RUNNING AND ADULT NEUROGENESIS:

DOES SEPTOHIPPOCAMPAL SONIC HEDGEHOG

PLAY A ROLE?

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ABSTRACT

This study aims to elucidate the molecular underpinnings of running-mediated neurogenesis. Running has long been associated with hippocampal theta oscillations critically dependent on medial septum and diagonal band of Broca (MSDB) afferents.

Specific lesions showed that septohippocampal cholinergic cells were not responsible for running-mediated neurogenesis (assessed with bromodeoxyuridine). mRNA and protein expression of a putative candidate sonic hedgehog (Shh) and its key downstream effectors were observed in the MSDB and hippocampus. Shh-immunopositive neuronal bodies in the MSDB, and its presumptive varicosities were present in the hippocampal neurogenic niche, in close association with stem cell markers. Disruption of axonal transport enhanced Shh-immunoreactivity in the MSDB, with a concomitant attenuation in the hippocampus. Retrograde tracing demonstrated that Shh was expressed mainly in septohippocampal GABAergic projection neurones. Pharmacological antagonism of Shh signalling, which did not impair baseline progenitor proliferation, abrogated the running-induced increase. Real-time PCR and immunoblotting determined that running activates the transcriptional response downstream of Shh signalling in the hippocampus.

A model is proposed whereby running evokes theta, and the subsequent release of Shh via septohippocampal GABAergic projections, giving rise to the increase in hippocampal neurogenesis.

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"…once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In adult centres the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be generated. It is for science of the future to change, if possible, this harsh decree."

Santiago Ramón y Cajal (1913, 1914/1991) Cajal's Degeneration and Regeneration of the Nervous System, J.DeFilpe and E.G.Jones, eds. Translated by R.M.May. *New York: Oxford University Press*

1. INTRODUCTION

1.1 ADULT NEUROGENESIS

For nearly a century neuroscientists embraced the prevailing tenet that unlike the skin, heart, liver, lungs, blood and other organs, the brain is a closed system with no regenerative capabilities. A decade ago, however, a groundbreaking paper established that the adult human brain does indeed possess the capacity to give rise to new neurones (Eriksson et al., 1998). This firmly dispels the original dogma and captures the imagination of both scientists and the public with the possibility that the central nervous system (CNS) can remodel its circuitry. That certain regions of the CNS can generate new newborn cells was in fact pointed out decades ago, without much fanfare, in autoradiographic [^{3}H]thymidine studies of rats, cats and song birds (Altman, 1962; Altman and Das, 1965; Kaplan and Hinds, 1977; Paton and Nottebohm, 1984).

The self-renewing cells are not found throughout the brain, but are restricted to two main germinal areas - the lateral ventricles, which contain cerebrospinal fluid (Lois and Alvarez-Buylla, 1993), and the hippocampus (Eriksson et al., 1998; Gould and Cameron, 1996; Gould et al., 1999b) , a region important for learning and memory (Squire et al., 2004). Animal models show that newly generated precursors have the ability to migrate: after a spell of proliferation the progenitors of the subventricular zone (SVZ) travel rostrally to the olfactory bulb to complete formation into interneurones, and those

found in the subgranular zone (SGZ) of the dentate gyrus (DG) will move radially into the granule cell layer to continue their differentiation into dentate granule cells (Alvarez-Buylla et al., 2002; Gage, 2002) (FIGURE 1-1). This thesis will centre on adult neurogenesis in the DG of the hippocampus *per se*.

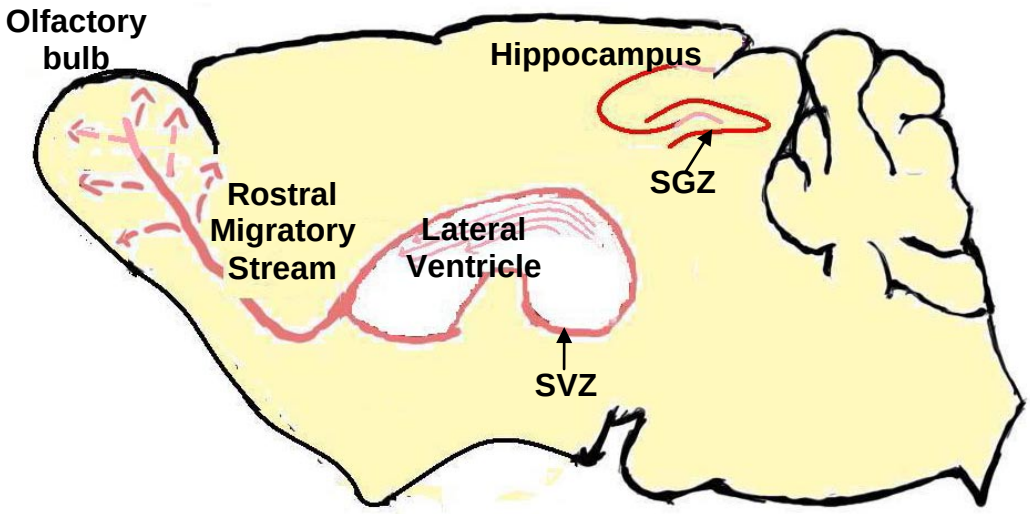


FIGURE 1-1 Neurogenesis in the adult rodent brain (adapted fromGage, 2002). Arrows point to the two neurogenic regions: the subgranular zone (SGZ) and subventricular zone (SVZ).

1.1.1 Stages of adult neurogenesis

Neurogenesis is a multi-step process, orchestrated at every phase by an intricate interplay of environmental cues (such as interacting cells, growth factors, axon guidance molecules, etc.) present in the microenvironment where the neural precursors reside. The specific pockets of cellular rejuvenation are termed as neurogenic niches.

Precursor cells along each stage of neurogenesis can be divided into various cell types, largely identified by their antigenic characteristics. Recent

advances in techniques like retroviral labelling with green fluorescence protein (GFP) also allow tracking of the maturation progress of cells over time.

The birth of new neurones does not occur in batches like a factory assembly line. The creation, maturation and eventual survival of an individual neurone in the SGZ are unique events at any one point of time. To sketch an outline of the developmental process, multipotent neural stem cells first go through intermittent cycles of division, giving rise to rapidly dividing precursor cells of limited renewal potential, which then go on to differentiate into various lineages. Half of the immature neurones perish before successfully migrating and evolving into fully functional neurones (FIGURE 1-2). The sustained production and elimination of cells in the DG are a testament of the brain's dynamic ability to remodel discrete networks throughout the entire lifespan. The defining characteristics of the cells at differential time-points are charted in Table 1-1.

1.1.1.1 Type I cells

Type I cells are the prototype neural stem cells: they are multipotent (having the potential to differentiate into various lineages e.g. neurones, astrocytes or oligodendrocytes) and self-renewing (possessing the ability to produce identical daughter cells) (Seri et al., 2001). These radial glia-like cells share morphological similarities with astrocytes. They have triangular somas in the SGZ with long apical processes across the granule cell layer (Filippov et al., 2003), and are immunopositive for an intermediate filament marker, glial fibrillary acidic protein (GFAP), which has long been used to identify

astrocytes. They also possess electrophysiological characteristics similar to astrocytes with delayed rectifying currents and low input resistance (Filippov et al., 2003; Fukuda et al., 2003). However, they do not express the calcium binding protein S100β, another marker for astrocytes (Steiner et al., 2004). Type I cells receive no synaptic input despite expressing $GABA_A$ and glutamate receptors (Wang et al., 2005).

