SEROTONIN AND SEROTONIN RECEPTORS IN NEURAL STEM AND PROGENITOR CELL PROLIFERATION

TAN CHEE KUAN FRANCIS

NATIONAL UNIVERSITY OF SINGAPORE

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TAN CHEE KUAN FRANCIS
(B.Sc.(Hons.), M.Sc., NUS)

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SUMMARY

Serotonin (5-HT) is a neurotransmitter that is also involved in embryonic development. Its imbalance is one of the known causes of pathological condition of depression. Treatment of depression using antidepressants is found to increase neural stem and progenitor cell (NSPC) proliferation and ablation of NSPC proliferation ablates the behavioural effects of antidepressants in rodents, thereby suggesting that proliferation and neurogenesis of NSPCs are essential to the effects of antidepressants. Many antidepressants increase availability of the serotonin by acting as selective serotonin reuptake inhibitors.

This thesis examines various aspects of serotonergic systems to determine the regulatory mechanisms by which serotonergic systems control NSPC proliferation. Serotonergic fibres are found in the neurogenic regions of the brain, namely the subgranular zone of the dentate gyrus and the subventricular zone of the lateral ventricles, suggesting the likelihood of direct serotonergic control of NSPC proliferation. The notion of direct serotonergic control was further reinforced by findings that exogenous addition of 5-HT to cultured NSPCs triggered an increase in NSPC proliferation and that NSPCs express a host of serotonin receptors..

Of the many 5-HT receptor subtypes that were found to be expressed in the NSPCs, this thesis focuses on 5-HT1A, 5-HT3 and 5-HT7 receptors. Previous reports suggested that the 5-HT1A receptor is one of the main receptor subtypes involved in the antidepressant-induced increase in NSPC

proliferation. However, the identification of new subtypes of serotonin receptors and the discovery of the cross-subtype activation of the 5-HT1A receptor agonist, 8-OH-DPAT, raises the possibility that the reported increase in NSPC proliferation may not be specific to 5-HT1A receptor activation. Despite the 5-HT1A receptor being previously reported as the site of action for 5-HT-induced NSPC proliferation, in this thesis it is shown that the selective 5-HT1A receptor agonist, 8-OH-PIPAT, failed to increase the NSPC proliferation whereas 8-OH-DPAT, a partial agonist for both 5-HT1A and 5-HT7 receptors, was able to increase NSPC proliferation. Moreover, AS-19, a selective 5-HT7 receptor agonist, was found to increase the NSPC proliferation in culture suggesting the likelihood that 8-OH-DPAT treatment increases NSPC proliferation through 5-HT7 receptor activation. NSPCs were also found to express functional 5-HT3A and 5HT3B receptors and direct treatment with 5-HT3 receptor selective antagonists was also able to increase NSPC proliferation both *in vitro* and *in vivo,* which supports the notion that antidepressants may increase NSPC proliferation through blockade of 5-HT3 receptors.

Besides 5-HT receptors, 5-HT biosynthesis was also examined. Some studies show that polymorphisms in the 5-HT biosynthesis enzyme, tryptophan hydroxylase (TPH), affect antidepressant treatment outcome suggesting that endogenous levels of 5-HT are one of the confounding factors in treatment of depression. In this thesis, it was found that *TPH1* and *TPH2* are expressed by NSPCs suggesting the possibility of self-regulation of proliferation. *TPH1* expression dropped upon NSPC differentiation showing NSPC specific

expression. Reduction in NSPC proliferation in TPH1 KO mice further pointed to the role of TPH1 in regulating and maintaining NSPC proliferation.

Taken together, NSPC proliferation may be regulated by the direct influence of serotonergic systems. To assist research on NSPCs, a method of cryopreservation of cultured NSPCs through serum and protein-free vitrification has also been optimized in this thesis.

LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine, serotonin
8-OH-DPAT	(±)-8-Hydroxy-2-dipropylaminotetralin hydrobromide
8-OH-PIPAT	(RS)-trans-8-Hydroxy-2-[N-n-propyl-N-(3'-iodo-2'-propenyl) amino]tetralin oxalate
ANOVA	analysis of variance
AS-19	(2S)-(+)-5-(1,3,5-Trimethylpyrazol-4-yl)-2-(dimethylami no)tetralin
bFGF	basic fibroblast growth factor
BMP	bone morphogenic protein
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CNPase	2', 3'-cyclic nucleotide 3'-phosphodiesterase
CNS	central nervous system
CUS	chronic unpredictable stress
DAPI	4',6-diamidino-2-phenylindole
Dcx	doublecortin
DNA	deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
EBSS	Earles balance salt solution
ECM	extracellular matrix
EDTA	ethylene-diamine-tetra-acetate

EG	ethylene glycol
EGF	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid
ERK	extracellular signal-regulated kinases
et al.	*et alter* (and others)
FGF	fibroblast growth factor
FN	fibronectin
GFAP	glial fibrillary acidic protein
GPCR	G-protein coupled receptor
HBSS	Hank's balance salt solution
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hTERT	human telomerase reverse transcriptase
IgG	immunoglobulin G
KCl	potassium chloride
KO	knockout
LIF	leukemia inhibitory factor
MAP2	microtubule associated protein
m-CPBG	1-(3-Chlorophenyl)biguanide hydrochloride
MDL72222	tropanyl 3,5-dichlorobenzoate
$MgCl_2$	magnesium chloride
NaCl	sodium chloride
NMDA	N-methyl-D-aspartate
NSF	novelty suppressed feeding test
NSPC	neural stem and progenitor cell

PBS	phosphate buffered saline
PCPA	*p*-chlorophenylalanine
PCR	polymerase chain reaction
PLO	poly-L-ornithine
PSA-NCAM	poly-sialated neural cell adhesion molecule
RNA	ribose nucleic acid
RT-PCR	reverse transcription polymerase chain reaction
S15535	1-(2,3-Dihydro-1,4-benzodioxin-5-yl)-4-(2,3-dihydro-1h-inden-2-yl)-piperazine
SEM	standard error of mean
SERT	serotonin transporter
SGZ	subgranular zone
Shh	Sonic hedgehog
Sox2	sex determining region Y box 2
SPSS	statistic package for social sciences
SVZ	subventricular zone
SR57227	1-(6-Chloro-2-pyridinyl)-4-piperidinamine hydrochloride
SSRI	selective serotonin reuptake inhibitor
TBS	tris buffered saline
TBS-T	tris buffered saline containing tween-20
TPH	tryptophan hydroxylase
Tris	tris(hydroymethyl)-aminomethane
TuJ1	βIII-tubulin
Wnt	wingless-type MMTC integration protein
Y-25130	*N*-(1-Azabicyclo[2.2.2]oct-3-yl)-6-chloro-4-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazine-8-carboxamide

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1. INTRODUCTION

1.1 Neural stem/progenitor cells and neurogenesis

The discovery of self-renewable, multipotent or totipotent stem cells has opened up an exciting field of research in regenerative medicine. This is especially true for embryonic stem cell research, which promises to offer a host of possibilities from whole organ regeneration to cell transplantation (Macchiarini *et al.*, 2008; *Keirstead et al.* 2005). However, the difficulty of generating specific cell types from embryonic stem cells has brought researchers to look at a less totipotent, more restricted type of stem cells, termed adult stem cells. These stem cells, such as haemopoietic, mesenchymal and neural stem cells, have more limited differentiation capability which allows them to only produce certain cell types that belong to the niche in which they are found (Watt and Driskell, 2010). One of the most interesting cell types among the newly discovered adult stem cells is neural stem and progenitor cells (NSPCs) due to it had long been thought that the brain was unable to generate any new cells upon the completion of postnatal development and the dogma that, upon brain damage, there will be no hope for recovery had long been accepted (Ramon y Cajal, 1928).

1.1.1 NSPCs – historical prespective

The first identification of the presence of NSPCs in the brain comes from the discovery through thymidine-H^3 incorporation into the dividing cells that the cells in certain regions of the brain can undergo proliferation and generate new neurons in rodents (Atlman, 1962). This paper is perhaps one of the earliest identifications of neurogenesis in the brain. However, the results were met with skepticism as others failed to find the same radionucleotide incorporation in the neurons (Schultze and Oehlert, 1960; Messier and Leblond, 1960). Later, more evidence followed from the discovery of similar phenomenon in other animals such as cats and song birds (Atlman and Chorover, 1963; Paton and Nottebohm, 1984). These discoveries did not generate much attention until Eriksson *et al.* (1998) discovered that these NSPCs are also found in the human brain and these cells are able to give rise to new neurons. This shows that the brain is still plastic in nature and brings forth the possibility that there are hopes of using the NSPCs in therapeutic cell transplantation. This hope is further enhanced by the discovery of that such NSPCs can propagate indefinitely, which suggests the life-long presence of NSPCs in adult brain (Reynold and Weiss, 1992; Kilpatrick and Bartlett, 1993).

In the adult brain, NSPCs are not widespread and are found to be restricted to only a few regions of the brain. The two main regions are the subependymal layer of the lateral ventricle walls covering the striatum (termed the subventricular

zone, SVZ) and the inner granular cell layer of the dentate gyrus of the hippocampus (termed the subgranular zone, SGZ) (Lois and Alvarez-Buylla, 1993; Eriksson *et al.*, 1998). Other regions of the central nervous system (CNS) that have been suggested to also contain NSPCs are the cerebellum and the spinal cord (Lee *et al.*, 2005; Dromard *et al.*, 2008). The presence of the NSPCs in these areas represents the need for continuous replacement or generation of new cells in these regions. For the NSPCs from the SVZ region, they are actively proliferating cells, which will migrate along the rostral migratory stream (RMS) along the surface of the lateral ventricles and ended up in the olfactory bulb, differentiating into interneurons (Gage, 2000). The NSPCs in the SGZ however, will mature and move radially into the granule cell layer where they will differentiate into the granule cells (Seri *et al.*, 2004).

There has been an interesting suggestion that the definition of neurogenic regions does not only encompass the areas that contain NSPCs but also the presence of the microenvironments that consist of cell-to-cell interactions and diffusible factors that promote neural development of the NSPCs and also the neurogenic potential that is capable of supporting transplanted NSPCs. This interpretation has lead to the suggestion of classifying the neurogenic regions into those supporting: (1) constitutive neurogenesis, where the larger population of NSPCs were found and where there are regions of active cell proliferation and neurogenesis; (2) potential neurogenesis, where smaller numbers of NSPCs have been isolated such as the rostro-caudal region of the anterior SVZ along

the neuraxis to the spinal cord and the dentate gyrus of the hippocampus; and (3) reactive neurogenesis, where neurogenesis can be induced by damage to the brain regions such as in the cortex and hippocampal CA1 region (Ortega-Perez *et al.*, 2007).

1.1.2 Identification of the neurogenic niche

By definition, the NSPCs are cells that are capable of self-renewal throughout the lifetime of the organism and capable of mutlipotent differentiation into neurons, astrocytes and oligodendrocytes (Gage, 2000). However, due to the lack of unambiguous markers, single NSPC is yet to be identified in the adult neurogenic niches (Morshead *et al.*, 1994). The general consensus among researchers is that there is a lack of an unique repertoire of markers that can be used as stem cell markers but current identification methods use a diverse set of markers that were shared with the non-stem cells. Therefore, up to this point, only subpopulations of cells can be identified and they may differ in characteristics such as antigenic profile, cell cycle stages, self renewal potential and differentiation potential. Based on the current established markers commonly used, neurogenesis has been broadly classified into a few stages.

1.1.3 Stages of neurogenesis

1.1.3.1 Quiescent neural progenitors

The quiescent neural progenitors, frequently known as the “true” neural stem cells, are the most primitive cell population in the neural stem cell niche (Bull and Bartlett, 2005; Seaberg and van der Kooy, 2002). In the hippocampal formation, they are glial fibrillary acidic protein (GFAP) and nestin expressing cells with triangular somata located at the SGZ and processes terminating in the molecular layer of the dentate gyrus (Mignone *et al.*, 2004). Due to their expression of GFAP, there have been suggestions that the neural progenitors arise from glial lineage (Krisegstein and Alvarez-Buylla, 2009). However, these cells do not express S100β, which is a marker for mature astrocytes (Steiner *et al.*, 2004). These cells are described as quiescent due to their low proliferation rate, with less than 2% of the cells being labeled by a 2hr BrdU pulse (Kronenberg *et al.*, 2003; Seri *et al.*, 2001). These quiescent neural precursor cells undergo asymmetric division, suggesting that they maintain the primitive precursor pool and were found to generate transit amplifying precursor cells upon mitosis (Encinas *et al.* 2006).

1.1.3.2 Transit amplifying neural progenitors

As described in the previous section, the transit amplifying neural progenitors arise from the asymmetric division of the quiescent neural progenitors. They are small oval shaped cells, typically around 10μm in diameter, found in both the SGZ and SVZ (Encinas *et al.*, 2006; Doetsch *et al.*, 2002). They are identified by nestin and Sox2 expression, but not GFAP or vimentin expression, as compared to quiescent neural progenitors (Brazel *et al.*, 2005; Ellis *et al.*, 2004; Kawaguchi *et al.*, 2001). These cells are highly proliferative as indicated by their ability for BrdU incorporation. About 20-25% of the cells are labeled in a 2hr BrdU pulse (Encinas *et al.*, 2006). However, they only have a capacity for a limited number of divisions and will not remain in this stage indefinitely (Basak and Taylor, 2009). These cells are found usually in clusters along the SGZ region of the dentate gyrus and in the SVZ regions of the lateral ventricles.

1.1.3.3 Neuroblast – type 1 and type 2

This class of cells arises from the transit amplifying neural progenitors. They cease to express nestin and Sox2 and express doublecortin (Dcx) and Poly-Sialated Neural Cell Adhesion Molecule (PSA-NCAM). They also started to express immature neuron markers such as β-tubulin (Tuj1) (Roskams *et al.*, 1998). Typically, these cells are post-mitotic cells which are morphologically similar to the transit amplifying cells with less than 1% being labeled with BrdU

(Seri *et al.,* 2004). Most of the neuroblasts are non-mitotic therefore it is likely that BrdU labeling observed is carry forward from the transit amplifying neural progenitor proliferation and maturation into neuroblasts. The neuroblast population can be further divided into type 1 and type 2 neuroblasts. They can be differentiated by their processes: type 1 neuroblasts typically have shorter (1-5 μm processes) whereas the type 2 neuroblasts have longer 20-50 μm processes). Another characteristic is that the type 2 neuroblasts express NeuN whereas the type 1 does not. Therefore, the type 2 neuroblast is likely to be a more mature form of the type 1 neuroblast, while both are post-mitotic neuronal precursor cells as they differentiate to become immature neurons (Encinas *et al.* 2006)

1.1.3.4 Immature and mature neurons

The immature neurons are larger cells as compared to the neuroblasts with somata of 15-20 μM across and their morphology is similar to that of the granule cells of the dentate gyrus. They have round somata with apical process that branches out in the molecular layer. They express the same markers as the type 2 neuroblasts and therefore can only be identified through morphological analysis. Upon maturation into mature neurons, they will move up into the granule cell layer with more developed apical dendrites and axons forming the mossy fibres. They cease to express the immature neuronal markers PSA-NCAM and Dcx and began to express the neuronal markers of the granule cell neurons.

GABAergic activation of the new neurons due to high chloride-dependent depolarization may help promote formation of GABAergic and glutamatergic synaptic inputs in these newly formed neurons (Ge *et al.*, 2006).

Identification of the markers of the various stages of neurogenesis allows clear delineation of the various stages of neural stem cells development.

1.1.4 Regulation of cell proliferation

The presence of continuous neurogenesis in both the SVZ and the SGZ suggests that the adult NSPCs are maintained throughout the life of the organism. There have been suggestions that Hedgehog signaling is present in the quiescent NSPC to establish and maintain the NSPC pool required for continuous neurogenesis (Ahn and Joyner, 2005; Balordi and Fishell, 2007; Han *et al.*, 2008). As mentioned in the previous sections, the NSPC pools that are capable of proliferation are the quiescent NSPCs, the transit amplifying cells and to a lesser extend the neuroblasts. There are a variety of pathological, physiological and pharmacological stimuli that are capable of regulating the cell proliferation rate during neurogenesis. Such factors include exercise, learning, seizures, stroke, aging, hormones and antidepressant treatments (Ming and Song, 2005; Steiner *et al.*, 2008; Hattiangady and Shetty, 2008; Zhao *et al.*, 2008). However, each of these factors affects different pools of neural progenitors. For example, neuroblasts proliferation can be promoted induced due

to kainic acid-induced seizures whereas treatment with the antidepressant, fluoxetine, targets both the neuroblasts and the transit amplifying progenitors (Jessberger *et al.*, 2005; Encinas *et al.*, 2006).

Various growth factors also affect the cell proliferation rate of the NSPCs. NSPCs, when dissociated from the brain, require the presence of growth factors such as epidermal growth factor (EGF) and basic fibroblast growth factor (FGF2) for long term survival and expansion in culture (Reynolds and Weiss, 1992; Kuhn *et al.*, 1997). However, these growth factors and their respective receptors are temporally regulated in development. For example, the EGF receptors are only express on the NSPCs at E14.5 whereas the FGF2 responsiveness appears much earlier in development (E8.5) (Tropepe *et al.*, 1999). Moreover, the maintenance of NSPC proliferation by EGF and FGF2 may also differ in their mechanisms as EGF is able to promote proliferation after expansion of the EGF-responsive pool of NSPCs as compared to the FGF2 responsive pool. One report also suggests that FGF2 inhibits neuronal lineage determination and thereby maintains the progenitor pool in the proliferative state (Chen *et al.*, 2007).This difference was suggested to be a result of control of cell cycle length by the growth factors (Gritti *et al.*, 1999).

Another growth factor that has been implicated in the maintenance of self-renewal of NSPCs is the cytokine ciliary neurotrophic factor that signals through the heterotrimeric receptor complex of CNTF receptor α, Leukemia Inhibitory

Factor (LIF) receptor β and gp130 subunits (Conover *et al.*, 1993; Shimazaki *et al.*, 2001). LIF is also routinely used for the maintenance of human NSPCs (Carpenter *et al.*, 1999). The activation of both the CNTF and LIF receptors can promote self renewal in NSPCs mediated through Notch signalling (Chojnacki *et al.*, 2003).

In order to influence the proliferation of the NSPCs, the growth factors involved may not need to be from the cells of the neurogenic niche. At the neurogenic niches, lies a vast network of blood vasculature which is closely juxtaposed to the NSPCs, the progenitors, the neurons and the glial cells. Thus growth factors could also be derived from the circulatory system (Palmer *et al.*, 2000). The vascular endothelial cells have been shown to secrete soluble factors that help to promote the proliferation of the NSPCs and inhibit their differentiation (Shen *et al.*, 2004). Interestingly, an angiogenic factor, vascular endothelial growth factor (VEGF) that can promote vascular endothelial growth is also capable of stimulating NSPC proliferation both *in vitro* and *in vivo* (Jin *et al.*, 2002).

Besides the host of growth factors, physiological activity such as exercise and learning can also promote the increase in cell proliferation. Voluntary exercise on running wheels has been shown to increase NSPC proliferation as compared to mice in the same enriched environment with immobilised running wheels (Ho *et al.*, 2009). However, in some cases, simply exposure to an enriched social and learning environment can also increase the proliferation of the neuroblasts and

the transit amplifying cells (Steiner *et al.*, 2008). These animals exposed to enriched environments are also shown to be able to better perform in learning and memory tasks such as the Morris water maze (Kempermann *et al.*, 1997).

Aging contributes to decreased cell proliferation. It has been shown that in aged rats, the number of $Sox2^+$ cells does not differ from that in young rats. However, when analysed together with markers of proliferation such as Ki-67 and BrdU incorporation, it was apparent that there is an increase in the quiescence of these $Sox2^+$ neural stem cells (Hattiangady and Shetty, 2008). Therefore, aging reduces the proportion of proliferating cells without affecting the quiescent neural stem cell pool.

Studies have also shown that stress can cause reduce NSPC proliferation. This is attributed to the presence of glucocorticoid stress hormones, such as cortisol in humans and corticosterone in rodents, reducing the NSPC proliferation. Administration of glucocorticoid hormones has been shown to reduce neurogenesis in rats (Cameron and Gould, 1994; Gould *et al.*, 1998; Karishma and Herbert, 2002). Removal of circulating adrenal steroids by adrenalectomy, on the other hand, is able to reverse the stress-induced decrease in neurogenesis (Cameron and McKay, 1999; Cameron *et al.*, 1998; Mirescu *et al.*, 2004).

Besides all the above mentioned factors, some morphogens are also capable of regulating the NSPC proliferation, albeit that these may be the downstream

signaling molecules that are directly activated by the physiological and behavioural effects mentioned above. Some of these morphogens such as bone morphogenetic proteins (BMPs), Notch, Noggin, Wingless-type MMTV integration (Wnt) and Sonic hedgehog (Shh) are members of the groups of developmental morphogens that are present during embryonic development (Breunig *et al.*, 2007; Fan *et al.*, 2004; Lai *et al.*, 2003; Babu *et al.*, 2007).

Notch signaling in NSPCs stimulates proliferation and self renewal (Breunig *et al.*, 2007). The Notch ligands, Jagged1 and Jagged2, bind to its extracellular domain, promoting cleavage of the Notch intracellular domain (NICD), which will translocates to the nucleus to modulate transcription of gene repressors, including the *Hes* and *Herp* genes, that downregulate expression of proneural genes and so inhibit neuronal differentiation (Kageyama and Ohtsuka, 1999; Iso *et al.*, 2003). It has also been shown that overexpression of NICD leads to the maintenance of NSPCs even under conditions that drive differentiation *in vivo* (Breunig *et al.*, 2007).

1.1.5 Regulation of neurogenesis and differentiation

Physiologically, generation of new neurons only occurs in the two neurogenic regions, the SVZ and the SGZ, whereas the astrocytes and oligodendrocytes are continuously being renewed throughout the central nervous system. Therefore, there must be specific signals that regulate the tight restriction of neuron

formation at these two neurogenic regions. In the dentate gyrus, the neuronal formation signals are modulated by Wnt-signaling (Lie *et al.*, 2005).

Glial differentiation, however, is regulated by the bone morphogenic protein (BMP) signaling cascade in both the SVZ and the SGZ (Lim *et al.*, 2000; Bonaguidi *et al.*, 2005). The BMP signals can be antagonized by noggin at the SVZ and neurogenesin-1 at the SGZ, which upon blockade of the BMP signaling; direct the differentiation process to neuronal differentiation (Lim *et al.*, 2000; Ueki *et al.*, 2003). Noggin is specifically expressed by the ependymal cells at the SVZ and neurogenesin-1 by the astrocytes and granule cells at the dentate gyrus and this expression serves to specifically block the BMP signaling to bring about neuronal differentiation in these two regions (Lim *et al.*, 2000; Ueki *et al.*, 2003).

Following initiation of differentiation, the newly formed neurons will be directed to migrate towards their designated location for neuronal integration. Generally the adult central nervous system is not permissive to neurite outgrowth and neuronal migration. Despite the inhibitory environment in the central nervous system, the new neurons of the SVZ are directed to migrate to their destination by a host of adhesion molecules, such as PSA-NCAM, β1-intergrin, Tenascin-R, and guidance signaling molecules, such as GABA, neuregulin and Slits. These molecules maintain the stability, mobility and direction of the neuronal migration (Ming and Song, 2005; Zhao *et al.*, 2008). As for the dentate gyrus, the newly formed neurons are maintained in the granule cell layer and migrate out from the

border with the hilus into the granule cell layer under the control of the molecule reelin (Gong *et al.*, 2007). More recent knockout and knockdown studies further identify that Dcx, Disrupted-in-Schizophrenia 1 (DISC1) and Nuclear distribution protein nudE-like 1 (NDEL1) are also involved in maintaining the neuronal migration pathways in SVZ and SGZ (Koizumi *et al.*, 2006; Duan *et al.*, 2007).

Growth factors also have the ability to influence the process of neurogenesis. It has been shown that FGF2 can enhance neuronal survival, differentiation, axonal growth and migration in cultured hippocampal granule cells (Lowenstein and Arsenault, 1996a; Lowenstein and Arsenault, 1996b). Intracerebroventricular infusion of FGF2 in middle-aged rats has also been show to enhance neurogenesis and promote dendritic growth (Rai *et al.*, 2007).

Another growth factor, insulin-like growth factor (IGF1), was also shown to promote generation of new neurons (Aberg, 2000; Anderson, 2002). Interestingly, overexpression of IGF1 locally in the hippocampus of the Ames dwarf mouse was able to act on the NSPCs at the dentate gyrus to increase neurogenesis and also activate anti-apoptotic signals (Sun, 2006). Neurogenesis in hippocampus has been suggested to be involved in learning and memory (Shors *et al.*, 2001; Synder *et al.*, 2005, Winocur *et al.*, 2006, Kee *et al.*, 2007). This IGF1-induced increase in neurogenesis might explain why Ames mice maintain their cognitive ability during aging as compared to age-related decline in cognition in normal mice (Sun, 2006).

1.1.6 Synaptic integration of the new neurons

Interestingly, the process of synaptic integration of the new neurons into existing neural networks follows the same steps as the embryonic and early neuronal developmental pathway. The neural progenitors and immature neurons need to be activated by the presence of ambient γ-aminobutyric acid (GABA) signals before they are capable of receiving any functional synaptic inputs (Ge *et al.*, 2007). It has been suggested that the new dentate granule cells need to be primed with GABAergic inputs for about one week after formation, followed by two weeks of glutamatergic induction before finally developing mature perisomatic GABAergic inputs (Esposito *et al.*, 2005).

Taking inference from the embryonic brain, GABA initially acts as an excitatory molecule by binding to $GABA_A$ receptors present on the NSPCs. This binding leads to an efflux of Cl^- ions causing depolarization and the subsequent activation of voltage-dependent calcium channels (Ben-Ari, 2002). As the NSPC matures and differentiates, the GABA signal switches from being excitatory to become inhibitory (LoTurco, 1995).

Following GABAergic priming, glutamatergic synapses are formed (Ben-Ari, 2007). N-methyl-D-aspartate (NMDA) receptor subunits NR1 and NR2B are expressed in quiescent neural stem cells and immature neurons in the DG

(Nacher *et al.*, 2007). In adult rodents, the activation of NMDA receptors by NMDA causes a drop in neural stem cell proliferation in the SGZ and blockade of the NMDA receptor using antagonists MK-801 and CGP37849 increased cell proliferation (Cameron *et al.*, 1995; Nacher *et al.*, 2003). This shows that glutamatergic signals at the quiescent neural stem cell stage inhibit cell proliferation. However, Tashiro *et al.* (2006) showed that the glutamatergic signals are required for neuronal survival in the newly generated neurons where retroviral knockout of NR1 *in vivo* caused a decrease in the survival of new neurons. This may suggest a dual mechanism by glutamatergic input, one to inhibit NSPC proliferation and the other to maintain cell survival during neuronal maturation.

Upon migration to the region where the synaptic pathways are to be integrated, these new neurons will contact pre-existing boutons that synapse with other neurons. However, as they mature, they will eventually form stable synapse with boutons that are devoid of other synaptic partners (Toni *et al.*, 2007).

1.1.7 Antidepressant treatments and neurogenesis

Recently, it has been discovered that some antidepressant and mood stabilizer therapies are able to increase neurogenesis. Treatments such as lithium, electroconvulsive seizure, monoamine oxidase inhibitors, norepinephrine-selective reuptake inhibitors and 5-HT-selective reuptake inhibitors (SSRIs) have

been shown to increase proliferation of NSPCs (Malberg *et al.,* 2000; Chen *et al.*, 2006). Using learned helplessness as an animal model for depression, it was shown that controllable stress caused less reduction in SGZ NSPC proliferation as compared to uncontrollable stress in male rats (Shors *et al.*, 2007). In another related study, Chen *et al.* (2006) discovered that SSRIs can reverse the behavioral effect of learned helplessness with the increased in SGZ NSPC proliferation. This suggests that the effects of depression and associated decreases in neurogenesis both involve 5-HT as a mediator.

1.2 The serotonergic system and neurogenesis

1.2.1 Role of 5-HT in brain development

Serotonin (5-HT) is sometimes called the “happy hormone” as it is known to activate serotonergic systems which give rise to a feeling of well being and elation. The serotonergic system has a widespread distribution in the CNS and it influences a host of different aspects of mammalian physiology ranging from the cardiovascular system, respiration, the gastrointestinal system (Kato *et al.*, 1999), pain sensitivity and thermoregulation to more centrally regulated functions such as circadian rhythm, aggression, appetite, sexual behavior, sensorimotor activity, cognition, mood, learning and memory (Miyata *et al.*, 2000; Nebigil *et al.*, 2000; Thorin *et al.,* 1990; Bazarevitch *et al.*, 1978; Kato *et al.*, 1999; Sodhi and Sanders-Bush, 2004). In fact, 5-HT has a dual role: it acts as a regulator of brain development during the embryonic stage and as a neurotransmitter in the mature brain.

The development of the embryonic brain follows the principles of refinement of experience, also known as the “use it or lose it principle”. The entire brain develops in totality and has more cells and more connections than are actually required by the fully developed brain. During the maturation period, the brain must determine which cells and which connections are required by the mature brain and maintain those. The rest of the cells and connections will be lost during

the process of brain maturation. Therefore, to determine which of the cells and connections are to be kept or removed, the process requires the activation and signaling of the neuronal connections. As 5-HT is present in the developing organism from a very early stage, it would be a good choice to use it as it is already present and functioning in cell signaling (Whitaker-Azmitia, 2001). In fact, 5-HT may be present as early as the blastocyst stage as embryonic stem cells also expressed TPH (Walther and Bader, 1999).

The importance of 5-HT in the developing brain can be seen from 5-HT depletion studies. Depletion of prenatal 5-HT delays the onset of neuron formation in the serotonergic terminal regions. It has been suggested that in the fetus, 5-HT functions to differentiate cortical and hippocampal neurons whereas in the adult brain, it is a neurotransmitter as well as regulating neuronal plasticity by maintaining the synaptic connections in the cortex and hippocampus (Azmitia *et al.*, 1995; Chen *et al.*, 1994; Mazer *et al.*, 1997).

5-HT has also being found to affect neural precursor cells. As mentioned previously, the neuronal precursor cells are found at the SVZ of the lateral ventricles and the SGZ of the hippocampus (Gould *et al.,* 1998). Both inhibition of 5-HT synthesis and selective lesions of serotonergic neurons caused a decrease in the number of newly generated cells in the SGZ as well as the SVZ (Brezun and Dasazuta, 1999).

1.2.2 5-HT biosynthesis and breakdown

5-HT is synthesized from L-tryptophan, which can be found across different species from lower plants to higher mammals. The tryptophan is first converted by 5-hydroxytryptophan via a rate limiting step mediated by the enzyme tryptophan hydroxylase (TPH), before being converted to 5-hydroxytryptamine (5-HT) by aromatic L-amino acid decarboxylase (or dopa decaryboxylase). 5-HT is broken down by monoamine oxidase and aldehyde dehydrogenase into 5-hydroxylindolacetic acid (5-HIAA). This byproduct of 5-HT breakdown is usually pass out in urine and can be used as a method of detection of 5-HT amounts in the body.

TPH, being the rate limiting enzyme in the biosynthesis of 5-HT, therefore determines the biosynthesis rate of the 5-HT via its enzyme levels and activity. Two different isoforms of TPH has been found: TPH1 is found mostly in the periphery in multiple tissue types whereas the more recently discovered TPH2 isoform is found specifically in the brain (Zhang *et al.*, 2004). The enzyme activity of the two isoforms are also varied with TPH1 having a higher enzyme activity. As 5-HT does not pass through the blood-brain barrier, the 5-HT synthesized within the central nervous system and at the periphery generally does not intermix (Erspamer, 1966). However, the tryptophan and the TPH product, 5-hydroxytryptophan, do cross the blood-brain barrier; therefore their levels can

generally affect the overall serotonergic systems in the brain (Zmilacher *et al.*, 1988).

1.2.3 The 5-HT receptors subtypes – properties and functions

To detect the serotonergic signals, there are the 5-HT receptors. The first 5-HT receptor was identified by Gaddum and Picarelli (1957). To date, there are a total of 16 different subtypes of 5-HT receptors identified. The classification of the 5-HT receptors into seven major family groups was done based on their animo acid sequence, pharmacology and intracellular signaling mechanisms (Gaddum and Picarelli, 1957; Hoyer *et al.*, 1994). The 5-HT receptors are mostly seven putative transmembrane domains, G-protein coupled metabotropic receptors except for the 5-HT3 receptor, which is a ligand-gated ion channel (Uphouse, 1997). The functions of these receptors in the brain are associated with specific physiological responses which modulate neuronal activity, neurotransmitter release and behavioural changes. These receptors often have distinct distributions in the brain and also specific downstream signal transduction pathways in the cells that express them. Each of these 5-HT receptors families will be reviewed below with a focus on their cellular distribution in the brain, pharmacology and their signal transduction pathway activation, which may affect NSPC proliferation and neurogenesis.

1.2.3.1 5-HT1 receptor family

The 5-HT1 receptor family consists of subtypes 1A, 1B, 1D, 1E and 1F. 5-HT1C receptor has been reclassified as the 5-HT2C receptor (Pazos *et al.*, 1984). The 5-HT1 receptor subtypes have high amino acid sequence homology and all are coupled negatively to adenylate cyclase via G-protein. The initial criteria of classification of 5-HT1 receptors was high affinity for 5-CT and methysergide, blockade by methiothepin and no blockade by selective antagonists of 5-HT2 and 5-HT3 receptors (Bradley *et al.*, 1996). However, with the current inclusion of the 5-HT1E and 5-HT1F receptors, which have low affinity for 5-CT and methiothepin, these criteria are to be realigned.

1.2.3.1.1 5-HT1A receptors

In vivo mapping of the 5-HT1A receptor distribution has been conducted by receptor autoradiography using ligands such as [^{3}H]-5-HT, [^{3}H]-8-OH-DPAT, [^{3}H]-WAY100635 and [^{125}I]-p-MPPI (Pazos and Palacios, 1985; Hoyer *et al.*, 1986; Kung *et al.*, 1995; Khawaja, 1995). High density of 5-HT1A receptors is found in limbic brain areas, such as the hippocampus, cingulated cortex, entorhinal cortex, lateral septum and the mesencephalic raphe nuclei. The 5-HT1A receptor mRNA message distribution also mirrors that of the results from the binding assays (Chalmers and Watson, 1991; Pompeiano *et al.*, 1992; Burnet *et al.*, 1995). It is also found that this distribution of the 5-HT1A receptor is similar

across species except that the distribution of 5-HT1A receptor in the hippocampal and cortical areas of human brain is different from that of rodent in that the human CA1 and middle laminae contain higher levels of 5-HT1A receptor mRNA whereas in the rat, the 5-HT1A receptor mRNA is more abundant in the dentate gyrus and deep laminae (Burnet *et al.*, 1995). *In situ* hybridization and immunohistochemistry shows the presence of 5-HT1A receptors in the cortical pyramidal neurons and in the pyramidal and granular neurons of the hippocampus (Pompeiano *et al.*, 1992; Burnet *et al.*, 1995). The 5-HT1A receptor has also been reported to be expressed by serotonergic neurons in the raphe nuclei, cholinergic neurons in the septum and glutamatergic neurons in the cortex and hippocampus (Francis *et al.*, 1992; Kia *et al.*, 1996a). Ultrastructurally, the 5-HT1A receptor can be found at the synaptic membranes and also extrasynaptically (Kia *et al.*, 1996b).

