium iodide (red) corresponding to image C (control culture) and D (2.5 mM VPA-treated culture). (E and F) Merged images of control and 2.5 mM VPA-treated cultures clearly shows nuclei expressing Ki67 are also positive for propidium iodide. There was a significant reduction of Ki67 positive cell number in VPA-treated culture compared to control culture. The cells stained for Ki67 and propidium iodide are indicated by arrows.

Control culture   **2.5 mM VPA culture**
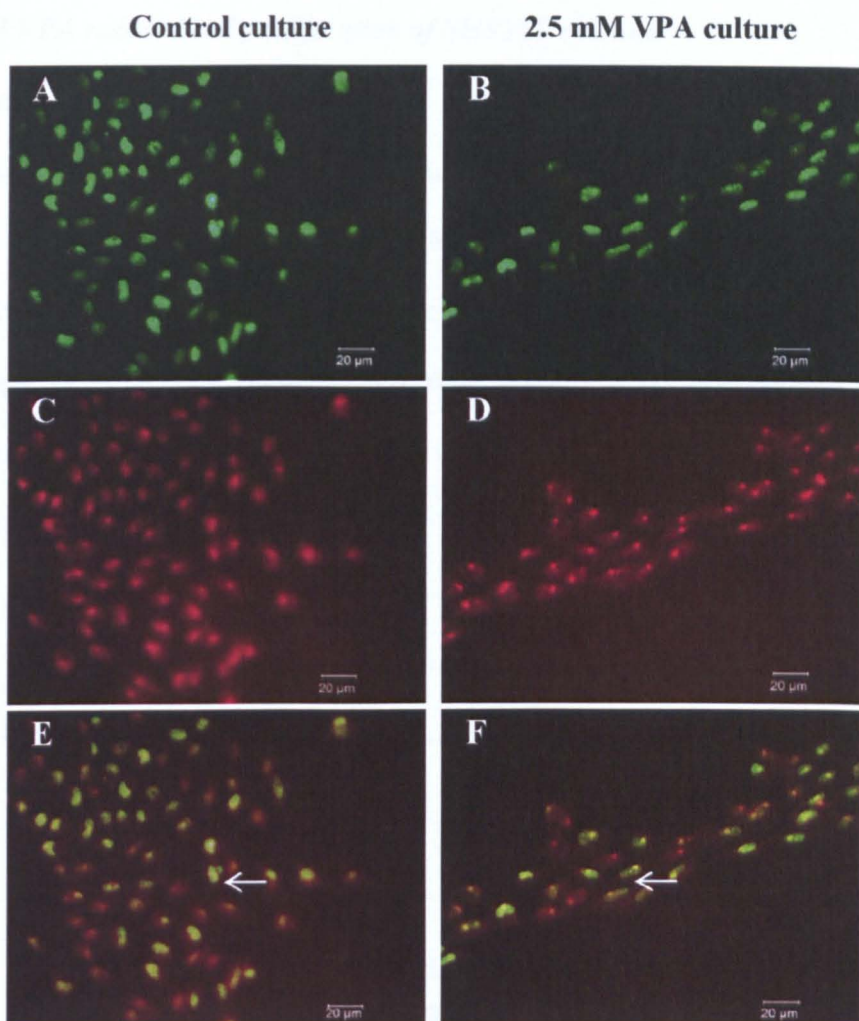
Figure 4.6 Fluorescent photomicrographs illustrating the effect of VPA treatment on Med1 cell proliferation expressing the division marker Ki67. In control (A) and 2.5 mM VPA-treated (B) cultures, Ki67 positive cells were immunostained green. Total nuclei counterstained with red nuclear dye, propidium iodide, control (C) and 2.5 mM VPA-treated cultures (D). Merged images of control (E) and 2.5 mM VPA-treated cultures (F) clearly shows nuclei expressing Ki67 were also positive for propidium iodide. The average number of Ki-67 positive cells in VPA-treated culture was significantly decreased in comparison with control culture. Arrows indicate the cells that are positive for Ki67 and propidium iodide.

4.3.3 VPA reduced cell proliferation of SHSY5Y cell line

To evaluate whether changes in cell proliferation of SHSY5Y cells occur during VPA treatment, the number of untreated SHSY5Y cells was compared with the cells treated with 1, 2 or 3 mM VPA for 72 hours (Figure 4.8). The results showed that there was a significant difference among the experimental groups ($p < 0.001$, one-way ANOVA, Figure 4.7). Interestingly, cell proliferation of SHSY5Y cells treated with 1, 2 and 3 mM VPA groups was significantly lower than untreated cells in a concentration dependent manner (** $p < 0.01$ and *** $p < 0.001$, one-way ANOVA, Figure 4.7). Treatment with VPA (1, 2 and 3 mM) for 72 hours, the inhibition rates of SHSY5Y cell proliferation were 32%, 55% and 64%, respectively.
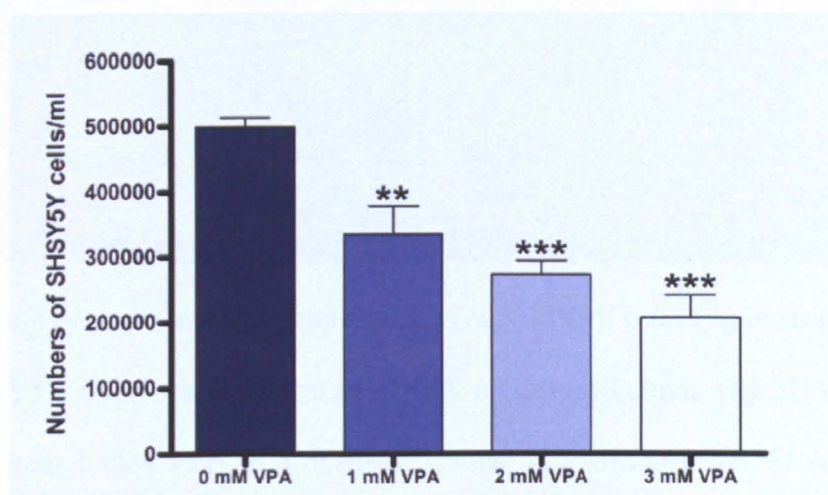
Figure 4.7 Bar graph illustrating the effect of varying concentration of VPA on the cell proliferation of SHSY5Y cell line. Numbers of SHSY5Y cells exposed to 1, 2 and 3 mM VPA were significantly reduced in comparison to control culture (** $p < 0.01$ and *** $p < 0.001$). Comparison of experimental groups showed that there was a significant difference between their means of total cell number ($p < 0.001$). All statistic analysis was assessed using one-way ANOVA.
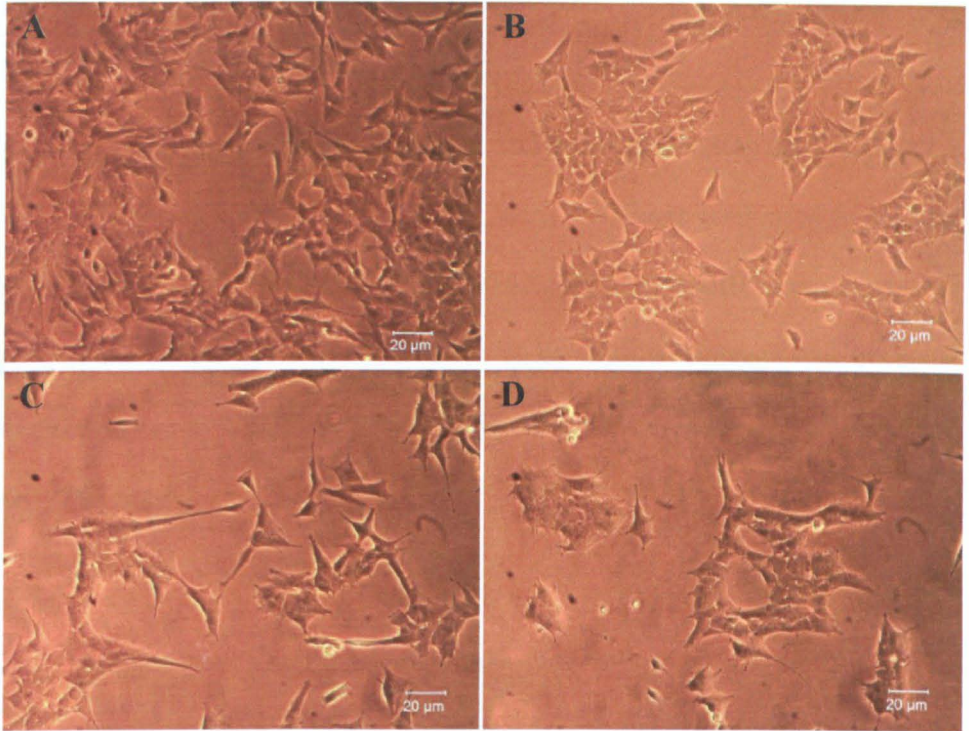
Figure 4.8 Phase contrast photomicrographs of SHSY5Y cells. (A) Photograph of SHSY5Y cells treated with 0 mM VPA or control culture. (B) SHSY5Y treated with 1 mM VPA at X20 magnification. (C) SHSY5Y treated with 2 mM VPA at X20 magnification. (D) SHSY5Y treated with 3 mM VPA at X20 magnification.