1.1.1.2 Type II cells

The most proliferative among all cell types, Type II cells serve as the transition phase between multipotency and lineage specialization (Steiner et al., 2006a). The cell bodies of type II cells are also in the SGZ, with their short plump processes oriented tangentially (Filippov et al., 2003; Kronenberg et al., 2003; Suh et al., 2007). Type II cells have higher input resistance than Type I cells (Fukuda, 2003). The progressive development of these progenitors can be subdivided into 2 phases: Type IIa and Type IIb, based on their immunoreactivity to specific cell markers. It is believed that Type IIb cells are lineage committed (Steiner et al., 2006a). The initial inputs to Type II cells are excitatory GABAergic synapses (Tozuka et al., 2005; Wang et al., 2005).

1.1.1.3 Type III cells

The expansion of the pool of these neuroblasts is not as prolific as the Type II cells. Type III cells display antigenic characteristics typical of a neurone, and do not express any glial cell markers. Radial migration into the granule cell area commences in this phase in which the cells proceed to their postmitotic development into neurones (Brandt et al., 2003).

1.1.1.4 Immature neurones

No longer in the neurogenic milieu of the SGZ, the new immigrant cells in the granule cell layer now face a harsh selection process in an unfamiliar environment. Cell death occurs at a constant and relatively high rate, and about 50% of the 1- to 4- week old newborn neurones perish (Biebl et al., 2000; Dayer et al., 2003). Programmed cell death plays a regulatory mechanism here, by eliminating excess new neurones to ensure a prescribed granule cell layer size and to determine that the eventual selected population will form proper neuroneal circuits (Kuhn et al., 2005). This apoptotic process does not affect preneuroneal progenitor cells (Kuhn et al., 2005).

The young granule cells possess different membrane properties from mature granule cells such as very high input resistance and greater paired-pulse facilitation, which is indicative of an increased probability of vesicle release (Schmidt-Hieber et al., 2004). These membrane properties make the young neurones more excitable than their neighbouring mature cells. The newly minted dendrites of the new neurones project out into the molecular layer (Wang et al., 2000) guided by scaffolds formed by radial processes of glia (Shapiro et al., 2007). They receive synaptic inputs through axosomatic, axodendritic, and axospinous synapses (Toni et al., 2007; van Praag et al., 2002). GABAergic inputs are now inhibitory, and the first glutamatergic inputs appear around this period (Ge et al., 2006). The changing synaptic connections further mature the neurone functionally and are crucial for the integration of young cells into the existing network (Ge et al., 2006).

1.1.1.5 Fully functional neurones

Having survived the period of high susceptibility to apoptosis, cell death appears to halt for the approximately 1-month old postmitotic neurones (Dayer et al., 2003). These fully mature cells are now part of the principal cells of the DG and are physiologically indistinguishable from their neighbours 7 weeks after cell division (van Praag et al., 2002). It was found from comparative electrophysiological recordings that similar to granule cells of the embryonic brain, adult born neurones have excitatory glutamatergic and inhibitory GABAergic inputs, and can fire action potentials in response to excitation (Laplagne et al., 2006).

These new neurones preferentially contact pre-existing boutons involved in synapses with other neurones but form synapses with boutons devoid of other synaptic partners as they mature over the next few weeks. The connectivity continues to change until at least 2 months indicating that full maturation of the connectivity of the adult-born neurone is reached between 60-180 days after cell division (Toni et al., 2007). Axonal outgrowth occurs later than the dendritic projections into the cellular layer (Shapiro et al., 2007) and projects into the hippocampal CA3 regions (Hastings and Gould, 1999; Markakis and Gage, 1999).

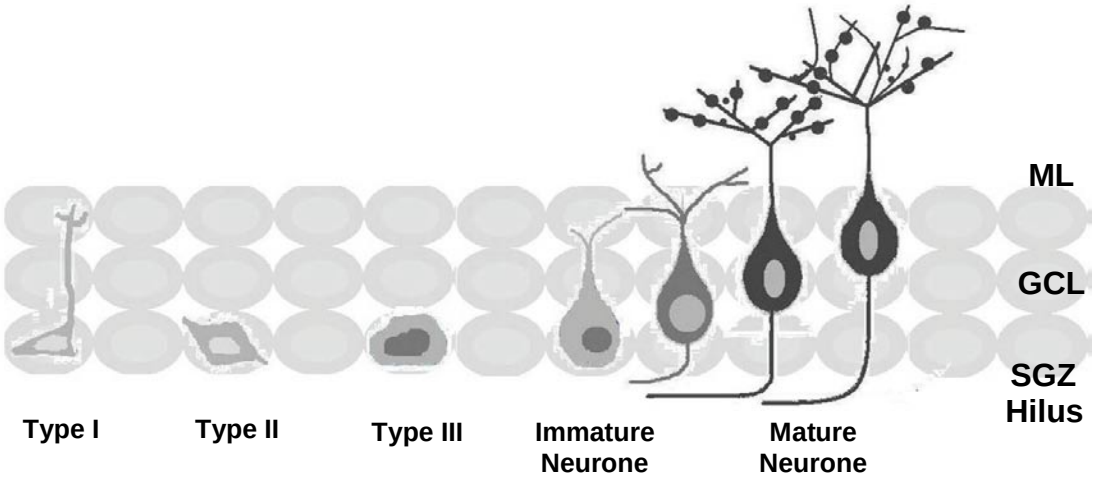


FIGURE 1-2 Stages of neurogenesis in the SGZ (adapted from Duan et al., 2008). The newborn cell residing in the subgranular zone (SGZ) will migrate across the granule cell layer (GCL), and extend its newly formed dendrites out into the molecular layer (ML).

Cell type	**Type I**	**Type IIa**	**Type IIb**	**Type III**	**Immature neurone**	**Mature neurone**
Cell age		1-3 days			2-3 weeks	>4 weeks
Stage of cell cycle	Quiescent	Mitotic			Postmitotic	
Defining characteristic	*Multipotent* radial glia -like stem cell; rare and slowly proliferating; Present in SGZ	Highest proliferative rate among all cell types but limited self-renewal	Highly proliferative but limited self-renewal. Differentiation into various lineages	Migrates to granule cell layer	50% die by apoptosis	Forms functional synapse with other neurones
Markers	GFAP+	DCX-	DCX-	DCX+	DCX+	DCX+
	Nestin+	Nestin+	Nestin+	Nestin-	NeuN+	NeuN+
	PSA-NCAM-	PSA-NCAM+	PSA-NCAM+	Prox1+	Prox1+	Prox1+
	Sox2+	Sox2+	Sox2+	Sox2-	Calretinin+	Calbindin+
	BLBP+	BLBP+	BLBP+	BLBP-	TuJ1+	TuJ1+
			NeuroD1-	NeuroD1+	NeuroD1+	Map2ab+
Synaptic inputs	-	Excitatory GABAergic			Initially inhibitory GABAergic, then glutamatergic	all
Input resistance	<100 MΩ	~50 –8500 MΩ		~5000–10000 MΩ	>1500 MΩ	~300 MΩ
Voltage-gated currrents	A type K+	K+, small Na+		K+, small Na+, T-type Ca2+	K+, Na+, T-type Ca2+	K+, Na+