5-HT1A receptor found presynaptically, classified as autoreceptors, are found to regulate the release of the 5-HT at these synaptic terminals (Miquel *et al.*, 1991). Stimulation of 5-HT1A autoreceptors inhibits the release of 5-HT to the synaptic terminals (Sharp and Hjorth, 1990). Therefore, some of the agonists of the 5-HT1A receptors exhibit a biphasic response in that they inhibit the release of 5-HT release by stimulating the 5-HT1A receptor and at the same time, the agonist stimulates the postsynaptic 5-HT1A receptors in place of the 5-HT. One such agonist is 8-OH-DPAT, which has been shown to bind to the 5-HT1A

autoreceptors at low doses whereas at high doses, it stimulates the postsynaptic 5-HT1A receptors (Hjorth and Magnusson, 1988).

Pharmacologically, the 5-HT1A receptor is unique in the 5-HT1 family and can easily be differentiated from the other members within the family using selective 5-HT1A receptor agonists such as 8-OH-PIPAT, 8-OH-DPAT, dipropyl-5-CT and gepirone (Hoyer *et al.*, 1994). There are also 5-HT1A receptor antagonists available, such as (S)-UH-301, WAY100135, NAD-299 and WAY100635 (Hillver *et al.*, 1990; Björk *et al.*, 1991; Johansson *et al.*, 1997; Fletcher *et al.*, 1993a,b, 1996). WAY100635 is by far, the most potent antagonist, although selectivity wise, NAD-299 is more superior (Johansson *et al.*, 1997; Fletcher *et al.*, 1996). Also, a new agonist S15535 is found to be a selective 5-HT1A presynaptic receptor (autoreceptor) agonist and at the same time a 5-HT1A postsynaptic receptor antagonist (Millan *et al.*, 1993 and 1994).

The 5-HT1A receptors couple negatively to adenylate cyclase via Gαi-proteins in guinea pig and rat hippocampal tissues and in transfected cell lines expressing recombinant 5-HT1A receptors (Boess and Martin, 1994; Albert *et al.*, 1996; Saudou and Hen, 1994). However, at the dorsal raphe, there are reports that suggest 5-HT1A receptors do not inhibit adenylate cyclase (Clarke *et al.*, 1996). There are also reports that suggest 5-HT1A activation stimulate adenylate cyclase at the hippocampal tissues (Shenker *et al.*, 1983; Fayolle *et al.*, 1988). However, these positive coupling are suggested to be attributed to the effects of

other 5-HT receptor subtypes, such as 5-HT7 receptors (Barnes and Sharp, 1999). Besides interaction with adenylate cyclase, the 5-HT1A receptor has also been shown to modulate intracellular Ca^{2+} and activate phospholipase C in cell lines transfected with 5-HT1A receptors (Albert *et al.*, 1996). However, these results may be dependent on the G-protein subunit and the effector proteins present in the particular cell line used as there is no evidence that this activation exist in the brain tissues (Albert *et al.*, 1996). The 5-HT1A receptor activation has also been reported to induce the secretion of S-100β from primary astrocytes in culture and this increase induced an increase in growth in neuronal cultures (Azmitia *et al.*, 1996; Riad *et al.*, 1994). This suggests a possible neurotropic role of 5-HT1A receptors in the brain (Riad *et al.*, 1994; Yan *et al.*, 1997; Azmitia *et al.*, 1996).

1.2.3.1.2 5-HT1B receptors

From autoradiography studies, the 5-HT1B receptor is found to be distributed with high density the globus pallidus, ventral pallidum, substantia nigra and entopeduncular nucleus of the basal ganglia (Verge *et al.*, 1986; Pazos *et al.*, 1985; Bruinvels *et al.*, 1993). However, the *in situ* hybridization studies shows some agreement with and some discrepancy from the binding studies, such as the presence of 5-HT1B receptor mRNA at the dorsal and median raphe nuclei, which is not shown in the binding studies, and for striatum which is consistent in both binding and mRNA studies (Boschert *et al.*, 1994; Doucet *et al.*, 1995;

Bruinvels *et al.*, 1994a,b; Jin *et al.*, 1992). This suggests that the 5-HT1B receptors are found both presynaptically and postsynaptically to the 5-HT neurons in which the receptors are synthesized at the cell body and transported to nerve terminals (Bruinvels *et al.*, 1994a,b; Boschert *et al.*, 1994).

5-HT1B receptors can be activated by potent agonist such as RU 24969, 5-CT and L-694247 and blocked by methiothepin and CP 93129 (Hoyer *et al.*, 1994). Although these compounds have affinity for other 5-HT receptors subtypes such as the 5-HT1A receptor, the affinity of these drugs are relatively low and therefore 5-HT1B receptors can be discriminated from the other 5-HT receptors. As there are structural similarities between 5-HT1B and 5-HT1D receptors, only high affinity and selective antagonists such as SB-244289 and SB-216641 can be used to distinguish between 5-HT1B and 5-HT1D receptors (Roberts *et al.*, 1997; Price *et al.*, 1997)

Similar to 5-HT1A receptors, 5-HT1B receptors are coupled negatively to the adenylate cyclase in cell culture when stimulated with forskolin and *in vivo* in the rat and calf substantia nigra (Adham *et al.*, 1992; Levy *et al.*, 1992; Bouhelal *et al.*, 1988; Schoeffter and Hoyer, 1989).

1.2.3.1.3 5-HT1D receptors

The 5-HT1D receptors are expressed at relatively low levels in the central

nervous system within the basal ganglia, the cortex, the hippocampus and spinal cord (Bruinvels *et al.*, 1993; Castro *et al.*, 1997). However, *in situ* hybridization has detected 5-HT1D receptor mRNA in various regions of the brain including the caudate putamen, nucleus accumbens, olfactory cortex, dorsal raphe nucleus and locus coeruleus but interestingly undetectable in some basal ganglia regions such as ventral pallidum, globus pallidus and substantia nigra where the autoradiography detected binding sites (Hamblin *et al.*, 1992a,b; Bruinvels *et al.*, 1994a,b). These data suggest that the 5-HT1D receptors are located predominantly on the synaptic terminals of both 5-HT and non-5-HT neurons away from the cell body.

Due to the 77% amino acid sequence similarity within the transmembrane region, the 5-HT1B and 5-HT1D receptors are almost indistinguishable by their drug binding profiles (Weinshank *et al.*, 1992). Therefore, most 5-HT1B receptor ligands also have relatively high affinity for 5-HT1D receptors (Pauwels *et al.*, 1996). However, there are some compounds, such as BRL-15572, which have been reported to have a higher affinity and more selectivity for 5-HT1D receptors as compared to 5-HT1B receptors (Price *et al.*, 1997). However, due to the lack of a highly selective agonist (BRL-15572 being an antagonist), the signal transduction mechanism driven by 5-HT1D receptors it is still not clear, although there are suggestions that it couples negatively to adenylate cyclase in cells transfected with 5-HT1D receptors (Weinshank *et al.*, 1992; Hamblin and Metcalf, 1991).

1.2.3.1.4 5-HT1E and 5-HT1F receptors

Less is known about the receptor distribution of these two receptors in terms of their distribution using autoradiography, however based on [^{3}H]-5-HT autoradiography it has been suggested that the 5-HT1E and 1F receptors are distributed in the entorhinal cortex, caudate putamen, claustrum, amygdale and hippocampus (Miller and Teitler, 1992; Bruinvels *et al.*, 1994c; Barone *et al.*, 1993). Moreover, in situ hybridization studies of 5-HT1E and 5-HT1F receptors detected the mRNA message in the same areas as the binding studies, suggesting that these receptors have a postsynaptic location (Bruinvels *et al.*, 1994a,b).

Both 5-HT1E and 5-HT1F receptors have similar pharmacological characteristics with high affinity for 5-HT and low affinity for 5-CT (Adham *et al.*, 1993a,b; Amlaiky *et al.*, 1992; Lovenberg *et al.*, 1993a,b). 5-HT1E receptors can be set apart from 5-HT1F receptors by their lower affinity for sumatriptan. Overexpression of 5-HT1E receptors has been shown to inhibit adenylate cyclase activity (McAllister *et al.*, 1992; Levy *et al.*, 1992; Adham *et al.*, 1994; Zgombick *et al.*, 1992). As for 5-HT1F receptor, the agonist LY344864 is found to be selective potent agonist in overexpression cell culture systems and, as for the 5-HT1E receptor, the 5-HT1F receptor is able to inhibit adenylate cyclase activity

(Phebus *et al.,* 1997; Amlaiky *et al.*, 1992; Johnson *et al.*, 1997; Lovenberg *et al.*, 1993a,b; Adham *et al.*, 1993a,b).

1.2.3.2 5-HT2 receptor family

The 5-HT2 receptor family consists of three members subtypes namely, 5-HT2A, 5-HT2B and 5-HT2C receptors which are similar in structure, pharmacology and signal transduction pathways. Although the 5-HT2 receptors have a high degree of structural similarity in their seven transmembrane domains, these domains are structurally different from the other 5-HT receptor families (Baxter *et al.*, 1995). The 5-HT2 receptors are all coupled positively to phospholipase C and are able to mobilize intracellular calcium.

1.2.3.2.1 5-HT2A receptors

The 5-HT2A receptor is found to be present in many forebrain cortical areas, such as neocortex, entorhinal cortex, pyriform cortex, claustrum, caudate nucleus, nucleus accumbens, olfactory tubercle and hippocampus with results from autoradiography ligand binding assays, in situ hybridization mRNA expression and immunohistochemistry being consistent with each other (Pazos *et al.*, 1985, 1987; Mengod *et al.*, 1990; López-Giménez *et al.*, 1997; Pompeiano *et al.*, 1994; Burnet *et al.*, 1995; Morilak *et al.*, 1993, 1994). This further suggests that the receptors are located postsynaptically to the 5-HT neurons. Beside

neurons, cultured astrocytes and glioma cells are also found to express 5-HT2A receptors (Deecher *et al.*, 1993; Meller *et al.*, 1997).

Pharmacologically, the 5-HT2A receptor is characterized by low binding affinity to 5-HT, high binding affinity to the agonists, 1-[2,5-dimethoxy-4-iodophenyl]-2-aminopropane (DOI), (-)-1-[2,5-dimethoxy-4-bromophenyl]-2-aminopropane (DOB), 2,5-dimethoxy-4-methylamphetamine (DOM), and 5-HT2 receptor antagonists, ICI 170809 and ritanserin. However, to differentiate the effects of 5-HT2A receptors from the other 5-HT2 receptors, selective agonists for 5-HT2A receptors, such as MDL 100907, and selective 5-HT2B and 5-HT2C receptor antagonists, such as SB206553 and SB200646A, can be used in conjunction (Sorensen *et al.*, 1993; Kehne *et al.*, 1996; Baxter *et al.*, 1995; Baxter, 1996).

Stimulation of 5-HT2A receptors has been shown to activate phospholipase C via G-protein coupling in 5-HT2A receptor expression cell lines and in brain tissues (Pritchett *et al.*, 1988; Julius *et al.*, 1990; Conn and Sanders-Bush, 1984; Stam *et al.*, 1992; Godfrey *et al.*, 1988). Stimulation of 5-HT2A receptors also causes an increase in brain-derived neurotrophic factor (BDNF) levels (Vaidya *et al.*, 1997). This increase has also been observed in response to antidepressant treatments, which suggests that BDNF may be one of the contributing factors to the therapeutic effects of antidepressants due to alteration of synaptic connections by BDNF (Duman *et al.*, 1997).

1.2.3.2.2 5-HT2B receptors

The 5-HT2B receptor expression is relative low and has been detected immunohistochemically in the cerebellum, lateral septum, dorsal hypothalamus and medial amygdala (Duxon *et al.* 1997). The 5-HT2B receptors have low affinity for ritanserin but high affinity for yohimbine as compared to 5-HT2A and 5-HT2C receptors (Bonhaus *et al.*, 1995). There are also potent selective agonists and antagonists for the 5-HT2B receptor available, such as BW723C86 and SB206553, respectively (Baxter, 1996; Baxter *et al.*, 1995).

It has been noted in heterologous expression systems that the 5-HT2B receptor can hydrolyse phosphatidylinositol (Wainscott *et al.*, 1993; Schmuck *et al.*, 1994; Kursar *et al.*, 1994). On top of this, some studies suggest that 5-HT2B receptors, during neural development, can mediate the mitogenic effects of 5-HT due to the expression of 5-HT2B receptor at the neural crest during embryonic development and developmental abnormalities occurring in 5-HT2B receptor knockout mice (Choi *et al.*, 1997).

1.2.3.2.3 5-HT2C receptors

The 5-HT2C receptor is expressed in the choroid plexus, pyriform, cingulate and retrosplenial, nucleus accumbens, olfactory nucleus, amygdale, hippocampus and the basal ganglia (Palacios *et al.*, 1991). The mRNA expression of 5-HT2C

receptors is in line with the ligand autoradiography, which suggests the postsynaptic location of the 5-HT2C receptor except for high levels of mRNA expression and low binding autoradiography at the lateral habenular nucleus, which suggests the 5-HT2C receptor may be presynaptic on the projection from the habenula (Abramowski *et al.*, 1995).

The pharmacology of the 5-HT2C receptor is close to that of the other two 5-HT2 receptors but can be distinguish by its high affinity for SB 200646A and lower affinity for the antagonists such as ketanserin, MDL 100907 and spiperone. As with the other two 5-HT2 receptors, activation of 5-HT2C receptor increases phospholipase C activity especially at the choroid plexus due to high 5-HT2C receptor expression there (Sanders-Bush *et al.*, 1988).

1.2.3.3 5-HT3 receptor family

The 5-HT3 receptor is a ligand-gated ion channel and the only non G-protein coupled receptor in the 5-HT receptor family. Its structure is consistent with the Cys-loop superfamily of pentameric proteins and the channel is permeable to Na^+, K^+ and Ca^{2+} ions (Maricq *et al.* 1991; Yang 1990; Hargreaves et al, 1994). It mediates mainly currents and membrane depolarization under physiological conditions. Activation of postsynaptic 5-HT3 receptors causes fast excitatory neural transmission in brain areas such as the lateral amydala and visual cortex; whereas presynaptic 5-HT3 receptors modulate release of dopamine and GABA

(Roerig *et al.*, 1997; Sugita *et al.*, 1992; Koyama *et al.*, 2000; van Hooft and Vijverberg, 2000). The 5-HT3 receptors can exist as homopentamers, with 5-HT3A receptors subunits, or heteropentamers, with mixed 5-HT3A and 5-HT3B receptor subunits (Davies *et al.*, 1999; Hanna *et al.*, 2000). The presence of the homomeric and heteromeric 5-HT3 receptors accounts for some of the heterogeneity of the responses after the activation 5-HT3 receptors (Yang *et al.*, 1992; Fletcher and Barnes, 1998; Hussy *et al.*, 1994).

The highest levels of 5-HT3 receptor expression are at the dorsal vagal complex of the brain stem (Pratt *et al.*, 1990). Other areas of 5-HT3 receptor expression are the amygdale, hippocampus and the superficial layers of the cerebral cortex (Parker *et al.*, 1996a). *In situ* hybridization experiments show that the 5-HT3 receptors mRNA is found within the hippocampus, piriform cortex and entorhinal cortex. Within the hippocampus, 5-HT3 receptor mRNA is found to be expressed by the GABAergic interneurons (Tecott *et al.,* 1993).

There are large numbers of pharmacologically selective ligands for 5-HT3 receptors, however pharmacological difference exist between inter-species 5-HT3 receptors. The effects of these ligands can differ in three orders of magnitudes in some cases, such as selective agonist m-CPBG differs in affinity between the rat and the rabbit 5-HT3 receptors in approximately 300-fold (Kilpatrick *et al.*, 1991). The 5-HT3 receptors antagonist MDL72222 also

displays lower affinity for 5-HT3 receptors in guinea pig (Kilpatrick and Tyers, 1992).

1.2.3.4 5-HT4 receptor family

The 5-HT4 receptors were initially identified from cultured neurons when assaying for stimulation of adenylate cyclase activity (Bockaert *et al.*, 1990). Initially, it was thought to be the only 5-HT receptor that can stimulate the adenylate cyclase, which was the pharmacological definition of the 5-HT4 receptor until the discovery of the 5-HT6 and 5-HT7 receptor subtypes (Fillion *et al.*, 1975; von Hungren *et al.*, 1975).

The 5-HT4 receptor was found to be present at high levels in the nigrostriatal and mesolimbic systems of the brain identified by using selective antagonists [^{3}H]GR113808 and [^{125}I]SB207710 in radioligand studies (Grossman *et al.*, 1993; Mengod *et al.*, 1996). The in situ hybridization studies also showed a similar mRNA distribution as the radioligand binding studies (Gerald *et al.*, 1995; Mengod *et al.*, 1996; Claeysen *et al.*, 1996). Two splice variants of the 5-HT4 receptors are present, the 5-HT4A receptor the expression of which is restricted to the striatum and the 5-HT4B receptor, which is widely expressed throughout the brain (Gerald *et al.*, 1995). This differential expression may indicate the functional difference in the different splice variants.

5-HT4 receptors, both native and heterologously expressed, are able to couple to adenylate cyclase (Claeysen *et al.*, 1996; Gerald *et al.*, 1995). Although, 5-HT4A and 5-HT4B receptors differ in the protein sequence at their C-terminal, which may affect G-protein coupling and phosphorylation desensitization of the receptor, no pharmacological differences have been reported (Gerald *et al.*, 1995; Claeysen *et al.*, 1996). It has also been shown that the 5-HT4 receptor activation is able to mediate increase in cAMP levels leading to phosphorylation of cAMP-dependent protein kinase (Fagni *et al.*, 1992).

1.2.3.5 5-HT5 receptor family

The 5-HT5 receptor subtype is one of the least understood subtypes of 5-HT receptor. The 5-HT5A and 5-HT5B receptor subtypes were identified from brain cDNA library screening of mouse and rat and shortly after from human cDNA library (Matthes *et al.*, 1993; Rees *et al.*, 1994; Erlander *et al.*, 1993),

From *in situ* hybridization, it has been shown that 5-HT5A receptor mRNA is widely distributed in the mouse and rat brain (Plassat *et al.*, 1992; Erlander *et al.*, 1993). The 5-HT5A receptor transcript was found in neurons of the cerebral cortex, the dentate gyrus and the pyramidal cell layer of the hippocampus, the granule cell layer of cerebellum and the tufted cells of the olfactory bulb (Plassat *et al.*, 1992). As for 5-HT5B receptor, in situ hybridization studies found transcripts to be present at supraoptic nucleus of the hypothalamus, medial and

lateral habenula, hippocampus, olfactory bulb, dorsal raphe nucleus, entorhinal cortex and piriform cortex (Wisden *et al.*, 1993; Erlander *et al.*, 1993). 5-HT5 receptors are members of the seven transmembrane domains G-protein coupled receptor family. It has been found that high overexpression of 5-HT5 receptors can result in an inhibition of adenylate cyclase (Francken *et al.*, 1998).

1.2.3.6 5-HT6 receptor family

The identification of 5-HT6 receptors was the result of non-stringency cDNA library screening for a seven transmembrane receptor to obtain a novel 5-HT sensitive receptor (Monsma *et al.*, 1993; Ruat *et al.*, 1993a,b). The 5-HT6 receptor was later identified to be a G-protein coupled receptor. The expression of the 5-HT6 receptor is generally confined to the CNS although some expression has been identified in the periphery (Ruat *et al.*, 1993a,b; Monsma *et al.*, 1993). In the brain, 5-HT6 receptor mRNA has been detected in the caudate nucleus within the striatum, the olfactory tubercles, hippocampus and nucleus accumbens (Monsma *et al.*, 1993; Ruat *et al.*, 1993a,b). Immunohistochemical analysis shows of 5-HT6 receptor distribution generally corresponds to the mRNA expression suggesting that the receptor is postsynaptic to 5-HT neurons as confirmed by electron microscopy (Gérald *et al.*, 1997).

A detailed pharmacological profile of 5-HT6 receptors was obtained using selective 5-HT6 receptor antagonists Ro04-6790 and Ro63-0563, which are

capable of passing the blood brain barrier (Sleight *et al.*, 1998). This ability greatly facilitates in vivo analysis of the 5-HT6 receptors. The 5-HT6 receptor transfected into cell lines and the native 5-HT6 receptor in mouse primary striatal neuron cultures were able to couple to a metabotropic signal transduction system that can stimulates adenylate cyclase activity (Ruat *et al.*, 1993a,b; Monsma *et al.*, 1993; Schoeffter and Waeber, 1994).

1.2.3.7 5-HT7 receptor family

The 5-HT7 receptor is the latest identified subtype of the 5-HT receptor family. It is found to be expressed in mouse, rat, guinea pig and human (Shen *et al.*, 1993; Lovenberg *et al.*, 1993a,b; Plassat *et al.*, 1993; Meyerhof *et al.*, 1993; Ruat *et al.*, 1993a,b; Tsou *et al.*, 1994; Nelson *et al.*, 1995). There are at least four spliced variants, namely 5-HT7A, 5-HT7B, 5-HT7C and 5-HT7D receptor, being identified for the 5-HT7 receptors with rat and human expressing only of the three variants. Although the 5-HT7C receptor mRNA can be synthesized from the human gene, no native 5-HT7C mRNA has been detected in the human tissues (Heidmann *et al.*, 1997).

The 5-HT7 receptor tissue expression revealed in both mRNA and ligand binding assays shows similar patterns suggesting that the 5-HT7 receptor is probably postsynaptic (Stowe and Barnes, 1998b; Gustafson *et al.*, 1996). The tissue expression profile indicates that the 5-HT7 receptor is expressed in the thalamus,

hypothalamus, hippocampus, cerebral cortex and amygdala (To *et al.*, 1995; Stowe and Barnes, 1998b; Gustafson *et al.*, 1996). No distinct splice isoform specific tissue expression patterns have been identified (Heidmann *et al.*, 1997, 1998; Stam *et al.*, 1997).

Both transfected and native 5-HT7 receptors are able to stimulate adenylate cyclase by coupling with Gαs-protein within the third intracellular loop of the protein (Bard *et al.*, 1993; Heidmann *et al.*, 1997 and 1998; Stam *et al.*, 1997; Obosi *et al.*, 1997). However, expression of 5-HT7A receptors in cell lines has also enabled the activation of the Gαs insensitive adenylate cyclases, AC1 and AC8, as well as the Gαs sensitive isoform, AC5 (Baker *et al.*, 1998). The activation of AC1 and AC8 caused an increase in the intracellular [Ca^{2+}] which may activate other signalling cascades within the cell (Baker *et al.*, 1998).

1.2.4 The requirement for multiple 5-HT receptor subtypes in brain

The occurrence of 5-HT and the 5-HT receptors originated even before the evolutionary separation between vertebrates and invertebrates and has resulted in a host of different subtypes of 5-HT receptors that continue to exist in the organisms today that must confer a certain advantage of a single ligand, multiple receptors system (Peroutka, 1994). Explanations such as that differences in the distribution of the 5-HT receptor subtypes in different cells leads to multifunctional roles played by 5-HT in neuronal function failed to explain the

presence of multiple receptor families as several 5-HT subtypes can be found in the same anatomical location (Pazo and Palacios, 1985; Pazo *et al.*, 1987). Therefore, a more complex explanation must exist for the presence of multiple subtypes of 5-HT receptors that continue to be conserved in evolution.

Closer examination of the cellular response to the neurotransmitter system suggests that it depends upon the neurotransmitter's interaction with a receptor followed by the formation of the ligand-receptor complex and the ligand-receptor complex interaction with an effector protein. Finally, in order for the interaction to produce a response, the overall cellular state must be conducive for leading to the effector protein activation (Uphouse, 1997). The effectiveness of the binding leading to the cellular response will be dependent on the affinity of the ligand receptor binding and the potency of the response. The affinity of the ligand to the receptor will determine the stability of the neurotransmitter receptor complex. The stability will determine the ability to activate the effector protein and initiate the signaling cascade.

However, if the cell contains more than one receptor at which the single neurotransmitter can act on, the final cellular response will be the result of the interactions of multiple signal cascades and this allow small regulatory modulation of the signaling cascade. This will also allow the same neurotransmitter to be used to send different cellular messages across different brain areas based on the host of receptors expressed in that area and

synchronize multiple physiological functions using the same environmental or physiological signal (Uphouse, 1997).

It was suggested that five characteristics of the 5-HT receptor system enables the modulation of a wide variety of possible neuronal function using one single neurotransmitter: (a) 5-HT has varying affinity and/or potency for the different receptor subtypes; (b) multiple transduction pathways are used by the different receptor subtypes; (c) receptor subtypes differ in their susceptibility to agonist-mediated desensitization/downregulation; (d) receptor subtypes interact in mediating cellular responses to the neurotransmitter; and (e) receptor subtypes respond differently to changes in the physiological environment. These five characteristics allow the cells to sense the quantitative status of 5-HT at the synapse which translate to different receptors being activated. The activation of that particular receptor will lead to the activation of the corresponding signalling cascade. The desensitization/downregulation of the receptors with the receptor subtype interactions provide a plausible mechanism for modulation of the 5-HT signals. These will lead to the organism being able to adapt to the changing physiological demands (Uphouse, 1997).

1.2.5 The 5-HT transporter

Another important protein that is involved in the regulation of 5-HT levels in the synapses is the 5-HT transporter (SERT; SLC6A4, solute carrier family 6

neurotransmitter transporter, member 4). The function of SERT is to reuptake the 5-HT at the synapses and returns it back into the serotonergic cells. This also serves to limit the duration for which 5-HT acts on the postsynaptic 5-HT receptors. There are allelic forms of the SERT genes commonly known as the long allele and the short allele which arise from a 44 base pair DNA deletion (Levinson, 2006). The short allele does not affect the individual SERT activity but rather cause a lower expression of the SERT molecules which affect the 5-HT reuptake activity in the cells (Lesch *et al.* 1996). SERT is one of the frequent targets of antidepressant drugs which act to increase the 5-HT availability at the synapses by inhibiting the reuptake process by inhibiting SERT activity (Malberg *et al.* 2000).

1.3 Serotonergic systems and NSPC proliferation

Depression is generally accepted to be caused by decreases in 5-HT levels in the brain as indicated by brain imaging and neuroendocrinological studies (Dursun *et al.*, 2001). This observation forms the neural basis of "the serotonin hypothesis" of depression. However, there is little consensus as to the whether the lack of 5-HT synthesis, insufficient release, excessive degradation within the synaptic cleft or the perturbation in intracellular events postsynaptically are the main biochemical causes of depression. As discussed in the previous section, some of the current antidepressants act as SSRIs to inhibit 5-HT reuptake process, thereby increasing the 5-HT availability at the synapses (Malberg *et al.*, 2000). The use of SSRIs as antidepressants has been effective in the treatment of depression (Potter *et al.*, 1991; Richelson, 1993; Cowen, 1993).

Interestingly, one observation on chronic treatment with SSRI-based antidepressants, such as fluoxetine, is that these drugs cause an increase in the NSPC proliferation (Malberg *et al.*, 2000; Santarelli *et al.*, 2003). A landmark paper by Santarelli *et al.* (2003) suggested that hippocampal NSPC proliferation is required in order for the antidepressant treatment to have behavioral effects. The experiments employed X-ray irradiation to ablate the proliferating neural progenitor cells at the SGZ and the SVZ while the non-proliferating cells are not affected. After ablation of the proliferating neural progenitor cells at the SGZ of mice, the effects of antidepressant treatments, including fluoxetine, were tested

using novelty suppressed feeding test (NSF). The NSF test assays the latency for the mice to approach and eat a familiar food in a brightly lit arena in the presence of a novel stimulus. It measures the stress-induced anxiety in the animal and it is a good test for chronic antidepressant treatment (Bodnoff *et al.*, 1988) that is routinely used in the pharmaceutical industry to screen for potential antidepressant drugs. Their result shows that there is a reduction of the latency time to feed for mice that went through chronic fluoxetine treatment. However, for the mice that have been X-ray irradiated at the SGZ region, the fluoxetine treatment failed to reduce the latency time significantly, indicating that the neural progenitor cell proliferation, specifically at the SGZ region, is required for the behavioral effects of fluoxetine (Santarelli *et al.*, 2003). The ablation of SGZ neural progenitor cell proliferation also affected the effects of fluoxetine in the chronic unpredictable stress (CUS) test, which is another test for anxiety. In this test, the mice were subjected to CUS which impairs self grooming in the mice, an effect that has been shown to be rescued by chronic antidepressant treatment (Griebel *et al.*, 2002).Chronic treatment with fluoxetine in SGZ irradiated mice was able to neither improve the coat condition nor improve the latency to groom, indicating that the recovery from the behavioural effects of CUS is also affected by ablation of neural progenitor cells at the SGZ (Santarelli *et al.*, 2003). The behavioral effects of fluoxetine are specific to hippocampal cell proliferation as ablation of proliferative cells in the SVZ or the cerebellum had no effect (Santarelli *et al.*, 2003).

The above experiments suggest that neural progenitor cell proliferation, and possibly neurogenesis, is an important process for the recovery from depression. It also implies that the control of cell proliferation of the neural progenitor cells can be regulated by the serotonergic systems as on of the antidepressants used in the study was the SSRI, fluoxetine. Studies show that 5-HT depletion can cause a decrease in neurogenesis at both the SVZ and the SGZ and preferential activation or blocking of some 5-HT receptors was able to regulate the neural progenitor cell proliferation (Brezun and Daszuta, 1999; Banasr *et al.*, 2004; Jha *et al.*, 2008).

With the development of selective agonists and antagonists against 5-HT receptors, these molecules can be used to preferentially activate and block specific subtypes of 5-HT receptors. These agonists and antagonists have been used as a tool to study the effects of activation and blocking of these receptors in an attempt to assess their effect on neural progenitor cell proliferation. It has been shown that administration of 8-OH-DPAT increases neural progenitor cell proliferation in rats, which the authors attributed to activation of 5-HT1A heteroreceptors (Banasr *et al.* 2004). Blockade of 5-HT1A receptors using the antagonists 1-(2-Methoxyphenyl)-4-(4-phthalimidobutyl)piperazine hydrobromide (NAN-190), 4-(2'-methoxy-phenyl)-1-[2'-(n-2"-pyridinyl)-p-iodobenzamido]-ethyl-piperazine (*p*-MPPI) and *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]- *N*-(2-pyridyl)cyclohexanecarboxamide (WAY-100635) can decrease cell proliferation (Radley and Jacobs, 2002). However, 8-OH-DPAT has been found to also be a

partial agonist at 5-HT7 receptors, which complicates the interpretation of the results (Wood *et al.*, 2000). It has also been suggested that the effects of the increase in number of BrdU labelled cells indicate an increase in the rate of cell cycling rather than an increase in the total pool of proliferating cells (Banasr *et al.*, 2004). The effects of 5-HT acting on the neural progenitor cells are suggested to be local as raphe neurons transplanted into the hippocampal regions can increase neural progenitor cell proliferation (Brezun and Daszuta, 2000). The 8-OH-DPAT-induced newly divided cells are able to mature and differentiate into neurons (Banasr *et al.*, 2004).

Besides the 5-HT1A receptor, the cell proliferation effects of other subtypes of 5-HT receptors have also been studied. 5-HT1B and 5-HT2C receptor agonists failed to increase neural progenitor cell proliferation, however, the 5-HT1B receptor agonist, sumatriptan, restored parachlorophenylalanine (PCPA) induced decreases in cell proliferation back to control levels suggesting that the 5-HT1B heteroreceptors also contribute to the increase proliferation (Banasr *et al.*, 2004). Acute treatment with the 5-HT2A/2C receptor antagonist ketanserin reduced proliferation of neural progenitor cells (Banasr *et al.*, 2004; Jha *et al.*, 2008). As ketanserin's affinity for 5-HT2A receptors is higher than for 5-HT2C receptors, it has been suggested that the decrease is caused by the blockade of the 5-HT2A receptor (Banasr *et al.*, 2004). However, upon chronic treatment for 7 days with ketanserin neural progenitor cell proliferation rate increased (Jha *et al.* 2008). This phenomenon may be attributed to the downregulation of 5-HT2 receptor

expression upon chronic treatment with the antagonist as has been previously observed in other systems (Toth and Shenk, 1994). Further evidence points towards 5-HT2A receptor blockade contributing to increased proliferation as treatment with antisense to 5-HT2A receptors produce mice that display behaviours consistent with an antidepressant effect suggesting that 5-HT2A receptors may play a more important role in regulation of neural progenitor cell proliferation (Sibille *et al.*, 1997).

These activations and inhibitions of neural progenitor cell proliferation through 5-HT receptor-mediated pathways have been suggested to be due to a convergence of the downstream signaling pathway with neuronal growth factors (Cowen *et al.*, 2007). It has been suggested that the activation of some 5-HT receptor subtypes can either activate regional expression of growth factors such as IGF-1, FGF-2 and S-100β or activation of the Akt and ERK pathways, which have been suggested to be involved in neurogenesis (Lauder, 1993; Lambert and Lauder, 1999; Cowen *et al.*, 2007; Aberg *et al.*, 2003).

1.4 Hypothesis and Aim

SSRI-based antidepressants used to treat depression have triggered the interesting phenomenon of increased NSPC proliferation and neurogenesis. This is not a side effect of the antidepressant treatment but rather the NSPC proliferation and neurogenesis is a requirement for the behavioral recovery of the depressive symptoms. This suggests the regulation of NSPC proliferation and neurogenesis by 5-HT release, 5-HT receptor subtype activation and SERT reuptake. Also, that 5-HT is a signaling molecule synthesized early in development may also suggest its role in stem cell proliferation and maturation. The requirement for NSPC proliferation and neurogenesis underlying the behavioural effects of depression treatments further suggests that the NSPCs may be participating in regenerating impaired neural networks. It also implied that the NSPCs may be constantly regenerating in the adult brain, but at a slower rate as compared to embryonic development. All these lines of evidence suggest that a control mechanism may be present to activate NSPC proliferation and neurogenesis as and when required. This activation may require either intercellular signals or signals generated from the neural networks involving 5-HT.