4.3.4 Western blot analysis of levels of receptor Notch1 expression in Epn1 and Med1 cell lines

To see if the reduction in cell proliferation brought about by VPA could be correlated with changes in Notch1, levels of this protein was quantified by Western blot.

Following 72 hours treatment, there was no Notch1 expression in 0, 1, 2 and 3 mM VPA-treated cultures in Epn1 and Med1 cell lines compared to Notch1 expression in the positive controls (C2C12 cell line) which was detected at approximately 120 KDa (Figure 4.9). These results suggest that receptor Notch1 was not expressed in either untreated or VPA-treated cells in Epn1 and Med1 cell lines.
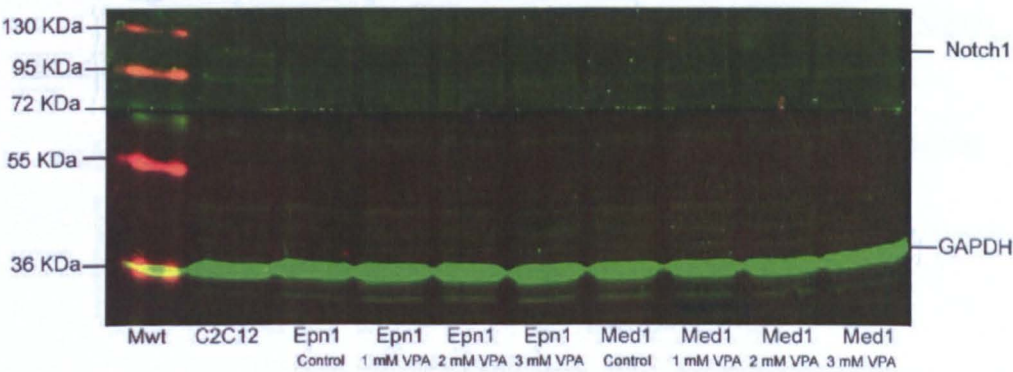
Figure 4.9 Photomicrographs illustrating the detection of Notch1 at 120 KDa. C2C12 cell line was used as a positive control (lane 2) for Notch1. GAPDH was detected at 36 KDa and used as a loading control. A representative blot revealed that there was no Notch1 expression in Epn1 and Med1 cells as indicated by comparing to Notch1 expression in C2C12 in lane 2.

4.3.5 VPA induced Notch1 expression of SHSY5Y cells

Western blot analysis of control and treated SHSY5Y cells was carried out to evaluate if VPA administration had any effect on the level of Notch1 expression. Following optimization of Notch1 antibody, immunoblot band was observed at 120 KDa (Figure 4.10). Intensities of each band was detected at 700nm and then normalized against intensities of GAPDH expression of each sample which were also observed at 800nm. The intensities of Notch1 expression in SHSY5Y cells exposed to 1, 2 and 3 mM were significant higher than non-treated cells (* $p < 0.05$ and ** $p < 0.01$, Figure 4.11). Levels of Notch1 expression also revealed statistical difference between these experimental groups ($p < 0.01$, Figure 4.11).
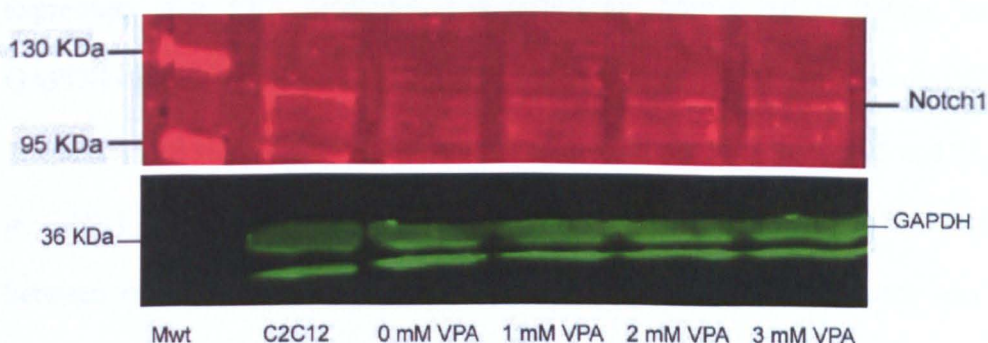
Figure 4.10 The effect of increasing concentrations of VPA on the expression of Notch1. Notch1 was detected at 120 KDa. A representative blot of Notch1 expression in SHSY5Y cells exposed to 0, 1, 2 and 3 mM VPA, respectively. C2C12 cell line was used as a positive control (lane 2) for Notch1. GAPDH was detected at 36 KDa and used as a loading control. The expression of Notch1 was increased in treated SHSY5Y cultures as indicated by the presence of darker bands compared with untreated culture.
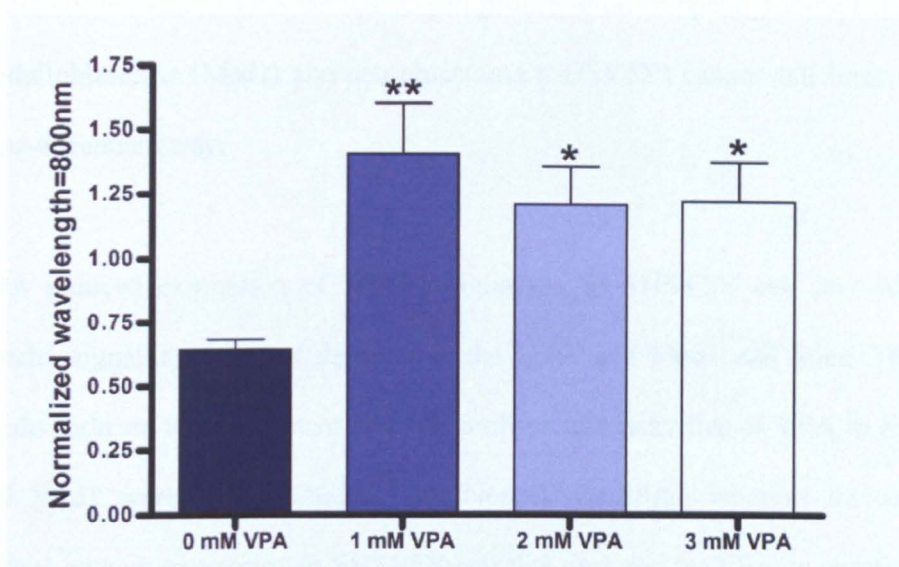
Figure 4.11 Protein analysis of Notch1 signalling expression in control and VPA-treated SHSY5Y cell line. Bar graph showed an assessment of Notch1 expression after VPA treatment. The results are normalized at 800nm to GAPDH expression. Levels of Notch1 expression in SHSY5Y cells exposed to 1, 2 and 3 mM were significant higher than non-treated cells (* $p < 0.05$ and ** $p < 0.01$). Levels of Notch1 expression also revealed statistically difference between these experimental groups ($p < 0.01$). Statistical significance was determined using a one-way ANOVA.