TABLE 1-1 Characteristics of adult-born neurones in the SGZ at different time-points

Abbreviations and key references: β-tubulin (TuJ1) (Parent et al., 1997); Brain lipid binding factor (BLBP) (Steiner et al., 2006a); Calbindin (Sloviter et al., 1989); Calretinin (Brandt et al., 2003); Doublecortin (DCX) (Filippov et al., 2003; Plumpe et al., 2006); Glial fibrilliary acidic protein (GFAP) (Filippov et al., 2003); Microtubule associated protein 2ab (Map2a) (Brazel et al., 2005; Steiner et al., 2006a); Nestin (Filippov et al., 2003; Lendahl et al., 1990; Mignone et al., 2004); Neurogenic differentiation 1(NeuroD1) (Steiner et al., 2006b); Polysialic acid neural cell adhesion molecule (PSA-NCAM) (Seki, 2002a, b; Seki and Arai, 1993); Prospero-related homeobox1 (Prox1) (Brandt et al., 2003); SRY(sex-determining region Y)-box 2 (SOX2) (Brazel et al., 2005; Steiner et al., 2006a; Suh et al., 2007)

1.1.2 Factors regulating neurogenesis

For a seemingly restricted region, the permissive SGZ niche is susceptible to a host of regulatory agents that affects neurogenesis at every stage. Being vestiges of the embryonic brain, it is fairly straightforward to imagine niches as microenvironments where developmental neurogenic qualities are retained, and where original neuromodulators are still at work.

Most studies investigating the mechanisms behind neurogenesis have been accomplished utilizing the thymidine analog bromodeoxyuridine (BrdU) as an *in vivo* marker of proliferating cells. BrdU can be visualized using immunohistochemical techniques and quantitatively assessed (Gould and Gross, 2002). The colocalization of BrdU-labelled cells with cell type-specific markers can be verified by orthogonal reconstruction of different planes captured by confocal microscopy (Gould and Gross, 2002).

In the context of this discourse, modulators of neurogenesis are broadly subdivided into (i) cellular and molecular factors, and (ii) physiological and behavioural factors. A list of these factors is given in TABLE 1-2.

1.1.2.1 Cellular and molecular factors

1.1.2.1.1 Glial cells

There is increasing documentation to suggest that glial cells, originally regarded as supporting cells, are instrumental in regulating neurogenesis (Ma et al., 2005).

Astrocytes are the most abundant of all glia. When extracted from the hippocampus and cultured, astrocytes were shown to spur the growth of progenitors and subsequently commit these progenitors to a neuroneal lineage (Song et al., 2002). Hippocampal astrocytes were also able to promote synapse formation of neurones derived from adult neural stem cells (Song et al., 2002). This is because astrocytes provide a lattice for the growth of axons and dendrites from newly generated neurones, as revealed through structural studies (Horner and Palmer, 2003).

Microglia, another non-neuroneal cell normally activated during CNS inflammation, is proposed to regulate the pro- and anti-neurogenic effects of immune cytokines in the DG niche (Battista et al., 2006). Microglia activation correlates with the presence of an anti-inflammatory cytokine transforming growth factor-β (TGFβ) and an increase in progenitor proliferation (Battista et al., 2006). Exposure of microglia to other cytokines such as interleukins also induces neurogenesis (Butovsky et al., 2007).

1.1.2.1.2 Growth factors

A growing body of evidence suggests a primary role for peptide growth factors such as basic fibroblast growth factor (FGF2), insulin-like growth factor-I (IGF1), granulocyte-colony stimulating factors (G-CSF), vascular endothelial growth factor (VEGF), erythropoietin, epidermal growth factor (EGF) and TGFβ in influencing neurogenesis. These ligands are detected in early stages of

development, and their expression persists postnatally into adulthood in the hippocampal DG (Bondy and Lee, 1993; Ozawa et al., 1996).

Specifically, FGF2 has been widely used to expand cultured neural progenitor cells from fetal and adult brains. In primary cultures of DG granule cells from neonatal rats, addition of FGF2 enhanced neuroneal survival and differentiation (Lowenstein and Arsenault, 1996b). FGF2 also increased axon number and length, and boosted migration (Lowenstein and Arsenault, 1996a). Infusions of FGF2 into the ventricles of middle aged rats increased neurogenesis and augmented dendritic growth (Rai et al., 2007). Some reports indicate that FGF2 inhibits neuroneal lineage determination and hence maintains the progenitor pool in a proliferative state (Chen et al., 2007). Another growth factor IGF1 has been shown to generate new neurones from adult hippocampal progenitors *in vitro* (Aberg et al., 2000; Anderson et al., 2002). The angiogenic factor VEGF can stimulate cell genesis in cortical cultures, and increase the overall production of neurones (Jin et al., 2002). *In vivo* experiments also show that intracerebroventricular injections of VEGF into the adult rat brain increased SGZ progenitor proliferation (Jin et al., 2002).

The source of these growth factors may or may not be intrinsic to the neurogenic niche. Underlying the region is a rich network of blood vasculature, where tight clusters of proliferating precursors, committed progenitors, neurones and glial cells are grouped (Palmer et al., 2000). The growth factors may derive from the

circulatory system. *In vitro*, soluble factors secreted by the vascular endothelial cells, components of blood vessels, promote self-renewal and neurogenesis in fetal neural stem cells (Shen et al., 2004).

The survival of newly generated neurones may also involve neurotrophins such as brain-derived neurotrophic factor (BNDF), nerve growth factor (NGF) and neurotrophin 3 (NT3). *In vitro*, NT3 but not BDNF significantly increases the number of newborn neurones (Babu et al., 2007). ICV infusions of NGF increase the proportion of both BrdU-positive and DCX-positive cells two weeks later (Frielingsdorf et al., 2007). In BDNF heterozygous mice ($BDNF^{+/-}$) and trkB (receptor of BDNF) dominant null mice, the number of new neurones born is considerably less (Sairanen et al., 2005).

1.1.2.1.3 Neurotransmitters

Afferents from other parts of the brain extend to postsynaptic neurones in the DG, releasing chemical messengers that facilitate neurognenesis. A couple of amino acid neurotransmitters, namely γ-aminobutyric acid (GABA) and glutamate, are the major forces behind excitatory-neurogenesis coupling.

In the embryonic brain, GABA initially acts as an excitatory molecule. GABA binds mostly to $GABA_A$ receptors present in the precursor cell, which has an elevated intracellular chloride (Cl^-) concentration, and hence a lower resting membrane potential. This leads to an efflux of Cl- ions and depolarization, and

subsequent activation of voltage-dependent calcium channels (Ben-Ari, 2002). Later, due to a drop in intracellular Cl^- concentration in the more mature cell, GABA switches from being excitatory to inhibitory (LoTurco et al., 1995).