This thesis aims to identify the 5-HT receptors and other related proteins of serotonergic systems that are involved in the regulation of NSPC proliferation. As many antidepressant treatments increase availability of 5-HT, this thesis

questions whether serotonergic neurons are directly acting on the NSPCs. The focus will be on which of the 5-HT receptor subtypes are expressed on the NSPCs and which of these receptor subtypes are involved in the regulation of NSPC proliferation. With the recent discovery of neuronal TPH and the finding that embryonic stem cells also express TPH, the notion of NSPCs synthesizing 5-HT and self-regulation of NSPC proliferation will also be examined. To allow a consistent pool of NSPCs to be used, this project also aims to establish a protocol for the cryopreservation of neural stem cells to allow storage of constant, high viability batches of the neural stem cells for research.

2. EFFECTIVE CRYOPRESERVATION OF NSPCS WITHOUT SERUM OR PROTEINS BY VITRIFICATION

2.1 Introduction

The extraction, expansion and maintenance of good quality and large quantities of primary NSPCs is both time consuming and expensive. While stem cells are able to self-renew, NSPCs cannot be maintained indefinitely by continuous passaging in culture. Long-term culture of NSPCs may cause telomere shortening in the NSPCs, which may lead to senescence and associated dysfunctions in proliferation and neurogenesis (Ferron *et al.*, 2004; Wright *et al.*, 2006). While telomere shortening can be avoided by immortalization with overexpression of human telomerase reverse transcriptase (hTERT), long-term culture of human neural progenitor cells immortalized in this manner can result in accumulation of karyotypic abnormalities (Bai *et al.*, 2004; Wang *et al.*, 2004). Such chromosomal abnormalities can in turn lead to abnormal proliferation and transformation of progenitor or stem cell characteristics, although this is not necessarily an inevitable consequence of all immortalization protocols (Hodges *et al.*, 2007; Li *et al.*, 2005; Pollock *et al.*, 2006). This could influence experimental results and their interpretation and, on clinical application, may be associated with increased risk of tumorigenesis. Therefore, the development of efficient cryopreservation techniques is essential as it ensures a constant supply

of cells and will be of benefit both to future clinical applications and current basic research into the functions and properties of NSPCs

Cryopreservation of NSPCs with sterile and reproducible protocols will be a prerequisite for quality assurance, storage, and distribution of the cells for research and even clinical use (Milosevic *et al.*, 2005). It is anticipated that sterile and xeno-free culture and storage protocols will be a requirement for the ultimate realization of the clinical application of NSPCs. As has been discussed in the fields of mesenchymal and embryonic stem cell research, the use of animal derivatives and other organic components increases the risk of contamination and batch-to-batch variability (Kassem *et al.*, 2004; Mallon *et al.*, 2006; Mannello *et al.*, 2007). In view of the requirement for sterility, it have been demonstrated that cryopreservation of biological material by vitrification can be achieved using only non-biological additives (Kuleshova and Lopata, 2002; Kuleshova *et al.*, 2004; Magalhães *et al.*, 2008; Tan *et al.*, 2007; Wu *et al.*, 2007).

The NSPCs can be maintained and propagated as neurospheres in culture. Each neurosphere consists of an aggregate of heterogeneous neural progenitor cells at different stages of development. Recent studies have shown that the inner core of neurospheres contain a mixture of partially differentiated and undifferentiated cells, while the outer layer consists of more “stem-like” cells (Campos, 2004). Extracellular matrix (ECM) is also present and supports the growth of the NSPCs by allowing for more cell-cell and cell-ECM interactions.

The presence of these cell-cell and cell-ECM interactions are of particular interest as they may model the likely interactions within the *in vivo* NSPC niche, which contains progenitor cells at different stages of maturity (Molofsky *et al.*, 2003). However, the presence of such three dimensional structures may possess a problem for conventional cryopreservation methodologies, especially as NSPCs plated at the same time and under the same culture conditions grow into neurospheres which differ in size and morphology.

Ideally, the cryopreservation techniques for storing NSPCs should allow for high cell viability, ability to maintain the sterility of the cultures, and should not impair the functional properties of the cells. Approaches to cryopreservation can be classified as freezing and vitrification. Cryoprotectants are used in both approaches. In the case of freezing, the aim is to reduce damage to cells by the formation of extracellular ice crystals rather than intracellular ice. In contrast, vitrification aims to avoid ice crystal formation on both cooling and warming by achieving glass-like solidification (Kuleshova and Lopata, 2002). Most studies have concluded that slow-cooling freezing protocols are suboptimal for cryopreservation of embryonic stem cells (Heng *et al.*, 2005). There have been interesting reports on rapid-cooling freezing cryopreservation of human and other primate embryonic stem cells avoiding conventional slow-cooling methods by directly plunging the cells into liquid nitrogen (Fujioka *et al.*, 2004; Reubinoff *et al.*, 2001; Richards *et al.*, 2004; Suemori *et al.*, 2006; Zhou *et al.*, 2004). Although these methodologies are preferred over slow-cooling, all of them employed

serum or proteins of human or animal origin. Additionally, there are various concerns over whether these protocols are able to achieve the glass-like vitreous state to cryopreserve the samples.

Commercial stem and precursor cells are frequently cryopreserved by slow-cooling freezing protocols (Milosevic *et al.*, 2005). Such freezing protocols may be detrimental to sensitive cells due to the damage caused by ice formation. Slow-cooling protocols are reported to have long-lasting adverse effects on embryonic stem cells (Katkov *et al.*, 2006). Commonly, dimethyl sulfoxide (DMSO) is used as a cryoprotectant and often fetal calf serum is also included (Carvey *et al.*, 2001; Hancock *et al.,* 2000; Milosevic *et al.*, 2005). DMSO introduced as a cryoprotectant in many freezing protocols can, under some circumstances, impair survival and influence differentiation (Jacob and Herschler, 1986).

Vitrification procedures have been established in clinical application for the cryopreservation of oocytes for fertility treatment and vitrification protocols have been shown to be superior to slow-cooling freezing protocols for the preservation of cells, tissues and organs (Kuleshova *et al.*, 1999; Kuleshova *et al.*, 2007). Recently, vitrification protocols have been reported for the cryopreservation of human and other primate embryonic stem cells (Fujioka *et al.*, 2004; Reubinoff *et al.*, 2001; Richards *et al.*, 2004; Suemori *et al.*, 2006; Zhou *et al.*, 2004). These protocols show improved survival compared to freezing protocols and did not

alter the pluripotency of the cells or produce karyotype abnormalities. Therefore, for the cryopreservation of embryonic stem cells, vitrification protocols are deemed to be superior to slow-cooling freezing protocols (Heng *et al.*, 2005).

Another limitation of most current cryopreservation protocols is that it is difficult to prevent contamination. In the field of assisted reproduction, it is well documented that pathogens survive cryopreservation and the cross-contamination can even occur during cryostorage (Mazzilli *et al.*, 2006; Steyaert *et al.*, 2000; Tomlinson and Sakkas, 2000). A simple strategy that has been developed to prevention contamination is the “straw-in-straw” arrangement (250 µl sterile straw placed in 500 µl straw) which allows biological samples to be cooled by direct immersion in liquid nitrogen and warmed by direct immersion in a water bath. This technique allows the maximum temperature distribution during the cryopreservation and recovery process, which aids in increasing survival of the cells. This strategy, which involves placing an inner straw within a sealed outer sealed straw, have been successful employed in the cryopreservation of embryos and the vitrification of encapsulated hepatocytes (Kuleshova *et al.*, 1999, 2004; Wu *et al.*, 2007). This protocol is both cost- and time-effective as it does not need expensive slow-cooling apparatus and requires only direct immersion into liquid nitrogen for cooling.

Hence in the present study, attempts to develop a protein- and serum-free vitrification protocol to cryopreserve NSPCs in the form of neurospheres will be

made using solutions, which vitrified on cooling and devitrify on warming. The methodology of vitrification, slow-cooling and rapid cooling freezing will also be investigated. The vitrified neurospheres will be assayed for structural integrity of neurospheres, cell survival, karyotype, and expression of neural stem cell markers, proliferative capacity, and the potential for multipotent neural differentiation.

2.2. Materials and Methods

2.2.1. Animals

The animals were obtained from the Centre for Animal Resources, National University of Singapore (NUS) and housed in the Animal Holding Unit, NUS, under a 12 hr light: 12 hr dark cycle, with *ad libitum* access to food and water. All animal procedures were conducted in accordance to the Institute Animal Care and Use Committee (IACUC) guidelines.

2.2.2. Culture of NSPCs

Pregnant C57BL/6J mice were deeply anaesthetized by intraperitoneal injection of pentobarbital (150 mg/kg) prior to dissection. The embryonic day 14-15 (E14-15) fetuses were surgically extracted and decapitated. The brains were removed, and lateral ventricle wall and hippocampal tissues were dissected in Solution 1 (1X Hank's Balanced Salt Solution, HBSS, Invitrogen, CA, USA; 30mM glucose, AMRESCO, OH, USA; 15mM HEPES, Invitrogen; pH 7.5). The tissues were dissociated enzymatically in 0.25% Trypsin-EDTA (Gibco, CA, USA) at 37^{o}C for 30 minutes, with trituration after the first 15 minutes. To stop trypsin digestion, two times the volume of Solution 3 (0.6mM BSA, Sigma-Aldrich, MO, USA; 20mM HEPES; 1X Earles Balanced Salt Solution, EBSS, Invitrogen; pH 7.5) was added and the digested tissues were sieved using 40μM cell strainers (BD

Biosciences, CA, USA). The collected cell suspension filtrates were centrifuged at 380xg for five minutes, followed by resuspension in 10ml of Solution 2 (0.5X HBSS; 0.9M sucrose, 1st Base, Singapore; pH 7.5) and centrifugation at 600xg for ten minutes. Subsequently, the cell pellet was resuspended in 2 ml of Solution 3 and gently transferred drop-wise to 12 ml of Solution 3, which is then centrifuged at 380xg for seven minutes to recover the cells. The cell pellet was resuspended in neurosphere medium, containing Dulbecco's modified Eagle's medium (DMEM) with Ham's nutrient mixture F12 (1:1 v/v, DMEM/F12, Gibco), supplemented with N2 supplement (Gibco), 20ng/ml epidermal growth factor (Invitrogen), 20ng/ml basic fibroblast growth factor (Chemicon, Tenecula, CA, USA), 100U/ml penicillin (Sigma-Aldrich) and 100ug/ml streptomycin (Sigma-Aldrich). NSPCs were cultured in 6-well dishes in a humidified 95% air / 5% CO_2 incubator at 37^{o}C.

The NSPCs were cultured till the neurospheres became macroscopically visible. To passage the neurospheres, the neurospheres were collected and incubated in TrypLE™ (Invitrogen) at 37^{o}C for two minutes. After two minutes, neurospheres were dissociated by gentle trituration and re-incubation for another three minutes. The trypsinisation was stopped by the addition of two times the volume of Solution 3. The single-cell suspension was then centrifuged at 380xg for five minutes, resuspended in 1ml of Solution 2, and followed by centrifugation at 600xg for ten minutes. Subsequently, the cell pellet was resuspended in 200 µl of Solution 3 and gently transferred drop-wise to 1 ml of Solution 3, which is then

centrifuged at 380xg for seven minutes. The final cell pellet was resuspended and re-plated in fresh neurosphere medium. The cells were passage at least three times before using for any of the experiments to ensure that other cell types had been excluded from the culture system.

2.2.3. Vitrification of neurospheres

In the vitrification experiments, third passage neurospheres were taken through a complete cooling-warming cycle. Controls were neurospheres from the same culture processed in parallel to the vitrification group and included (a) untreated neurospheres and (b) a solution control in which the neurospheres were treated with the cryopreservation solutions but without undergoing the cooling-warming procedure. All experimental comparisons were performed at least in triplicate on three different batches of neurospheres isolated from fetuses from different pregnant females. For vitrification, neurospheres were first transferred to a 1.5 ml microcentrifuge tube and allowed to settle to the bottom of the tube. Culture medium was replaced with 10% (v/v) ethylene glycol (EG) in medium. The neurospheres were allowed to sink to the bottom of the tube to permit changing of the solution. The 10% (v/v) EG in medium was replaced with 25% (v/v) EG in medium, the tube was systematically tapped on the bench of the hood to facilitate sedimentation of the neurospheres. To minimize dilution of the final vitrification solution, the neurosphere pellet was then transferred to a second tube containing the final vitrification solution, which consisted of 40% (v/v) EG,

0.6M sucrose (the final concentration of 40% (v/v) EG, 0.6 M sucrose constitute 39.63 wt% EG 0.535m sucrose resulting in a 57.95% (w/w) total solute concentration) in medium. Under the experimental conditions, 39.63 wt% EG 0.535m sucrose is capable of undergoing vitrification. The neurospheres in the vitrification solution were aspirated into a 250 µl sterile plastic straw. The 250 µl straw was in turn placed inside a 500 µl sterile straw, which was then heat sealed using an impulse sealer. For vitrification the straws were then immersed into liquid nitrogen. As previously reported by Kuleshova et al. (1999), a cooling rate of 400 °C/min was achieved in the 250 µl straw within the 500 µl straw in the "straw-in-straw" configuration. Solution controls were exposed to the same solutions in a 15 ml centrifuge tube but without undergoing the cooling-warming procedure. The total duration of the procedure was approximately 11 min with the duration of each step being approximately being equal. All equilibration steps, including introduction to the vitrification solution, were performed at room temperature (23 ± 2°C).

2.2.4. Freezing of neurospheres by rapid-cooling

To determine whether the employment of vitrification solutions for cryopreservation of neurospheres was advantageous in comparison to other cryopreservation protocols, a rapid "freezing" protocol was evaluated. As in the vitrification protocol, the neurospheres were exposed to cryopreservation solutions in a stepwise manner, drawn into plastic straws and rapidly frozen by

immersion in liquid nitrogen (-196 °C). This stepwise equilibration procedure, including introduction of neurospheres to the final cryopreservation solution, was performed at room temperature (23 ± 2°C). However, the total solute concentration of the final cryopreservation solution was reduced by application of 37% (v/v) EG, 0.6 M sucrose in medium. Lowering the total solute concentration should compromise the ability of the solution to support vitrification, a stable glass-like state (or amorphous state), and result instead in freezing by rapid-cooling. This solute concentration appeared to still provide protection against cell death and reduce toxicity to NSPCs. Thus, we also compared 30% (v/v) EG, 0.6 M sucrose, which conferred the benefit of further reducing the toxicity of the final cryopreservation solution to the cells. The total solute concentrations for 37 % (v/v) EG 0.6 M sucrose (36.8 wt% EG 0.535m sucrose) and 30 % (v/v) EG 0.6 M sucrose (30 wt% EG 0.535m sucrose) are 55.08% (w/w) and 48.34% (w/w), respectively. Both solutions vitrify on cooling but devitrify on warming.

2.2.5. Freezing of neurospheres by slow-cooling

Vitrification was also compared to a slow-cooling freezing protocol. For slow-cooling freezing, third passage neurospheres were collected and re-suspended in medium supplemented with 10% (v/v) DMSO. The slow-cooling was as described by Milosevic et al. (2005) with minor modifications. Briefly, the resuspended neurospheres were transferred to a cryogenic vial (Nalgene, NY, USA) and were frozen to -70 °C with a cooling rate of 1 °C/min in a rate-

controlled freezing system (Planer Kryo10, series III, Planner Products Ltd, UK). At the end of cooling cycle the samples were stored in liquid nitrogen (-196 °C).

2.2.6. Warming of neurospheres and dilution of cryoprotectant

To recover the neurospheres, the straws or vials containing the neurospheres were warmed by immersion for 30 s in a water bath at 38 ± 0.5 ºC with continuous agitation. As previously reported, a warming rate of 650 °C/min was achieved in the "straw-in-straw" configuration (Kuleshova *et al*., 1999). For recovery from vitrification and from rapid-cooling freezing, the neurospheres were then expelled into a 15 ml centrifuge tube containing 1 M sucrose in medium. The concentration of the sucrose was decreased to 0.7 M by dilution with 0.25 M sucrose in medium and then by 0.175 M on each subsequent step by dilution with medium. The total time for the dilution procedure was approximately 15 min (5 min for the first step and 2 min for each subsequent step). All treatments were performed at room temperature (23 ± 2°C). During the dilution process, sedimentation of neurospheres took place at the final dilution step at a low concentration 0.175 M of sucrose. The neurospheres were then allowed to sink and excess solution was removed. The neurospheres were then washed in neurosphere culture medium and placed in the incubator for 30 min after which they were transferred to fresh culture media for continuous culture. To ensure complete retrieval of neurospheres, the recovery solution was observed under the microscope. If neurospheres were found to be present, they were retrieved,

washed and added to the main pool. Supplementary flushing of the straw with 1 M sucrose recovery solution did not retrieve any more neurospheres. For recovery from slow cooling, the samples were diluted 1:10 with neurosphere culture medium. The neurospheres were then retrieved in neurosphere culture medium and cultured routinely until further analysis.

2.2.7. Observation of neurosphere integrity and measurement of cell viability

Each sample was examined before and after vitrification by optical microscopy for the presence of damaged neurospheres and free cells. Survival after thawing was measured after overnight recovery using a LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen) according to the manufacturer's instructions. In brief, the neurospheres were incubated with a cocktail of calcein AM and ethidium homodimer-1. In live cells, the non-fluorescent calcein AM is converted to green-fluorescent calcein, after the acetoxymethyl ester is hydrolyzed by intracellular esterases. The red-fluorescent ethidium homodimer-1 on the other hand is actively excluded by live cells and so only accumulates in dead cells where it labels the nucleic acids in the nucleus. The labelled neurospheres were imaged by sequential scanning using confocal microscopes (LSM510 META, Carl Zeiss Microimaging GmbH, Germany and Fluoview 1000, Olympus, Japan). The live neurospheres were optically sectioned with z-axis steps of 5 μm to take 16 to 28 confocal micrographs of each neurosphere for cell counting. An investigator blind

(with special thanks to Lee KH) to the treatment conditions counted the number of dead cells (red) and live cells (green). For each neurosphere, 5 areas outside of the necrotic core were randomly selected for counting. Each area counted contained at least 200 cells. In total, at least 1,000 cells were counted for each neurosphere.

To verify the cell viability obtained by counting cells from optical sections of images, confocal images of cells from dissociated neurospheres were obtained for the control and vitrification group. Neurospheres were dissociated with 0.25% trypsin-EDTA for 5 min at 37°C. The reaction of trypsin-EDTA was stopped by adding fresh neurosphere medium. Cells were stained with LIVE/DEAD Viability/Cytotoxicity Kit and confocal images obtained. Additionally, the viability of cells in neurospheres was measured quantitatively by using an automated cell viability analyzer (Vi-CELL, Beckman Coulter, Inc., CA, USA). Control and vitrified neurospheres were dissociated by trypsin-EDTA. The sample cup containing cell suspension was loaded onto the Vi-CELL. The Vi-CELL system utilizes the trypan blue dye exclusion method to determine cell viability and provides precise, unbias automated cell counting from 50 images within 2 min. A sample size of 4×10^5 to 11×10^5 cells were analysed for viability in each count.

2.2.8. Karyotyping of NSPCs (in collaboration with Assoc Prof Hande MP and Dr Poonepalli A)

The cryopreserved neurospheres were tested for chromosomal abnormalities by karyotyping as previously described (Hancock *et al*., 2000). Briefly, the neurospheres were treated with 7 mg/l colcemid (Sigma-Aldrich) three days post recovery from vitrification in order to arrest cells at the metaphase stage. The neurospheres were then dissociated to single cells using 0.25 % of trypsin-EDTA (Invitrogen). The NSPCs were recovered by centrifugation and the cytoplasm osmotically expanded in 0.03M sodium citrate solution. The cells were then fixed with 3:1 methanol: glacial acetic acid and spread onto a Superfrost Plus slide (Esco, Portsmouth, USA). The chromosomes were stained using DAPI. Twenty metaphase spreads per sample were evaluated for chromosomal rearrangements and the chromosome number and integrity were analyzed.

2.2.9. Assay for NSPC Markers

To determine whether the vitrification affected the NSPC state, dissociated NSPCs were plated on poly-L-ornithine/fibronectin-coated coverslips in a monolayer and maintained for 3 days in neurosphere medium. The NSPCs were then fixed by treatment with 4% paraformaldehyde for 20 min. Immunostaining was conducted sequentially using anti-Sox2 (AB5603, Chemicon) and anti-nestin (MAB353, Chemicon) antibodies for identification of neural stem or progenitor

cell markers. Fluorescence-conjugated Alexa Fluor secondary antibodies (Invitrogen) were used to visualize the primary antibodies and the coverslips were counterstained with DAPI in Prolong Antifade Gold mounting medium (Invitrogen). The immunostained cells were then imaged by sequential scanning with a confocal microscope (LSM 510, Carl Zeiss Microimaging GmbH).

2.2.10. Cell Proliferation Assay

In order to assay the rate of cell proliferation of the NSPCs, the control neurospheres were washed thoroughly using culture medium, prior to dissociation and staining, mimicking the dilution process of the vitrified group for procedure consistency. The neurospheres were dissociated using 0.25% trypsin-EDTA solution and the cells were replated on poly-L-ornithine/laminin-coated coverslips. The cells were allowed to adhere to the coverslips and 5-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich, MO, USA) was then added to the culture medium to a final concentration of 10 μM and incubated with the dissociated NSPCs for 24 h. The cells were then fixed with 4% paraformaldehyde for 20 min. Immunostaining was conducted to detect the BrdU incorporation using an anti-BrdU antibody (1:100, BRD.3, Neomarkers, Lab Vision Corporation, CA, USA) and detected using Alexa Fluor 555 conjugate secondary antibody (Invitrogen). The cells were counterstained with DAPI in the mounting medium to reveal all nuclei. The cells were imaged by sequential scanning with a confocal microcope (Fluoview 1000, Olympus, Japan). An investigator blind (with special thanks to

Lee KH) to the treatment conditions counted the number of BrdU-immunoreactive cells and DAPI-positive nuclei and the number of BrdU-positive cells was expressed as a percentage of the total cell count.

2.2.11. Assay for Multipotent Differentiation

To assay for multipotent differentiation into neurons, astrocytes, and oligodendrocytes, dissociated NSPCs were plated on poly-L-ornithine/laminin-coated coverslips. Differentiation was induced with 0.5% fetal calf serum (Hyclone Laboratories Inc., UT, USA) in neurosphere medium without EGF and bFGF. After 1 day, the medium was replaced with DMEM/F12 with N2 supplement and the cells were allowed to differentiate for another 6 days. The differentiated cells were then fixed by treatment with 4% paraformaldehyde for 20min. Immunostaining was conducted sequentially using an anti-MAP2a+b antibody (MAB378, Chemicon) for identification of neurons, an anti-glial fibrillary acidic protein (GFAP) antibody (Z0334, Dako, Denmark) for identification of astrocytes and an anti-CNPase antibody (MAB326R, Chemicon) for identification of oligodendrocytes. Fluorescence-conjugated Alexa Fluor secondary antibodies (Invitrogen) were used to visualize the primary antibodies and the coverslips were counter-stained with DAPI. The differentiated cells were then imaged by sequential scanning with a confocal microscope (Fluoview 1000, Olympus). The numbers of neurones, oligodendrocytes and astrocytes were counted by an

investigator blind (with special thanks to Lee KH) to the treatment and expressed as a percentage of the total cell count.

2.2.12. Statistical analysis

All statistical analyses were performed using SPSS version 12 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± SEM. Statistical analysis was by Student's t-test comparison with the controls. In case of statistically significant differences, ANOVA was used to determine which groups statistically differed from each other. Differences were considered significant if $P < 0.05$.

2.3. Results

2.3.1. Effects of vitrification on neurosphere integrity and viability

The integrity of neurospheres was preserved by vitrification. Figure 2.1 shows samples of neurospheres before (A) and after (B) vitrification sampled from the total population of neurospheres vitrified (C). All the neurospheres recovered from vitrification remained intact and no remaining free cells were found in the decanted recovery solution indicating that the vitrified neurospheres were not destroyed by the vitrification process (Fig. 2.1B,C). Additionally, the vitrification procedure improved the quality of the culture as free cells, which were not capable of forming neurospheres were removed during the vitrification procedure.

After overnight recovery, the vitrification solution alone (Fig. 2.2B) did not have any appreciable adverse effect on the structural integrity of the neurospheres on visualisation by confocal microscopy as compared to that seen in the untreated control samples (Fig. 2.2A,B). A small number of red-fluorescent dead cells were evident in both untreated and vitrification solution treated neurospheres but there was no significant effect of vitrification on cell survival compared to the untreated group (Fig. 2.2A,B inset; Fig. 2.2D).

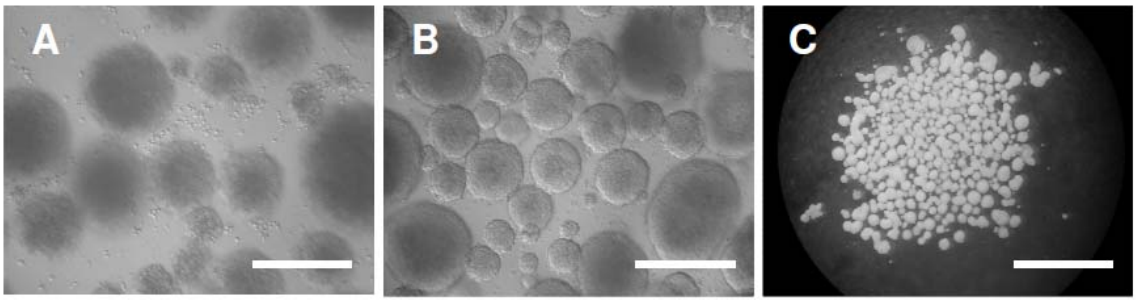

Figure 2.1. Photomicrographs showing the structural integrity of neurospheres undergoing a vitrification-warming cycle. Transillumination optical microscopy images of neurospheres sampled from (A) untreated control culture prior to vitrification and (B) immediately after warming following vitrification. (C) Reflectance optical microscopy image with white-light epillumination of the total population of neurospheres vitrified in a representative experiment immediately after warming following vitrification. The scale bars indicate 250 μm in A and B and 7 mm in C. (published in Cryoletters 28(6), 445-460)

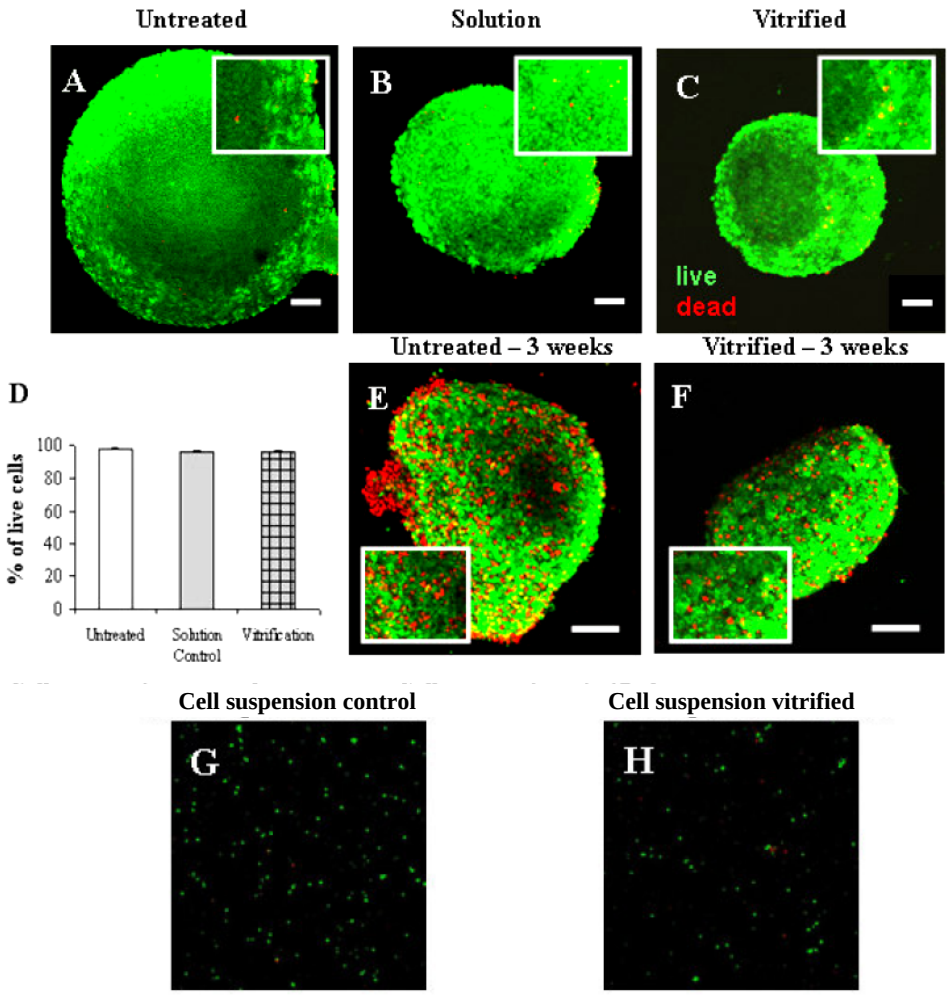

Figure 2.2. Short-term and long-term cell viability and structural integrity of neurospheres undergoing vitrification. Confocal micrographs of representative neurospheres (A) in untreated control culture, (B) after treatment with the cryopreservation solutions without undergoing the cooling-warming procedure and (C) after recovery from a complete cooling-warming cycle. The neurospheres were labelled with a LIVE/DEAD Viability/Cytotoxicity kit. Green fluorescence indicates live cells, while red fluorescence indicates dead cells. The presence of small numbers of red-fluorescent dead cells is evident at higher magnification (inset boxes). (D) Viability was quantified by counting the number of live cells and expressing it as a percentage of the total cells. The data are mean ± SEM of three replicates. There were no significant differences. Confocal micrographs of neurospheres in (E) untreated control culture and (F) 3 weeks after recovery from a complete vitrification-warming cycle taken at matched time points. The scale bars represent 100 µm. (G,H) Cell suspension from dissociated neurospheres showing the viability of cells. G – untreated control; H – vitrified samples after recovery from a complete cooling-warming cycle. Images A,B,C,D,G,H were taken on the next day following treatment. (published in Cryoletters 28(6), 445-460)

Likewise, structurally the vitrified neurospheres (Fig. 2.2C) showed no significant difference from those untreated and treated only with the cryopreservation solutions. Again a small number of red-fluorescent dead cells were evident in the vitrified neurospheres (Fig. 2.2C inset) but the LIVE/DEAD assay did not reveal any significant effect of vitrification on viability, which was 96.5 ± 0.72 % (mean ± SEM) after vitrification and 97.6 ± 0.96 % in untreated control culture (Fig. 2.2D). Similarly, cells from dissociated neurospheres from both the control and vitrified samples also showed high viability (Fig. 2.2G,H).

The measures of cell viability obtained through counting of cells from confocal optical sections were further confirmed by the quantitative data obtained from dissociated neurospheres with a Vi-CELL Cell Viability Analyzer. The Vi-CELL analysis reported 89.9 ± 2.3% viability in control samples and 88.7 ± 1.3 % viability in samples vitrified at passage three (P3). Vitrification of cells from advanced passages (P5 and P6, n=3) did not significantly alter viability with 89.3 ± 1.8% viability in control and 89.2 ± 5.1 % viability in vitrification group. Although, the viability recorded by the LIVE/DEAD assay in the intact neurospheres was marginally higher than that reported by the Vi-CELL analysis in dissociated neurospheres, all three approaches confirmed that the viability of the cells in the vitrified samples did not differ from that of the cells in the untreated control cultures.

After 3 weeks in culture following recovery from vitrification and, prior to passaging, neurospheres were again sampled (Fig. 2.2F). The structural integrity of the neurospheres was maintained. Overall cell viability in the intact neurospheres had declined to approximately 80 %, even in the untreated controls (Fig. 2.2E), however there was no difference in the cell viability in the neurospheres from the untreated control culture and the vitrified samples (Fig. 2.2E,F).

Thus, three methods, namely, cell counts from confocal optical sections, confocal images of stained cells from dissociated neurospheres and cell viability obtained with a Vi-CELL analyzer have shown no significant effects of vitrification on neurosphere viability.

2.3.2. Effects of different cryopreservation techniques on neurosphere integrity and viability

Vitrification was compared to both rapid-cooling freezing and the more commonly applied slow-cooling freezing method of cryopreservation. Rapid-cooling freezing with 37% (v/v) EG, 0.6 M sucrose in medium disrupted the structure of the neurospheres (Fig. 2.3B). The disruption of the structural integrity of the neurospheres was even more severe on slow-cooling freezing (Fig. 2.3C). Despite the structural disruption during rapid-cooling freezing the LIVE/DEAD assay only revealed occasional red-fluorescent dead cells and there was no

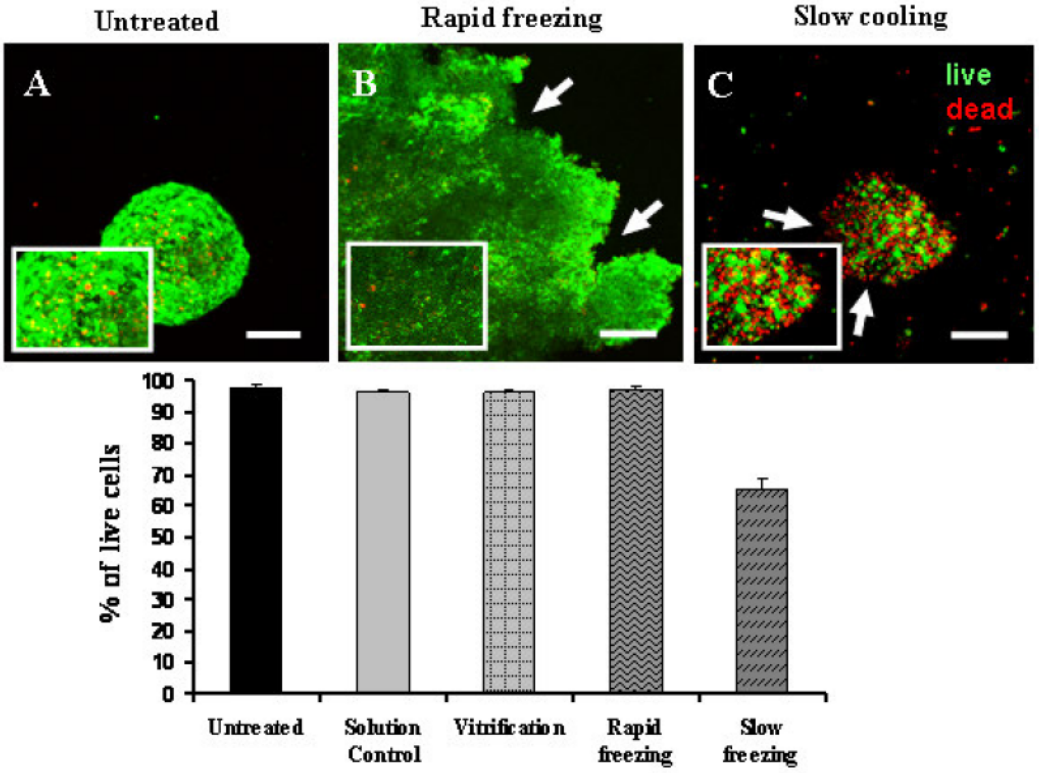

Figure 2.3. Comparison of the cell viability and structural integrity of neurospheres undergoing cryopreservation by various methods. Confocal micrographs of representative neurospheres (n=3): (A) untreated, (B) after recovery from rapid-cooling freezing in liquid nitrogen in 37% (v/v) EG, 0.6 M sucrose and (C) after recovery from conventional slow-cooling freezing in 10% (v/v) DMSO. The neurospheres were labelled with a LIVE/DEAD Viability/Cytotoxicity kit. Green fluorescence indicates live cells, while red fluorescence indicates dead cells. The structural integrity of the neurospheres is compromised in (B) and (C); the arrows indicate compromised integrity of neurospheres most likely caused by formation of ice. In (C) the neurospheres were fragmented. In (B) the presence of small numbers of red-fluorescent dead cells is evident at higher magnification (inset). In (C) large numbers of red fluorescent dead cells are evident. The scale bar for A and C represents 100 µm, while that for B represents 50 µm. (D) Viability was quantified by counting the number of live cells and expressing it as a percentage of the total cells. The data for the untreated control and vitrified group from Figure 2.2 are shown for comparison. The data are mean ± SEM of three replicates. * $P < 0.001$ Student's t-test against untreated control. (published in Cryoletters 28(6), 445-460)

significant decrease in viability compared to the untreated and vitrified groups (Fig. 2.3B inset; Fig. 2.3D). In contrast, slow-cooling freezing greatly increased the number of red-fluorescent dead cells and cell viability fell significantly to 65.4 ±3.8 % ($p < 0.001$; Fig. 2.3C,D).