4.4 Discussion

In the current study, VPA decreased cell proliferation of ependymoma (Epn1), medulloblastoma (Med1) and neuroblastoma (SHSY5Y) cancer cell lines in a dose-dependent way.

VPA induced expression of Notch1 signalling in SHSY5Y cell line while Notch1 signalling was not detected in the Epn1 and Med1 cell lines. These results indicate that the potent anti cell proliferative activities of VPA in Epn1 and Med1 were not correlated with Notch1 signalling whereas increased Notch1 protein expression in SHSY5Y cell line, induced by VPA, is correlated with its anti cell proliferation effect.

VPA decreased cell proliferation of Epn1, Med1 and SHSY5Y

In the current study, the *in vitro* antiproliferative effect of VPA in Epn1, Med1 and SHSY5Y was concentration dependent (1, 2 and 3 mM, respectively). This is in agreement with evidence that human neuroblastoma (SHSY5Y and SK-N-BE(2)) cell proliferation is inhibited by VPA (1, 2 and 4 mM) in a dose-dependent manner (Stockhausen et al., 2005). Treatment with VPA (0.5 – 2 mM) also results in growth inhibition of human thyroid cancer cells (Shen et al., 2005). Similarly, a previous study shows that at doses of 0.5 – 3 mM VPA decrease growth of thyroid cancer cells (Catalano et al., 2005). More than 50% of cell proliferation of human medulloblastoma cell lines (D283-MED and DAOY) is attenuated by VPA at therapeutic safe doses (0.6 and 1 mM) (Li et al., 2005). VPA (0.5 – 50 mM) suppresses M14 melanoma cell proliferation in a dose-dependent manner. M14 cell growth is inhibited about 25% and 60%

after treated with 1 mM VPA for 24 and 72 hours, respectively. Moreover, it has been found 50% growth inhibition or ID_{50} at the concentration of 2.99 mM for 72 hours (Valentini et al., 2007). Treatment of DMS53 human small cell lung cancer cells with doses ranging from 1 to 10 mM VPA shows a dose-dependent inhibition of cell proliferation (Platta et al., 2008). Additionally, cell viability of rat hepatoma cell line (FaO) is significantly decreased by exposing to VPA greater than 300 μM in a dose-dependent reduction. Interestingly, 60% of FaO cell viability is impaired at a maximal concentration of 700 μM VPA after 144 hours (Phillips et al., 2003). In addition to the *in vitro* study, daily i.p. administration of 400 mg/kg VPA for 4 weeks significantly suppresses growth of two medulloblastoma (D283-MED and DAOY) xenografts in severe combined immunodeficient mice (Li et al., 2005). Also, daily i.p. injection of VPA (1.25 mM/kg) induces growth inhibition and attenuates metastasis of MT-450 breast cancer cells in animal experiments (Gottlicher et al., 2001).

Besides the anti-cell proliferative activities of VPA, the reduction of viable cells in the current study could be due to an increase of cell death caused by VPA. A report has shown that VPA (0.6 and 1 mM) enhances cell cycle arrest and apoptosis in human medulloblastomas (D283-MED and DAOY) (Li et al., 2005). Treatment of rat hepatoma cells with concentrations of 400 to 600 μM VPA induces apoptosis, which was investigated by chromatin condensation and DNA fragmentation assay (Phillips et al., 2003). Furthermore, induction of apoptosis in carcinoma cells, transformed hematopoietic progenitor cells, and leukemic blasts from AML patients is occurred after exposure to as low as 0.2 mM or 1 mM VPA for 72 hours (Gottlicher et al., 2001). It has been also

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189

demonstrated that M14 melanoma cells treated with 2.5 and 5 mM VPA are enhanced 50% and 80% of apoptosis compared to untreated cells (Valentini et al., 2007). The anti cell proliferative influence of VPA on the cell cycle is detected to be in the G1 phase (Martin and Regan, 1991, Greenblatt et al., 2007). Similarly, M14 human melanoma cells, human ovarian carcinoma cells and human multiple myeloma cells are progressively arrested at the G_0/G_1 phase of the cell cycle after treatment with VPA (Takai et al., 2004b, Valentini et al., 2007, Kaiser et al., 2006). At a dose of 1 mM VPA, which is slightly greater than the therapeutic serum concentration used in epileptic patients, cells are arrested in G1 phase (Catalano et al., 2005). Additionally, exposure to doses of 0.5 and 1.5 mM VPA decreases pro survival gene expression by 10% to 60%, but induces proapoptosis gene expression 23% to 85% in human thyroid cancer cells (Shen et al., 2005).

VPA is also known as a histone deacetylase (HDAC) inhibitor which causes hyperacetylation of the N-terminal tails of histones H3 and H4 *in vitro* and *in vivo* (Gottlicher et al., 2001, Li et al., 2005). This induces transcription activity, up-regulates cell cycle arrest and apoptosis. It has been found that VPA inhibits cell proliferation and enhances cell cycle arrest and apoptosis which is associated with increased expression of p21 (a cyclin-dependent kinase inhibitor) (Li et al., 2005, Takai et al., 2004a, Kaiser et al., 2006). This tumour suppressor protein plays an important role in blocking cell proliferation and cell cycle arrest in the G1 phase (Mai et al., 2005). Moreover, VPA, functions as a HDAC inhibitor, causes suppression of cell proliferation and induction of apoptosis in human multiple myeloma KM3 cells *in vitro* via down-regulating

vascular endothelial growth factor (VEGF) receptor (Dong et al., 2007). VEGF is a protein that binds to receptor tyrosine kinases on endothelial cells to promote angiogenesis (Jin et al., 2002). Lack of VEGF expression caused by VPA indirectly induces inhibition of proliferation, migration and tube formation of endothelial cells (Michaelis et al., 2004).