Drawing parallels from the embryonic brain, Type II progenitors in the SGZ similarly receive GABAergic inputs (Tozuka et al., 2005). By triggering spontaneous GABAergic synaptic events, Type II cells are depolarized, causing increased intracellular calcium concentration and induction of NeuroD expression (Tozuka et al., 2005). NeuroD is a transcription factor that drives neuroneal differentiation (Liu et al., 2000). Addition of $GABA_A$ receptor antagonists elevates progenitor proliferation, while $GABA_A$ receptor agonists elicit the opposite effect, increasing differentiation of newly born neurones, further cementing the evidence that GABAergic inputs promote activity-dependent neuroneal differentiation (Tozuka et al., 2005). Other reports show that injection of $GABA_A$ receptor agonist into the rodent brains do not affect the survival of newborn cells (Karten et al., 2006), but rather increase dendritic length and complexity (Ge et al., 2006). Initial GABA-induced depolarization is crucial for ensuing inhibitory GABAergic and excitatory glutamatergic synaptic inputs in newly generated neurones (Ge et al., 2006).

Glutamatergic synapses are formed after GABAergic synapses in the embryonic brain (Ben-Ari et al., 2007). The dentate granule cells receive most of the excitatory glutamatergic inputs from the entorhinal cortex. In adult rodents, the

activation of N-methyl-d-aspartate receptors (NMDAR) by the agonist NMDA resulted in a drop in cell division in the SGZ. In contrast, intraperitoneal injections of NMDA receptor antagonist led to an increase in cell birth in both young adult (Cameron et al., 1995) and middle-aged rats (Nacher et al., 2003). NMDA receptor subunits NR1 and NR2B are expressed in Type I precursor cells and immature neurones in the DG (Nacher et al., 2007). An elegant experiment in which retrovirus-mediated gene knockout of NMDAR in a single-cell reduces neuroneal survival, only to be rescued by NMDAR antagonist application that blocks receptors of surrounding functional neurones demonstrates that glutamatergic inputs may be important for extending the lifespan of newly generated neurones (Tashiro et al., 2006).

The regulatory effects of acetylcholine amino acid from cholinergic inputs to the DG will be elaborated more in Chapter 2 of this dissertation.

1.1.2.1.4 Steroid hormones

Many studies have revealed that glucocorticoid stress hormones are major dampeners of progenitor proliferation (Cameron and Gould, 1994). Removal of circulating adrenal steroids by adrenalectomy reverses the stress-induced decline in neurogensis in DG (Cameron and McKay, 1999; Tanapat et al., 2001).

Sex hormones, another class of steroids, generate and sustain new cells differentially in adult female and male rodents. One of the earlier observations

comes from female rats, which possess a greater number of newborn cells in the DG compared to male rats, and which the cell count fluctuates at different periods of the oestrus cycle (Tanapat et al., 1999). Cell proliferation is decreased by ovariectomy but can be reversed by progesterone (Tanapat et al., 2005). Acute estrogen treatment likewise induces cellular proliferation (Tanapat et al., 2005). Estrogen receptor agonists also enhances cell genesis (Mazzucco et al., 2006). Interestingly, estradiol too stimulates progenitor proliferation in middle aged male mice (Saravia et al., 2007). Unlike estrogen, androgens targets neurogenesis at a later time point. Cell survival was decreased for castrated rats, but prolonged in male rats injected with testosterone and one of its derivatives, dihydrotestosterone (Spritzer and Galea, 2007).

1.1.2.1.5 Morphogens

Properties of the embryonic brain are conserved in specialized niches. As such, developmental morphogens such as Notch, bone morphogenetic proteins (BMPs), Noggin, Sonic hedgehog (Shh), Wingless-type MMTV integration (Wnt) have all been implicated in the regulation of neurogenesis (Babu et al., 2007; Breunig et al., 2007; Fan et al., 2004; Lai et al., 2003).

For instance, Notch1 signalling acts like a switch between Type I, Type IIa and Type IIb cells (Breunig et al., 2007) in postnatal mice. Another developmental protein, BMP4 and its signalling antagonist Noggin are expressed in the SGZ of adult DG (Fan et al., 2004). Antisense Noggin infusion into the ventricles reduced

DG progenitor proliferation (Fan et al., 2004). Another member of the BMP family, BMP2 inhibited neurogenesis in monolayer precursor cell culture from adult mouse DG (Babu et al., 2007).

Shh is a potent mitogen of multipotent adult hippocampal progenitor cells *in vitro* (Babu et al., 2007; Lai et al., 2003). *In vivo*, viral delivery of Shh in the hippocampus increases progenitor division and subsequently the number of newborn neurones in the granule cell layer (Lai et al., 2003) whereas pharmacological blockade of Shh signalling reduces proliferation (Banerjee et al., 2005). Wnt signalling affects neuroneally restricted Type IIb precursors (Pozniak and Pleasure, 2006). Wnt3 proteins are secreted by astrocytes in the DG hilus and cause increases in the total number of immature neurones (Lie et al., 2005).

1.1.2.2 Behavioural and physiological factors

1.1.2.2.1 Aging

Our brains, like other parts of the body, deteriorate over time. Not surprisingly, neurogenesis decreases with increasing age, attributed by different groups either to slower precursor proliferation, migration or differentiation (Hattiangady and Shetty, 2008; Kempermann et al., 1998b; Kuhn et al., 1996);. The turnover of the newly generated cells, characterized by rates of apoptosis, also slows down with increasing age (Heine et al., 2004). The observations of age-related decline in neurogenesis were replicated outside laboratory settings, in different species of

wild-living rodents that presumably receive more environmental stimuli than their laboratory counterparts (Amrein et al., 2004).

Recently, a study in primates suggested that the decline in neurogenesis precedes aging and the subsequent decline in synaptic plasticity may lead to the drop in cognitive functions associated with old age (Leuner et al., 2007).

1.1.2.2.3 Antidepressant treatments

The "neurogenic theory of depression" (Drew and Hen, 2007) was formulated upon a collective body of studies implicating aberrant hippocampal circuitry and dynamics in depression (Meltzer et al., 2005). For example, significantly reduced hippocampal volume is observed in depressed patients (Videbech et al., 2004). A wide spectrum of antidepressants and mood stabilizer therapies, each utilizing different pharmacological pathways, such as lithium (Chen et al., 2000), electroconvulsive seizures, monoamine oxidase inhibitors, norepinephrine-selective reuptake inhibitors and serotonin-selective reuptake inhibitors, have been shown to elevate progenitor cell proliferation in rodents (Encinas et al., 2006; Malberg et al., 2000) and primates (Perera et al., 2007). In stress-induced behavioural depression modelled by learned helplessness in rats, a serotonin-selective reuptake inhibitor inhibitor treatment reversed the learned helplessness behaviour (Chen et al., 2006). Activation of various serotonin receptors, e.g. 5-HT1A, 5-HT2A, have been reported to mediate the mechanisms of serotonin on adult hippocampal neurogenesis (Banasr et al., 2004; Santarelli et al., 2003). In

addition, ablation of neurogenesis in the hippocampus reversed behavioural effects of antidepressants in rodents (Santarelli et al., 2003).