As 37% (v/v) EG, 0.6 M sucrose in medium appeared to protect against cell death, although not against loss of the structural integrity of the neurospheres, we also compared rapid-cooling freezing at lower solute concentration, 30% (v/v) EG, 0.6 M sucrose in medium. There was markedly increased cell death (77.1%±4.5; $p < 0.001$) with 30% (v/v) EG, 0.6 M sucrose compared to 37% (v/v) EG, 0.6 M sucrose (Fig. 2.4).

2.3.3. Karyotyping of neurospheres after vitrification

To investigate whether the process of vitrification causes any chromosomal abnormalities in NSPCs, the vitrified neurospheres were karyotyped. A total of 20 metaphase spreads were analyzed in each sample. All samples displayed a complete set of 40 chromosomes without any structural deviation from the norm for murine cell karyotypes. Figures 2.5 show representative sets of chromosomes from untreated and vitrified NSPCs.

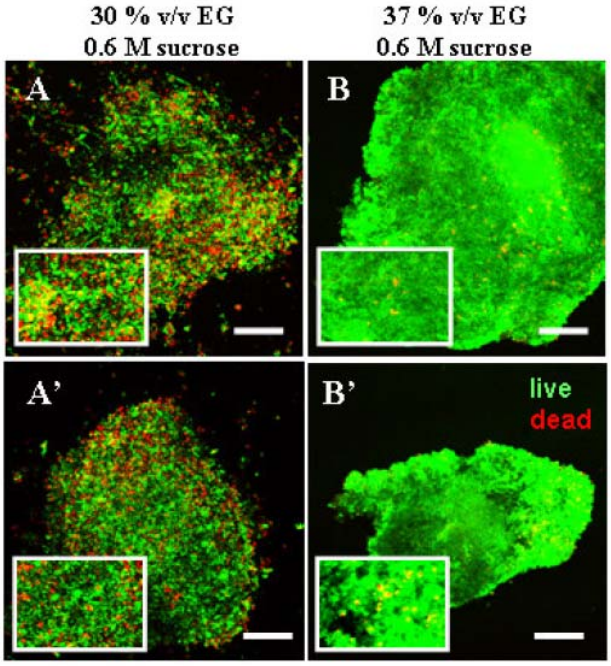

Figure 2.4. Comparison of the cell viability and structural integrity of neurospheres undergoing rapid-cooling freezing with different total solute concentrations. Confocal micrographs of representative neurospheres recovered from rapid-cooling freezing in liquid nitrogen in 30% (v/v) EG, 0.6 M sucrose (A, A') and (B, B') 37% (v/v) EG, 0.6 M sucrose. The neurospheres were labeled with a LIVE/DEAD Viability/Cytotoxicity kit. Green fluorescence indicates live cells, while red fluorescence indicates dead cells. There are more dead cells present in (A) than in (B). The scale bar represents 100 μm. (published in Cryoletters 28(6), 445-460)

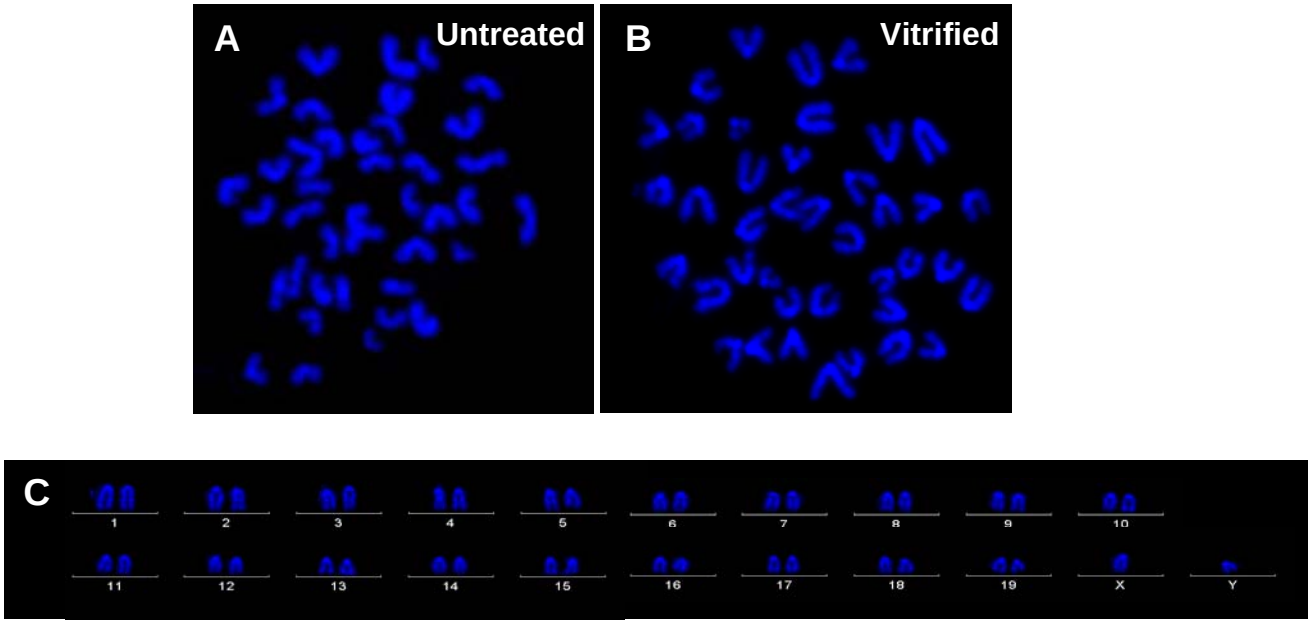

Figure 2.5. Karyotyping of NSPCs in untreated and vitrified neurospheres. Representative examples of metaphase spreads from (A) untreated neurospheres and (B) neurospheres after recovery from a complete vitrification-warming cycle. Chromosomal number and structural integrity of the chromosomes from the untreated and vitrified neurospheres were analysed to identify possible chromosomal abnormalities due to the vitrification process. (C) A typical alignment of the chromosome pairs of a vitrified sample. There is no evidence of chromosomal abnormality. (published in Cryoletters 28(6), 445-460)

2.3.4. Effects of vitrification on expression of stem cell markers

To investigate whether the process of vitrification had an effect on the stem or progenitor cell state of NSPCs, expression of the neural stem or progenitor cell markers, nestin and Sox2, was assayed. The cells were plated on poly-L-ornithine and fibronectin-coated coverslips and allow to attached and recover for 3 days. The vitrified NSPCs were found to express nestin and Sox2 (Fig. 2.6A). To investigate whether the NSPCs would lose their stem/progenitor cell state upon further passaging, the neurosphere cultures were passaged three times and the assay was conducted again. After three passages, the NSPCs were still found to express nestin and Sox2. This indicates that the neural stem or progenitor cell state was maintained for at least three passages after vitrification (Fig. 2.6B).

2.3.5. Effect of vitrification on the rate of proliferation

The proliferation rate of the vitrified NSPCs was compared with the untreated group by a BrdU incorporation assay. The proliferation rate over 24 h was calculated as the percentage of BrdU-immunoreactive cells in the total DAPI-stained cell count. Vitrification did not significantly alter the proportion of cells labelled with BrdU compared to the untreated control (Fig. 2.7). Quantification of the BrdU-positive cells did not reveal any significant change in the rate of proliferation in vitrified NSPCs compared to the untreated control (Fig. 2.7C).

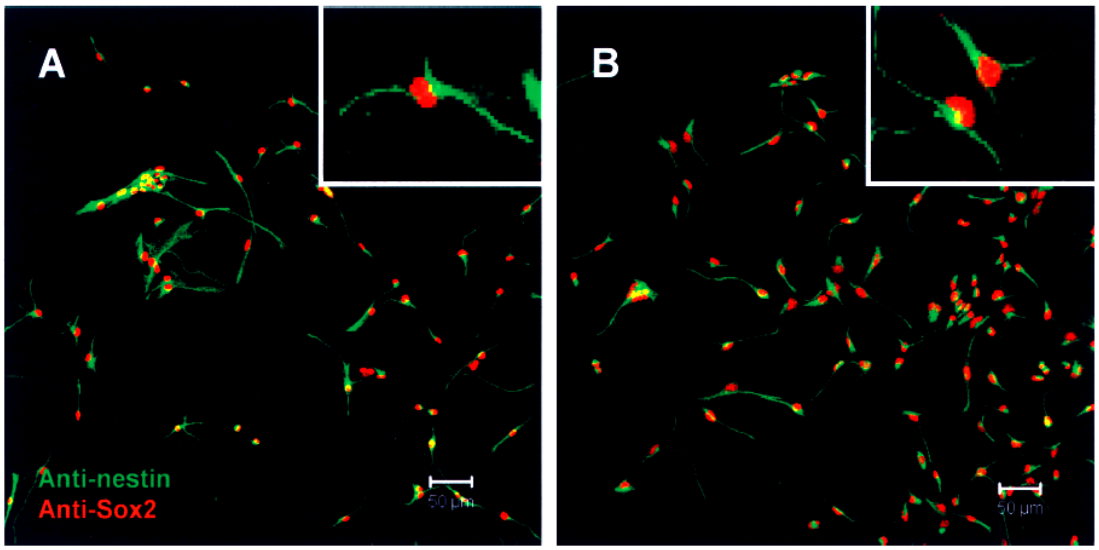

Figure 2.6. Vitrified NSPCs maintain expression of NSPC markers. Vitrified NSPCs were plated as a monolayer and immunostained using anti-nestin (green) and anti-Sox2 (red) antibodies. NSPCs cultured (A) after vitrification and (B) three passages after vitrification. All cells were double-stained for nestin and Sox2. Representative examples of double-stained cells at higher magnification are inset. The scale bar represents 50 μm. (published in Cell Transplantation 18, 135-144)

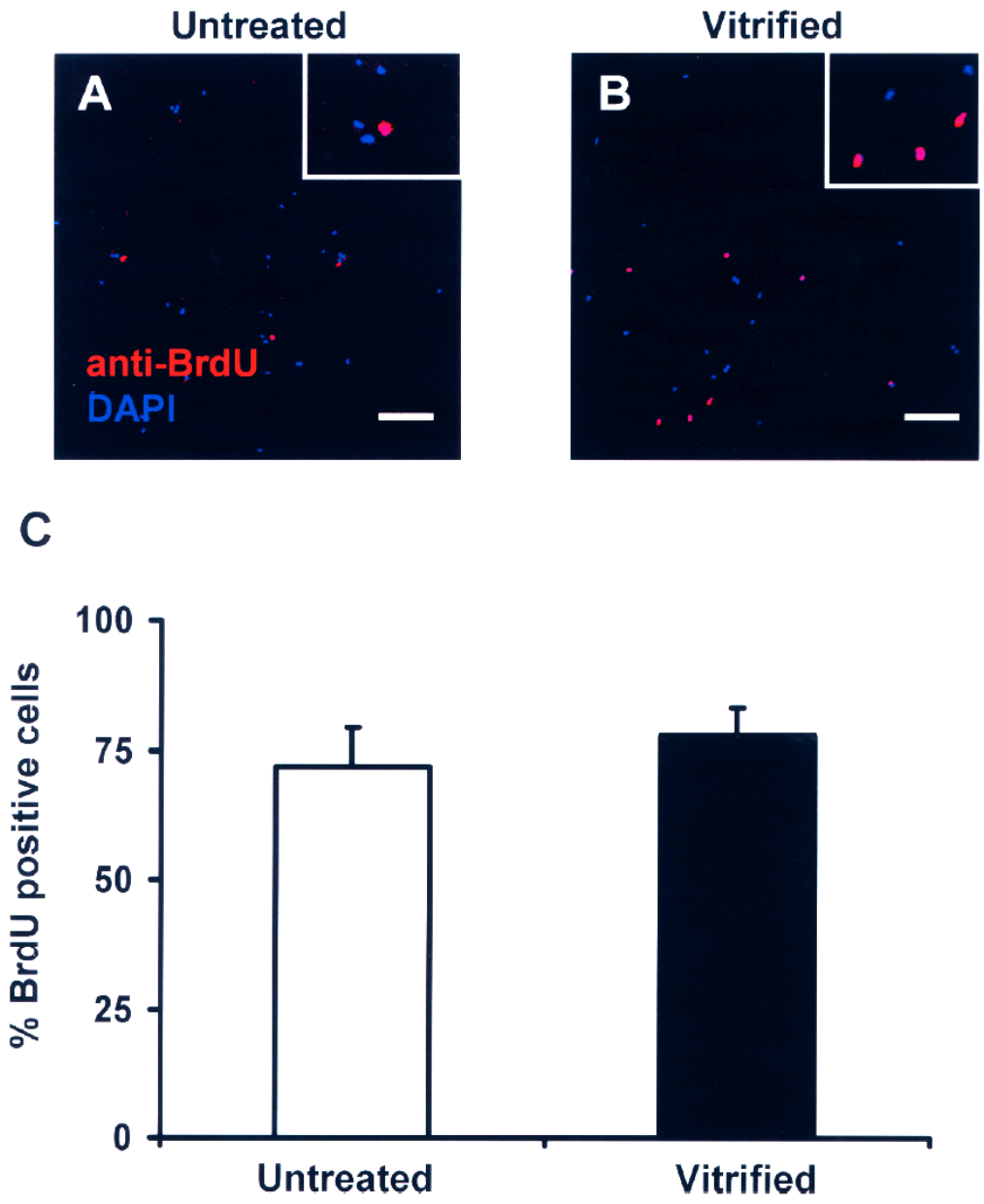

Figure 2.7. BrdU cell proliferation assay of NSPCs after vitrification. NSPCs dissociated from neurospheres (A) untreated, and (B) after recovery from a complete vitrification–warming cycle. The dissociated cells were plated on poly-L-ornithine and laminin coated coverslips and assayed for proliferation by BrdU incorporation assay. The cells with BrdU incorporation were identified using an anti-BrdU antibody (red) and the cell nucleus were counterstained with DAPI (blue). Representative examples of BrdU positive cells at higher magnification are inset. The scale bar represents 80 µm. (C) The number of cells undergoing division within 24 h was counted and expressed as a percentage of the total DAPI-positive cell count. The data are mean ± SEM of five replicates. There were no significant differences. (published in Cell Transplantation 18, 135-144)

2.3.6. Multipotent Differentiation after Vitrification

The neurons, astrocytes, and oligodendrocytes were identified by immunostaining with cell type-specific markers (Fig. 2.8). Both untreated and vitrified NSPCs differentiated into cells expressing either GFAP or MAP2a+b and GFAP or CNPase (Fig. 2.8A-D). The number of each of the three cell types was quantified as a percentage of the total cell number counted by DAPI counter staining. Both untreated and vitrified NSPCs were able to differentiate into 7–11% neurons, 80–86% astrocytes, and <1% oligodendrocytes (Fig. 2.8E). There were no significant differences in the differentiation in untreated and vitrified cells.

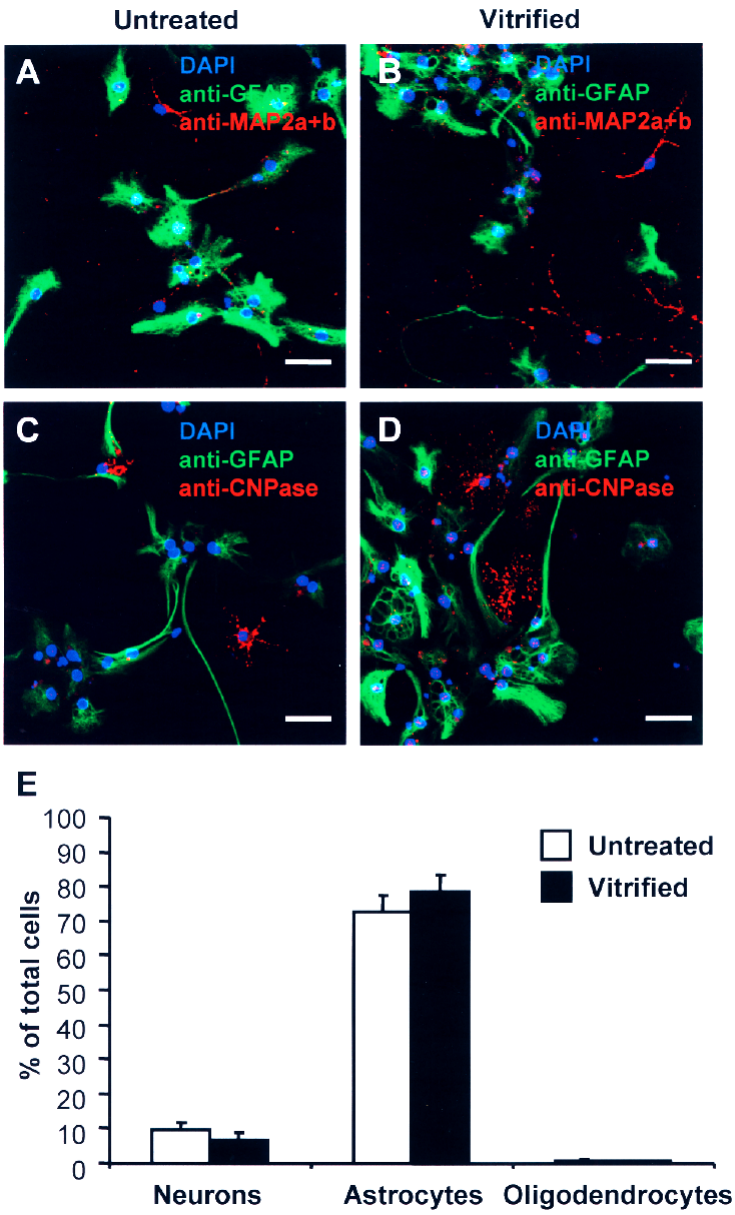

Figure 2.8. Multipotent differentiation of untreated and vitrified NSPCs. (A and C) untreated control; (B and D) NSPCs after recovery from a complete vitrification–warming cycle. The neurospheres were differentiated with 0.5% fetal calf serum and double immunostained for (A and B) astrocytes and neurons using anti-GFAP (green) and anti-MAP2a+b (red) antibodies, respectively, and (C and D) astrocytes and oligodendrocytes using anti-GFAP (green) and anti-CNPase (red) antibodies, respectively. Nuclei were counterstained with DAPI (blue). The scale bar represents 20 μm. (E) Percentage of the neurons, astrocytes and oligodendrocytes differentiated from untreated and vitrified NSPCs expressed as a percentage of the total DAPI-positive cell number. The data are mean ± SEM of three replicates. There were no significant differences between the untreated and vitrified groups. (published in Cell Transplantation 18, 135-144)

2.4. Discussion

The application of vitrification and freezing (slow-cooling and rapid cooling) in protein and xeno-free cryopreservation of NSPCs cultured as neurospheres was investigated. The vitrification protocol was found to be superior to both rapid-cooling freezing and slow-cooling freezing for the preservation of the structural integrity of neurospheres. The cryopreservation of complex systems will be important for regenerative medicine but has been a challenge because freezing protocols can disrupt the three-dimensional structure of cell aggregates and tissues (Fashy *et al.*, 2006). Future clinical applications of NSPCs may include the use of three-dimensional structures for nerve bridging or filling of lesion cavities in spinal cord injury or traumatic brain injury. It is therefore important to develop cryopreservation protocols that do not disrupt the integrity of three-dimensional cell aggregates. Also, if cryopreservation protocols are to serve as the foundation for the development of protocols for clinical cell transplantation, it is also important that they should be free from foreign proteins and sera so as to avoid any possible risk of contamination (Mallon *et al.*, 2006). Xeno-free cryopreservation protocols have sometimes replaced animal proteins with human proteins, but ideally even human proteins would be eliminated to prevent all possibility of contamination (Richard *et al.*, 2004).

The importance of the employment of vitrification solutions with sufficiently high total solute concentration has still not been widely appreciated. In the past,

cryopreservation solutions with lower solute concentrations than required to achieve vitrification were explored as a means of preventing ice formation during the cooling process only as the aim is to reduce the toxicity arise from high concentration of cryoprotectant. However, with the realization that low toxicity cryoprotectants such as sugars and polymers could be used to increase solute concentrations, higher solute concentrations can now be use to ensure vitrification and devitrification. EG is regarded as a promising cryoprotectant due to its high permeability and relatively low molecular weight (62 Da) (Valdez *et al.*, 1992). Sucrose is an easily soluble, cheap cryoprotectant additive with little or no toxicity and, in contrast to EG, is a non-penetrating cryoprotectant. Previously, reports have shown that the use of mixtures of penetrating and non-penetrating cryoprotectants in certain proportions is a feasible approach for cryopreservation of human oocytes, encapsulated hepatocytes, and self-assembled aggregates (Kuleshova and Lopata, 2002; Kuleshova *et al.*, 2004; Wu *et al.*, 2007; Magalhães *et al.*, 2008). Although stepwise introduction and removal of cryoprotectants insured minimum osmotic changes to the neurospheres, direct immersion into liquid nitrogen in protocols employing solutions which do not form stable glass (either 30% (v/v) EG 0.6 M sucrose or 37% (v/v) EG 0.6 M sucrose) did not lead to complete protection. Thus, cryopreservation of the structure of the neurospheres was more efficient with the higher solute concentration of our vitrification solution.

Cell viability was an important assessment for the efficiency of the developed vitrification protocol. The cell viability was assayed semi-quantitatively by counting cells from optical sections, qualitatively via confocal images of stained cells from dissociated neurospheres and quantitatively using a Vi-CELL analyzer. For the semi-quantitative confocal imaging, the number of cells counted in each sampled area was not identical as the neurosphere structure varies with depth and the size of the neurospheres ranged from 90 to 924 μm in diameter such that smaller neurospheres could be imaged through their entirety, while larger neurospheres could only be imaged to a depth of approximately 140 μm (28 optical sections at 5 μm z-axis intervals starting from approximately 5 μm into the neurosphere). In the case of larger neurospheres, the centre of some optical sections appeared dark as the core is compact and consists of necrotic cells to a large extend (Bez *et al.*, 2003). The presence of the necrotic core is likely due to limitation of nutrient penetration during culture and is not an artefact of cryopreservation. That there was no rupture of the neurospheres by ice crystals observed suggests that the cryoprotectant permeated the entire neurosphere allowing the vitrification procedure to preserve even the core. To minimize sampling variability the areas sampled for counting in the larger neurospheres were in the outer regions of the neurosphere and avoided the necrotic core. Although counting the LIVE/DEAD cells in the intact neurospheres resulted in a higher estimate of viability than the Vi-CELL analysis of dissociated neurospheres, all three methods found no difference in cell viability in untreated control cultures and the cultures recovered after vitrification.

The neurospheres remain intact and that the cells within the intact neurospheres remain viable is most important for the perpetuation of neurosphere culture after recovered from vitrification. The measures of cell viability reported here are estimates of the viability of the cells within the recovered neurospheres. However, it is also interesting to know whether neurospheres are lost during vitrification. Since number of neurospheres in each sampled varied from 150 to 300 neurospheres of various sizes, it was not feasible to count the neurospheres prior to vitrification as it was necessary to minimize the time of exposure outside the incubator (Fig. 2.1C). It was therefore not possible to directly measure the recovery of neurospheres after vitrification. However, microscopic observation of the recovery solution offered no evidence of fragment neurospheres or free cells indicating that all the vitrified neurospheres were recovered. Recovery was facilitated by tapping of the tube to accelerate sedimentation of the neurospheres. Supplementary flushing of the straw with recovery solution did not recover additional neurospheres. Together these observations indicate that there is near complete recovery of the vitrified neurospheres. On the other hand, as the culture appears healthier and devoid of free cells after vitrification, it seems that vitrification acted as a selection regime after which only healthy and intact neurospheres remained in the culture dish for subsequent cultivation while small debris, diffuse neurospheres with poor cell-cell contact and compromised free cells which were not able to form spheres were removed during sedimentation in the tubes containing 10% and 25% EG.

The present data on the integrity of neurospheres suggests that the vitrification protocol described here may be a useful starting point for the development of cryopreservation protocols for NSPCs in three-dimensional complex structures. As alkaline pH values greater than pH 9 have been shown to be detrimental to the extracellular matrix that holds NSPCs together as neurospheres (Sen *et al.*, 2004), the pH of the cryoprotectant solution may be crucial. The pH of the vitrification solution used in the experiment is around pH 8.0 ± 0.2. Also in this vitrification protocol, the time of exposure to liquid vitrification solutions never exceeds 3 minutes, except during the dilution steps on recovery after warming when exposure is not more than 15 minutes while sucrose containing solutions are employed in decreasing concentrations.

Careful component selection is vital to avoid toxic effects in cryopreservation solutions. The NSPCs vitrification method tested in this paper employed the use of EG and sucrose as cryoprotectants to provide a low toxicity vitrification solution (Kuleshova *et al.*, 2000; Valdez *et al.*, 1992; Wu *et al.*, 2007). In the current protocol, sucrose serves to dehydrate the cells by increasing the osmolarity of the vitrification solution and the use of EG in the vitrification protocol did not cause an observable toxic effect. To reduce the toxicity of the final solution, it might be thought that the total solute concentration could be reduced without harm to viability and integrity of structured cultures. However, this study proved this concept is not applicable to neurospheres. Although the results

obtained for rapid “freezing” using a solution of 3% reduction in total solute concentration showed no significant decrease in cell viability, further decrease in the total solute concentration resulted in a marked decrease in cell viability (Fig. 2.4). The fundamental difference between rapid-cooling freezing and vitrification is that higher solute concentrations are used in vitrification to ensure amorphous state on cooling or warming while rapid-cooling freezing uses lower solute concentrations that permit the formation of ice crystals. However, as solute concentrations approach the vitrification threshold, ice formation on quenching reduces before ice formation on thawing. Therefore, the high cell viability shown in Figure 2.3B could have implied a near-vitrification reduction in ice formation during cooling-thawing cycle. A similar study was previously conducted a study on stepwise reduction of total solute concentration in cryopreservation solutions and its effects on cell survival and found that decreases in total solute concentration of 4% (v/v) from the vitrification threshold had no significant adverse effect on cell survival, whereas a decrease in total solute concentration of 7% (v/v) did, most probably due to the formation of ice during cooling-thawing cycle (Wu *et al.*, 2007). Consistent results were obtained in the current study whereby a decrease in total solute concentration of 10% (v/v) has led to markedly reduced cell viability (Fig. 2.4A,A’). Rapid-cooling freezing itself is not conducive to maximal cell survival and therefore not a viable cryopreservation procedure for structured cultures such as neurospheres.

The absence of any evidence for changes in the karyotype suggests that vitrification of NSPCs is also not associated with chromosomal abnormalities. A potential application of vitrification would be to preserve NSPCs at early passages to avoid chromosomal abnormalities due to excessive passaging. Therefore, it is of importance that the process of vitrification itself does not cause chromosomal abnormalities. Post-thaw chromosome abnormalities of human cells are of great concern. For example, human oocytes have a complex subcellular structure, which can be damaged easily by brief cooling to room temperature (Bernard and Fuller, 1996; Pickering *et al.*, 2003). This impact on the meiotic spindle and subsequent chromatid nondisjunction is a limitation for application of slow-cooling protocols (Bernard and Fuller, 1996). Most importantly an increase in chromosome abnormalities after fertilization of frozen and thawed human oocytes was also found (AlHasani *et al.*, 1987; Bernard and Fuller, 1996). It has also been reported that vitrification does not cause chromosome abnormalities of human oocytes (Kuleshova *et al.*, 1999). Meiotic normality and good preservation rates have also been achieved with vitrification of animal oocytes (Isachenko and Nayudu, 1999).

Expression of nestin and Sox2, two commonly used neural stem or progenitor cell markers, was studied to ensure that the state of the NSPCs was not affected by vitrification. Both the markers were detected in NSPCs recovered from vitrification and also three passages after recovery from vitrification. This shows that the vitrification procedures did not alter the expression of these important

stem cell markers. There was also no evidence for stimulation of spontaneous differentiation of NSPCs following recovery from vitrification, which had been mentioned as a problem following cryopreservation of embryonic stem cells in a protocol involving the use of dimethylsulfoxide (DMSO) (Ji *et al.*, 2004). This problem could be attributed to the role of DMSO in promoting differentiation, mechanical and osmotic stresses, and other chemical and physical factors (Buchanan *et al.*, 2004; Ishiguro *et al.*, 2004). Maintenance of progenitor or stem cell properties following vitrification is likely to be important in clinical application as expansion of the NSPCs will probably be required to reach the desired cell number for cell replacement therapy.

It is essential that NSPCs maintain their multipotency after cryopreservation. Retention of the potential for differentiation is an important assessment because the multipotency of the NSPCs in neurospheres is sensitive to alteration of culture conditions. Furthermore, the ability of these NSPCs differentiate into all mature neural cell types for integration into existing neural networks is likely to be vital to transplantation for cell replacement therapy as different diseases may require the replacement of different cell types, for example oligodendrocytes would be required for cytotherapy for multiple sclerosis (Merrill and Scolding, 1999) while neuronal differentiation would be required for repair of hypoxic and ischemic damage in the central nervous system (Park *et al.*, 2002). The differentiation of vitrified neurospheres was assessed and it was observed that the capacity for mulitpotent differentiation was preserved. There was no

significant difference in the proportion of the three cell types between untreated and vitrified NSPCs. Thus, these results indicate that the vitrification process does not affect the multipotent differentiation of NSPCs

Vitrification would offer a cost-effective alternative to slow-cooling freezing. The slow-cooling freezing method is inaccessible to many research laboratories as it requires expensive machinery in order to accurately maintain the cooling rate and it also has its limitations. In a recent study, the efficacy of different cooling rates for the cryopreservation of human embryonic stem cell (hESC) colonies was assessed (Yang *et al.*, 2006). In this study, a controlled rate of 0.5°C/min combined with cryopreservation solutions comprising 10% DMSO and 30% fetal bovine serum was found to be optimal. This selected protocol resulted in a post-thaw viability of 54% recorded on day 5 in culture that by day 9 had propagated to 80% of the initially preserved population. Although these hESC were found to be mainly undifferentiated and with normal karyotypes, with prolonged culture, non-induced differentiation still occurred.

Another comprehensive study was conducted on the effectiveness of slow-cooling method for cryopreservation of murine neural precursor cells (NPCs) (Milosevic *et al.*, 2005). In this study, various combinations of cryopreservation solutions consisting of penetrating (DMSO, EG and glycerol) and non-penetrating cryoprotectants (trehalose) were assessed. To enhance survivability of NSPCs co-culture of NSPCs with fibroblasts growth factor-2 and epidermal growth factor

prior to “freezing” was employed. The percentage of surviving cells 24 h after thawing for NSPCs treated with 10% DMSO, 10% EG, 10% DMSO + 0.2M trehalose, and some other combinations added with caspase inhibitors was not significantly different from that of the untreated control. However, after 1 week of thawing, the percentage of surviving cells for all treated NSPCs was significantly lower than the control. The results showed that the freezing approach is not able to preserve cell survival at longer culture durations. A mere 25% of clonogenic survival observed in NSPCs treated with 10% DMSO and 10% glycerol (compared to control) has further substantiated the inability of freezing to preserve the colony forming ability of these cells. Here, we have shown that after 3 weeks of continuous culture following vitrification cell viability was consistently preserved (Fig. 2.2E). This result suggests that the method presented here is better than those previously described and therefore suggest that vitrification may be an effective and feasible means to cryopreserve these sensitive cells.

In this chapter, a cost-efficient and simple protocol for the cryopreservation of neurospheres by vitrification has been discussed. The rapid cooling freezing protocols did not allow the maintenance of the structural integrity of the neurospheres, suggesting that this approach could not be considered further for the cryopreservation of this, as well as other structured cultures. As there was severe structural disturbance and partial lost of cell viability, it might be concluded that conventional freezing is inappropriate for use in stem cells research. In contrast, vitrification preserved structural integrity of neurospheres

and cell viability. These cells recovered from the complete vitrification-warming cycle were free of chromosomal abnormalities, stem cell characteristics unchanged with the maintenance of their capacity for multipotent differentiation. Additionally, the employment of the “straw-in-straw” methodology also allows sterility to be maintained during the entire process. While it is uncertain whether neurospheres will be the preferred culture system for potential future clinical application of NSPCs (Jensen and Parmar, 2006), the ability to preserve the unsupported three-dimensional structure of neurospheres by vitrification suggests that this approach could be considered as a method to the preservation of NSPCs in three-dimensional microfabricated structures or scaffolds (e.g., nerve conduits for spinal cord repair) (Teng *et al.*, 2002). With the complete avoidance of products of human or animal origin, this protocol can serve as starting point for development of protein- and serum-free vitrification protocols for the cryopreservation of human stem cells, especially human neural stem cells that may eventually be used in clinical settings. This established protocol will also be useful for the research which requires a constant supply of NSPCs in the subsequent chapters in the thesis.

3. SEROTONERGIC FIBRES AND 5-HT RECEPTORS – IMPLICATIONS FOR NSPC PROLIFERATION

3.1 Introduction

Treatment with SSRI-based antidepressants has been shown to trigger increases in NSPC proliferation. This induction is likely brought about by the increased availability of 5-HT at the synapses due to the inhibition of the 5-HT reuptake inhibitors on the serotonergic neurons. However, the exact mechanism by which 5-HT induces the increase in cell proliferation is not clear.