VPA induced expression of Notch1 receptor in SHSY5Y but not in Epn1 and Med1

Notch1 has multifunctional roles in regulating cellular differentiation, development, proliferation and survival in the animal kingdom. Notch1 in human cancer cells can function as either a tumour suppressor or an oncogene (Kunnimalaiyaan and Chen, 2007, Allenspach et al., 2002). Therefore, determination of an association between the antiproliferative effect of VPA and expression of Notch1 receptor in human Epn1, Med1 and SHSY5Y cell lines may throw some light on the role of Notch1 in different cancers and whether VPA is exerting its effect through this signalling pathway. The results show VPA inhibited cell proliferation after exposure to 1, 2 and 3 mM VPA in a dose-dependent manner in all the cancer cell lines but only SHSY5Y cells expressed Notch1. Treatment with VPA significantly up regulated Notch1 expression in SHSY5Y cells but had no stimulatory effect on expression in the other cell lines. The induction of Notch1 expression in SHSY5Y in the present study is in accordance with the suggested tumour suppressor function of Notch1 signalling in these and other cancer cells (Stockhausen et al., 2005, Greenblatt et al., 2008, Greenblatt et al., 2007). In addition, VPA can induce apoptosis in medullary thyroid cancer (MTC) and carcinoid cancer cells

(Greenblatt et al., 2008, Greenblatt et al., 2007) and neuroblastoma cells (Stockhausen et al., 2005). Moreover, inhibition of medullary thyroid cancer and small cell lung cancer cells is caused by Notch1 signalling via cell cycle arrest in G1 phase associated with p21 activation (Sriuranpong et al., 2001, Kunnimalaiyaan et al., 2006). Both the reduced proliferation and increased apoptosis are suggested to be effects of increased Notch1 signalling (Greenblatt et al., 2008).

In the present investigation VPA treatment produced a dose-dependent decrease in cell proliferation. However the effect of VPA did not seem to produce a dose-dependent effect on Notch1 expression as the lowest doses of VPA produced similar increases in Notch1 to the higher doses. It is possible that lower doses of VPA may show a more graded effect on Notch1 expression but we have not investigated whether their direct association between induction of Notch1 and cell proliferation.

In contrast to the induction of Notch1 protein expression by VPA in SHSY5Y cells, Notch1 protein expression was not detected in both VPA-treated and untreated Epn1 and Med1 cells in the present study. Not all cancers express Notch 1 for example it is low or absent in prostate cancer, and it is not present in all neuroendocrine tumours (NETs) (Kunnimalaiyaan and Chen, 2007).

The lack of expression and induction of Notch1 in Epn1 and Med1 strongly suggests that Notch signalling is not playing a part in the growth of these cancers.

Besides the tumour suppressive function, it has been revealed that Notch1 in human cancer cells can act as a tumour oncogene (Kunnimalaiyaan and Chen, 2007). For instance, Notch1 induces cell proliferation and inhibits cell cycle arrest in human T-cell neoplasia and medulloblastoma (DAOY and PFSK cell lines) (Kunnimalaiyaan and Chen, 2007, Fan et al., 2004). In addition, Notch1 plays a crucial role in promoting cell proliferation and the formation of cancer stem cell-like cells in human glioma (Zhang et al., 2008b). Recently, Notch1 has been reported to inhibit VEGF receptor expression, disrupting vessels and decreasing tumour viability in murine skin. This suggests that Notch1 is required for expression of VEGF-induced angiogenesis (Funahashi et al., 2008). In addition, it has been demonstrated that Notch1 is involved in angiogenesis in embryonic vascular development *in vivo* (Rehman and Wang, 2006). Up-regulation of Notch1 in SHSY5Y cells by VPA might induce cell cycle arrest and increase p21, causing inhibition of cell proliferation. Furthermore, the induction of Notch1 receptor by VPA might inhibit VEGF receptor expression, causing inhibition of angiogenesis and tumour growth. Altogether, Notch1 signalling appears to play a tumour suppressor role in SHSY5Y cells in the present study. The lack of Notch1 signalling in Epn1 and Med1 led to future investigation of its potential role in influencing proliferation and/or survival in these cancers. Thus, understanding of Notch signalling in regulating normal development and malignant transformation may be essential for novel cancer therapeutics.

In summary, the effect of VPA was shown to inhibit cell proliferation in Epn1, Med1 and SHSY5Y cells. The antiproliferative effect of VPA is associated with up-regulation of Notch1 signalling in SHSY5Y cells; however, Notch1 protein was not found in Epn1 and Med1 cells. These findings clearly indicate that the effect of anti cell proliferation activities of VPA might modulate cancer cell growth via Notch1 signalling pathway in SHSY5Y but not in Epn1 and Med1. Therefore, these findings suggest that anti-tumour activities of VPA mediated Notch1 signalling pathway might serve as an effective drug for a novel therapeutic option in neuroblastoma treatment.

Chapter 5:

General discussion

5.1 Introduction

The principle aim of this present project research was to use a rodent model to test the effect of VPA on hippocampal dependent spatial and recognition memory and to see if any changes in cognition could be correlated with changes in neurogenesis in the sub granular zone (SGZ) of the hippocampal dentate gyrus. This was done firstly to see if patient reports of cognitive impairment could be modelled in this rodent model and secondly to investigate if a mechanism causing these deficits could be found in the process of neurogenesis in the hippocampus.

To further investigate the impact of VPA, neural stem cells (NCS) were isolated and together with three neural cancer cell lines and these were treated in vitro, to determine the effects of the drug on their proliferation and expression of stem cell markers.

The main findings of this project were as follows:

1. Administration of VPA (300 mg/kg) twice daily for 10 days produced an impairment in the performance of the novel location discrimination test which is a test of hippocampal dependent spatial memory but did not impair performance in the contextual fear conditioning test (Chapter 2).

2. Treatment *in vivo* also reduced proliferation of cells in the SGZ of the dentate gyrus (Chapter 2).

3. Chronic *in vivo* VPA treatment caused reduced levels of the neurotrophin BDNF and the receptor Notch1 in the hippocampus but did not significantly reduce DCX levels (Chapter 2).

4. Exposure to VPA (0.3 and 1 mM) for 48 hours significantly suppressed neurosphere formation and cell proliferation of adult hippocampal NSCs *in vitro* (Chapter 3).

5. Quantitative RT-PCR showed that the reduction in proliferation of adult hippocampal NSC was correlated with a reduction of Notch1, Sox2 and nestin gene expression after exposure to 1 mM VPA but not 0.3 mM VPA (Chapter 3).

6. Treatment of VPA (1, 2 and 3 mM) reduced cell proliferation of Epn1, Med1 and SHSY5Y cells in a concentration-dependent manner (Chapter 4).

7. Treatment of VPA (1, 2 and 3mM) induced expression of Notch1 receptor protein in SHSY5Y as shown by Western blotting. However, VPA did not alter Notch1 protein expression in Epn1 and Med1 cells. Notch1 expression was not detected in untreated SHSY5Y cells (Chapter 4).

Overall the results of the present project provide evidence that VPA treatment can produce a cognitive impairment, confirming previous patient reports. The decline in spatial memory is associated with a suppression of adult hippocampal NSC proliferation which VPA can produce both *in vivo* and *in vitro*. Both cells (*in vitro*) and hippocampal tissue show a reduction in the expression of the NSC markers; Notch1 (*in vivo* and *in vitro*), Sox2 and Nestin (*in vitro*) after VPA treatment. Additionally, VPA reduced BDNF levels within the hippocampus. As both NSC proliferation and BDNF are components of the process of hippocampal neurogenesis their reduction after VPA treatment provides a plausible mechanism to explain the cognitive decline.

In human patients, VPA is a widely prescribed medication; trade names include Depakane, Valproate and Valrelease (Vadney and Kraushaar, 1997). Its primary uses are to prevent seizures caused by epilepsy and as a mood stabiliser in bipolar disorders (Lagace et al., 2004, Henry, 2003). It is normally taken orally using capsules resistant to degradation in the stomach. It has low systemic toxicity, the major problems are liver failure in young children and some reports of pancreatitis (Henry, 2003, Straussberg et al., 1998). Patient web sites (http://www.psycheducation.org/depression/meds/valproate.htm) and published studies, however report a range of side effects including hair loss, weight gain (Henry, 2003) and memory loss (Vermeulen and Aldenkamp, 1995, Straussberg et al., 1998, Goldberg and Burdick, 2001). Higher doses are given to epileptic patients compared to bipolar patients. The dose for epileptic patients is determined empirically starting with a low dose and increasing it until seizures are prevented. Dosage range is from 750 mg per day up to 3000 mg per day (Ristic et al., 2006, Park-Matsumoto and Tazawa, 1998, Jamora et al., 2007). The average of these doses is approximately 25 mg/kg.