1.1.2.2.4 Neurological disorders

The birth of new cells can be regulated by physiopathogenic events. For instance, neurogenesis is increased following induced epileptic seizures (Jessberger et al., 2005; Parent and Murphy, 2008; Parent et al., 1997; Scharfman et al., 2000);. An acute bout of seizure induced by kainic acid showed that the cell types affected are Type I, IIa and III cells and that a single seizure event can have lasting effects on adult neurogenesis (Steiner et al., 2008). Seizures also affect morphology and localization of the newborn cells, with dispersion of granule cell layer, and neurones abnormally positioned in the hilus and inner molecular layer of DG (Jessberger et al., 2005; Parent and Murphy, 2008; Scharfman et al., 2000). A recent work by Bartlett and colleagues show that pilocarpine- evoked status epilepticus activates a latent pool of hippocampal progenitor cells by depolarization activity (Walker et al., 2008).

Ischaemia-induced stroke in rodents can likewise increase neurogenesis in the SGZ (Jin et al., 2001; Liu et al., 1998). Another example of pathology-altered neurogenesis is Alzhiemer's Disease, where loss of cholinergic function is associated with reduced neurogenesis (Amaral and Kurz, 1985; Kaneko et al., 2006; Kotani et al., 2006; Mohapel et al., 2005).

Recently, cognitive impairments associated with schizophrenia has been linked to defective adult neurogenesis. In the hippocampus of human schizophrenic patients, a decline in precursor cell proliferation has been observed (Reif et al., 2006). Animal knock-out models of Disrupted in Schizophrenia 1 (DISC1), a gene associated with schizophrenia, also show reduced neural progenitor proliferation (Duan et al., 2007; Mao et al., 2009). Conversely, atypical antipsychotics increase newborn cells in the DG (Kodama et al., 2004).

1.1.2.2.5 Drugs of abuse

The acute and chronic usage of social drugs such as nicotine (Abrous, 2002), alcohol (Crews et al., 2006; Ieraci and Herrera, 2007; Nixon and Crews, 2002; Rice et al., 2004) and illegal drugs of the opioid family, like morphine and heroin (Arguello et al., 2008; Eisch et al., 2000), cannabis and cocaine (Andersen et al., 2007; Dominguez-Escriba et al., 2006; Eisch et al., 2008; Venkatesan et al., 2007) have all been implicated in the inhibition of hippocampal neurogenesis.

1.1.2.2.6 Learning

Neurogenesis in the hippocampus is postulated to be involved in learning and memory (Adlard et al., 2005b; Kee et al., 2007; Shors et al., 2001; Snyder et al., 2005; Winocur et al., 2006). Conversely, learning and memory influence neurogenesis. Hippocampal-dependent learning tasks such as trace eyeblink conditioning and spatial memory training extend the life of newly generated

granule cells for prolonged periods of time (Gould et al., 1999c; Leuner et al., 2004).

Housing rodents in an enriched environment provides opportunities for socialization, learning and physical activity, and also increases the survival of newly generated neurones (Bruel-Jungerman et al., 2005; Kempermann et al., 1998a; Kempermann et al., 1997; van Praag et al., 1999b). Further investigations revealed that mice exposed to an enriched environment for merely a day had increased proliferation of Type IIb lineage committed cells and Type III neuroblasts, and hence a higher number of postmitotic cells (Steiner et al., 2008). Not only that, the animals were able perform better in the Morris water maze, a test for learning (Nilsson et al., 1999)and had enhanced long-term memory (Bruel-Jungerman et al., 2005).

	Renewal of mitotic cells	Promotion of lineage	Survival of newly generated cells
INCREASE	Sonic hedgehog (Shh) (Lai et al., 2003)	FGF-2 (Babu et al., 2007; Palmer et al., 1999)	Ciliary neurotrophic factor (CNTF) (Chen et al., 2007; Lowenstein and Arsenault, 1996b)
	IGF1 (Aberg et al., 2000; Trejo et al., 2001)	Shh (Ahn and Joyner, 2005; Babu et al., 2007; Machold et al., 2003)	BDNF (Linnarsson et al., 2000; Sairanen et al., 2005; Scharfman et al., 2000)
	Fibroblast growth factor (FGF-2) (Palmer et al., 1995)	Endothelial cell growth factor (ECGF) (Babu et al., 2007)	NMDA receptor activation (Deisseroth et al., 2004; Tashiro et al., 2006)
	Epidermal growth factor (EGF) (Kuhn et al., 1997)	GABAergic excitation (Tozuka et al., 2005)	Anti-depressants (Chen et al., 2000; Malberg et al., 2000; Sairanen et al., 2005; Santarelli et al., 2003)
	Selective serotonin reuptake inhibitors (Santarelli et al., 2003)	Neurotrophin-3 (NT3) (Adlard et al., 2005b; Babu et al., 2007; Chang et al., 2003)	Hippocampal-dependent learning (Gould et al., 1999a; Leuner et al., 2004)
	Seizures (Banerjee et al., 2005; Bengzon et al., 1997; Jessberger et al., 2005; Parent and Murphy, 2008)	Brain-derived neurotrophic factor (BDNF) (Bull and Bartlett, 2005; Chang et al., 2003)	Granulocyte-colony stimulating factor (G-CSF) (Schneider et al., 2005)
	Estrogen (Ormerod and Galea, 2001; Tanapat et al., 2005; Tanapat et al., 1999)	Wnt3 (glial-induced neurogenesis) (Lie et al., 2005)	Glial cell interaction (Song et al., 2002; Toda et al., 2000)
	Glial cell interaction (Song et al., 2002)	Neurogenesin-1 (Ng1) (factor secreted by astrocytes (Ueki et al., 2003)	Cholinergic innervation (Cooper-Kuhn et al., 2004)
	Endothelial cell interaction (Shen et al., 2004)	Glial cell interaction (Song et al., 2002)	Running (van Praag et al., 1999a)
	Running (van Praag et al., 1999b)		Enriched environment (Brown et al., 2003; Kempermann et al., 1998a; Kempermann et al., 2002; Kempermann et al., 1997, 1998b; Nilsson et al., 1999)
			Wnt3 (Lie et al., 2005)

	Renewal of mitotic cells	Promotion of lineage	Survival of newly generated cells
DECREASE	Glucocorticoids (Gould et al., 1997; Gould et al., 1998; Tanapat et al., 1998; Tanapat et al., 2001)	Bone morphogenic protein 2 (BMP2) (Babu et al., 2007*)gliogenic*	Nicotine (Abrous et al., 2002)
	Stress, mediated by Learned helplessness (Chen et al., 2006)	Leukemia inhibitory factor (LIF) (Babu et al., 2007)	Chronic morphine (Eisch et al., 2000; Mandyam et al., 2004)
	Binge alcohol (Crews et al., 2006; Ieraci and Herrera, 2007; Nixon and Crews, 2002; Rice et al., 2004) and chronic alcohol exposure (He et al., 2005)	Chronic alcohol exposure (He et al., 2005)	Kainic acid induced seizures (Magloczky and Freund, 1993; Pollard et al., 1994)
	Chronic morphine (opiates) (Arguello et al., 2008; Eisch et al., 2000; Mandyam et al., 2004)		Chronic mild stress (Lee et al., 2006)
	Nicotine (Abrous et al., 2002)		Chronic alcohol exposure (He et al., 2005)

TABLE 1-2 Factors regulating adult hippocampal neurogenesis

With the advancement in experimental techniques, the last decade has seen an explosion of literature with regards to the numerous, and at times confusing signals involved in modulating neurogenic responses. The implications are exciting –innumerable strategies can be thought up to tap into the potential pool of precursor cells and their regulatory factors, and their manipulation to non-neurogenic regions in order to facilitate regeneration. This can either be in the form of *de novo* cellular replacement or the stimulation of self-repair via engineering of intracellular signalling.