It is conceivable that there may be two ways in which the 5-HT may transmit signals to the NSPCs to induce an increase in NSPC proliferation. (1) The increase in the availability of 5-HT at the serotonergic nerve endings may activate a downstream neural network which eventually reaches the NSPC and regulates its increase in NSPC proliferation. (2) The increase in the availability of 5-HT at the serotonergic synapses may act directly on the NSPCs, suggesting that 5-HT is able to regulate NSPC proliferation directly. This scenario requires the presence of 5-HT receptors on the NSPCs to perceive the serotonergic signals.

Hippocampus is found to be innervated by serotonergic neurons as suggested with early lesion experiments that show hippocampal 5-HT contents are greatly

reduced by lesioning of the raphe nuclei in rats (Jacobs *et al.*, 1974). Further work by Moore and Halaris (1975) using autoradiography tracing of the serotonergic neuron projections from the raphe nuclei further confirm the presence of serotonergic innervations into the hippocampus. As the hippocampus is innervated by serotonergic neurons, the direct interactions of serotonergic synapses acting directly on the NSPCs will be explored. In order for the serotonergic synapses to be directly acting on the NSPCs, they must be located at or near the neurogenic regions of the brain. Investigations will be conducted on location of the serotonergic terminals in the SGZ and the SVZ to identify whether the serotonergic synapses are likely close to the neurogenic regions of the brain. At the serotonergic synapses, the 5-HT signal must also be able to be perceived by the NSPCs and trigger the increase in NSPC proliferation. Through *in vitro* isolation of primary NSPC cultures, the direct effects of 5-HT on the NSPCs will be studied and the 5-HT receptors subtypes expressed by the NSPCs will also be identified.

3.2. Materials and Methods

3.2.1. Tissue preparation

Adult female C57BL/6J mice aged 6-8 weeks old were anaesthetized with an overdose of pentobarbital (Nembutal), then transcardial perfused with 4% paraformaldehyde in 0.1 M of phosphate buffer (pH 7.4). After which the brains were extracted and postfixed overnight in the same fixative. The forebrain containing the lateral ventricles and the hippocampi of the mice were then sectioned using a vibratome (Vibroslice, World Precision Instruments) at a thickness of 40 μm prior to immunohistochemical assays. The tissue sections stored in phosphate buffered saline (PBS) at 4°C until use.

3.2.2 Immunohistochemistry

To label the serotonergic nerve fibres, immunofluorescence labeling was carried out on the free- floating sections. The sections were first blocked with 10% goat serum before anti-serotonin antibody (1:100; MAB352, Chemicon, Temucula, CA) in 10% goat serum was added to the sections and incubated overnight at 4^{o}C. The secondary antibodies used was Cy2 labeled goat anti-rat (1:200, Jackson ImmunoResearch, PA, USA). The sections were mounted with Pro-Long Anti-Fade reagent (Molecular Probes, Invitrogen) before being coverslipped. The

sections were then imaged on a Laser Scanning Confocal Microscope (LSM 510, Zeiss MicroImaging GmbH).

3.2.3 Cell proliferation assay of **5-HT** *treated NSPCs*

To determine whether exogenous addition of 5-HT to cultured NSPCs is able to influence cell proliferation rate; a cell proliferation assay was performed using the CyQuant NF Cell Proliferation Assay (Invitrogen). NSPCs were isolated as indicated in Section 2.2.2. Third passage neurospheres obtained were dissociated using the same procedure as for cell passaging. Resuspended single cells were sieved with 40 μM cell strainers and counted using a haemocytometer (Neubauer, Germany). NPCs were then plated at 1000 cells/well on poly-L-ornithine (PLO, Sigma-Aldrich) and fibronectin (FN, Gibco) coated 96-well, black-walled, flat transparent bottom culture dishes in neurosphere medium. The cells for each assay run were plated from the same batch of cells to maintain plating homogeneity. The cells were precultured for 72 hours prior to addition of treatment solution. Seventy two hours after addition of the 5-HT, CyQuant NF Cell Proliferation Assay reagents were added according to manufacturer's instructions and incubated at 37ºC. After 1 hour incubation, the first reading was taken using a fluorescence plate reader (Infinite M200, Tecan, Zürich, Switzerland) at an excitation wavelength of 490nm and an emission wavelength of 530nm. Each treatment was done with three independent biological repeats and each biological repeat was conducted with three technical repeats.

3.2.4. Statistical Analysis

After subtracting the background, the fluorescence intensity, which was linear to the cell number, was expressed as a ratio to the control without treatment and the SEM is calculated from three independent biological repeats. The 5-HT-treated samples were compared with the control and analyzed using Student's t-test with $P<0.05$ was considered as statistically significant. All data are reported as mean ± SEM.

3.2.5 RNA Extraction and Reverse Transcription PCR (RT-PCR)

Total RNA was extracted, treated with DNaseI (DNase I recombinant, RNase-free; Roche Diagnostic GmbH, Mannheim, Germany) and purified according to the manufacturer's instructions for PureLink™ Micro-to-Midi Total RNA Purification System (Invitrogen) from passage 3 NSPCs isolated from fetal hippocampal and SVZ from Section 3.2.3. Quantitation of RNA was performed with Quant-iT™ RNA Assay Kit (Invitrogen). Approximately 1 µg of total RNA was reverse transcribed with oligo(dT) primers using the ImProm-II™ Reverse Transcription System (Promega, WI, USA).

The reverse transcribed cDNA was used as a template for PCR using AmpliTaq Gold DNA polymerase (Applied Biosystems) according to manufacturer's

instructions. PCR primers were designed to be intron spanning to eliminate the presence of false positive from genomic DNA contamination. The primer sequences for the respective genes and their annealing temperature used were stated in Table 3.1. The PCR reactions were conducted at 94^{o}C for 30 sec, annealing at the primers' respective temperature for one min and 72^{o}C for one min for 35 cycles. The PCR products were separated in 1.5% agarose gel, stained with ethidium bromide, and then visualized under UV irradiation.

Table 3.1 – Primer sequence, annealing temperature and amplicon size of 5-HT receptors, SERT and Sox2

Gene	Forward Primer	Reverse Primer	Annealing Temperature	Amplicon size
5-HT1A receptor	5'-GGAGCGGGCACCAGCTTCGGAACA-3'	5'-CACTGTCTTCCTCTCACGGGCCAA-3'	62°C	203bp
5-HT1B receptor	5'-AAGAAACTCATGGCCGCTAGGGAG-3'	5'-GCGTATCAGTTTGTGGAACGCTTG-3'	57°C	252bp
5-HT1D receptor	5'-TACAAACACCTCAGAGCTACCAAGC-3'	5'-TTTAAAGCCAGAGACAAAAAGAAACAG-3'	59°C	251bp
5-HT1F receptor	5'-CCTGCCACACCACGGTATTC-3'	5'-TGATCGCAGCGATCACGA-3'	60°C	200bp
5-HT2A receptor	5'-GGGTACCTCCCACCGACAT-3'	5'-AGGCCACCGGTACCCATAC-3'	60°C	252bp
5-HT2B receptor	5'-CAGAAGACATGTGATCACCTGATC-3'	5'-TGTAATCTTGATGAATGCAGTAGCC-3'	60°C	474bp
5-HT2C receptor	5'-GCTCCGCTGGGCGATT-3'	5'-CACAAGGAGTGAGCGCACC-3'	60°C	251bp
5-HT3A receptor	5'-GATCGGTACCCCCCTCATTG-3'	5'-GTCCTGAGGTCCTCCAACATG-3'	60°C	301bp
5-HT3B receptor	5'-CAACGTAGTGATCCGCAGATGT-3'	5'-CCGCTCCTCATAGAGGAATTTG-3'	59°C	301bp
5-HT4 receptor	5'-ATCGCATGAGGACAGAGACC-3'	5'-GCCACCAAAGGAGAAGTTGC-3'	62°C	400bp
5-HT5A receptor	5'-GGCTCCACTGCTATTTGGCT-3'	5'-CACGTATCCCCTTCTGTCTGG-3'	60°C	303bp
5-HT5B receptor	5'-TTCTACCTGCCTCTAGCGGTG-3'	5'- GGCTGATGAGCTCCGTCAG-3'	61°C	301bp
5-HT6 receptor	5'-GTCCGGCGTCACCTTTTTC-3'	5'-CAGTCACATACGGCCTGAGCT-3'	60°C	318bp
5-HT7 receptor	5'-GGCCTCGATCATGACCCTG-3'	5'-CCTGGCGGCCTTGTAAATC-3'	63°C	301bp
SERT	5'-ACATCTGGCGTTTTCCCTACAT-3'	5'-TTTTGACTCCTTTCCAGATGCT-3'	58°C	525bp
Sox2	5'-AAGTACACGCTTCCCGGAGGCTTG-3'	5'-AGTGGGAGGAAGAGGTAACCAC-3'	55°C	412bp

3.3. Results

3.3.1 Serotonergic fibres are found in the neurogenic regions of the mouse brain

To determine whether serotonergic fibres were present in the neurogenic regions of the brain where the NSPCs were found, we conduct immunohistochemistry on the neurogenic regions of the brain using anti-serotonin antibody. Results indicated that there were serotonergic fibres around the regions where NSPCs were normally located in both the subgranular zone (SGZ) in the dentate gyrus of the hippocampus (Fig. 3.1A) and the subventricular zone (SVZ) of the lateral ventricles (Fig. 3.1B). There were also areas of intense staining on the serotonergic fibres, which may suggest areas of high density of 5-HT. These may be the areas rich in serotonergic synapses and some of these areas are found closed to the SGZ and the SVZ regions (Fig. 3.1)

3.3.2 5-HT is able to induce an increase in cell proliferation of cultured NSPCs

The above results suggest the possibility that the serotonergic fibres may synapse or terminate at or close to the NSPCs and release 5-HT. This may be a plausible mechanism by which serotonergic signals regulate NSPC proliferation as suggested by the effects of treatment with SSRIs inducing increases in NSPC

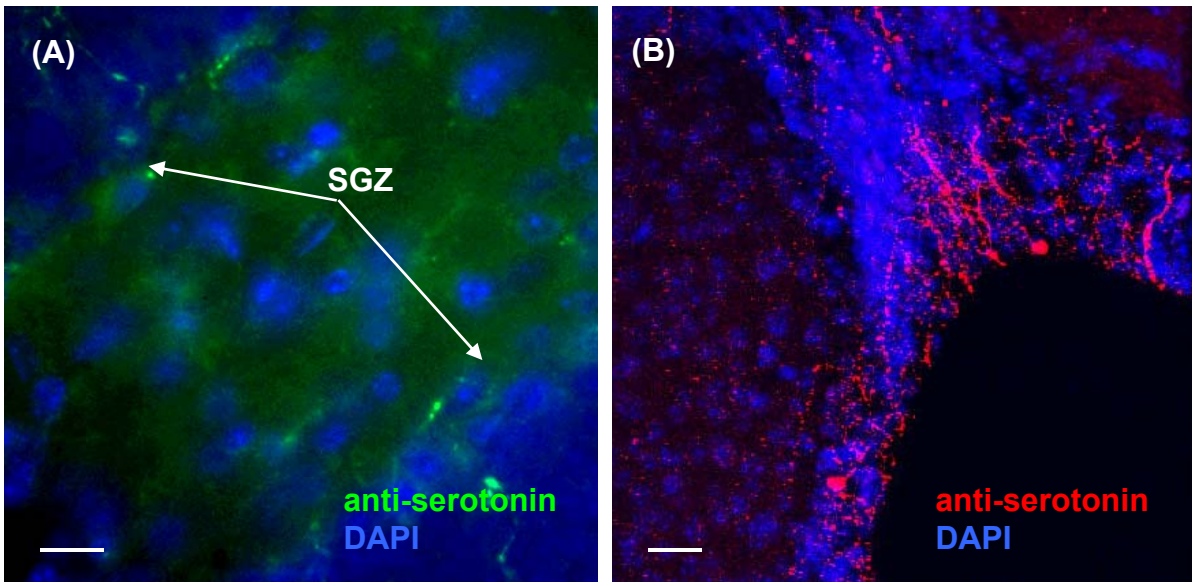

Figure 3.1. Immunostaining of serotonergic fibres/terminals in the (A) subgranular zone of the dentate gyrus and (B) subventricular zone. Serotonergic projections and serotonergic nerve endings were immunostained using anti-serotonin antibody in the dentate gyrus of the hippocampus and the SVZ region of the lateral ventricles. The sections were counter-stained with DAPI to locate the position of the cells. The scale bar represents 10 μm.

proliferation. Therefore to ascertain whether 5-HT could bring about an increase in NSPC proliferation, we isolated NSPCs from the hippocampus and the lateral ventricles of fetal C57BL/6 mice and subjected the cells to different concentrations of exogenous 5-HT. It was observed, visually and through cell proliferation assay, that by directly treating the cells with 1-10 µM of 5-HT were able to increase the cell proliferation by 20-30% as compared to control (Fig. 3.2). However, concentrations higher than 10 µM caused a decrease in NSPC proliferation, which may be attributed to toxicity.

3.3.3 5-HT receptors are expressed on NSPCs

The presence of serotonergic fibres/terminals in the neurogenic regions of the brain and the ability of 5-HT to induce an increase in cell proliferation suggest that the NSPCs were able to pick up 5-HT signals. These data suggest the presence of 5-HT receptors expressed on the NSPCs.

To determine the presence of 5-HT receptors and SERT on the NSPCs, reverse transcriptase PCR was employed to determine the transcriptional expression of the 5-HT receptors on the NSPCs. Primers were designed for the 14 subtypes of 5-HT receptors namely, 5-HT1A, 1B, 1D, 1F, 2A, 2B, 2C, 3A, 3B, 4, 5A, 5B, 6, 7 receptor and SERT. The RT-PCR was conducted using cultured hippocampal NSPCs and SVZ NSPCs total RNA as a template. The results shows that both hippocampal NSPCs and SVZ NSPCs expressed 5-HT1A, 1B, 1D, 1F, 2A, 2B,

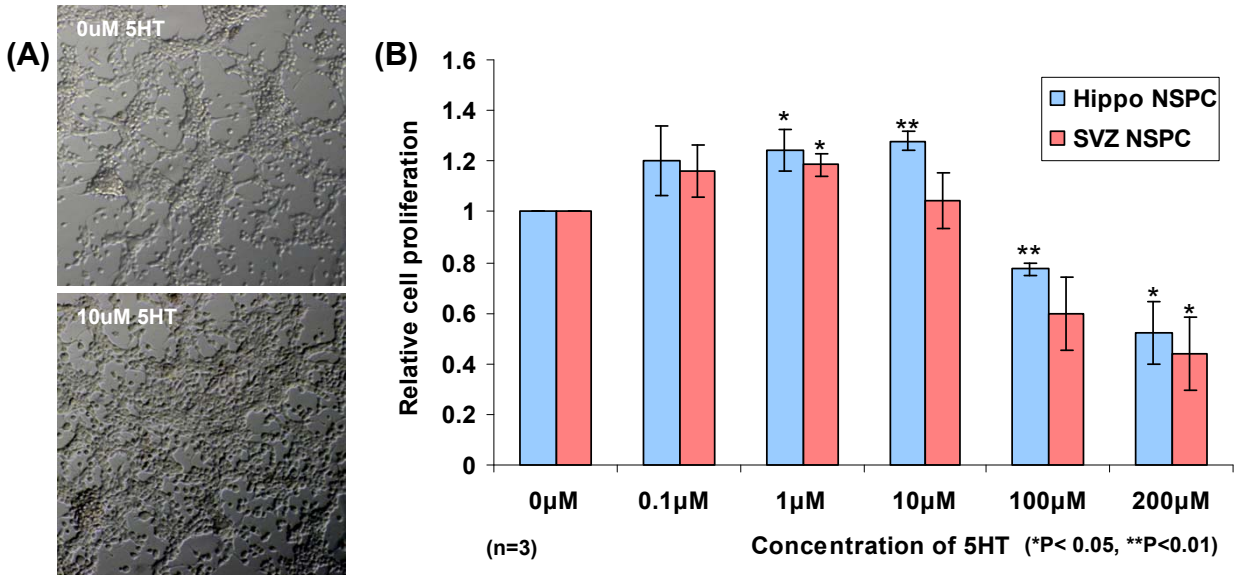

Figure 3.2. NSPC proliferation can be induced by 5-HT. NSPCs were plated at a density of 1000 cells per well in a 96 well dish and treated with different concentrations of 5-HT for 3 days to observe the changes in the rate of proliferation. (A) Microscopy showing the density of cells after 3 days of treatment between the untreated and the NSPCs treated with 10µM serotonin. (B) CyQuant cell proliferation assay was conducted and the relative proliferation under the different treatments were plotted against the control for both hippocampal (Hippo) and subventricular zone (SVZ) NSPCs.

2C, 3A, 3B, 4, 5A, 5B, 6, 7 receptor and SERT (Fig. 3.3). Expression of the NSPC maker, Sox2, was used to show that the population of cells in the neurospheres contained NSPCs.

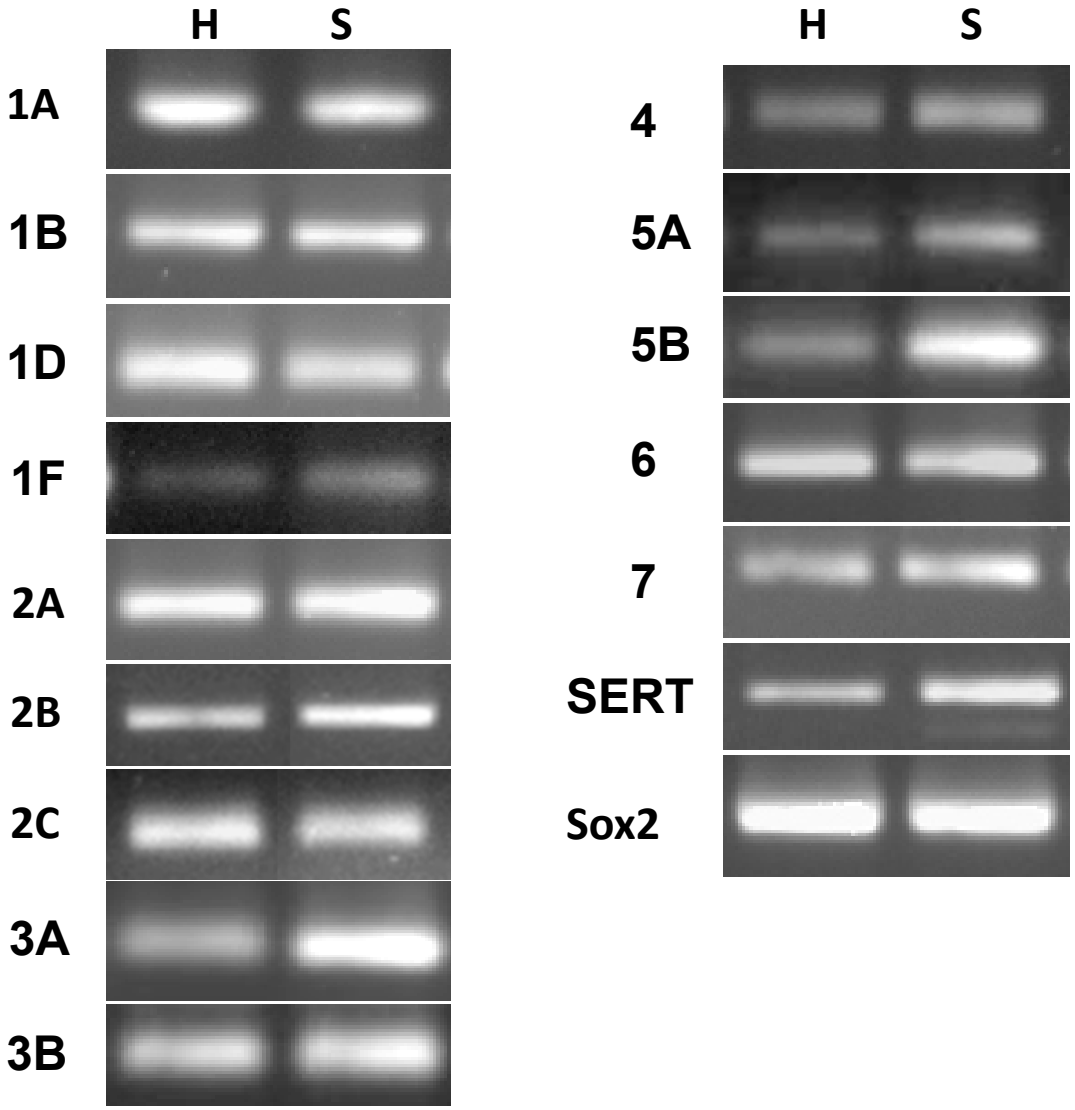

Figure 3.3. Expression of 5-HT receptors in cultured neurospheres from E14 fetuses. RT-PCR was conducted on total RNA isolated from cultured fetal neurospheres extracted from H – hippocampus and S – subventricular zone of the lateral ventricles using specific primers designed to amplify the various subtypes of 5-HT receptors and SERT. Sox2 is a marker that is expressed in NSPCs. All amplicons of the expected size were sequenced to confirm their identities.

3.4. Discussion

The results presented here suggest that serotonergic fibres in the neurogenic regions of the brain may directly interact with NSPCs. The NSPCs are suggested to be able to receive 5-HT signals using the host of 5-HT receptors subtypes they expressed as detected at transcript level. Exogenous addition of 5-HT to the primary NSPCs is able to increase in their proliferation although, at this point, it is not clear which of the receptors are involved in the regulation of the NSPC proliferation as a large number of 5-HT receptors are expressed by the NSPCs.

Studies on the regulation of adult neural progenitor/stem cell proliferation and neurogenesis by antidepressants have focused on the roles 5-HT receptors and the 5-HT transporters. However, it is not known whether these effects are mediated by direct actions of 5-HT receptors expressed by the neural progenitor cells or by indirect effects through other synaptic pathways. Previous studies have provided some clue as to whether the serotonergic fibres are present in the hippocampus, however, their cellular resolution was usually too low to determine whether the fibres are close to or synapse at the NSPCs (Schmitt et al, 2007). Therefore, we determine if the serotonergic fibres were present at the neurogenic regions, namely the SGZ and the SVZ, of the brain near, and perhaps synapsing, on the NSPCs. The presence of these serotonergic fibres, suggests the possibility that the serotonergic fibres may act directly on the neural progenitor cells to bring about an increase in cell proliferation. This also suggests that the neural progenitor cells are able to pick up the 5-HT signals released from these

serotonergic fibres. In fact, exogenous addition of 5-HT was shown to increase in the NPC proliferation *in vitro* further pointing to the fact that these serotonergic fibres act directly on the NPCs.

The NSPCs expressed the transcript for a large number of subtypes of 5-HT receptors. As the RT-PCR was done on the pool of NSPCs, it is unknown as to whether each of the NSPC expresses all the subtypes of 5-HT receptors assayed in the study. This will require single cell RT-PCR to provide such cellular resolution. Further detection and studies also need to be conducted to examine the protein expression and functionality of the 5-HT receptors in the NSPCs. However, it has been suggested that having a host of subtypes of 5-HT receptors will allow fine tuning of the response of the cell to the 5-HT signal depending on the amount of 5-HT available (Uphouse, 1997). The concentration of 5-HT at the synapse will also determine which of the receptors will be more preferential activated due to each of the 5-HT receptor have different affinity to the 5-HT. It is not hard to conceive that perhaps 5-HT at the basal level produces a certain functional signal whereas an increase in the availability or the release of 5-HT from neighboring serotonergic neurons may evoke an increase in NSPC proliferation rate. More work will have to be done to delineate the function of each of the subtype of 5-HT receptors and their role in regulation of NSPC proliferation.

4. INDUCTION OF NSPC PROLIFERATION – EFFECTS OF 5-HT1A AND/OR 5-HT7 RECEPTORS

4.1 Introduction

In the previous chapter, it has been show that the exogenous addition of 5-HT can increase NSPC proliferation and it has also been suggested that NSPCs can receive 5-HT signals as they expressed a host of 5-HT receptors. From the time of the discovery that SSRI's can increase NSPC proliferation, studies have suggested that specific activation some 5-HT receptor subtypes using agonists can also induced NSPC proliferation (Radley and Jacobs, 2002; Banasr *et al.,* 2004; Jha *et al.*, 2008). However, no attempts were made to identify whether these agonists act directly on the NSPCs or activate the NSPCs via neural networks downstream of the serotonergic neurons.

It has been shown that treatment with the 5-HT receptor agonist, 8-OH-DPAT, is able to increase NSPC proliferation rate in the neurogenic regions of the brain in mice (Banasr *et al.*, 2004). 8-OH-DPAT is suggested to act specifically at 5-HT1A receptors to increase the cell proliferation. The 5-HT1A receptor has been reported to be expressed in limbic brain areas such as the hippocampus, lateral septum, cortical regions and mesencephalic raphe nuclei (Barnes and Sharp, 1999). Functionally, the 5-HT1A receptor is a G-protein coupled receptor (GPCR) that is capable of causing neuronal hyperpolarization upon activation due its

coupling to the K^+ channels (Nicoll *et al.*, 1990). It is also known to negatively regulate adenylate cyclase in the hippocampus but not in the raphe nuclei (Clarke *et al.*, 1996).

However, the use of pharmacological agonist 8-OH-DPAT to activate the 5-HT1A receptor is not without caveats. It is found that 8-OH-DPAT is also a partial agonist at the 5-HT7 receptor (Wood *et al.*, 2000). The 5-HT7 receptor is a GPCR that is highly expressed in the thalamus, hypothalamus and hippocampus with lower expression also found in the cortex and amygdala (To *et al.*, 1995; Gustafson *et al.*, 1996; Stowe and Barnes, 1998). The 5-HT7 receptor is known to stimulate adenylate cyclase through coupling with Gαs (Bard *et al.*, 1993; Obosi *et al.*, 1997). Due to the fact that 8-OH-DPAT also activates the 5-HT7 receptors, it is conceivable that the increase in NSPC proliferation reported in Banasr *et al.*'s (2004) study may be also due to the activation of the 5-HT7 receptor instead of the 5-HT1A receptor. Furthermore, there is evidence that adenylate cyclase activity increases cellular cAMP, which can potentially trigger the activation of ERK, which in turn leads to the activation of the cell proliferation pathway (Stork and Schmitt, 2002).

It is also noted that both 5-HT1A receptors and 5-HT7 receptors are expressed in the hippocampus, which is one of the neurogenic regions in the brain. Serotonergic fibres are found to be present in the hippocampus near the dentate gyrus as evident in the immunostaining of serotonergic fibres near the

neurogenic regions of the brain in Chapter 3 and also by Schmitt *et al.,* (2007) showing axonal projections in the dentate gyrus expressing serotonin transporters. This suggests the possibility that the serotonergic fibres act directly on the NSPCs to regulate the increase in the NSPCs during antidepressant treatment through either 5-HT1A and/or 5-HTR7 receptors.

In this chapter, attempts will be made to examine whether the activation of the 5-HT1A or the 5-HT7 receptor is important in the induction of NSPC proliferation. Investigation will be done on the expression of 5-HT1A and 5-HT7 receptors on NSPCs and the effects of their activation on NSPC proliferation using specific agonists. Identification of which receptor induces NSPC proliferation may help us to understand the likely mechanism of by which the action of antidepressants leads to the increase in NSPC proliferation and recovery from depression.

4.2. Materials and Methods

4.2.1. Immunocytochemistry of NSPCs

To determine the expression of 5-HT1A and 5-HT7 receptors in NSPCs, dissociated NSPCs were plated on poly-L-ornithine (Sigma-Aldrich) and fibronectin (Gibco) coated coverslips for 2 hr. The cells were then fixed by treatment with 4% paraformaldehyde for 20 min followed by permeabilization using PBS with 0.1% Triton X-100. Immunostaining was conducted using anti-5-HT1A receptor antibody (Santa Cruz, CA, USA) and anti-5-HT7 receptor antibody (Santa Cruz) in 10% donkey serum in PBS overnight at room temperature. Fluorescence-conjugated Alexa Fluor secondary antibodies (Invitrogen) were used to visualize the primary antibodies and the coverslips were counterstained with DAPI.

4.2.2. Cell proliferation assay of agonist-treated NSPCs

To determine whether activation of the 5-HT1A and/or 5-HT7 receptors is able to influence NSPC cell proliferation rates, a cell proliferation assay was performed using CellTiter 96® AQueous One Solution Reagent (Promega, Madison, US). Third passage neurospheres were dissociated using the same procedure as for cell passaging. Resuspended single cells were counted using a haemocytometer (Neubauer, Germany) and plated at 1000 cells/well in 100µl volume on poly-L-

ornithine (Sigma-Aldrich) and fibronectin (Gibco) coated 96-well flat transparent culture dish in neurosphere medium. The cells for each assay run were plated from the same batch of cells to maintain plating homogeneity. The cells were pre-cultured for 3 days prior to addition of treatment solution in neurosphere medium for 3 days. CellTiter 96® AQueous One Solution reagent was added according to manufacturer's instructions and incubated at 37ºC. After 1 hour incubation, the first reading was taken using a spectrophotometric plate reader (Infinite M200, Tecan) at 490nm with reference wavelength at 630nm. Subsequently second, third and fourth readings were taken every 1 hour incubation at 37ºC.

The cells were treated with (±)-8-Hydroxy-2-dipropylaminotetralin hydrobromide (8-OH-DPAT; 0.1μM, 1μM, 2.5μM, 5μM; Tocris, Missouri, USA); (RS)-trans-8-Hydroxy-2-[N-n-propyl-N-(3'-iodo-2'-propenyl)amino]tetralin oxalate (8-OH-PIPAT; 0.1μM, 1μM, 2.5μM, 5μM; Tocris), (2S)-(+)-5-(1,3,5-Trimethylpyrazol-4-yl)-2-(dimethylami no)tetralin (AS-19; 0.1μM, 1μM, 2.5μM, 5μM; Tocris); 1-(2,3-Dihydro-1,4-benzodioxin-5-yl)-4-(2,3-dihydro-1H-inden-2-yl)-piperazine (S15535; 1nM, 10nM, 100nM, 1μM, 10μM, 100μM; Sigma-Aldrich).

4.2.3. Statistical Analysis

Each drug treatment was repeated using 3 independent batches of cells obtained from different pregnant mice with triplicate samples each. Mean absorbance computed, which was linear to the cell number, was expressed as a ratio to the

control without treatment and the SEM is calculated from three independent biological repeats. The treated samples were compared with the control and analyzed using Student's t-test with $P<0.05$ was considered as statistically significant. All data are reported as mean ± SEM.

4.2.4. In vivo BrdU cell proliferation assay

Adult male C57BL/6 mice aged 6 to 8 weeks were group-housed with free access to food and water (12 hr light/dark cycles). The mice were given 5-7 days of habitat acclimatization before application of the drug treatment regime. The mice were treated with 8-OH-DPAT (4hr at 1mg/kg; Tocris); fluoxetine (14 days at 5mg/kg/day; Tocris) and 8-OH-PIPAT (3 days and 14 days at 1mg/kg/day; Tocris). Saline (vehicle control) or treatment drugs were intraperitoneally administered for the duration stipulated in the results section. After the treatment period, the animals were injected with 300mg/kg of 5-Bromo-2-deoxyuridine (BrdU; Sigma-Aldrich) 2 hours prior to sacrificing by anaesthetic overdose and transcardinal perfusion with 4% paraformaldehyde. The extracted brains were post-fixed overnight in ice cold 4% paraformaldehyde before paraffin embedding and microtome sectioning. Six µm brain slices were collected from the hippocampus and attached to 1.5% gelatin coated slides. The sections were then first permeabilized and treated with 4M HCl and trypsin (1 mg/ml in PBS, 10 min, 37˚C) before blocking and detection using anti-BrdU antibody (1:100; Neomarkers, Fremont, CA). Secondary detection was done according to

manufacturer's instruction using Vectastatin® Elite ABC kit (Vector Laboratories). One section was counted for every 30μm for the whole hippocampus and the total number of BrdU positive cells was expressed as a ratio against the total perimeter of the dentate gyrus of the hippocampal sections. The mean of three mice treated with drugs was compared with the mean of three mice treated with the saline using Student's t-test with $P<0.05$ considered as significant. All data are reported as mean ± SEM.

4.3. Results

4.3.1. 5-HT1A and 5-HT7 receptors were expressed on NSPCs

In Section 3.3.3, the 5-HT1A and 5-HT7 receptors were found to be expressed on the NSPCs at the RNA level. To further confirm the presence of the 5-HT1A and 5-HT7 receptors at the protein level, we conducted immunocytochemistry on NSPCs using double immunostaining. Double immunocytochemistry also verified that both 5-HT1A and 5-HT7 receptors can be found on the same NSPCs (Fig. 4.1).

4.3.2. Acute administration of the 5-HT1A/5-HT7 receptor agonist, 8-OH-DPAT, but not the selective 5-HT1A receptor agonist, 8-OH-PIPAT, increased cell proliferation in vitro and in vivo

To determine whether the activation of 5-HT1A receptor is the main effect that drives the increase in NSPC proliferation, we study the effects of 8-OH-DPAT and 8-OH-PIPAT on NSPC proliferation. Cell proliferation assay was conducted on cultured hippocampal and SVZ NSPCs treated with either 8-OH-DPAT or 8-OH-PIPAT. Results showed that 8-OH-DPAT was able to bring about a significant increase in NSPC proliferation upon a 3 day acute treatment for SGZ NSPC, but this was not the case for 8-OH-PIPAT (Fig. 4.2).

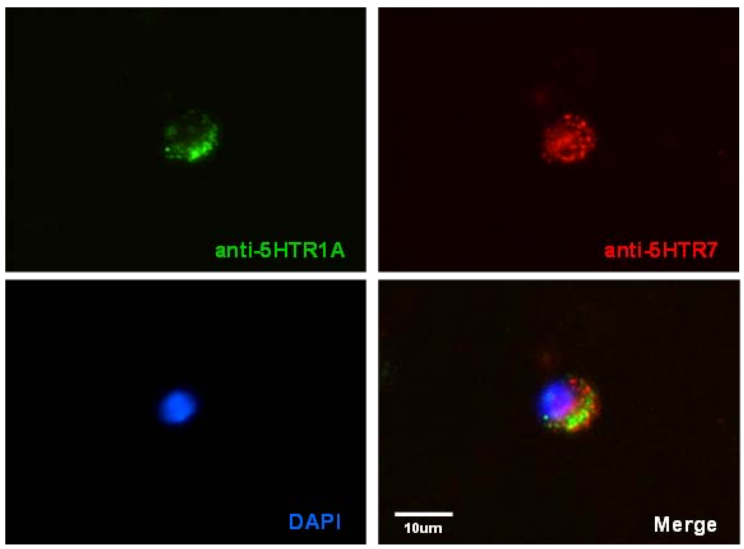

Figure 4.1. Expression of 5-HT1A and 5-HT7 receptors on NSPCs. Double immunocytochemistry of 5-HT1A and 5-HT7 receptors was conduced using anti-5-HT1A and anti-5-HT7 receptor antibodies to detect the presence of the receptors on the same NSPC. The scale bar indicates 10μm.