The dose of VPA given in the present study (300 mg/kg, by i.p. injection twice daily for 10 days) was chosen as it has previously been shown to prevent seizures in spontaneously epileptic rats (Nissinen and Pitkanen, 2007) and a similar dosing regime (the same series of injections but VPA also given in drinking water) has been used in recent investigations of the action of VPA in preventing Kainic acid induced seizures in rats (Jessberger et al., 2007, Hsieh et al., 2004). From this it is likely that this dose is approximately equivalent to the clinically effective dose given to epileptic patients to prevent seizures.

Conversion of the dose given in the present study to a human equivalent dose (Reagan-Shaw et al., 2008) gives a figure of 113.5mg/kg which is somewhat higher than the 13 mg/kg - 50mg/kg dose range given to patients.

5.2 Cognitive effects of VPA in the adult hippocampus

Studies in the present thesis examined the effect of VPA on hippocampal-dependent memory using an animal model. Memory was tested using two established behavioural tests; the novel object location (NOL) and the contextual fear conditioning tests (Chapter2). In the NOL task, the results showed that rats receiving VPA displayed significant impairments in their ability to discriminate between the familiar and novel locations of objects compared to control animals. This is taken to indicate an impairment in spatial working memory (Dix and Aggleton, 1999, Mustafa et al., 2008).

In contrast, treated rats, did not show a significant difference in the duration of freezing behaviour after exposure to an aversive stimulus in the contextual fear conditioning task.

5.2.1 The NOL test

The NOL task uses the naturally spontaneous behaviour of rats that tend to explore novel stimuli without the requirement of positive or negative reinforcements, i.e. food and foot shock. These reinforcements can cause arousal or stress (Ennaceur and Delacour, 1988, Dere et al., 2007). The novelty-preference paradigm used in this study is more closely related to

human location memory as observed in everyday life (Ennaceur and Delacour, 1988).

In terms of spatial memory, the hippocampus is involved in consolidating and recalling memories. As might be expected, the hippocampus shows an increase in activity when animals are exploring a novel context compared to a familiar context (Aggleton and Brown, 1999). These observations indicate the importance of this brain region in performance of the NOL task.

Lesion studies of the hippocampus have further demonstrated the critical role this structure plays in carrying out the NOL. In rodents, animals with lesions to the dentate gyrus and CA3 are unable to perform the NOL test (Lee et al., 2005). This suggests that the connection between dentate gyrus and CA3, which uses the dentate gyrus granule cell axons, is essential for this type of spatial memory. Rats with hippocampal lesions show a significant NOL deficit when a 5 minute inter-trial interval is used (Dere et al., 2007). This length of delay, which was used in the current investigation, between initial exposure to the arena and recall, requires the hippocampus to consolidate the spatial memory. The NOL test was used in the present investigation as it was suspected that deficits in dentate gyrus neurogenesis could be the cause of any cognitive deficits. Other spatial memory tests could have been used, for example the novel object recognition test or Morris water maze. Lesions to the hippocampus do not always lead to deficits in the performance of these tests (Saxe et al., 2006, Shors et al., 2002) indicating that other brain regions are being used in the performance of these tests. In addition the Morris water maze

requires training over 8 days. It has been shown that this training itself affects hippocampal neurogenesis as both an increase and a decrease in neurogenesis at different time points during the learning procedure have been reported (Dupret et al., 2007). This would significantly complicate analysis of the effects of VPA on neurogenesis which was one of the objectives of this investigation.

The present results showed that treated animals were significantly reduced in their performance in the NOL test. This would indicate that the dentate gyrus and CA3 connection is impaired leading to a reduction in memory. As discussed later this may relate to the reduction in cell proliferation in the SGZ.

5.2.2 Contextual fear conditioning test

Contextual fear conditioning is modelled using contextual (conditional) stimuli to predict aversive (unconditional) stimuli such as foot shock (Maren, 2001, Fanselow, 2000, Fendt and Fanselow, 1999). The present test followed the procedure of Resstel (Resstel et al., 2006) where the contextual stimulus is the box in which the animal receives the shocks. Association between shocks and context is tested the following day by measuring the degree of freezing behaviour. Performance in this test is thought to require input from several brain regions including the hippocampus, amygdala and brain stem (periaqueductal grey matter) (Fanselow, 2000). As such it is not as hippocampal specific as the NOL but provides information on the functions and integration of several brain regions associated with this type of learning. Importantly, lesions to the hippocampus do not prevent performance in this test

(Shors et al., 2002). Animals treated with VPA did not show any reduction in the amount of freezing behaviour when placed back into the box where they have received shocks the day after receiving shocks. This result is different from that obtained with the NOL test and there are several possible reasons for this.

In some instances, there are some confounding factors which prevent learning in contextual fear conditioning. The aversive stimuli, per se, can produce confounding factors such as stress (Ennaceur and Delacour, 1988) that might make the interpretation of the effect of VPA on memory difficult.

This test can be performed in a variety of ways. The most common methods are either to lesion or drug treat the animals before training (pre-training lesion) (Shors et al., 2002, Ko et al., 2009) or after training (post-training lesion) but prior to re exposure to the box (Maren and Fanselow, 1997). In the present study, the contextual fear conditioning was done after treatment (pre training drug treatment). Pre-training lesions do not produce as strong a contextual conditioning impairment compared with post-training lesions. This is because in the pre training design, the contextual stimuli can be directly associated with foot shock in the amygdala without the processes of consolidation of the contextual memory in the hippocampus (Maren, 2001). In contrast, massive impairments are produced if hippocampal lesions are given after training (Maren, 2001, Anagnostaras et al., 2001).

Additionally, the preshock period (i.e. the time the animal spends in the foot shock box prior to receiving a shock) in this study was 1 minute, which is shorter than a typical preshock period of 2-3 minutes used in some publications (Fanselow, 2000, Saxe et al., 2006). The length of the preshock period, used by the rat to explore the context prior to a shock delivery, seems to be important for acquiring contextual conditioning. In addition the frequency of the shocks used in this study is greater than the typical shock (1-4 shocks).

However the experimental protocol used in the present study has worked with other investigators in this School and the present results may indicate differences in the type of memory affected by VPA.

There are several reports in the literature that contextual fear conditioning does not always correlate with other behavioural tests. For instance, animals with depleted hippocampal BDNF show deficits in novel object recognition (NOR) but do not show an impairment in contextual fear conditioning (Heldt et al., 2007). Similarly animals with the hippocampus lesioned by ibotanic acid microinjection show deficits in spatial memory (Morris water maze) but not in contextual fear conditioning in mice (Yoon and Gaiano, 2005). This may indicate that the contextual fear conditioning test is measuring different aspects of memory from these other tests.

5.3 Effects of VPA on cell proliferation of adult hippocampal NSCs both in
vivo and in vitro

Adult neurogenesis in the dentate gyrus is required for hippocampal-dependent

spatial memory (Dupret et al., 2008, Imayoshi et al., 2008). Most recent papers

show that reducing adult hippocampal neurogenesis causes deficits in spatial

memory. This is most clearly demonstrated where conditional knock out of

adult neural stem cells produces impaired performance in spatial tasks

(Imayoshi et al., 2008, Jessberger et al., 2009, Dupret et al., 2008). However, a

recent paper using mice in which the cyclin D2 gene had been knocked out,

show no deficits in several hippocampal-dependent behavioural tests

(Jaholkowski et al., 2009). One explanation of this contradictory result may be

that these mice have never had any adult neurogenesis (which uses cyclin D2

in cell division) whereas the previously described studies reduce or eliminate

adult neurogenesis in animals which have had hippocampal neurogenesis prior

to the conditional knock out. It is possible that animals which have never

experienced adult neurogenesis have found other ways to process and

consolidate spatial memories.