It remains for us to make sense of underpinning mechanisms behind neurogenesis and I shall attempt to contribute to the ever-expanding literature throughout the next few chapters. The next two parts of the Introduction (1.2 and 1.3) will provide a more in-depth insight into the latest findings pertaining to more directly to my research, while Chapters 2 – 5 will cover my findings (with the methodologies employed explained within each chapter).

“Mens sana in corpore sano”

Decimus Lunius Luvenalis (otherwise known as Juvenal) (lst AD) *Satires X*: 356-64

1.2 RUNNING AND NEUROGENESIS

The simple behavioural act of running causes a striking increase in neurogenesis (van Praag et al., 1999a; van Praag et al., 1999b). It does not merely facilitate cellular plasticity, it also brings about a host of beneficial brain changes at various levels that are worthwhile mentioning, and will be briefly touched on in the next few pages.

1.2.1 Running and cellular plasticity

In 1999, van Praag and colleagues, in search of neurogenic factors among the many variables of an enriched environment, added running wheels to the cage to allow the mice to run *ad libitum* (van Praag, 2008; van Praag et al., 1999a). The results were astounding: running increased cell division and the numbers of newborn neurones by nearly two-fold. Subsequently, other researchers reported the same robust phenomenon (Brown et al., 2003; Fabel et al., 2003; Kitamura et al., 2003; Kronenberg et al., 2006; Overstreet et al., 2004; Trejo et al., 2001; Van der Borght et al., 2007). The effects of running are the same regardless of voluntary or forced running (Uda et al., 2006; Wu et al., 2007). Interestingly, the neurogenic response to running is restricted to the DG, but not the SVZ (Brown et al., 2003).

Running specifically increases the population of Type II rapidly proliferating progenitor cells (Kronenberg et al., 2003). Running can also induce the rare

event of division in Type I multipotent cells (Suh et al., 2007). Another study showed that a single day of physical activity suffices to elevate numbers of both Type IIa and Type IIb lineage-determined progenitors (Steiner et al., 2008).

Cell turnover is reported to concurrently increase as a result of physical activity (Kitamura and Sugiyama, 2006). However, seemingly conflicting data show that running has a survival promoting effect on newly generated neurones, marked by increase in DCX and calretinin expression (Kronenberg et al., 2006). The survival effect is a result of long term running (≥ 3 weeks) (Kronenberg et al., 2006; Stranahan et al., 2006). Continuous running, however, downregulates progenitor proliferation to baseline levels in mice (Kronenberg et al., 2006). The downregulation is also visible in spontaneously hypertensive rats(Naylor et al., 2005), which exhibit habitual running behaviour (Shyu and Thoren, 1986).

The neurogenic effects of exercise also extend from mothers to their offspring. Voluntary wheel running resulted in the birth of more granule cells in pups (Bick-Sander et al., 2006). In aging mice, exercise can abate the age-dependent decline in cell genesis and neuroneal production (Kronenberg et al., 2006; van Praag et al., 2005).

1.2.2 Running and structural/synaptic plasticity

Neural network remodelling is not based solely on incorporation of new neurones but necessarily involves synaptogenesis. Running influences the morphology of

the granule cell population within the DG, in the form of significant dendritic elongation and complexity together with a denser network of spines, as revealed by Golgi staining (Eadie et al., 2005; Redila and Christie, 2006). In addition, exercise facilitates synaptic plasticity. Long term potentiation (LTP) is a model of synaptic plasticity (Bliss and Gardner-Medwin, 1973). Running is associated with an increase in DG LTP (van Praag et al., 1999a), attributed to enhanced potentiation in response to theta (Farmer et al., 2004). An increase in LTP can similarly be caused by forced treadmill exercise (O'Callaghan et al., 2007).

A growing body of evidence indicates that the changes in synaptic plasticity could in part be mediated by growth factors and/or their cross talk signalling. Many studies have demonstrated that exercise is linked to (i) higher BDNF gene expression (Berchtold et al., 2002; Farmer et al., 2004; Neeper et al., 1996) and protein expression levels (Adlard et al., 2005a; Soya et al., 2007) (ii) upregulation of downstream regulatory proteins, including cAMP response binding protein (CREB), phosphorylated calcium/calmodulin protein kinase II (CAMKII) and phosphorylated mitogen-activated protein kinase II (MAPKII) (Vaynman et al., 2003)and (iii) a rise in vesicular budding protein synapsin I expression(Adlard et al., 2005a; Vaynman et al., 2004a; Vaynman et al., 2006). Another study suggested that running induces higher levels of IGF1 that interacts with BDNF to produce a synergistic effect (Ding et al., 2006). Blocking IGF1 receptors led to not only a drop in BDNF mRNA expression levels, but also CAMPKII, MAPKII and synapsin I expression (Ding et al., 2006).

1.2.3 Running and learning and memory

As abovementioned, running facilitates both hippocampal cellular plasticity, and synaptic plasticity. The latter is widely considered as one of the major mechanisms underlying learning and memory (Martin et al., 2000; Neves et al., 2008). Hence, it is hardly surprising that exercise is associated with benefits in brain functions.

Animal studies have demonstrated that exercise can improve spatial memory (van Praag et al., 1999a; Vaynman et al., 2004b). In mice expressing a double mutant form of amyloid precursor protein, a hallmark of AD, extended voluntary physical activity reduced extracellular amyloid-β plagues in the cortical and subcortical regions, which is correlated to enhanced learning (Adlard et al., 2005b).

Exercise is associated with prevention of age-related decline in cognitive functions. Epidemiology studies showed that exercising reduced risks of cognitive impairment, and of developing dementia, and Alzheimer's disease (AD) (Friedland et al., 2001; Larson et al., 2006; Laurin et al., 2001). Functional magnetic resonance imaging studies of elderly subjects revealed that exercise is positively correlated to brain regions associated with executive functions such as planning, goal maintenance, working memory, multi-tasking and inhibitory control (Colcombe and Kramer, 2003; Kramer et al., 2003). Higher levels of physical

fitness in elderly participants are also associated with increased hippocampal volume, and better spatial memory (Erickson et al., 2009).

1.2.4 Factors underlying running-mediated neurogenesis

Here, I shall direct the attention of the reader back to the phenomenon of running-induced neurogenesis. Given that running can generate such a robust response in cell genesis, many attempts have been made to elucidate the cellular and molecular mechanisms behind this simple behavioural act. The possible factors are discussed here, though a conclusively convincing causal factor remains to be identified.