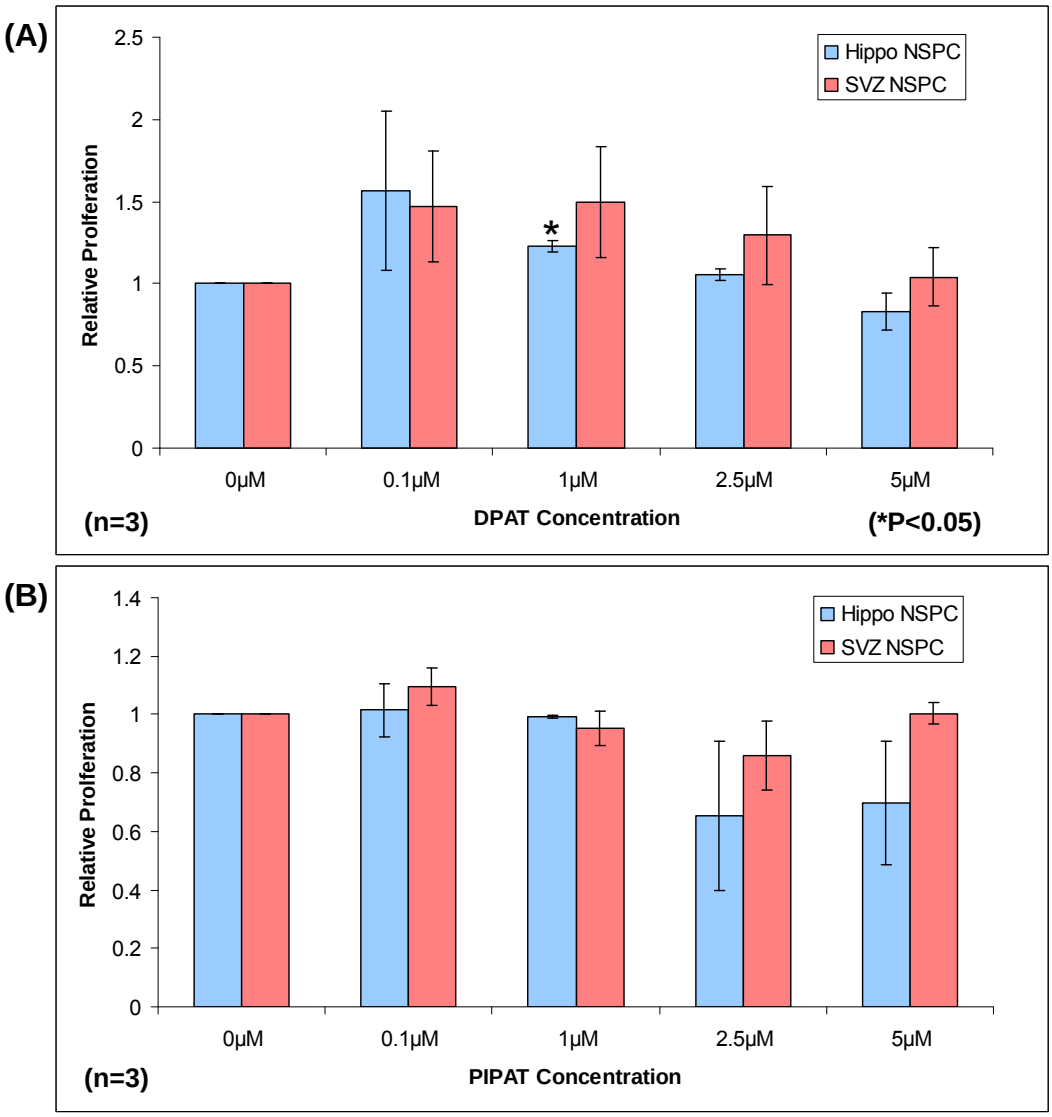

Figure 4.2. Treatment of cultured NSPCs with (A) 8-OH-DPAT and (B) 8-OH-PIPAT. Cultured NSPCs from the hippocampus (Hippo) and subventricular zone (SVZ) were treated with the 5-HT1A/7 receptor agonist, 8-OH-DPAT, and the specific 5-HT1A receptor agonist, 8-OH-PIPAT, before being subjected to CellTitre 96 AQueous cell proliferation assay. The relative proliferation rates were expressed as a ratio against the control.

To further ascertain that this effect was replicable *in vivo*, we treated the mice with acute treatment of either 8-OH-DPAT or 8-OH-PIPAT. Our results show that acute administration (6 hr) of 8-OH-DPAT is able to increase cell proliferation in NSPCs of mice (Fig. 4.3). However, 8-OH-PIPAT, a specific agonist to the 5-HT1A receptor, failed to increase cell proliferation in the acute treatment (3 days; Fig. 4.4). However, 8-OH-PIPAT is able to increase cell proliferation to similar levels as fluoxetine on chronic 14 day treatment in mice (Fig 4.4).

4.3.3. The 5-HT7 receptor specific agonist, AS-19, can increase NSPC proliferation in vitro

The failure of selective 5-HT1A receptor agonist 8-OH-PIPAT to increase NSPC proliferation upon acute treatment, suggests that 8-OH-DPAT may have acted at the 5-HT7 receptor in the study by Banasr et al. (2004). This suggests the likelihood that the activation of the 5-HT7 receptor will increase NSPC proliferation, therefore a 5-HT7 receptor agonist, AS-19, was tested on the cultured NSPCs. The treatment of cultured NSPCs with AS-19 shows a significant increase in NSPC proliferation on acute treatment for 3 days (Fig. 4.5).

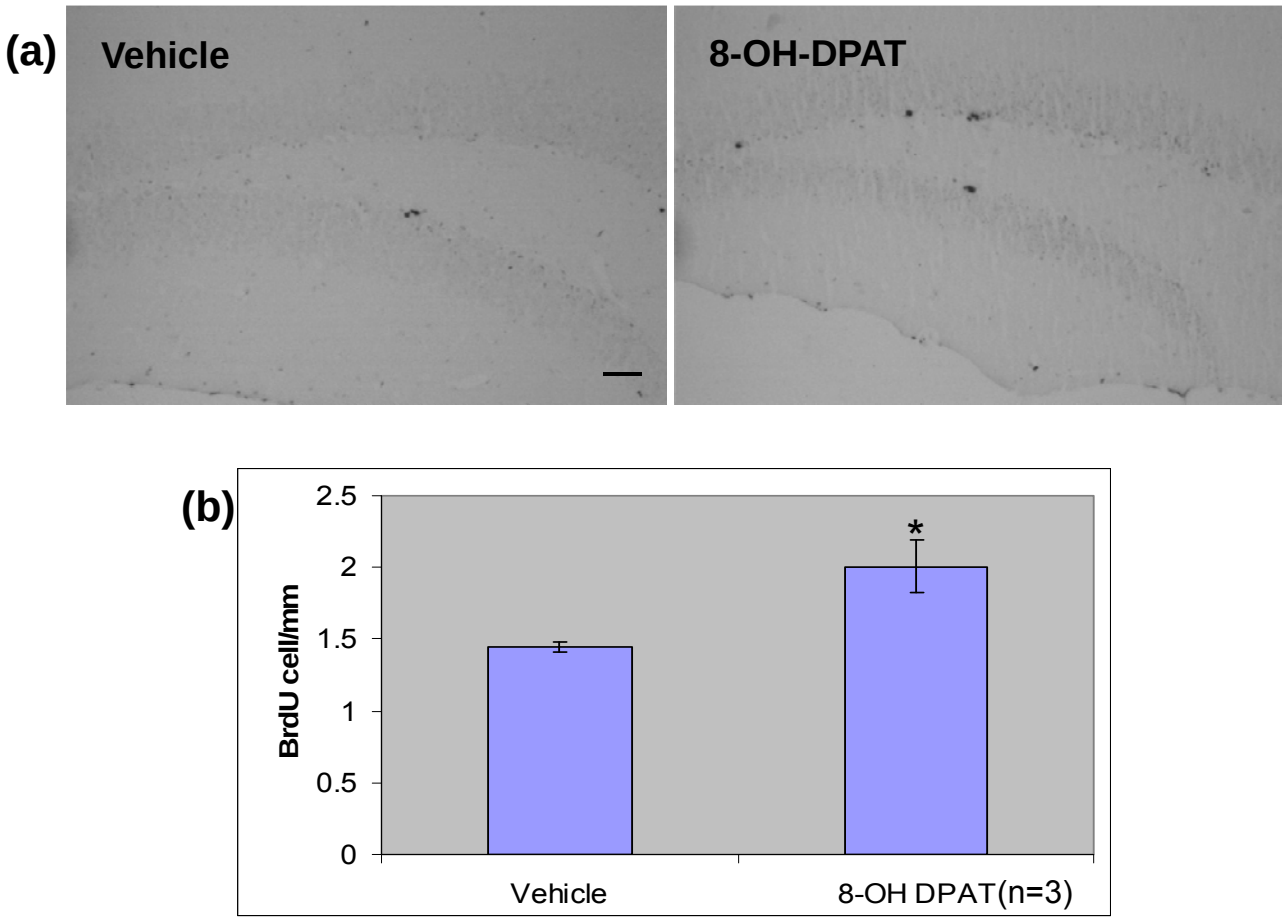

Figure 4.3. Treatment of mice with a mixed 5-HT1A and 5-HT7 receptor agonist. (a) Mice were injected with vehicle or 8-OH-DPAT (n=3 for each treatment). After 4 hours, the mice were injected with BrdU and perfused another 2 hours later. The brains were embedded in paraffin and sectioned at 6μm throughout the entire hippocampus on a microtome. Every 5th section was selected for immunostaining with anti-BrdU antibody. **(b)** The total number of BrdU immunoreactive cells was expressed relative to the length of the perimeter of the subgranular zone. The scale bar represents 100μm. (*P<0.05)

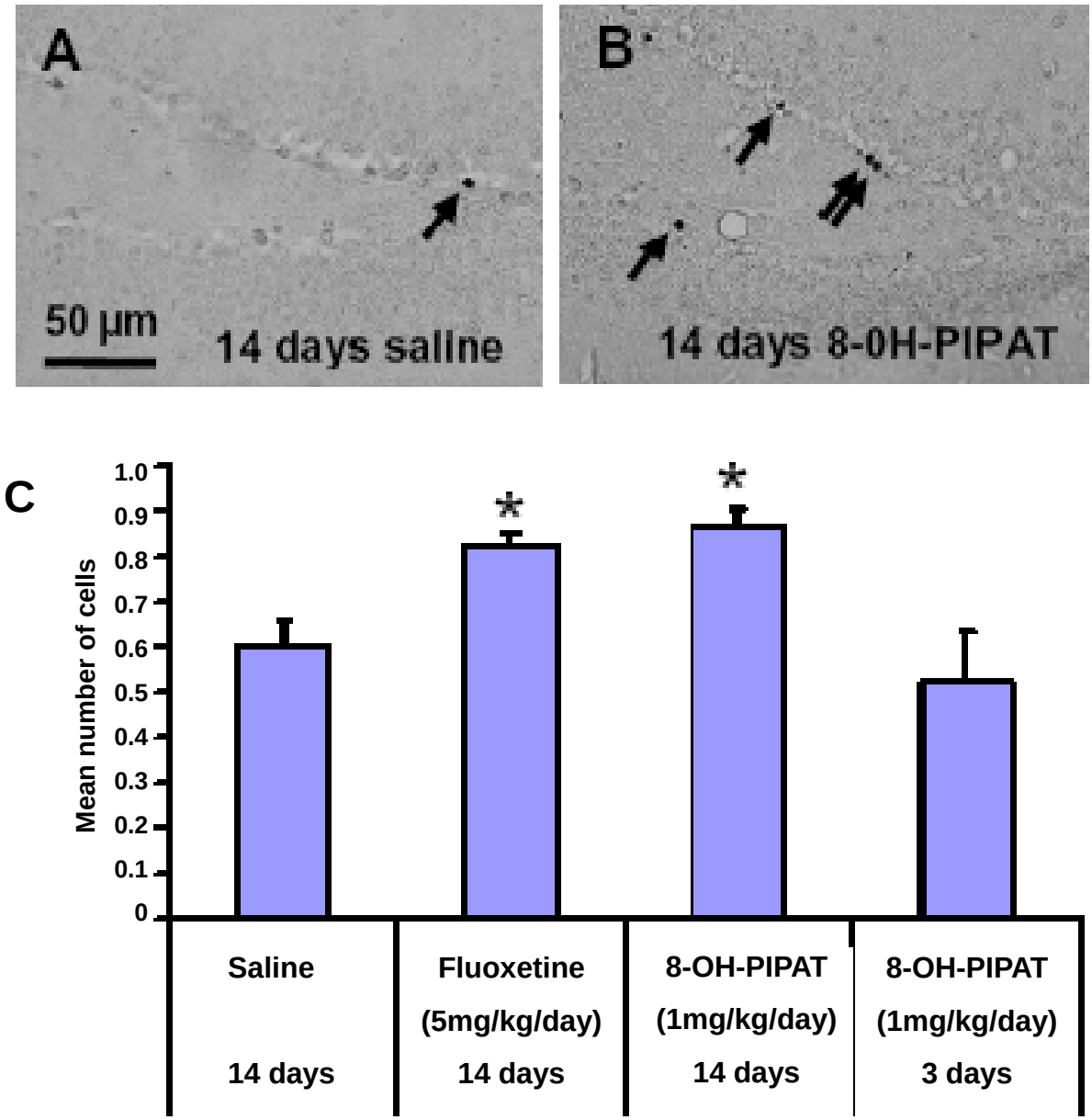

Figure 4.4. Chronic but not acute treatment with a 5-HT1A receptor agonist can increase NSPC proliferation in the dentate gyrus. Four mice for each group were injected with vehicle, fluoxetine or 8-OH-PIPAT for a duration of either 3 days or 14 days, after which the mice were injected with BrdU and perfused another 2 hours later. BrdU was detected by immunohistochemistry and the total number of BrdU immunoreactive cells was expressed relative to the length of the perimeter of the subgranular zone. This work is done by Ou L and Dawe GS (Tan *et. al.,* 2007, Poster at Neuroscience 2007).

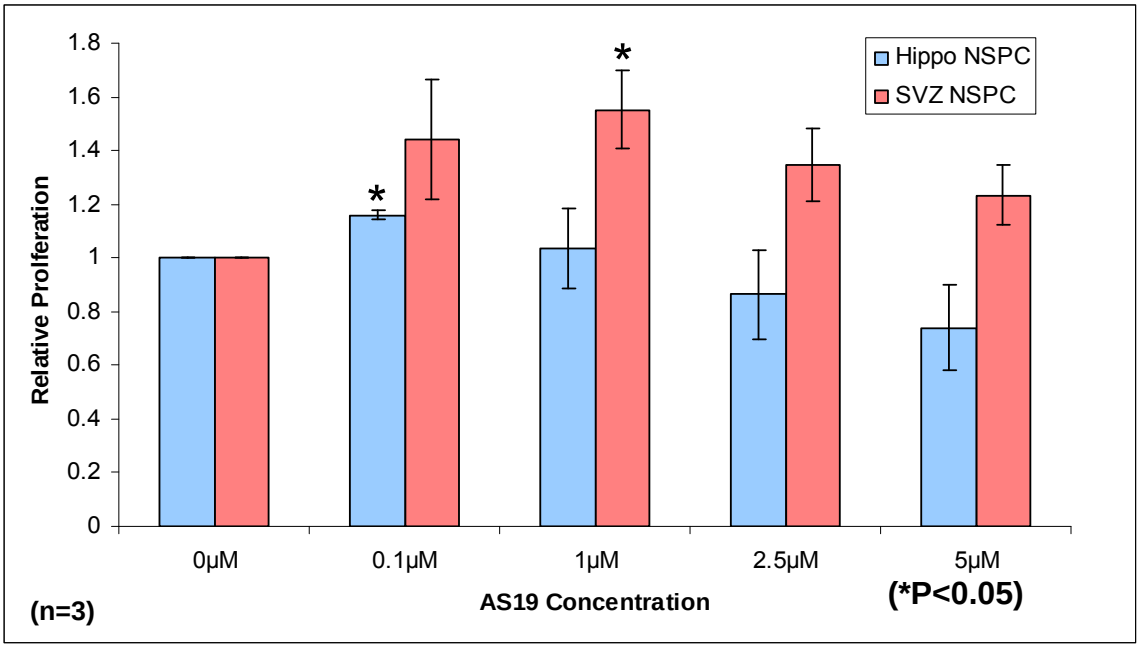

Figure 4.5. Treatment of cultured NSPCs with the selective 5-HT7 receptor agonist, AS-19. Cultured NSPCs from the hippocampus (Hippo) and subventricular zone (SVZ) were treated with the 5-HT7 receptor agonist, AS-19, for 3 days before being subjected to CellTitre 96 AQueous cell proliferation assay. The relative proliferation rates were expressed as a ratio against the control.

4.3.4. The 5-HT1A autoreceptor may also be a target for induction of NSPC proliferation

To determine whether the activation of the 5-HT1A autoreceptor could mediate an increase in NSPC proliferation, cultured NSPCs were treated with S15535. Results of our studies showed that activation of the 5-HT1A autoreceptor using S15535, which is a 5-HT1A autoreceptor specific agonist and a post-synaptic 5-HT1A receptor antagonist, was able to bring about an increase in cell proliferation, however the increasing trend is not statistically significant ($P>0.1$; Fig. 4.6).

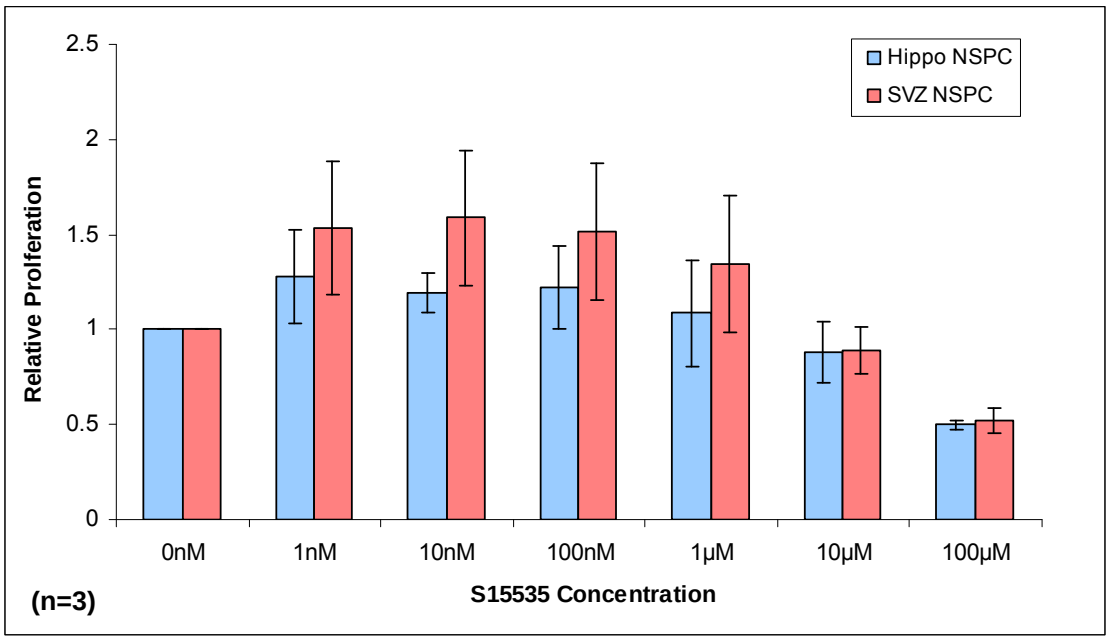

Figure 4.6. Treatment of cultured NSPCs with the 5-HT1A autoreceptor agonist/postsynaptic antagonist, S15535. Cultured NSPCs from the hippocampus (Hippo) and subventricular zone (SVZ) were treated with the 5-HT1A autoreceptor agonist/postsynaptic antagonist, S15535, for 3 days before being subjected to CellTitre 96 AQueous cell proliferation assay. The relative proliferation rates were expressed as a ratio against the control.

4.4. Discussion

NSPCs may be able to receive serotonin signals from the serotonin receptors they expressed, as was suggested in this chapter by investigation of the 5-HT1A and 5-HT7 receptors. Previous papers have suggested that 5-HT1A receptor activation by 8-OH-DPAT can bring about an increase in NSPC proliferation *in vivo* (Banasr *et al.*, 2004). However, 8-OH-DPAT also has moderate activity at the 5-HT7 receptor (Wood *et al.*, 2000). Our results shown that the specific 5-HT1A receptor agonist, 8-OH-PIPAT, was unable to increase the NSPC proliferation on acute treatment both *in vitro* and *in vivo*. Only upon 14 days of chronic treatment was 8-OH-PIPAT is able to produce an increase in NSPC proliferation to a similar level as chronic treatment with the antidepressant, fluoxetine. We also showed that by directly activating the 5-HT7 receptor using a specific agonist, AS-19, with 3-day acute treatment, we were able to increase NSPC proliferation *in vitro*. As the 5-HT1A receptor may also be an autoreceptor, the NSPC was treated with S15535 which activates 5-HT1A autoreceptor at the same time blocked the 5-HT1A postsynaptic receptor. An increasing trend in NSPC proliferation was observed, but further test needs to be conducted as the increase is not statistically significant.

As the 5-HT1A receptor was one of the first serotonin receptors studied for its activation of NSC proliferation *in vivo*. Banasr *et al.* (2004) suggested that NSPC proliferation can be increase with the acute 5-HT1A receptor activation by 8-OH-

DPAT. In our experiments, it was observed that the NSPC expressed 5-HT1A receptors. However, in our experiments we failed to increase NSPC proliferation when we acutely treated the cultured NSPCs or the mice with 8-OH-PIPAT. However, upon repeating the experiments with 8-OH-DPAT, the treatment was able to increase the NSPC proliferation. As 8-OH-DPAT is a partial agonist at the 5-HT7 receptor, therefore we suspect that the mode of action of 8-OH-DPAT in modulating NSPC proliferation may be through the 5-HT7 receptor. The presence of 5-HT7 receptors expressed on NSPCs further supports the feasibility of this view.

Examining the functional difference between the 5-HT1A and 5-HT7 receptors suggest that both the receptors mediate very different responses in cellular signaling. The 5-HT1A receptor is suggested to be coupled negatively to adenylate cyclase through Gαi/αo protein whereas the 5-HT7 receptor activation stimulates adenylate cyclase through the Gαs protein (Albert *et al.*, 1996; Bard *et al.*, 1993). It has been reported that stimulation of adenylate cyclase increases cAMP levels and cAMP has been implicated in increasing cell proliferation through the activation of ERK. This cAMP mediated cell proliferation has so far always been mediated by GPCRs coupled with Gαs and not others, further suggesting that activation of 5-HT7 receptor is mediating the increase in NSC proliferation (Stork and Schmitt, 2002). Indeed, the 5-HT7 receptor activation has been shown to increase adenylate cyclase activity through the cAMP-GEF pathway to activate ERK (Lin *et al.*, 2003).

Furthermore, activation of the 5-HT1A receptors has been suggested to inhibit the release of 5-HT. This is likely to be via the effects of activation of the 5-HT1A autoreceptors (Sharp and Hjorth, 1990). However, our experiments suggest that the 5-HTR1A autoreceptor agonist, S15535, may be capable in increasing the NSPC proliferation, however not statistically significant. This could be due to the mixed effect of acting partially as a 5-HT1A postsynaptic antagonist at the same time the activation of the autoreceptors. Further test could be conducted on the blockade of the 5-HT1A postsynaptic receptors to delineate the effects caused by S15535. Moreover, the presence of the 5-HT1A autoreceptor may also suggest the possibility that NSPCs are capable of synthesizing and releasing 5-HT.

One of the possible mechanisms of agonist-induced NSPC proliferation is that the activation of the receptor may cause the release of growth factors from other neuronal cell types. It has been noted that activation of 5-HT1A receptor cause astrocytes to release S-100, which is a growth factor (Whitaker-Azmitia *et al.,* 1990). Although, the S-100 protein has not been implicated in NSPC proliferation, it has been implicated in the proliferation of Schwann cells (Xu *et al.*, 2009). As such, we cannot discount the fact that the other surrounding cell types may release other growth factors which promote the NSPC proliferation. However, most of the experiments are done on cultured NSPCs which allow the elimination of the effects from non-NSPC cell types, thereby confirming the fact

that the increase in proliferation is induced purely by the activation of the receptors expressed on the NSPCs.

In conclusion, our *in vitro* and *in vivo* data suggest that the activation of the 5-HT7 receptor, but not the activation of the postsynaptic 5HT1A receptor that was previously suggested, can bring about the increase in the NSPC proliferation.

5. 5-HT3 RECEPTOR – PROSPECTS OF 5-HT ACTIVATED CURRENTS AFFECTING NSPC PROLIFERATION

5.1 Introduction

The 5-HT3 receptor is the only subtype of 5-HT receptor that is an ion channel and not a G-protein coupled receptor as the case for all the other subtypes of 5-HT receptor. Being a ligand gated ion channel permeable to sodium and calcium ions, upon activation by 5-HT, it could be capable of depolarizing the membrane, which would bring about action potentials within the NSPCs and may also bring about other cellular and physiological changes. The 5-HT3 receptors in the CNS are implicated in functions such as emesis, cognition and anxiety. 5-HT receptor immunoreactivity is also very abundant postsynaptically in the hippocampus (Miquel *et al.*, 2002).

As shown in the results presented in Chapter 3, both 5-HT3A and 5-HT3B receptors are found to be expressed in the NSPCs. Together with their implication in anxiety and depression, this expression profile makes the 5-HT3 receptors a suitable target for the study of activation of NSPC proliferation. Several antidepressants have been known to act as antagonists at the 5-HT3 receptor and to block its Na^+ and Ca^+ currents (Eisensamer *et al.*, 2003). Several of these antidepressants are also capable of inducing an increase in NSPC

proliferation (Santarelli *et al.*, 2003); suggesting the possibility that 5-HT3 receptors can be a target of modulation of NSPC proliferation.

In this study, the role of the 5-HT3 receptor in the NSPC proliferation will be examined. The expression and function of 5-HT3 receptors in NSPCs will be investigated first. As antidepressants that act as 5-HT3 receptor antagonists are at the same time able to activate the NSPC proliferation, the possibility of 5-HT3 blockade inducing the increase in NSPC proliferation will also be examined.

5.2. Materials and Methods

5.2.1. Immunocytochemistry of NSPCs

To determine the expression of 5-HT3 receptor in NSPCs, dissociated NSPCs were plated on poly-L-ornithine (PLO) and fibronectin (FN) coated coverslips and grown overnight. The cells were then fixed by treatment with 4 % paraformaldehyde for 20 minutes followed by permeabilization using PBS with 0.1% Triton X-100. Immunostaining was conducted using anti-5-HT3 receptor antibody (Santa Cruz, CA, USA) and anti-Sox2 antibody (1:1000; Chemicon, CA, USA) in 10% donkey serum in PBS overnight at room temperature. Fluorescence-conjugated Alexa Fluor secondary antibodies (Invitrogen) were used to visualize the primary antibodies and the coverslips were counterstained with DAPI. The differentiated cells were then imaged by sequential scanning with a confocal microscope (LSM 510, Carl Zeiss Microimaging GmbH).

5.2.2. Electrophysiological recordings of 5-HT3 activated currents in NSPCs

The 5-HT3 receptor activated currents were recorded in the whole cell configuration from cultured NSPCs plated on PLO and FN coated coverslips and grown for at least 3 days to allow recovery from trypsinization and cell growth. Whole-cell voltage clamp configuration was conducted with the membrane potential clamped at -10mV as the resting potential of the NSPCs in current

mode was -7.57±1.21 mV (n=79). The currents were recorded with borosilicate pipettes with resistances of 2–6 MΩ which were pulled from borosilicate glass capillaries (Boralex) with a Flaming Brown micropipette puller (P-81 Sutter Instrument Co., CA, USA). The pipettes were filled with an internal solution containing (in mM):130 KCl, 1 $MgCl_2$, 5 EGTA, 10 HEPES, 2 K-ATP with the solution adjusted to pH 7.2 and to 290 mOsm with sucrose. The standard external solution contained (in mM): 150 NaCl, 5 KCl, 1.1 $MgCl_2$, 2.6 $CaCl_2$, 10 HEPES, 10 glucose, with the solution adjusted to pH 7.4 and to 310 mOsm. NPCs were clamped using a MultiClamp 700A amplifier (Molecular Devices Corp., Sunnyvale, CA, USA) in conjunction with a Digidata 1322A interface (Axon Instruments, Union City, CA, USA) at a holding potential of –10 mV. Currents were recorded and analyzed using pCLAMP 9.2 software (Molecular Devices Corp).

5.2.3. Cell proliferation assay of 5-HT3 receptor agonist- and antagonist-treated NSPCs

To determine whether activation or blockade of the 5-HT3 receptors isable to influence the cell proliferation rate, a cell proliferation assay was performed using CellTiter 96® AQueous One Solution Reagent (Promega). Third passage neurospheres were dissociated using the same procedure as for cell passaging (Chapter 2, Section 2.2.2). Resuspended single cells were counted using a haemocytometer (Neubauer, Germany) and plated in neurosphere medium at

1000 cells/well in a 100 µl volume on poly-L-ornithine (Sigma-Aldrich) and fibronectin (Gibco) coated 96-well flat transparent culture dishes. The cells for the assay were plated from the same batch of cells to maintain plating homogeneity. The cells were pre-cultured for 3 days prior to addition of treatment solutions in neurosphere medium for 3 days. CellTiter 96® AQueous One Solution reagent was added according to the manufacturer's instructions and incubated at 37ºC. After 1 hour incubation, a first reading was taken using a spectrophotometric plate reader (Infinite M200, Tecan) at 490nm with the reference wavelength at 630nm. Subsequently second, third and fourth readings were taken each hour under continued incubation at 37ºC.

The cells were treated with the agonists 1-(3-Chlorophenyl)biguanide hydrochloride (m-CPBG; 0.05uM, 0.5uM, 5uM, 50uM) and 1-(6-Chloro-2-pyridinyl)-4-piperidinamine hydrochloride (SR57227; 0.05uM, 0.5uM, 5uM, 50uM) and the antagonists *N*-(1-Azabicyclo[2.2.2]oct-3-yl)-6-chloro-4-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazine-8-carboxamide (Y-25130; 0.05uM, 0.5uM, 5uM, 50uM) and Tropanyl 3,5-dichlorobenzoate (MDL72222; 0.05uM, 0.5uM, 5uM, 50uM). The drugs were purchased from Tocris Biosciences (Tocris, Missouri, USA). The concentrations chosen were in the range reported in other literature: m-CPBG (Lee *et al.,* 2005; Turner *et al.,* 2004); SR57227 (Edwards *et al.,* 1996); Y-25130 (Haga *et al.,* 1993) and MDL72222 (Middlemiss *et al.*, 1992).

5.2.4. Statistical Analysis

Each drug treatment was repeated using three independent batches of cells obtained from different pregnant mice each with triplicate samples. Mean absorbances were computed and one-way ANOVA was performed to find any differences between the drug concentration within the biological repeats. If there were any significant differences, Dunnett's statistics was computed to compare each concentration with the control.

5.2.5. In vivo BrdU cell proliferation assay

Adult male C57BL/6 mice of 6 to 8 weeks were group-housed with free access to food and water (12 hr light/dark cycles). The mice were given 5-7 days of habitat acclimatization before the drug application regime. Saline (vehicle control) or 1mg/kg Y-25130 were intraperitoneally administered once daily for a period of 14 days. After 14 days, the animals are injected with 300mg/kg BrdU (Sigma-Aldrich) 2 hr prior to sacrifice. The mice were then transcardial perfused with 4% paraformaldehyde. The extracted brains were post fixed overnight in ice cold 4% paraformaldehyde before equilibration in 30% sucrose in PBS for cryoprotection prior to sectioning. Twenty μm brain slices were collected from the hippocampus and attached to 1.5% gelatin coated slides. The sections were then first permeabilized and treated with 4M HCl and trypsin (1 mg/ml in PBS, 10 min, 37˚C) before blocking and detection using anti-BrdU antibody (1:100;

Neomarkers, Fremont, CA). Secondary detection was done according to manufacturer's instruction using Vectastatin® Elite ABC kit (Vector Laboratories). Three representative sections for each region were selected and the total number of BrdU-positive cells was expressed as a ratio against the total perimeter of the dentate gyrus of the hippocampal sections. The mean of three mice treated with Y-25130 was compared with the mean of three mice treated with the saline control using Student's t-test at 95% significance. All data are reported as mean ± SEM.

5.3. Results

5.3.1. NSPCs expressed 5-HT3 receptors

In Chapter 3, the results indicated that NSPCs expressed 5-HT3A and 5-HT3B receptors at the RNA level. The expression of 5-HT3 receptors at the protein level was further confirmed by immunocytochemistry using anti-5-HT3 receptor antibodies that were not specific to any of the 5-HTR3 subtypes. Results show that the 5-HT3 receptor was expressed in NSPCs which also co-expressed the NSPC marker, Sox2 (Fig. 5.1).

5.3.2. Functional study of the 5-HT3 receptor using patch clamp analysis

As the 5-HT3 receptor functions as a ligand-gated ion channel, to determine whether the 5-HT3 receptor expressed on the NSPCs were functional, patch clamp analysis was conducted to assess the receptor channel function in the NSPCs (with the help from Dr Li Shao and Ms Deng Hongmin) (Fig 5.2). The resting potential of stem cells examined in the current clamp mode was -7.57±1.21 mV (n=79), therefore the holding potential of whole cell recording was set at -10mV. The majority of the examined cells (56.25%, 9/16) were sensitive to 5-HT, which typically induced outward currents. 43.75% (7/16) of the cells were insensitive to 5-HT. The cells sensitive to 5-HT were also activated by SR57227, which is a 5-HT3 receptor specific agonist, and outwards currents were induced.

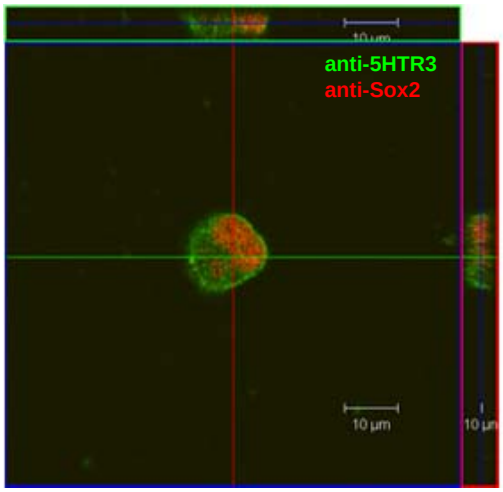

Figure 5.1. Expression of 5-HT3 receptors in NSPCs. Immunocytochemistry for 5-HT3 receptors was conducted using an anti-5HT3 receptor antibody which targets both 5-HT3A and 5-HT3B receptors. The cells were colabelled with anti-Sox2 antibody which is a NSPC marker. The scale bar represents 10µm.