Drug treatment with VPA in the present study is closer to studies using

conditional knock outs where existing adult neurogenesis is reduced rather than

where it has never occurred.

VPA used in the present study showed similar results to other anti-mitotic

drugs, e.g. MAM and chemotherapy (Mustafa et al., 2008, Mignone and

Weber, 2006, Bruel-Jungerman et al., 2005). All of these drugs reduce

proliferation in the SGZ which then correlates with deficits in spatial memory. The mechanism by which proliferation is reduced is different with each drug and this may have differing effects on stem cell numbers and the time course of recovery.

A reduction in cell proliferation will lead to a reduction in new neurons in the dentate gyrus (Brown et al., 2003, Imayoshi et al., 2008). Nearly all studies where proliferation is reduced show a cognitive effect (Raber et al., 2004, Drapeau et al., 2003, Seigers et al., 2008, Imayoshi et al., 2008). We can not prove a direct connection between a decrease in cell proliferation and a cognitive deficit but the impact of VPA on SGZ proliferation is a plausible explanation. VPA can affect other parts of the brain for example by disturbing the GABAergic pathways in the basal ganglia system and VPA has been found to accumulate in the substantia nigra, superior and inferior colliculus, and medulla as well as the hippocampus (Masmoudi et al., 2000). The action of VPA in these other brain regions may also contribute to its cognitive effects.

Having demonstrated that VPA can reduce proliferation of hippocampal neural stem cells *in vivo* it was decided to look at the effects of VPA on these cells *in vitro*. In this way the direct effects of VPA on these cells may be seen without the effects of surrounding tissues. The adult hippocampal NSC culture in this thesis showed that treatment with 0.3 and 1 mM VPA resulted in a significant inhibition of neurosphere formation and cell proliferation (Chapter 3). The concentration of VPA used in this experiment is similar to the human therapeutic serum concentration (0.35 – 1.04 mM or 50 – 150 mg/l) (Hao et al.,

2004). These indicate that the anti-mitotic effect of VPA can act in both lower (0.3 mM) and upper (1 mM) levels of antiepileptic range. Thus, these observations warrant the anti-mitotic effect of VPA on adult hippocampal NSCs both *in vivo* and *in vitro* and indicate that VPA may be having a direct effect on the proliferation of these cells *in vivo*.

Although VPA appears to have clear antiproliferative effects, through its action as a histone deacethylase (Bredy and Barad, 2008, Dong et al., 2007), there are reports of it increasing neural stem cell proliferation *in vivo* and *in vitro*. Feeding mice with sodium valproate-supplemented (20 mg/kg) chow for 6 weeks induces cell proliferation in the dentate gyrus of the adult hippocampus (Hao et al., 2004). No behavioural tests were carried out in this study and the different method of dosing makes comparison with the present study difficult. In the same study, VPA (0.8 mM) increased the proliferation of embryonic cortical derived cells in culture (Hao et al., 2004). This dose is within the range used in the present study and is difficult to reconcile with the results presented here except to say that the age and brain region are different. It is possible that VPA might have different effects with different ways of administration, concentrations and cell types.

5.4 Correlation between VPA treatment-induced alteration in memory and neurogenesis

The study of the effect of VPA in this thesis attempted to assess the correlation between hippocampal memory changes and cellular effects induced by VPA both *in vivo* and *in vitro*. The present findings reveal that spatial memory

deficits corresponded with inhibition of cell proliferation in SGZ of the hippocampal dentate gyrus using an animal model (Chapter 2) and reduction of hippocampal NSCs in culture (Chapter 3). The *in vitro* study shows that VPA directly suppressed hippocampal NSC proliferation. The reduction of cell proliferation in this study might play a key role in memory alterations as previous studies have shown that over 70% of proliferating cells in the SGZ contributing neurons in the dentate gyrus (Zhao et al., 2006). Taken together, this has led to the conclusion that cellular effects relating to neurogenesis might be the underlying neural memory mechanism being affected by the VPA treatment and causing memory changes.

Besides its antiproliferative activity, VPA has been demonstrated to be involved in increasing apoptosis (Blaheta et al., 2005). For example, VPA has been reported to increase apoptosis of hippocampal progenitor cells as evaluated by staining with propidium iodide (Hsieh et al., 2004). Moreover, apoptosis and long-term newborn neuron survival have been shown to be involved in hippocampal-dependent memory and learning mediated by neurogenesis (Dobrossy et al., 2003). In the process of neurogenesis such as proliferation, differentiation, survival and apoptosis of newborn neurons has been involved in diverse phases of hippocampal-dependent learning and memory (Abrous et al., 2005). During training in the Morris water maze test, there is an enhancement of survival of newly generated neurons which were born a few weeks earlier. It is likely that these neurons are required for encoding, long-term storage and recall of memory as they show increased activity during the training period (Kee et al., 2007). In contrast, cells born

during the training period show an increase in apoptosis. These cells appear not to participate in the formation learning network connections (Dupret et al., 2007). This process might be contributing to memory consolidation. After that, generation of newborn neurons is increased in the late phase of learning for compensating and resetting availability of the dentate gyrus to obtain novel memory (Abrous et al., 2005). Therefore, future studies to examine the effects of VPA on neurogenesis should incorporate an analysis of apoptosis and long-term newborn neuron survival in the dentate gyrus to assess whether side effects of VPA might be more specific to apoptosis or survival of newly generated neurons or mature neurons.

5.5 Effects of VPA on cellular changes

The effect of VPA on neuronal differentiation was determined using DCX and BDNF immunoblotting in the hippocampus and frontal cortex (Chapter 2). Administration of VPA did not produce any alterations in levels of BDNF and DCX protein expression in the frontal cortex. In the frontal cortex, it might seem that DCX levels paralleled those of BDNF, indicating that VPA does not affect neuronal differentiation, survival and proliferation of newborn neurons in this region. Cells generated in the subventricular zone migrate forward in the rostral migratory stream to supply interneurons in the olfactory bulb (Abrous et al., 2005).

Conversely in the hippocampus, VPA decreased levels of BDNF protein but not levels of DCX. This indicates that changes of BDNF did not correspond with levels of DCX protein expression in the hippocampus. VPA is known to

accelerate neuronal differentiation in the hippocampus (Hsieh et al., 2004). It is possible that VPA has decreased the proliferation of dividing cells in the SGZ and enhanced differentiation of non-dividing precursors. This could maintain the levels of DCX during the period of the experiment. It would be useful for future studies to see if this level of DCX is maintained over longer time periods. Thus, BrdU and NeuN immunostaining will prove indispensible to confirm whether VPA induces neuronal differentiation and/or decreases survival of newborn neurons as well as suppressing cell proliferation of the adult hippocampal NSCs (Chapter 2 and 3). The role of VPA in affecting the various aspects of neurogenesis may prove to be a useful field to investigate.