1.2.4.1 Growth factors

The physiological effects of exercise are well known. During physical activity, the heart pumps harder and there is increased blood flow to the rest of the body, including the brain. Several lines of evidence indicate that circulating growth factors (e.g. VEGF, FGF2, and IGF-1) released by muscular tissues during exercise may play important roles in mediating neurogenesis. Firstly, MRI in human subjects showed that there is a correlation between hippocampal blood flow and neurogenesis (Perera et al., 2007). Secondly, as aforementioned, the DG neurogenic niche is in close proximity to capillaries (Palmer et al., 2000) and cell genesis occurs in response to exogenous applications of vascular growth factors (Cao et al., 2004; Jin et al., 2002). Thirdly, exercise elevates gene expression of these blood-borne cytokines (Ding et al., 2006; Gomez-Pinilla et

al., 1997) and their peripheral inhibition resulted in less neurogenesis in runners (Fabel et al., 2003; Trejo et al., 2001).

Other studies suggest, however, that it may be difficult to reconcile these findings. Running does not increase vascularisation to the DG (van Praag et al., 2007). Running did not bring about a change in vascular permeability in the brain as well, even with the addition of the permeability-enhancing factor VEGF (Fabel et al., 2003). One plausible reason could be the notoriously selective blood-brain barrier, constituted by the tight junctions formed by capillary endothelial cells and astrocyte foot processes (Goldstein, 1988), constituting the. The putative blood-borne growth factors may not be able to cross the barricade of interendothelial junctions.

Apart from their extrinsic counterparts, intrinsic neurotrophins appear to be attractive candidates for running-mediated neurogenesis, given their prominent effects on synaptic plasticity. Nevertheless, their roles in neural progenitor proliferation remain to be established.

1.2.4.2 Beta-endorphins

The “runner’s high” is a feeling of euphoria in some athletes engaging in strenuous aerobic activity and is associated with the release of β-endorphins (Boecker et al., 2008; Morgan, 1985). β-endorphins are secreted by the pituitary gland and released into the blood steam where they bind to μ-opioid receptors,

which are also found in the hippocampus (Ableitner and Schulz, 1992; Mansour et al., 1994). *In vitro* and *in vivo* studies show that addition of opioid receptor antagonist reduces progenitor proliferation (Persson et al., 2004; Persson et al., 2003). In β-endorphin knock out mice, running does not increase progenitor proliferation (Koehl et al., 2008).

These results however, conflict with the reduced neurogenesis observedwith administration of exogenous μ-opioid receptor agonists such as morphine and heroin (Eisch et al., 2000; Mandyam et al., 2004) and increased neurogenesis in μ-opioid receptor knock out mice (Harburg et al., 2007) and β-endorphin knock out mice (Koehl et al., 2008). Hence, the modulatory role in β-endorphins in this aspect remains controversial.

1.2.5 Functional Implications of running-mediated neurogenesis

Given the prominent impact physical activity has on neurogenesis, and its immense potential in therapeutic cellular regeneration in neurological diseases (Bjorklund and Lindvall, 2000a, b; Eriksson, 2003; Horner and Gage, 2000; Jessberger and Gage, 2008; Magavi and Macklis, 2001), it is perhaps prudent, at this point of time, to play devil's advocate and question the functional significance of running-mediated neurogenesis. Or, to take a step backward and question, what exactly are new neurones for?

Strictly speaking, from an economic sense, it is very “costly” to make new neurones. It takes about a month for a new neurone to be generated, during which most of its precursors would have been rejected and eliminated after their transverse migration into another environment; and after which the young neurone must be securely integrated into the existing circuit amongst older granule cells while reaching out to form appropriate connections. It would have been more “cost effective” to just make use of existing neurones and manipulate extant synaptic plasticity rather than maintain neurogenesis for a lifetime.

Kempermann posited a plausible theory for the function for neurogenesis, which he calls the “neurogenic reserve theory” (Kempermann, 2008). Drawing from experimental outcomes in which physical and cognitive activity stemmed the decrease in progenitor proliferation due to age (Kronenberg et al., 2006; van Praag et al., 2005), the theory asserts that “activity preserves the potential for cell-based plasticity by maintaining [hippocampal] adult neurogenesis in an activated state” (Kempermann, 2008). Physical exercise and learning work in a complementary fashion: the fomer to generate a pool of rapidly proliferation new cells, and the latter to prolong the survival. Kempermann proposed that running is a non-specific activator for specific cognitive events, especially in animals where locomotion and learning are inseparable in real life. Sustained physical activity and cognitive challenges will maintain a pool of neurones that will allow that brain to accommodate novel complexities if the need arises (Kempermann, 2002).

At any rate, for the purposes of this discourse, a parsimonious conclusion that can be drawn is that and running, especially in animals, is closely associated to learning, and that the role of neurogenesis is inextricable from the function of the hippocampus.

“I do not think the human preoccupation with periodic processes is accidental. While there is something inherently fascinating about all cyclical processes in both animate and inanimate systems, biological oscillators have a special emotional immediacy. These rhythmic processes provide beautiful examples of profound elegance, simplicity and effectiveness of biological regulation.”

P. E. Rapp (1987) Why are so many biological systems periodic? *Progress in Neurobiology* pp. 270

1.3 THE HIPPOCAMPUS AND THETA

1.3.1 Functions of the hippocampus

The function of neurogenesis is fundamentally linked to that of the hippocampus, arguably one of the most studied regions of the brain. One of the first glimpses on the role of the hippocampus came from clinical observations of H.M (Scoville and Milner, 1957). To relieve his epileptic seizures, H.M underwent surgery to remove his medial temporal lobe, including his hippocampi. The surgical procedure left H.M. with anterograde amnesia, the inability to transfer short-term memory to long-term memory. In rodents, where memory is related to spatial processing in the hippocampus, the firing of hippocampal “place cells” when an animal is at a specific location in the environment or navigating its surroundings, helps it encode and store a neural representation of space (O'Keefe and Conway, 1978; O'Keefe and Dostrovsky, 1971). Other comprehensive studies ranging from lesions, pharmacological, electrophysiological, imaging, and MRI scans have likewise provided correlative evidence that learning and memory involves the hippocampus (Broadbent et al., 2004; Shrager et al., 2007; Squire et al., 2004).

Although the exact molecular mechanisms behind hippocampal memory formation have yet to be proven beyond a reasonable doubt, there is strong evidence favouring the synaptic plasticity model proposed by Richard Morris who posits that induction of appropriate activity-dependent synapses by LTP is an

essential and adequate requisite (Martin et al., 2000; Martin and Morris, 2002; Neves et al., 2008).

1.3.2 Structure of the hippocampus

The hippocampus is located deep within the medial temporal lobe of the cerebral cortex. So named by the Italian anatomist Julius Caesar Aranzi back in the 16th century due to its resemblance to a seahorse, the structure of the hippocampus is well conserved throughout mammalian species. The hippocampal formation comprises of four subfields, dentate gyrus and the cornu ammonis regions namely CA3, a miniscule zone CA2, and CA1. Within the hippocampus proper, pyramidal neurones, with extensive dendritic spines, comprise the bulk of the neurones amidst interneurones (Spruston, 2008). The neurones are arranged in a lamellar manner, with several series of strata. Within the DG itself, the only principal cells are the granule cells.