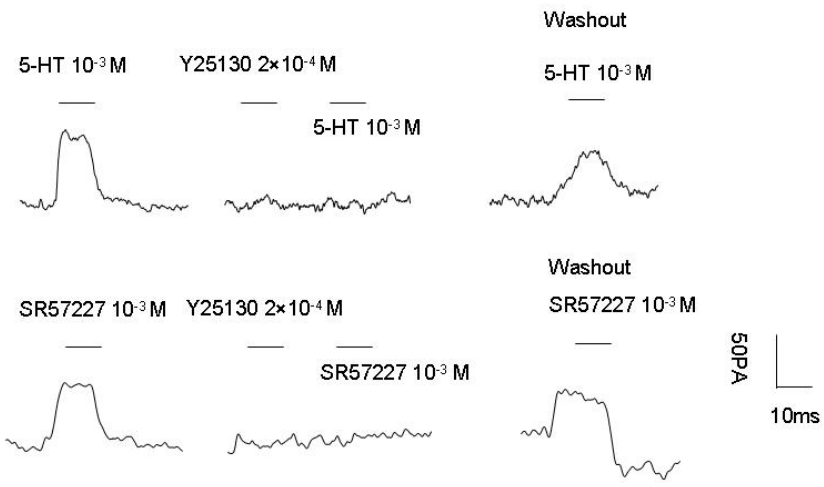

Figure 5.2. 5-HT3 receptor currents recorded from the NSPCs upon activation of the receptor by 5-HT or the agonist SR57227. Both 5-HT-activated currents and SR57227-activated currents could be completely blocked by the specific 5-HT3 receptor antagonist Y25130.

The average of 5-HT (1mmol/L) -activated currents was 44.32±19.6pA. Both 5-HT-activated currents and SR57227-activated currents could be completely blocked by Y25130 (200μmol/L), which is specific 5-HT3 receptor antagonist.

5.3.3. 5-HT3 receptor agonists and antagonists on NSPC proliferation in vitro

To identify whether the 5-HT3 receptor played a role in the neural progenitor proliferation, the cultured NSCs were treated with 5-HT3 receptor agonists (m-CPBG and SR57227) and antagonists (Y-25130 and MDL 72222). Treatment with the agonist, m-CPBG, was able to significantly increase the cell proliferation rate at concentrations of 0.05μM and 5μM whereas another agonist, SR57227, failed to increase the cell proliferation rate (Fig. 5.3A and B). Treatment with the antagonists, Y-25130 and MDL72222, was able to significantly increase cell proliferation at 0.5μM and 0.05μM respectively (Fig. 5.3C and D). Higher concentrations caused the NSPCs to decrease cell proliferation suggesting that high concentration of SR57227, Y-25130 and MDL72222 might be toxic to the cells (Fig. 5.3B, C and D).

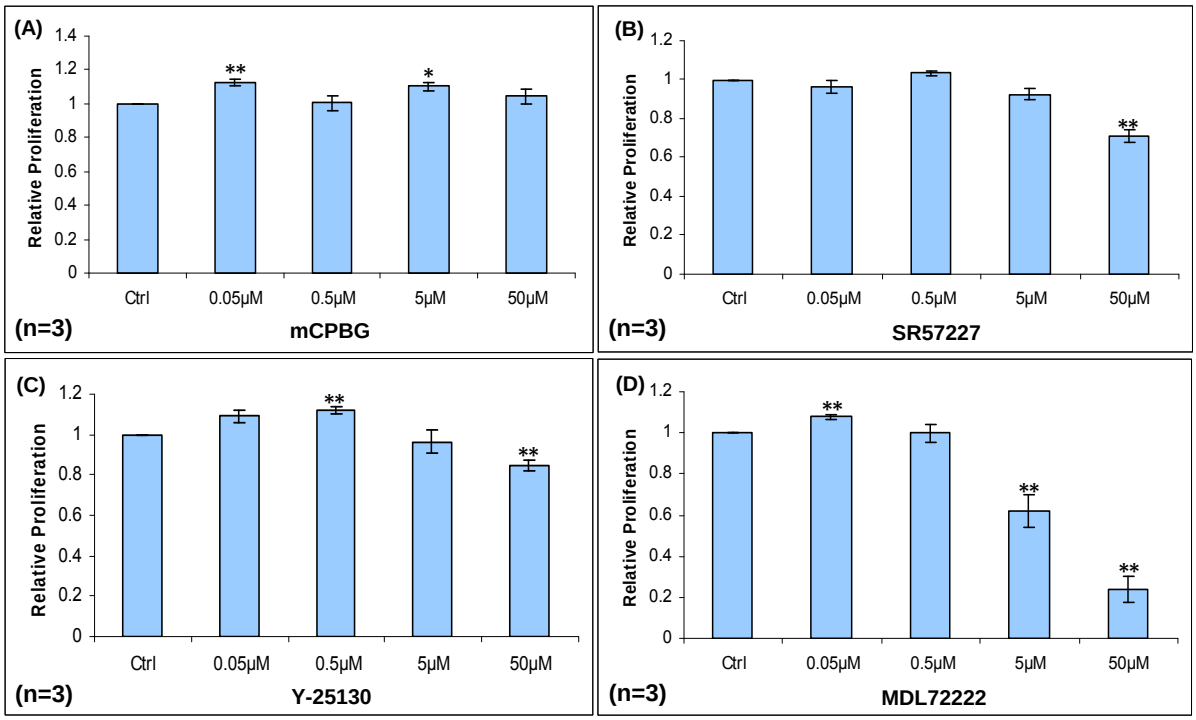

Figure 5.3. Cell proliferation assay of hippocampal NSPCs treated with 5-HT3 receptor agonists and antagonists. NSPCs were treated for 3 days with various concentrations of 5-HT3 receptor agonist (A) mCPBG and (B) SR57227, and antagonist (C) Y-25130 and (D) MDL72222 before being assayed for changes in cell proliferation using CellTiter 96 Aqueous One cell proliferation assay. Results were normalised within the assay groups and the relative proliferation of three biological repeats were plotted and analysed. For data where ANOVA shows significant difference (95% confidence), Dunnett's tests against control were performed. The values are expressed as mean ± SEM. (*$p<0.05$, **$p<0.01$)

5.3.4. The 5-HT3 receptor antagonist, Y-25130, is able to induce an increase in NSPC proliferation in vivo

Since treatment with 5-HT3 receptor antagonists was consistently able to increase NSPC proliferation *in vitro*, an assay to determine whether one of the antagonists Y-25130 was also able to increase cell proliferation in mice *in vivo* was performed. To assay whether the blockade of 5-HT3 receptor can also change the NSPC proliferation *in vivo*, mice were treated for 14 days with Y-25130 and compared with the saline-treated control mice. The Y-25130 treated mice show a significant increase in the BrdU incorporation in the dividing NSPCs as compare to control, indicating an increase in the cell proliferation rate due to induction by the specific blockade of 5-HT3 receptors (Fig. 5.4).

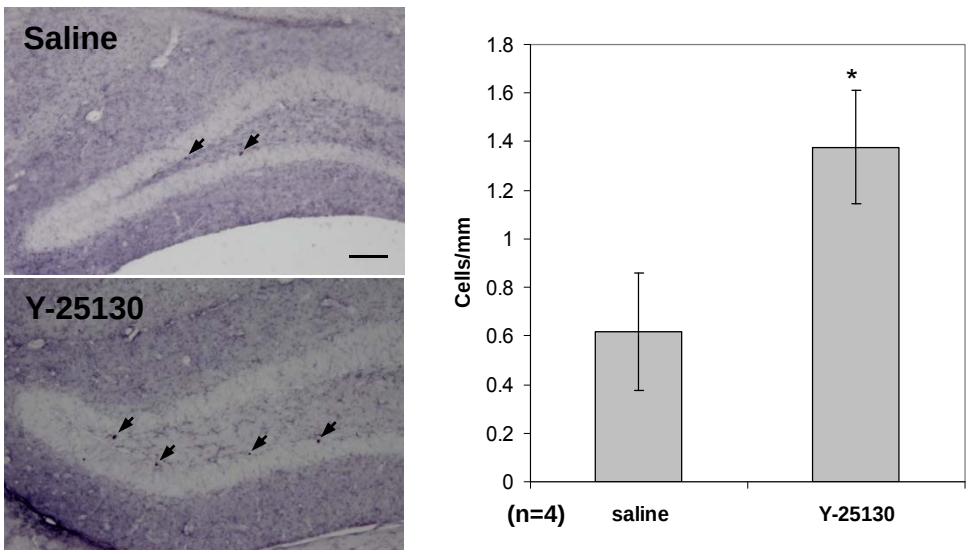

Figure 5.4. BrdU cell proliferation assay in mice treated with the 5-HT3 receptor antagonist Y-25130. Four mice for each group were intraperitoneally injected with either saline or Y-25130 (1mg/kg) daily for a period of 14 days. After that, they were subjected to BrdU cell proliferation assay. The number of BrdU immunopositive cells were expressed relative to the length of perimeter of the dentate gyrus. Students t-test analysis was conducted on the mean of the three biological replicates. The values are expressed as mean ± SEM. (*$p<0.05$). The scale bar represents 200μm.

5.4. Discussion

The results presented show that NSPCs expressed 5-HT3 receptors. These channels are functional and are able to produce 5-HT-induced currents activated both by 5-HT and also by a specific 5-HT3 receptor agonist, SR57227. These currents can be blocked by the application of the 5-HT3 receptor specific antagonist, Y-25130. Application of the 5-HT3 receptor antagonists, Y-25130 and MDL72222, increased cell proliferation of cultured NSPCs, however application of the agonists, mCPBG and SR57227, gave variable results. One of the 5-HT3 receptor antagonists tested, Y-25130, was also shown to be able to increase the NSPC proliferation rate after 14 day treatment of mice *in vivo*.

Interest in the ability of 5-HT reuptake inhibitor to increase neurogenesis in NSPCs has lead to interest in the various 5-HT receptors expressed on the NSPCs. In this chapter, the expression of the 5-HT3 receptor in mouse NSPCs was examined. The mRNA expression studies discussed in Chapter 3 were limited to the 5-HT3A and 5-HT3B receptor subtypes as no other 5-HT3 receptor subtypes have been identified in rodents (Brady *et al.,* 2007). However, in humans, there are reports of another three different subtypes of 5-HT3 receptor, namely 5-HT3C, 5-HT3D and 5-HT3E receptors (Niesler *et al.*, 2003; Karnovsky *et al.*, 2003). Our microarray analysis of human fetal NSPCs also shows that besides 5-HT3A and 5-HT3B receptors, human fetal NSCs also expressed the 5-HT3D receptor subtype (unpublished data). 5-HT3A receptors are able to form

pentaheteromeric ion channel pores with either the 5-HT3B or 5-HT3D receptor subunit. The 5-HT3A receptor's ability to form homomeric and heteromeric complexes enables change in the mechanisms underlying desensitization, sensitivity to endogenous agonist and time required for resensitization (Hapfelmeier *et al.*, 2003).

The 5-HT3 receptor is the only ion channel in the 5-HT receptor family. The expressed 5-HT3 receptors on the NSPCs are functional channels. Being ligand-gated ion channels, they are able to be activated by 5-HT and specific agonists to allow transmembrane currents. In our investigation, we found that the currents were only detected in 56.25% (9/16) of cells patched. This suggests that the expression of the 5-HT3 receptors on the NSPCs may be dynamic. A complete blockade of 5-HT currents was also observed when the 5-HT3 antagonist, Y-25130, was applied suggesting that some NSPCs might only functionally express 5-HT3 receptors. As NSPCs in culture are asynchronized and each cell may be at a different cell cycling stage at any moment, this suggests that the expression or function of 5-HT3 receptors may change at different stages of the cell cycle or between the actively dividing stage and the quiescent stage. This hypothesis can be further tested by collecting the patched single NSPCs for both with and without currents; and conducting single cell RT-PCR to find out the specific cell cycle stages of the collected NSPCs by profiling the marker genes expressed at a specific cell cycle stage.

The proliferation of the NSPCs has been implicated in treatment of depression as the proliferation of the NSPCs is important for the behavioural effects of antidepressants in rodents (Santarelli *et al.*, 2003). Since the 5HT3 receptor is expressed on NSPCs, proliferation may be affected by the specific activation and blockade of these receptors on NSPCs. Reports have suggested that the effects of the antidepressants may be attenuated by co-administration of the 5-HT3 receptor agonist, mCPBG, in an animal model of depression (Nakagawa *et al.,* 1998). However, mCPBG alone is not able to affect the duration of immobility in the force swim test, which is a behavioral model sensitive to antidepressant activity in rodents. However, the addition of a selective 5-HT3 receptor antagonist ICS205-903 is able to significantly decrease the duration of immobility in force swim test and this effect can be attenuated by the administration of mCPBG (Nakagawa *et al.,* 1998). Incidentally, in our experiments, the 5-HT3 receptor antagonists showed consistent induction of an increase in the rate of proliferation of the NSPCs. Further reports by Eisensamer *et al.* (2003) also showed that the antidepressants, desipramine, imipramine, trimipramine, fluoxetine, reboxetine and mirtazapine, are able to act as antagonists at the 5-HT3A receptor to effectively block 5-HT-induced Na^{+} and Ca^{2+} -currents in a dose dependent manner. Treatments with antidepressants fluoxetine and imipramine have been shown to also increase the cell proliferation of NSPCs in mice models, further suggesting that the inhibition of the 5-HT-induced currents leading to an increase in NSPC proliferation may be one of the mechanisms of the antidepressant effect (Santarelli *et al.*, 2003). As such, this suggests that selective 5-HT3 receptor

antagonists may be able to act as antidepressants as our experiment shows that Y-25130 can also increase in NSPC proliferation in mice and NSPC proliferation is essential to the behavioral effects of antidepressants (Santarelli *et al.*, 2003).

Treatment with 5-HT3 receptor agonists, mCPBG and SR57227, produced some puzzling results. Treatment with SR57227 had no significant effect on the cell proliferation of the NSPCs. However, treatment of the cultured NSPCs with mCPBG, although also an agonist, brought about a more varied effect with an increase in NSPC proliferation at 0.05µM and 5µM mCPBG treatment. This suggests that there may be a difference in the mode of action of mCPBG at different concentrations. Hapfelmeier *et al.* (2003) have shown that the mCPBG can induced a concomitant open-channel block at both homomeric 5-HT3A receptors and heteromeric 5-HT3A/B receptors which resulted in a bell shaped dose-response curve for current amplitude. The increase in NSPC proliferation by treatment with mCPBG in our experiments may be an effect of open channel blockade produced by the prolong treatment with mCPBG (3 days). The receptor resensitization for mCPBG induced currents is slower than resensitization by 5-HT. Also, mCPBG is able to evoke a tail current in cells expressing the 5-HT3A/B heteromeric form of the receptor, which is not present in the 5-HT-induced current. These effects induced by mCPBG treatments may have caused the induction of proliferation and resulted in the different response to that produced by the other agonist, SR57227.

Our results have shown that NSPCs express functional 5-HT3 receptors and their blockade can increase NSPC proliferation both *in vitro* and *in vivo*. These data point towards the use of selective antagonists at the 5-HT3 receptor as possible antidepressants. However, in this study we have yet to conduct behavioral testing to further ascertain whether the use of 5-HT3 receptor antagonists can bring about a reduction in the behavioral effects of depression. Therefore, more test will have to be performed to determine the whether the 5-HT3 receptors antagonists can be used as an effective antidepressant in the future.

6. PROSPECTS OF SELF REGULATION OF PROLIFERATION THROUGH 5-HT – TRYPTOPHAN HYDROXYLASE EXPRESSION IN NSPCs

6.1 Introduction

In Chapter 3, the results indicated that the exogenous addition of 5-HT can bring about an increase in the NSPC proliferation. This experiment simulates the presence of serotonergic neurons releasing 5-HT to the NSPCs, activating the 5-HT receptors, which bring about an increase in NSPC proliferation. 5-HT, besides being a neurotransmitter, is also involved in other roles such as morphogenesis during early embryonic development and both in fetal and adult neurogenesis (Di Pino *et al.*, 2004; Gaspar *et al.*, 2003; Lauder, 1993; Whitaker-Azmitia and Azmitia, 1994). One report suggests that embryonic stem cells express tryptophan hydroxylase (TPH), which are the rate limiting enzymes 5-HT synthesis (Walther and Bader, 1999). Considering that NSPCs can be considered as immature cells, there is a possibility that NSPCs may be capable of synthesizing 5-HT.

Results from the previous chapters also suggest that the NSPCs express 5-HT receptors and these receptors function to regulate neurogenesis. These 5-HT receptors have also been implicated in the regulation of the neurotransmission, morphogenesis and pathological disease such as depression due to 5-HT dysregulation (Murphy *et al.,* 1998; Bonnin *et al.,* 2006). The “serotonin

hypothesis" suggested that imbalance of 5-HT levels leads to the onset of depression and that restoration of the balance, together with the resulting increase in neurogenesis, is able to help recovery from the depressive state (Santarelli *et al.*, 2003).

Therefore, it seems that one of the regulatory mechanisms that control NSPC proliferation would likely to be 5-HT levels, which would determine whether the 5HT receptors on the NSPCs are triggered. Since TPH is the rate limiting enzyme in the 5-HT biosynthesis pathway, it would be natural to examine the presence of the TPH in the NSPC to provide evidence of the possibility of biosynthesis and self-regulation from 5-HT release.

The recent discovery of neuronal TPH2 raises the question of whether both the isoforms of TPH are expressed in NSPCs, as they are in embryonic stem cells. Although TPH1 and TPH2 share sequence homology, their expression pattern and regulation differs, this suggests a possibility of different functions (Nakamura and Hasegawa, 2007). TPH1 is expressed mainly in the periphery and the pineal gland. On the other hand, TPH2 is exclusively expressed in the neuronal cell types, more specifically at the median and dorsal raphe nuclei (Walter *et al*., 2003; Malek *et al.*, 2005; Sakowski *et al*., 2006, Nakamuara *et al.*, 2006). Besides being differentially expressed, the biochemical properties of TPH1 and TPH2 are also different. TPH2 is more soluble and has a higher molecular weight as compared to TPH1 (Invernizzi, 2007) and TPH1 has a 4-fold higher enzymatic

activity and higher substrate affinity compared with TPH2 (Nakamura *et al*., 2006). There is also evidence that polymorphism in the TPH1 gene may be associated with suicidal behaviour and antidepressant response in patients with unipolar major depression (Mann *et al.*, 1997; Nielsen *et al.*, 1998; Rujescu *et al.*, 2003; Bellivier *et al.*, 2004; Peters *et al.*, 2004).

Therefore, in this chapter, the expression of TPH1 and TPH2 in the NSPCs were explored. Attempts will be made to examine how their expression in NSPCs relates to their likely implications for NSPC proliferation and neurogenesis.

6.2. Materials and Methods

6.2.1. RNA Extraction and Reverse Transcription PCR (RT-PCR)

Total RNA was extracted, treated with DNaseI (DNase I recombinant, RNase-free; Roche Diagnostic GmbH) and purified from each of the NSPCs which have been isolated from fetal hippocampus and SVZ according to the manufacturer's instructions for PureLink^{TM} Micro-to-Midi Total RNA Purification System (Invitrogen). Quantitation of RNA was performed with Quant-iT^{TM} RNA Assay Kit (Invitrogen). Approximately 1 μg of total RNA was reverse transcribed with oligo(dT) primers using the ImProm-II^{TM} Reverse Transcription System (Promega, WI, USA).

The reverse transcribed cDNA was used as a template for PCR using AmpliTaq Gold DNA polymerase (Applied Biosystems) according to manufacturer's instructions. PCR primers were designed to be intron spanning to eliminate the presence of false positive from genomic DNA contamination. The primer sequences for *TPH1* are (forward: 5'-TCCCAAGATTGCCTGTAAAC-3', reverse: 5'-TAGCCCTGGCTCAGACTGA-3') and *TPH2* are (forward: 5'-CAAAGACGACCTGCTTGC-3', reverse: 5'-TGACTGCATTGTTGCTACACC-3'). The PCR reactions were conducted at 94^{o}C for 30 seconds, annealing temperature 55^{o}C for one minute and 72^{o}C for one minute for 35 cycles. The

PCR products were separated in 1.5% agarose gel, stained with ethidium bromide, and then visualized under UV irradiation.

6.2.2. Western blotting

Total protein was extracted from the NSPCs using protein extraction buffer containing 50mM Tris-HCl pH 7.4, 150mM NaCl, 20mM Na-EDTA, 1% Igepal CA-630, 10% glycerol. The protein concentration was determined using BCA Dye (Pierce Biotechnology, Rockford, IL). Twenty µg of protein was separated by 12% SDS-PAGE. The protein was then electro-transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The efficiency of the protein transfer was verified by staining with MemCode Reversible Protein Stain Kit (Pierce Biotechnology). The membranes were subsequently blocked with 5% non-fat skimmed milk in TBS-T with shaking at 4^{o}C overnight. Anti-TPH1 or anti-TPH2 antibodies (1:1000, Chemicon, CA, USA) and anti-β-actin antibody (Sigma-Aldrich) in 1% non-fat skimmed milk in TBS-T were incubated with the membrane with shaking overnight at 4^{o}C. The membranes were then washed six times in TBS-T for 5 minutes each time. The membranes were incubated with ImmunoPure Goat Anti-Rabbit IgG, Peroxidase Conjugated (1:50,000; Pierce Biotechnology) in 1% non-fat skimmed milk in TBS-T for one hour at room temperature. The membranes were then washed six times in TBS-T for 5 min each time. Detection of the protein bands were done by incubating the

peroxidase substrate SuperSignal West Femto (Pierce Biotechnology) with membrane for 5 min and exposing the X-ray film to the membrane.

6.2.3. Immunocytochemistry of undifferentiated and differentiated NSPCs

To determine the expression of TPH1 and TPH2 in undifferentiated NSPCs and upon multipotent differentiation of NSPCs, dissociated NSPCs were plated on poly-L-ornithine and fibronectin coated coverslips. Differentiation was induced with 0.5% fetal calf serum (Hyclone Laboratories Inc.) in neurosphere medium without EGF and bFGF. The cells were allowed to differentiate for a further 14 days with samples collected on the start of differentiation, day 1, 3, 5, 7 and 14 after the addition of differentiation medium. The cells were then fixed by treatment with 4 % paraformaldehyde for 20 minutes, followed by permeabilization using PBS with 0.1% Triton X-100. Immunostaining was conducted sequentially using anti-TPH1 or TPH2 (1:500; gift from Dr. Donald M. Kuhn; Sakowski *et al.,* 2006) with anti-vimentin antibody (1:200; Chemicon) or anti-PSA-NCAM antibody (1:400; Chemicon) in 3% BSA in PBS overnight at room temperature. Fluorescence-conjugated Alexa Fluor secondary antibodies (Invitrogen) were used to visualize the primary antibodies and the coverslips were counterstained with DAPI. The differentiated cells were then imaged by sequential scanning with a confocal microscope (LSM 510, Carl Zeiss Microimaging GmbH).

6.2.4. Cell proliferation assay of PCPA treated NSPCs

To determine whether exogenous addition of PCPA to cultured NSCs was able to influence the cell proliferation rate, a cell proliferation assay was performed using CyQuant NF Cell Proliferation Assay (Invitrogen). Third passage neurospheres were dissociated using the same procedure as for cell passaging. Resuspended single cells were sieved with 40 μm cell strainers and counted using a haemocytometer (Neubauer). NPCs were then plated at 1000 cells/well on poly-L-ornithine (Sigma-Aldrich) and fibronectin (Gibco) coated 96-well black-walled flat transparent bottom culture dish in neurosphere medium. The cells for the assay were plated from the same batch of cells to maintain plating homogeneity. The cells were pre-cultured for 72 hours prior to addition of treatment solution. 72 hours after the 5-HT or PCPA addition, CyQuant NF Cell Proliferation Assay reagents were added according to manufacturer's instructions and incubated at 37ºC. After 1 hour incubation, first reading was taken using a fluorescence plate reader (Infinite M200, Tecan, Zürich, Switzerland) at excitation wavelength of 490nm and emission of 530nm. Each treatment was done with three independent biological repeats and each biological repeat was conducted with three technical repeat.

6.2.5. Statistical Analysis

After subtracting the background, the fluorescence intensity, which was linear to the cell number, was expressed as a ratio to the control without treatment and the SEM is calculated from three independent biological repeats. The PCPA-treated samples were compared with the control and analysed using Student's t-test. $P<0.05$ was considered as statistically significant. All data are reported as mean ± SEM.

6.2.6. Analysis of cell proliferation in TPH1 KO mice

A single high dose of BrdU (Sigma-Aldrich) dissolved in saline was intraperitoneally injected into the mice at a concentration of 300 mg/kg. Two hours after the BrdU injection, the mice were anaesthetized with an overdose of pentobarbital (Nembutal) and then transcardially perfused with 4% paraformaldehyde in 0.1 M of phosphate buffer (pH 7.4), after which the brains were extracted. The hippocampi of the mice were then cryosectioned using a cryostat (Leica Microsystems) at a thickness of 20 μm and mounted onto poly-L-lysine coated slides prior to immunohistochemistry. The sections were then first permeabilized and treated with 4M HCl and trypsin (1 mg/ml in PBS, 10 min, 37˚C) before blocking and detection using anti-BrdU antibody (1:100; Neomarkers, Fremont, CA). Fluorescence-conjugated Alexa Fluor secondary antibodies (Invitrogen) were used to visualize the primary antibodies and the

slides were mounted on Prolong Anti-fade mounting medium with DAPI (Invitrogen). The sections of the hippocampus were sampled at 20μm per every 100μm for the whole hippocampus. The total number of BrdU positive cells was expressed as a ratio against the total length of the perimeter of the dentate gyrus of the hippocampal sections. The mean of four TPH1 KO mice (gift from Prof. Dr. Micheal Bader; Walther *et al.,* 2003) were compared with the mean of four wild-type and analyzed using Student's t-test. $P<0.05$ was considered as statistically significant. All data are reported as mean ± SEM.

6.3. Results

6.3.1. NSPCs expressed both TPH1 and TPH2

To determine whether the NSPCs were also capable of producing 5-HT, RT-PCR was conducted on total RNA isolated from NSPCs. Results showed that the NSPCs expressed both TPH1 and TPH2, which are the essential rate-limiting enzymes in the 5-HT biosynthesis pathway (Fig. 6.1A). Western blot analysis shows that the protein expression of TPH1 was higher in the NSPCs extracted from the hippocampus as compared to SVZ (Fig. 6.1B and C), whereas TPH2 levels were comparable for both cell types (Fig. 6.1B and C). The expression was further confirmed by immunocytochemistry using anti-TPH antibodies specific to TPH1 and TPH2 isoforms (Fig. 2D). The expression suggested that the NSPCs produced 5-HT and might therefore be capable to self-regulating proliferation using 5-HT as a signal.

6.3.2. Inhibition of 5-HT production reduced NSPC proliferation

To show that 5-HT production was important for NSPC proliferation and that proliferation might be self-regulated by the NSPCs, *p*-chlorophenylalanine (PCPA) was used to inhibit TPH activity and 5-HT production in the cultured NSPCs. Treatment with PCPA at 2.5mM was observed to reduce the number of NSPCs (Fig. 6.2A) and measurements by cell proliferation assay showed that

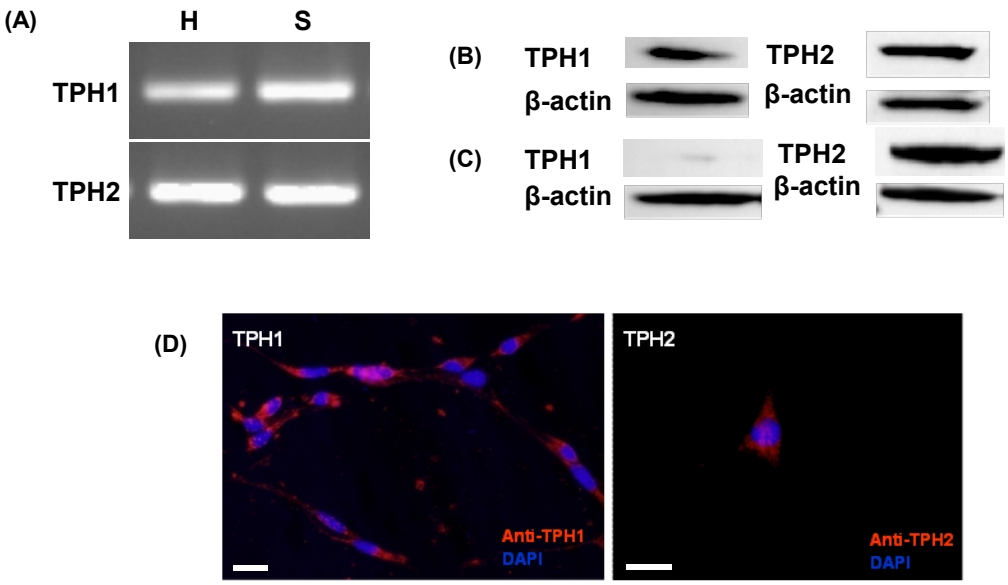

Figure 6.1. Expression of TPH1 and TPH2 in NSPCs. (A) RT-PCR of total RNA isolated from NSPCs and amplified using TPH1 and TPH2 specific primers. Western blotting showed the protein expression of TPH1 and TPH2 in NSPCs isolated from (B) hippocampus and (C) subventricular zone. (D) anti-TPH1 and anti-TPH2 immunostaining of hippocampal NSPCs plated as a monolayer. The scale bar in each micrograph represents 10μm.

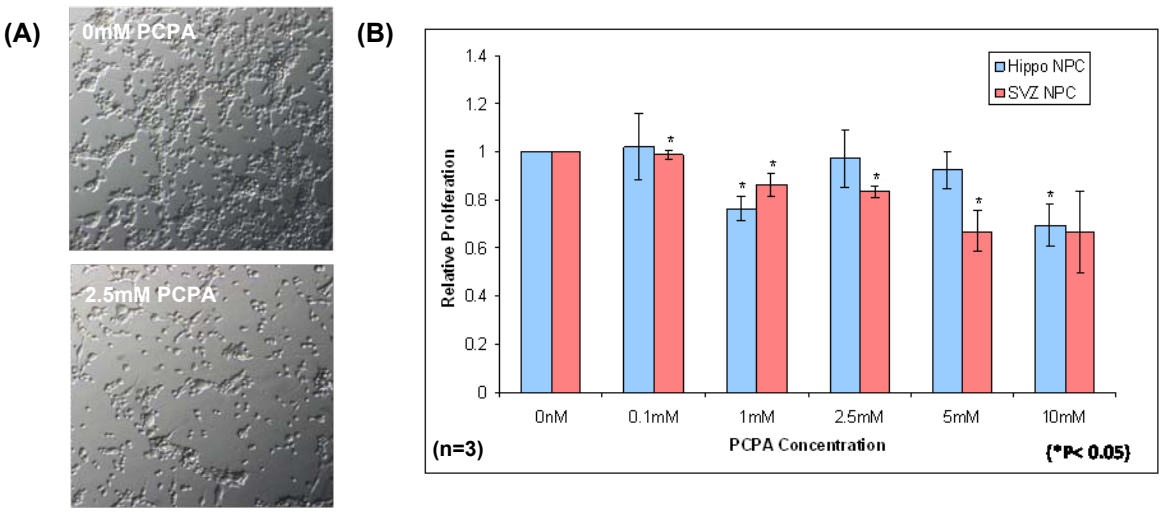

Figure 6.2. 5-HT depletion decreases NSPC proliferation in culture. NSPCs were plated at a density of 1000 cells per well in a 96 well dish and treated with different concentrations of PCPA for 3 days to observe the changes in the rate of proliferation. (A) Microscopy showing the density of cells after 3 days of treatment. (B) CyQuant cell proliferation assay was conducted and the relative proliferation in NSPCs isolated from hippocampus (Hippo) and subventricular zone (SVZ) with different treatments were plotted against the control.

1mM PCPA was able to inhibit the NSPC proliferation significantly (Fig. 6.2B). Inhibition of 5-HT production using PCPA reduced the cell proliferation rate of the cultured NSPCs suggesting that the 5-HT produced by the NSPCs was required for the maintenance of NSPC proliferation.

6.3.3. Expression of TPH1 and TPH2 during differentiation of NSPCs

Since it was possible that the 5-HT was important in the maintaining the NSPCs in the proliferative state, we decided to check the profile of the TPH1 and TPH2 expression during the process of differentiation. We plated the NSPCs on a monolayer and induced them to differentiate for 3 and 7 days before extracting the mRNA from the cells to check for expression of *TPH1* and *TPH2* using RT-PCR. Results showed that, upon differentiation, *TPH1* expression dropped on the third day of differentiation and was hardly detectable after 7 days of differentiation (Fig. 6.3). *TPH2* expression, however, persisted after differentiation for 7 days (Fig. 6.3).

This result was further confirmed using immunofluorescence staining using anti-TPH1 and anti-TPH2 antibodies together with anti-vimentin and anti-PSA-NCAM antibodies. Vimentin was expressed in NSPCs and upon differentiation its expression decreased. PSA-NCAM on the other hand increased in expression upon NSPC differentiation and its expression persisted from the neuroblast stage to the immature neuron stage.

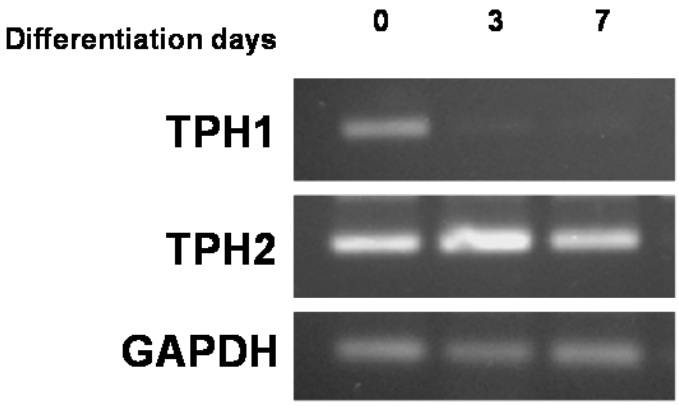

Figure 6.3. Expression of TPH1 and TPH2 in during differentiation of NSPCs. NSPCs were plated as a monolayer and induced to differentiate using 0.5% fetal bovine serum. mRNA was isolated from the undifferentiated NSPCs, NSPCs differentiated for 0 day, 3 days and 7 days. RT-PCR was conducted using specific primers to TPH1 and TPH2 transcript.