5.6 Influence of VPA on cell proliferation associated with expression of Notch1 receptor

Notch signalling pathway has been known to have potential roles in the normal development of many tissue and cell types, such as effects on differentiation, survival, and/or proliferation. Notch1 signalling is also involved in regulating differentiation, survival and/or proliferation of cancer cells (Allenspach et al., 2002). Changes in Notch1 signalling might be involved in the antiproliferative effects of VPA in different ways. In this project, changes in Notch1 expression were observed to correlate with reductions in cell proliferation during VPA treatment in the animal model (Chapter 2), the adult hippocampal NSC culture (Chapter 3) and one of the cancer cell lines (Chapter 4). The results in the animal model demonstrate that VPA (300 mg/kg, two times a day), which inhibited cell proliferation, also reduced Notch1 protein levels in the hippocampus. The *in vitro* studies showed that VPA reduced cell proliferation

in all cell types tested (cancer cell lines Epn1, Med1, SHSY5Y and primary hippocampal NSC), at both higher and lower doses. VPA reduced Notch1 expression in adult hippocampal NSC, increased Notch1 expression in SHSY5Y and was undetectable in Epn1 and Med1 cells. These effects were seen only at the higher dose in the NSCs. Notch signalling is "context dependent" and can increase or decrease cell proliferation in different types of cell (Kunnimalaiyaan and Chen, 2007). Activation of the Notch signalling pathway is thought to mediate an increase in cell proliferation by adult neural progenitors (Wang et al., 2009). Reduction in the levels of Notch1 may therefore contribute to the antiproliferative effects of VPA in adult hippocampal NSCs.

In contrast some cancer cell lines have been shown to up regulate Notch 1 in response to VPA treatment which correlated with suppression of their proliferation (Stockhausen et al., 2005). Treatment of SHSY5Y cells with VPA in the present project reduced their proliferation and this was associated with an increase in Notch1 expression. The other two cancer cell lines, Epn1 and Med1, did not appear to express Notch1 either before or after treatment with VPA (Chapter 4).

These results clearly suggest that Notch1 signalling might play a role in influencing cell proliferation of SHSY5Y and NSC cells. The action of increasing Notch appears to have opposite effects in these two types of cell. It is unclear whether VPA is directly affecting Notch1 expression or whether it is down stream in a signalling pathway involving Sox2 as shown in possible

signalling cascades illustrated in Chapter 3. Notch expression however is not required for the antiproliferative effects of VPA, as shown by its effect on Epn1 and Med1. It is unclear how much of the effect of VPA on proliferation in NSC and SYSY5Y cells is due to its action on Notch expression. Further work which reduces Notch signalling in these cell types could be used to investigate the role of this signalling pathway in the proliferation of these cells. The action of VPA in reducing neural cell proliferation irrespective of their expression of Notch1 may make it an effective drug in treating a range of neural tumours including neuroblastoma. It is however likely that the use of VPA in this way will result in cognitive effects as has been described by a variety of other chemotherapy agents (Mustafa et al., 2008).

5.7 Influence of VPA on expression of stem cell markers

Stem cell makers such as Notch1, Sox2, nestin and c-Myc have been known to be involved in maintaining stemness, neural stem cell proliferation, cell survival and neurogenesis and they also prevent cell differentiation (Breunig et al., 2007, Nagao et al., 2008, Cauffman et al., 2009). The effect of VPA on a stem cell maker, Notch1 gene expression, was determined *in vivo* and *in vitro* where VPA treatment reduced levels of Notch1 expression. The effect of VPA on Sox2, nestin and c-Myc expression was examined to investigate a correlation of these markers and Notch1. The results showed that VPA treatment as well as reducing Notch1, also reduced Sox2 and nestin expression however it did not change c-Myc expression. Sox2 and nestin are detected in NSCs and transient amplifying cells (Kempermaan, 2006, Kempermann, 2008). These results suggest that VPA is reducing a range of stem and transit

amplifying cell markers as well as reducing cell proliferation. This could be caused by VPA pushing the cells to differentiate. These stem cell markers are believed to be linked in a variety of signalling pathways. There is evidence that nestin expression is regulated by Notch1 (Mellodew et al., 2004). Moreover, it has been reported that both Notch1 and Sox2 which are involved in maintaining neural stem and progenitor cells and inhibiting neuronal differentiation, are also able to control each others expression (Yan et al., 2009, Qu and Shi, 2009). It is not clear whether VPA is independently affecting each of these genes or whether the reduction in expression of one gene is causing effects on the expression of other stem cell genes. In conclusion, VPA reduces the expression of key stem cell markers which is associated with a reduction in the proliferation of these cells.

5.8 Summary

In summary, the findings in this thesis provide strong evidence showing that administration of VPA, a widely used treatment of epilepsy and bipolar disorder has behavioural and cellular effects in an animal model. The behavioural effects are a deficit in at least one measure of spatial memory and confirm patient reports of the cognitive effects of this drug. Action of the drug on the hippocampus is strongly suggested as the behavioural test used is known to measure hippocampal function. This conclusion is further supported by the cellular and chemical changes which appear to be specific to this brain region. VPA treatment reduced cell proliferation in the SGZ, lowered BDNF and Notch 1 levels in the hippocampus but did not affect DCX. Although the lack

of effect on DCX is puzzling, overall VPA appears to have a significant detrimental effect on adult hippocampal neurogenesis.

Further studies of adult hippocampal NSCs both *in vitro* show that VPA can reduce Notch1, Sox2 and nestin gene expression. This indicates that VPA can have direct affects on NSCs.

The present study contributes to understanding the pathology and causes of VPA-induced cognitive deficits. In addition to the effect of VPA on NSC proliferation, VPA treatment potently decreased SHSY5Y tumour cell growth at a dose at the upper limit of the therapeutic range for epilepsy. The results also revealed that VPA increased expression of Notch1 in these cells in contrast to the results from hippocampal NSCs. This present study may lay the groundwork for predicting drug responsiveness and guiding the development of future clinical epileptic and tumour therapies.

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236

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APPENDIX

A1.1 Immunofluoresent staining reagents

4% PFA

To make up 50 ml of 4% PFA, 2g of PFA powder was dissolved in 45 ml of dH$_2$O. To this solution, 10µl of 5M NaOH was added and then heated in a water bath at 55-60°C until the powder had dissolved. Once dissolved 5 ml of 10X PBS was added and the solution was adjusted to a pH of 7.0 using a pH meter. To prepare 0.5% PFA, 1.25 ml of 4% was diluted in 3.75 ml of PBS.

Coating of slides with APES

The glass microscope slides were first soaked in 10% DECON overnight. Following overnight incubation the slides were washed in running tap water at 60°C for 1 hour to remove the detergent and then rinsed in distilled water H$_2$O (dH$_2$O). To ensure the slides were cleaned, they were dried at 60°C overnight. Following incubations, the slides were immersed into a solution of 2% APES (3-Aminopropyltriethoxysilane) (Sigma-Aldrich, Inc, St.Louis, USA) in ethanol for 2 minutes. The slides were then washed 2X in ethanol followed by one wash in acetone and left to dry overnight at 37°C.

The following solutions were used throughout this thesis during the process of Western blotting.

Lysis Buffer pH 7.6

20 mM Tris (Invitrogen, CA, USA)	12.1 g
1 mM Ethylene Glycol Tetraacetic Acid (EGTA)	1.9 g
(Sigma-Aldrich, Inc, St.Louis, USA)	
320 mM Sucrose (BDH Laboratory Supplies, Poole, UK)	51.7 g
0.1% Triton X100 (Sigma-Aldrich, Inc, St.Louis, USA)	500 µl
1 mM NaF (Sigma-Aldrich, Inc, St.Louis, USA)	0.021 g
10 mM Beta glycerophosphate	1.08 g
(Sigma-Aldrich, Inc, St.Louis, USA)	

The above reagents were dissolved in 500 ml of dH_2O and the solution was then adjusted to a pH of 7.6. When homogenizing the samples, 1 complete mini protease inhibitor tablet (Roche Diagnostics, Germany) was dissolved in 10ml of lysis buffer.