Upon differentiation, the expression of the TPH1 started to decrease from 1st day of differentiation and continue to decrease during differentiation (Fig. 6.4). The expression of vimentin started to decrease in a slower fashion with some of the cells still expressing vimentin after 14 days of differentiation (Fig. 6.4A, 6.5A). Moreover, the expression of PSA-NCAM increased upon differentiation starting from day 1 (Fig. 6.4B, 6.5B). However, due to the fact that PSA-NCAM was only expressed in young neurons, its expression was only observed in about 10-20% of the total cells. TPH2 expression was relatively weak in the immunostaining. Upon differentiation, TPH2 expression persisted throughout NSPC proliferation, however, it was found to be localized in the nuclei in most of the cells (Fig. 6.5).

6.3.4. Analysis of cell proliferation in TPH1 KO mice

As TPH2 is reported to be predominantly expressed in the brain while TPH1 is reported to be expressed at the peripheral nervous system (Zhang *et al.*, 2004), we attempt to identify the role of TPH1 expression in the NSPCs and its likely effect on NSPC proliferation. TPH1 might be pivotal in the induction of cell proliferation as it was known to have a higher enzymatic activity and its presence might play an important role in self- regulation of cell proliferation of the NSPCs. We had obtained TPH1 knockout (KO) mouse and used it to analyze the proliferation of the NSPCs in the hippocampus. Results indicated that the cell proliferation rate of the TPH1 KO mice in the dentate gyrus of the hippocampus was significantly lower than that in wild-type littermates (Fig. 6.6A and B).

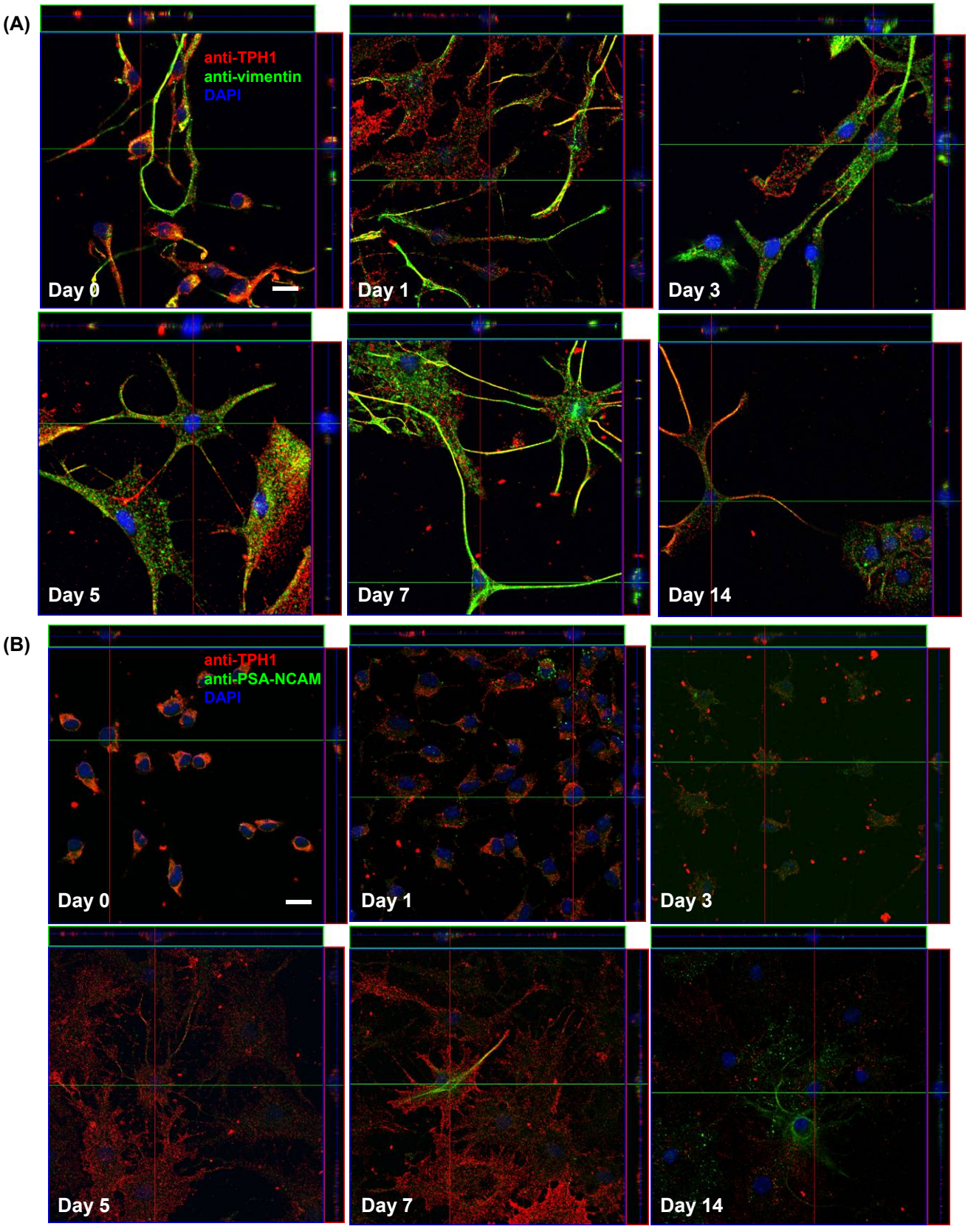

Figure 6.4. Expression pattern of TPH1 upon differentiation of hippocampal NSPCs. Hippocampal NSPCs were differentiated for various durations and immunostained with (A) anti-TPH1 and anti-vimentin and (B) anti-TPH1 and anti-PSA-NCAM. To show the progression of differentiation, vimentin is used as a marker for NSPCs and PSA-NCAM is used as a marker for developing neuroblasts. The scale bar represents 10μm.

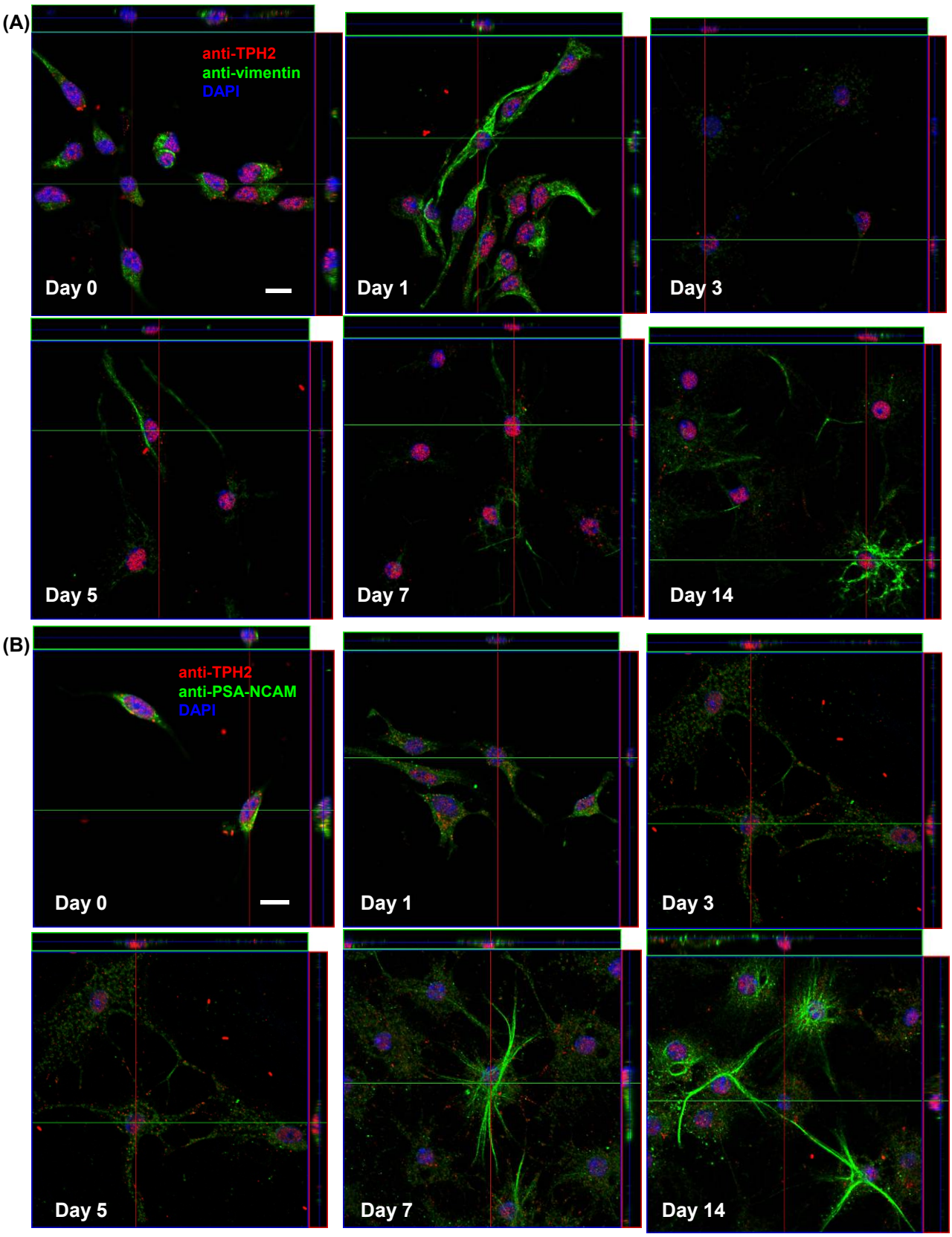

Figure 6.5. Expression pattern of TPH2 upon differentiation of hippocampal NSPCs. Hippocampal NSPCs were differentiated for various durations and immunostained with (A) anti-TPH2 and anti-vimentin and (B) anti-TPH2 and anti-PSA-NCAM. To show the progression of differentiation, vimentin is used as a marker for NSPCs and PSA-NCAM is used as a marker for developing neuroblasts. The scale bar represents 10μm.

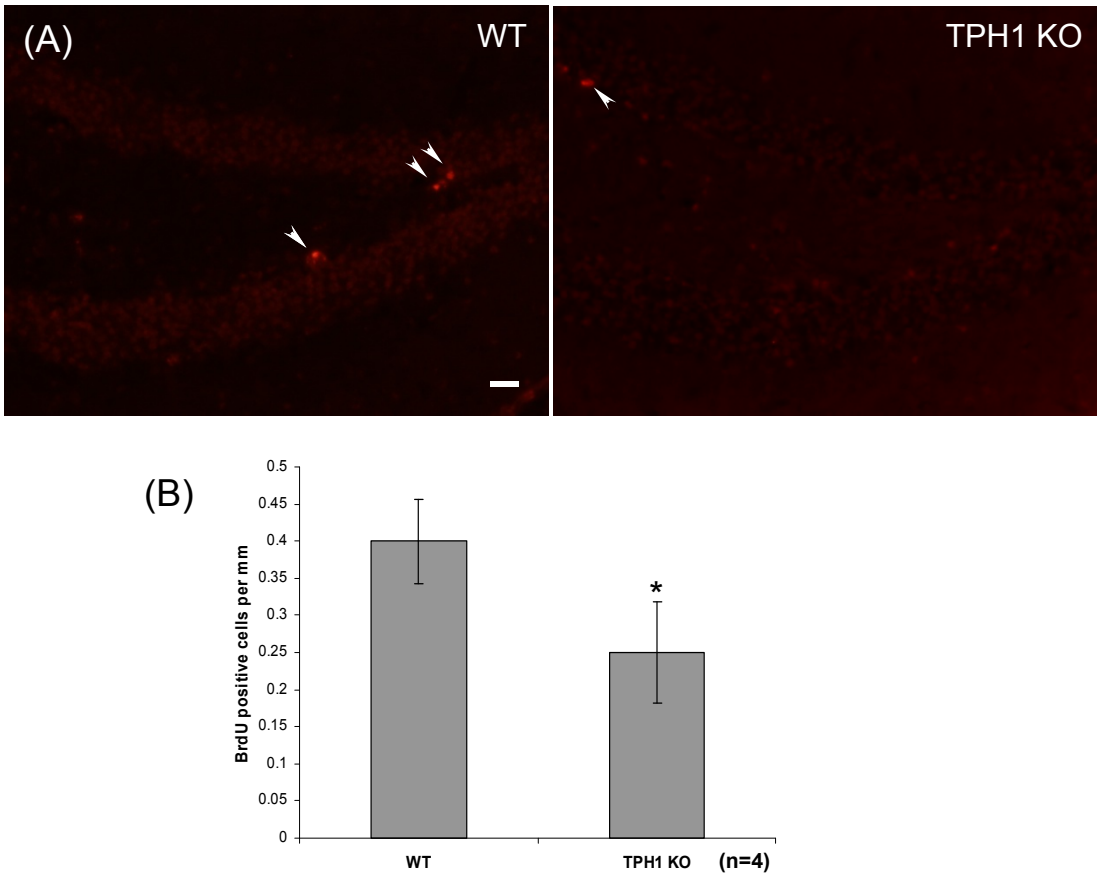

Figure 6.6. Effect of TPH1 KO on NSPC proliferation in the dentate gyrus. BrdU was injected intraperitonally into four wild-type and four TPH1 KO mice for 2hr to allow BrdU to incorporate into the dividing NSPCs before sacrifice. The sections were assayed for BrdU incorporation against the total length of the perimeter of the dentate gyrus. Statistical analysis was done using Students t-test. (A) BrdU immunopositive cells at the dentate gyrus of the WT and TPH1 KO mice. (B) NSPC proliferation rate between WT and TPH1 KO mice. (*P<0.01). The scale bar represents 50μm.

6.4. Discussion

The results presented have shown that serotonin can induced NSPC proliferation and that serotonin may be released from the NSPCs as they expressed both the "peripheral" TPH1 and the "neuronal" TPH2. Proliferation of cultured fetal NSPCs can be inhibited by non-specifically inhibiting TPH activity using PCPA, further implicating the likely regulatory function of serotonin in NSPC proliferation. The likelihood of NSPCs using TPH, specifically TPH1, as the main TPH for maintaining serotonin levels is further suggested by the fact that TPH1 expression is rapidly downregulated at both the mRNA and protein levels upon differentiation whereas TPH2 expression levels remain high throughout. This suggests the requirement of TPH1 expression and a high serotonin level in maintaining the NSPC proliferative state. With the availability of the TPH1 KO mice, we have shown that TPH1 KO causes a decrease in the proliferation rate of the NSPCs at the dentate gyrus of the TPH1 KO mice brain.

Serotonin has been known to be a morphogen in early brain development and as shown by the fact that embryonic stem cells also expressed both TPH1 and TPH2 (Walther and Bader, 1999). This suggests the possibility that tight regulatory control of serotonin levels may be required during the development process and this mechanism has been retained in the NSPCs. This is not surprising as NSPCs are still in the process of development and they are able to generate new cells, which are capable of differentiation into neurons,

oligodendrocytes and astrocytes. That exogenous addition of serotonin can bring about an increase in NSPC proliferation suggests that the neural progenitors are able to receive serotonin signals, implying that serotonin receptors are expressed on the neural progenitors (Fig. 3.2 and 3.3). Indeed, one study has shown that 5HT1A receptor is expressed on the adult NSPCs in mice (Benninghoff *et al*., 2002). Our own studies have also found the presence of some of the serotonin receptor subtypes expressed on the NSPCs further pointing to the fact that serotonin signals can be perceived by the NSPCs supporting the notion of self-regulating mechanism of cell proliferation (Fig. 3.3).

It has been shown in many studies that SSRI based antidepressants are able to induce an increase in NSPC proliferation (Duman *et al*., 2001, Encinas *et al*., 2006). These studies have been focusing on increasing the availability of the serotonin at the synapses to correct the serotonin imbalance that causes depression. Moreover, Santarelli *et al.* (2003) had shown that NSPC proliferation and neurogenesis is a requirement for the recovery from depression. With the discovery of TPH1 and TPH2 expression in the NSPCs, this further suggests the possibility that SSRIs may act directly on the NSPCs through the serotonin transporter (SERT). This action may increase the availability of the serotonin acting on the NSPCs as they are likely to also produce serotonin with or without the presence of the serotonergic synapses from the serotonergic neurons projecting from another site. However, a recent study by Schmitt *et al.* (2007) failed to show the expression of the SERT in adult NSPCs isolated from adult

mice. Our results, however, suggest that the NSPCs may express SERT only during fetal stage (Fig. 3.3). Furthermore, studies from SERT KO mice also suggest that knocking out SERT does not affect the NSPC proliferation rate of young adult mice as compared to WT. However, the NSPC proliferation rate was increased in the NSPCs from SERT KO aged mice as compared to control (Schmitt *et. al*., 2007). Schmitt *et al.* (2007) also noted that the failure to find SERT expression in primary adult NSPCs suggests that the reuptake process may not play a role in the regulation of neurogenesis. SSRI's effect on the neural progenitors, however, may be also due to the non-specific effects of SSRI's acting on other receptors or channels (Tytgat *et al*., 1997; Dierk *et al*., 2002; Eisensamer *et al*., 2003).

Therefore, the failure to find SERT expression in the adult NSPCs reinforces the likelihood that regulation of neurogenesis under normal physiological conditions may be controlled by the levels of serotonin. Expression of TPH1 and TPH2 in NSPCs further support this notion. As "neuronal" TPH2 is noted to be widely expressed in the brain, "peripheral" TPH1 expression in the central nervous system is less commonly observed. The expression of TPH1 is especially of significance as it is known to be more active serotonin biosynthesis enzyme as compared to TPH2 (Nakamura *et al*., 2006). TPH1's preferential expression in the NSPCs and its downregulation during the process of differentiation provides a likely mechanism of regulation of serotonin levels surrounding the NSPCs. It is likely that upon differentiation, the levels of TPH1 are downregulated as the

proliferative state of the NSPCs no longer needs to be maintained. The expression of TPH1 and TPH2 in the primary fetal neural progenitors is not restricted to mouse as our microarray data from our unpublished studies shows that *TPH1* and *TPH2* are also expressed in the primary human NSPCs and that *TPH1* expression levels are higher than those of *TPH2*.

We also studied the effects of TPH1 KO on NSPC proliferation in TPH1 KO mice. As TPH1 is predominantly expressed in the periphery, these TPH1 KO mice lack serotonin in the gut, the blood and the pineal gland. However, as for the brain, there are only minor reductions in the steady-state serotonin levels in the serotonergic regions (Walther and Bader, 2003). These mice allow us to examine the effects of TPH1 KO on the NSPCs with minimal concerns of the changes in the proliferation rate of the NSPCs are due to the TPH1 KO affecting other serotonergic pathways, as the predominant serotonin production in the brain is from the TPH2 isoform. We were able to observe a decrease in NSPC proliferation in the TPH1 KO as compared to the wild-type littermates suggesting that the knocking out *TPH1* reduces the serotonin levels surrounding the NSPCs, thereby reducing their proliferation rate. This may constitute a plausible mechanism for the NSPCs to maintain their constant rate of proliferation without relying on external stimuli. Also, external induction of cell proliferation may be activated simply by increasing or decreasing the levels of TPH1. However, further study will be needed to determine whether such a suggested mechanism exists.

Clinical studies on TPH1 polymorphisms in humans also suggest that TPH1 influences the efficacy of treatments for depressions despite the fact that TPH1 is not the predominant “neuronal” TPH in the brain. Population studies suggest that TPH1 polymorphism are associated with depression, anxiety and comorbid depression and anxiety in postpartum women (Sun *et al*., 2004). A218C allele of TPH1 has also been suggested to be associated with increase susceptibility to bipolar disorder (Bellivier *et al*., 1998). Also, another study suggest that the patients with some allelic forms of TPH1 failed to respond to fluoxetine antidepressant treatment suggesting that TPH1 polymorphisms may be crucial to the use of serotonin based treatments in depression (Peters *et al*., 2004). This also suggests that TPH1 is important in the treatment process of depression and that recovery process may be related to the NSPC neurogenesis. However, contradicting studies reporting no influence of TPH1 polymorphisms on depression are also present (Furlong *et al*., 1998; Frisch *et al*., 1999; Serretti *et al*., 2001)

In conclusion, serotonin biosynthesis in NSPCs may be a plausible mechanism of controlling the NSPC proliferation. This mechanism may constitute self-regulating of serotonin biosynthesis modulated through TPH1 expression. Upon differentiation, which renders the NSPCs non-proliferative, there is a downregulation of TPH1 expression, which is reminiscent of the pattern of expression during development. This is similar to the expression reported in

embryonic stem cells during maturation suggesting that morphogenic mechanisms may also operate in the same manner in more restrictive adult progenitor cells.

7. GENERAL DISCUSSION, FUTURE STUDIES AND CONCLUSION

7.1 General Discussion

This study started from the finding that treatments using SSRI-based antidepressants are able to increase NSPC proliferation and the effectiveness of these antidepressants is dependent on this increase for its behavioral effects (Santarelli *et al*., 2003). This suggests that serotonin may be a regulator of NSPC proliferation, either by directly acting on the NSPCs or indirectly using other neuronal pathways to activate proliferation. This thesis examines the hypothesis that treatment with SSRI antidepressants increases the availability of the serotonin at the serotonergic synapses which directly acts on the NSPCs, thereby inducing an increase in NSPC proliferation. The presence of serotonergic nerve fibres at the neurogenic regions of the brain and the ability of exogenous serotonin able to induce NSPC proliferation in culture provided that evidence. Pointing further to direct serotonergic induction is the presence of a large number of 5-HT receptors subtypes expressed on the NSPCs to perceive the serotonergic signals.

However, the presence of a large number of 5-HT receptor subtypes also complicates the issues of locating which receptors are involved in the regulation of NSPC proliferation. As suggested by Uphouse (1997), the presence of a large number of 5-HT receptor subtypes confers a certain advantage to the fine modulation of the 5-HT signals. As 5-HT is the natural ligand which activates all

5-HT receptors, the overall downstream signaling event would be difficult to predict with the presence of large numbers of different 5-HT receptor subtypes. Among all these 5-HT receptors, almost all the receptors are G-protein coupled receptors. However, different 5-HT receptors coupled to different G-protein subtypes activate very different signaling pathways. Some receptors have also been suggested to couple to different G-protein subtypes in different cell types (Albert *et al*., 1996).

Therefore, in order to determine which of the 5-HT receptors subtypes is implicated in the regulation of NSPC proliferation, the 5-HT receptors are activated or blocked using agonists and antagonists before assaying for NSPC proliferation. A host of experiments were conducted on the cultured NSPCs, which provide a clean and clear-cut system for us to assay proliferation without other considerations of additional effects that these agonists and antagonists have *in vivo*. In the *in vivo* cell proliferation model, we would not be able to discount the fact that these agonists or antagonists do not only act on the NSPCs, as they could also act on 5-HT receptors on other cell types and synaptic pathways which may be a confounding factor to our study. Also, we have established a protein and xeno-free vitrification system that will enable us to cryopreserve the NSCs for experimental use (Tan *et al*., 2007; Kuleshova *et al*., 2009). This protocol allows the cryopreservation of intact neurospheres which can be retrieved with high viability, no chromosomal abnormalities, and intact stem cell function with the ability to proliferate normally and differentiate into the

neuronal cell types. This established vitrification protocol can also be adapted in the future for clinical applications for human NSPC therapeutics.

Out of the 5-HT receptors expressed by the NSPCs, 5-HT1A, 5-HT3 and 5-HT7 receptors have been chosen for the targets of the study of NSPC proliferation. Both 5-HT1A and 5-HT3 receptors have prior been implicated in the modulation of NSPC proliferation. 5-HT1A receptors have been shown to promote the NSPC proliferation upon activation using a 5-HT1A agonist, 8-OH-DPAT (Banasr *et al*., 2004). 5-HT3 receptor has been shown to be blocked by administration of some antidepressants and these same antidepressants are also able to induce NSPC proliferation (Eisensamer *et al*., 2003; Santarelli *et al*., 2003). 5-HT7 receptor is been chosen as the commonly used 5-HT1A receptor agonist 8-OH-DPAT has been shown to be a partial agonist to 5-HT7 receptor which require a more detail study to delineate whether 5-HT1A or 5-HT7 receptor activation causes NSPC proliferation. In the study, it has been determined that activation of 5HT7 receptor and the blockade of 5HT3 receptor are able to induce an increase in NSPC proliferation. However, contradictory to prior report, 5-HT1A receptors are not involved in the induction of NSPC proliferation (Banasr *et al*., 2004).

The NSPCs have also being found to express TPH1 and TPH2, which suggests that NSPCs are capable of synthesizing 5-HT. Together with previous results, this suggests that the regulation of extracellular serotonin levels may be a mechanism for controlling NSPC proliferation. The presence of serotonin

synthesis enzymes in the NSPCs provides the possibility of self-regulation of proliferation. Among the two TPH isoforms, TPH1 has a higher synthesis activity and it is expressed highly in the peripheral nervous system and rarely in the CNS. Its presence in the NSPCs suggests the likelihood that high serotonin levels are required for the maintenance of NSPCs in their proliferative stage. This is especially significant as upon induction of differentiation, the levels of *TPH1* drop, suggesting that serotonin levels need to be maintained only at the proliferative, non-differentiated stage. Proliferation assays conducted on TPH1 KO mice showed that the TPH1 KO caused a significant reduction of proliferation, further implying the need for TPH1 activity for maintenance of proliferation. These results are made more significant as in the TPH1 KO mice, the levels of brain serotonin has not been globally altered due to the presence of the more dominant neuronal TPH2 (Walther and Bader, 2003). This suggests that the effect of the TPH1 KO on the NSPC proliferation is likely to be confined to the neurogenic regions and not cause by the knockout of *TPH1* changing the other neuronal pathways.

7.2. Future Studies

More work needs to be done in order to clearly delineating the relationships between the serotonergic system and NSPC proliferation. This is especially so when NSPCs were shown to express a large number of 5-HT receptors at the transcriptional level. Furthermore, the NSPCs also express TPH1 and TPH2,

suggesting that the NSPCs may be releasing 5-HT, which may self-regulate the NPSCs own proliferation further complicating the mechanism of action.

The serotonergic fibres innervating near the subgranular zone of the dentate gyrus and the subventricular zone of the lateral ventricles were shown to be close to the regions where the NSPCs and neurogenesis occurs. However, the resolution of the contact between the serotonergic neurons and the NSPCs can be further enhanced by employing triple immunostaining of the serotonergic fibres using anti-serotonin antibody, synaptic markers such as synapsin or synaptotagmin and a stem cell marker such as Sox2 or nestin. The immunostaining and in addition electron microscopy would allow further examination of the serotonergic fibres contact on the NSPCs at the ultrastructural level.

To further examine these likely mechanisms of control of NSPC proliferation, experiments can be conducted to investigate the dynamics of 5-HT receptor expression. NSPCs may consist of a pool of cells at different divisional stages which, at each stage, may express a subset of the 5-HT receptors. Single cell RT-PCR can be conducted on the NSPCs to allow the examination of whether all the NSPCs express the different 5-HT receptors. Cell division stage markers such as PTEN, c-fos, PCNA and ki67 could be used to identify the specific cell division stages of the NSPCs. These results can be further verified using western blotting and immunostaining.

Pharmacological methodologies used in these studies sometimes present certain challenges in terms of interpretation due to the non- or partial selectivity of the agonists and antagonists used. A more direct way will be to knockdown and perhaps overexpress the specific 5-HT receptors to examine the effects of the knockdown and overexpression on the overall proliferation of the NSPCs. Furthermore, knocking down a receptor followed by addition of the agonist will allow the examination of whether the agonist effect is specific to the particular 5-HT receptor. These knockdown NSPCs can also be used to assay for the changes in the NSPC proliferation rate due to the knockdown.

The 5-HT receptor, being a G-protein coupled receptor, worked by modulating the activity of adenylate cyclase. This suggests that the likely signaling cascade in the NSPC with the activation of particular 5-HT receptors. The adenylate cyclase would also be able to influence the phosphorylation states of ERK1/2, which has been implicated in cell proliferation. The phosphorylation states of ERK1/2 can also be measured using western blotting, which will indicate the effects of the agonist on the NSPC proliferation.

As this thesis utilize heavily the methodology of MTS assay, which measures the end point increase in the number of cells, it would be worthwhile to also examine the proliferation of the NSPCs using another methodology to further confirm the effects on the serotonergic systems are specific to proliferation. BrdU

incorporation assay or measuring the presence of Ki-67 positive cells could also provide another cell proliferation index for the measurement.

In the findings of the presence of TPH1 and TPH2 expression in the NSPCs, it was suggested that the NSPCs might be capable of releasing 5-HT. However, in this study, we have yet to directly examine such a notion. To further examine this, HPLC or 5-HT ELISA could be conducted on the culture medium of the NSPCs to check if 5-HT has been release into the medium by the NSPCs in culture. Also, PCPA can be treated to the NSPCs to inhibit the TPH activity and examine the level of 5-HT in the medium again to show that the 5-HT levels are a direct consequence of TPH1 and TPH2 expression. With the availability of the TPH1 mice, we can further examine the difference in levels of the 5-HT being release into the medium between the wild type and the TPH1 NSPCs, further examine the importance of TPH1 expression in the NSPCs.

7.3. Conclusion

Taken together, all these results suggest the role of 5-HT in regulating NSPC proliferation (Figure 7.1). 5-HT release from NSPCs may be used as a basal trigger for maintenance proliferation as NSPCs are capable of self-renewal at a fixed proliferative rate. Direct 5-HT release onto the NSPCs, either by the serotonergic neurons near the neurogenic regions or by the modulation of serotonin release from the NSPCs themselves may trigger an increase in proliferation. The increase and decrease in proliferation can be modulated by the

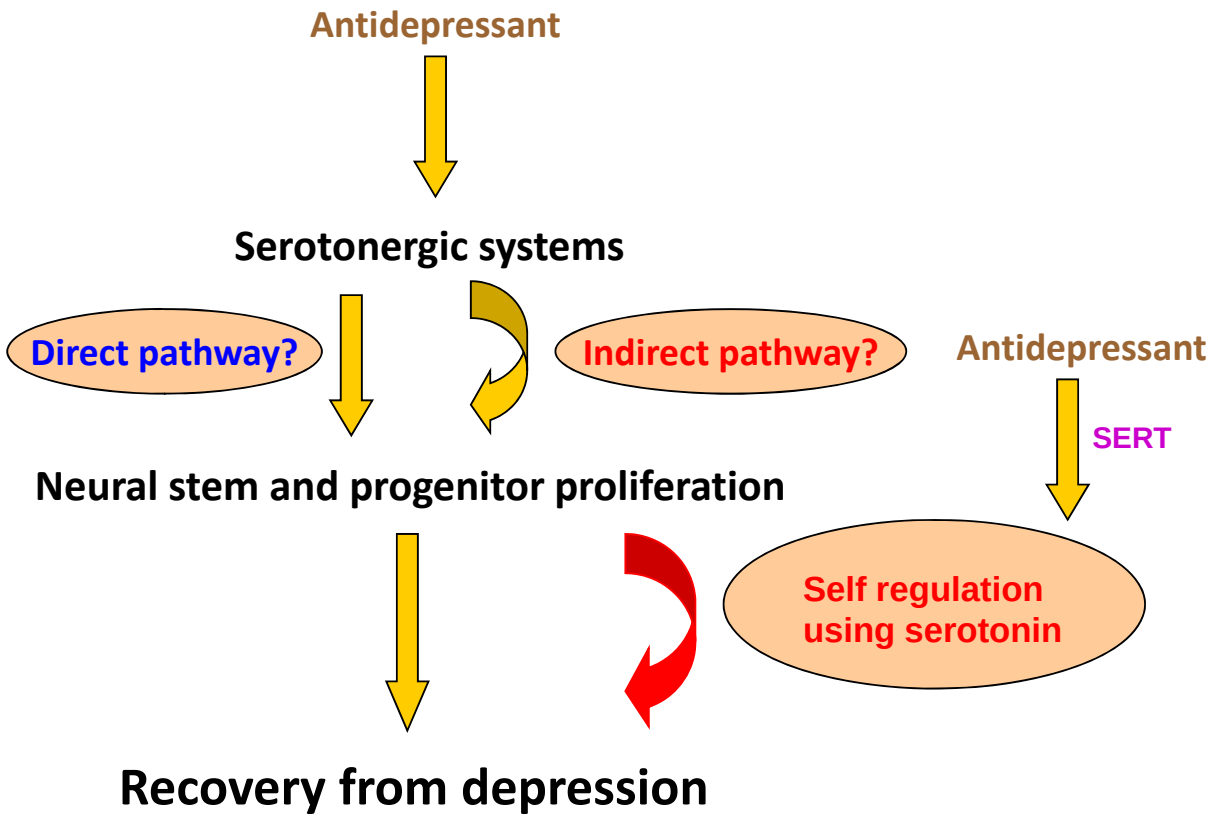

Figure 7.1. Summary of the effects of serotonergic systems and serotonin on NSPC proliferation. Antidepressants were known to act as SSRIs, which will increase the available serotonin at the serotonergic synapses by inhibiting SERT activity. The increase in serotonin will directly act on the NSPCs, which express 5-HT receptors, or indirectly, through indirect pathways involving other neurotransmitter systems, to affect NSPC proliferation. Alternatively, as NSPCs express both TPH1 and TPH2 suggesting that they are capable of synthesizing serotonin, basal NSPC proliferation may be self-regulated by controlling serotonin release. As SERT is also expressed on NSPCs, antidepressants may act directly on the SERT on the NSPCs to increase the available serotonin to act on the 5-HT receptors on the NSPCs. The increase in NSPC proliferation is a requirement for the behavioral effect of recovery from depression.

levels of 5-HT, as is evident from the TPH1 KO studies. The controls of NSPC proliferation is most likely modulated through the balance of signals received by the host of 5-HT receptors, with 5-HT3 and 5-HT7 receptors being two of the receptors that we have shown to be involved in this modulation of NSPC proliferation.

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9. APPENDIX

9.1 Publications

Tan FCK, Lee KH, Gouk SS, Magalhães R, Anuradha P, Hande M, Dawe G, Kuleshova L. 2007. Optimization of cryopreservation of stem cells cultured as neurospheres: Comparison between vitrification, slow-cooling and rapid cooling, "freezing" protocols. *Cryo Lett.* 28(6): 445-460.

Kuleshova LL[1], Tan FCK[1], Magalhães R, Gouk SS, Lee KH, Dawe GS. 2009. Effective Cryopreservation of Neural Stem or Progenitor Cells Without Serum or Proteins by Vitrification. *Cell Transplant.* 18: 135-144.

[1] **These authors contributed equally to this work.**

9.2 Poster presentation

Tan FCK, Lai J, Ou L, Nagarajah R, Dawe GS. 2007. Role of serotonin receptors in neural progenitor cell proliferation. Poster Presentation at Neuroscience 2007, San Diego, USA.