2X SDS-PAGE loading buffer

0.5 M Tris (Invitrogen, CA, USA)	2.5 ml
Glycerol (Sigma-Aldrich, Inc, St.Louis, USA)	2.0 ml
10% SDS (Sigma-Aldrich, Inc, St.Louis, USA)	2.0 ml
dH_2O	2.5 ml
Beta mercaptoethanol (Sigma-Aldrich, Inc, St.Louis, USA)	1.0 ml
2.5% Bromophenol Blue (Sigma-Aldrich, Inc, St.Louis, USA)	40µl

6X SDS-PAGE loading buffer

12% SDS (Sigma-Aldrich, Inc, St.Louis, USA)	1.2 g
30% Glycerol (Sigma-Aldrich, Inc, St.Louis, USA)	3 ml
603 mM DTT (Sigma-Aldrich, Inc, St.Louis, USA)	0.93 g
2.5% Bromophenol Blue (Sigma-Aldrich, Inc, St.Louis, USA)	80 µl
1.5 M Tris pH 6.8	8.33 µl

The above components were dissolved in 10 ml of dH_2O.

Lowry solution A

100 mM NaOH	2 g
(Fisher Chemicals UK Limited, Loughborough, UK)	
7 mM SDS (Sigma-Aldrich, Inc, St.Louis, USA)	1 g
187 mM $NaCO_3$	10 g

(Fisher Chemicals UK Limited, Loughborough, UK)

The above compounds were dissolved in 500 ml of dH_2O

Lowry solution B

1.0% $CuSO_4$ (Fisons Scientific Apparatus, Loughborough, UK)

2.0% NaK tartrate (BDH Laboratory Supplies, Poole, UK)

To prepare a working solution of Lowry AB, 100 µl of 1.0% $CuSO_4$ and 100 µl of 2.0% NaK tartrate was added to 20 ml of Lowry solution A.

10X Electrophoresis buffer

250 mM Tris (Invitrogen, CA, USA)	30.0 g
1.92 M Glycine	144 g
35 M SDS (Sigma-Aldrich, Inc, St.Louis, USA)	10 g

The above components were dissolved in 1ltr of dH_2O. To prepare a working solution of 1X Electrophoresis, 50 ml of 10X buffer was diluted in 450 ml of dH_2O.

Transfer buffer

25 mM Tris (Invitrogen, CA, USA)	30.3 g
192 mM Glycine (BDH Laboratory Supplies, Poole, UK)	144 g

The above compounds were dissolved in 8ltr of dH_2O and 2ltr of methanol after which the solution was stored at 4°C.

TBST 0.1% Tween

25 mM Tris (Invitrogen, CA, USA)	30.29 g
125 mM NaCl	73.12 g

(Fisher Chemicals UK Limited, Loughborough, UK)

The above compounds were dissolved in 1ltr of dH_2O which was then adjusted to a pH of 7.6. After the correct pH had been obtained the solution was made up to 10ltr to which 10ml of Tween 20 (Sigma-Aldrich, Inc, St.Louis, USA) was added.

Summary of BSA dilution for constructing a standard curve during Lowry determination of protein concentration.

Tube Number	Sample concentration (mg/ml)	BSA (μl)	dH$_2$O (μl)
1	0	0	200
2	0.05	10	190
3	0.1	20	180
4	0.15	30	170
5	0.2	40	160
6	0.25	50	150
7	0.3	60	140
8	0.35	70	130
9	0.4	80	120
10	0.45	90	110
11	0.5	100	100

Summary of the components for gel preparation

Components	Component volumes (ml)			
	4% Stacking gel	8% Resolving gel	10% Resolving gel	12% Resolving gel
Distilled H$_2$O	2.440	3.776	4.100	3.400
30% acrylamine	0.520	2.144	3.300	4.000
Resolving gel buffer 1.5M Tris-HCL		2.000	2.500	2.500
Stacking gel buffer 0.5M Tris-HCL	1.000			
10% Sodium dodecyl sulphate (SDS)	0.040	0.080	0.100	0.100
10% Ammonium Per Sulphate (APS)	0.020	0.040	0.050	0.050
Tetramethylethylenediamine (TEMED)	0.005	0.008	0.010	0.010

A1.3 Haematocytometry

In cell counting, the cell suspension was mixed thoroughly and then transferred 10 µl of it and mixed with 10 µl trypan blue in a vial. Transfer a sample of the suspension immediately to the edges of both chambers of an Improved Neubauer Hemocytometer and let the suspension run out of the pipette and be drawn under the coverslip by capillary action. The fluid should run only to the edges of the grooves. Did not overfill or underfill the chamber, it might change its dimension, due to alternations in the surface tension. Cells lying within the central large square in both halves of the chamber were counted. Cells that lie on the top and left-hand lines but not including the bottom and right-hand lines were counted in order to avoid counting the same cell. The cell (viable cells) concentration per ml could be calculated by using the following formula.

$$c = (n \times d)/v$$

c was cell concentration (cells/ml)

n was the number of cells counted

v was the volume counted (ml)

d was the dilution of cell suspension

If only the central was used, the depth of the chamber is 0.1 mm and v is 0.1 mm^2, or 1×10^{-4} ml. the formula becomes $c = (n \times d)/10^{-4}$, or $c = n \times d \times 10^4$.

If only the five diagonal squares within the central 1 mm^2 were counted (i.e., 1/5 of the total), this equation became $c = n \times 5 \times d \times 10^4$.

Total cells = cells/ml x original volume of cell suspension from which the sample was taken.

A1.4 Neural stem cell medium

Dulbecco's Modified Eagles Medium/Ham's F12	48 ml
(DMEM/F12 1:1, Invitrogen, CA, USA)	
Neurobasal media (Invitrogen, CA, USA)	48 ml
N2 supplement (Invitrogen, CA, USA)	1 ml
B27 (Invitrogen, CA, USA)	2 ml
0.5% Pen/Strep (Sigma-Aldrich, Inc, St.Louis, USA)	0.5 ml
EGF (Sigma-Aldrich, Inc, St.Louis, USA)	20 ng/ml
FGF-2 (Invitrogen, CA, USA)	20 ng/ml

A1.5 Quantitative PCR

50X TAE buffer, pH 8.0

250 mM Tris-acetate (Invitrogen, CA, USA)	121 g
0.5 M EDTA, pH8.0	28.6 g

Tris-acetate was dissolved in 250 ml of dH_2O and then the solution was stirred to dissolve. EDTA was added and measure in graduated cylinder and added dH_2O to 500 ml. To prepare a working solution of 1X TAE, 10 ml of 50X buffer was diluted in 490 ml of dH_2O.

1.4% agarose gel in TAE

For 50 ml of 1.4% agarose gel, 0.7g agarose (Invitrogen, CA, USA) was added into 50mL 1X TAE. The mixer was heated in a microwave 1 minute to get into solution, did not allow to boil over. If crystals were not dissolved, swirled and heated for additional 20 seconds-1 minute. After microwaving, 5µL of ethidium bromide solution was added. The mixer was cooled down before

pouring into the gel plates, made sure the gel plates taped and contained the well combs prior to pouring. The gel was allowed to set about 20 minutes.

Sequencing of primers

Sequencing of pCS2+MTmNotchIC

The following mouse Notch1 cDNA sequence (Mus Musculus Notch gene homolog 1, Accession number NM_008714) was obtained from the NCBI Blast database, http://www.ncbi.nib.gov/BLAST/Blast.cgi. The red text indicates homologous sequence obtained from the sequencing of pCS2+MTmNotchIC using SP6 and T7 primers.