The hippocampus receives connections from several subcortical and cortical structures, such as the anterior thalamic nuclei, the mammillary bodies of the hypothalamus, and the adjacent entorhinal cortex (EC). Another major input into the hippocampus is septal region of the basal forebrain, which is the subject of scrutiny in this dissertation (FIGURE 1-3).

One of the ways of studying the hippocampus is by monitoring the extracellular field potentials, which reflects the inherent properties of local neurones and

synaptic activity. The collective patterns of neuroneal activity offer clues on the reciprocal relationship between behaviour and cellular physiology. In the rat hippocampus, the most prominent network patterns are theta (3-12 Hz), gamma (40-100 Hz) and ultra-fast (>80 Hz) bands (Ben-Ari et al., 2007; Bland and Oddie, 2001; Buzsaki, 2002; Buzsaki et al., 2003). The focus of this discourse will be on theta waves, as it is closely associated with locomotion (Bland and Vanderwolf, 1972; Kramis et al., 1975; Vanderwolf, 1969; Vanderwolf and Heron, 1964).

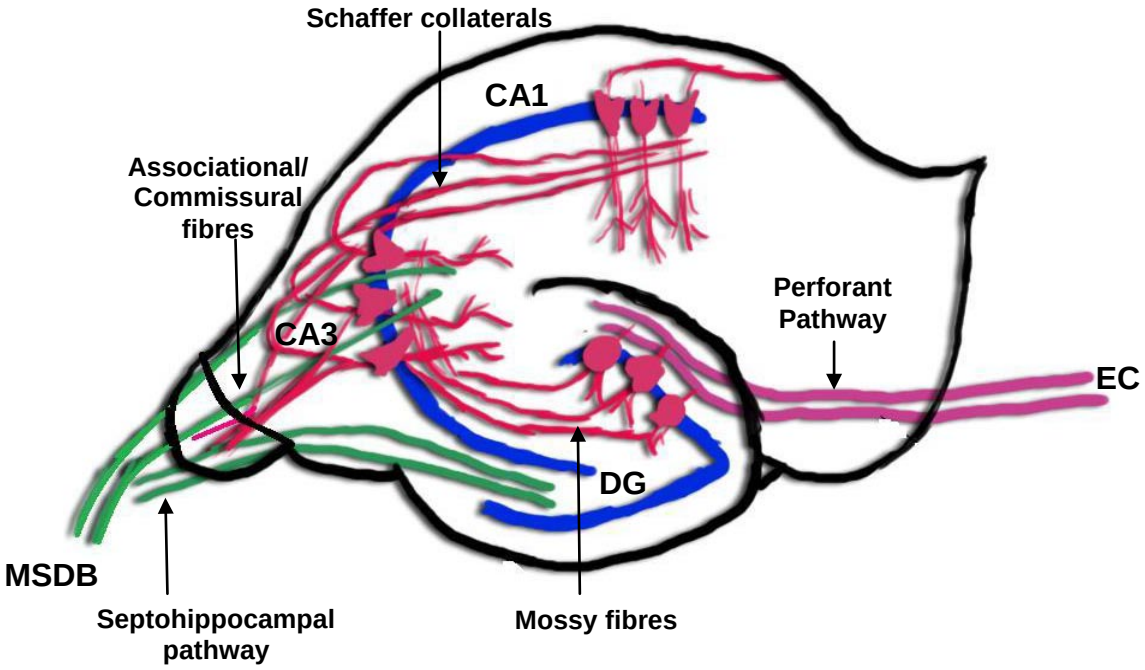


FIGURE 1-3 Major pathways of the hippocampus (adapted from Neves et al., 2008). The DG receives input from the EC through the perforant pathway. The granule cells then project via their axons (mossy fibres) to CA3 region. The CA3 pyramidal neurones then send axons to the ipsilateral CA1 pyramidal cells via the Schaffer Collateral pathway and contralateral CA3 and CA1 pyramidal cells via the Associational/Commissural fibres. Another extrahippocampal source of input to the DG and CA3 comes from the medial septum and diagonal band of Broca (MSDB) through the septohippocampal pathway.

1.3.3 Theta rhythm

Merely a few years before observations were first made on H.M., Green and Arduini described a rhythmic electroencephalographic (EEG) activity found in the hippocampi of rabbits, cats and monkeys (Green and Arduini, 1954). This oscillatory pattern, known as the theta rhythm, is the largest synchronous signal that can be recorded in the normal EEG of mammals and ranks among the most researched EEG phenomena (Bland, 1986, 2004; Burgess and O'Keefe, 2005; Buzsaki, 2002; Kahana et al., 2001; Stewart and Fox, 1990). The hippocampus is a “current generator” of theta rhythm i.e. it possesses transmembrane currents that can give rise to large amplitude extracellular potentials in the recorded field (Buzsaki, 2002). Most studies focus on EEG recording at the hippocampal fissure, the border between stratum lacunosum-moleculare of the CA1 and stratum moleculare of the DG, where the largest amplitude theta rhythm occurs (Hasselmo, 2005).

Theta in the hippocampus can be classified into 2 types, based on pharmacological characteristics (Kramis et al., 1975). Type I theta is resistant to muscarinic receptor antagonist atropine, and occurs during voluntary movement such as eating, drinking, grooming and running. Type II theta is abolished by atropine and is present only during immobility or urethane anaesthesia (Kramis et al., 1975). Theta rhythms are very pronounced during learning and memory (Kahana et al., 2001; Olvera-Cortes et al., 2004; Raghavachari et al., 2001; Vertes, 2005). Conversely, lesions reducing theta power (Rawlins et al., 1979)

have produced severe learning deficits in rats (Givens and Olton, 1994; Givens and Olton, 1990; Winson, 1978). Moreover, theta oscillations in CA1, Schaffer collateral pathway and dentate gyrus can effectively induce LTP (Holscher et al., 1997; Huerta and Lisman, 1993; Pavlides et al., 1988). There is also ample data to support the role of theta rhythm in sensorimotor integration(Bland, 2004; Bland and Oddie, 2001).

Theta is associated with free running (O'Keefe and Dostrovsky, 1971; Skaggs et al., 1996; Vanderwolf, 1969), running wheel (Buzsaki et al., 1983; Hyman et al., 2003) and treadmill running (Brankack et al., 1993; Fox et al., 1986). The speed of running is also positively correlated to the frequency of Type I theta to a certain extent (Kramis et al., 1975).

1.3.4 The septohippocampal system and theta

“Rhythm generator” refers to the synaptic inputs contributing to oscillatory pattern and frequency (Buzsaki, 2002). The medial septum and diagonal band of Broca (MSDB) is one of the four major regions of the basal forebrain septal region and projects to the hippocampal formation by way of the fimbria/fornix. The MSDB has long been regarded as the pacemaker of theta (Stewart and Fox, 1990) as early as 50 years ago when lesions of the rhythmically bursting cells of the septal nuclei abolished hippocampal theta (Green and Arduini, 1954; Petsche et al., 1962). Some reports have since indicated the supermammillary nucleus (Borhegyi and Freund, 1998; Borhegyi et al., 1998) and the posterior

hypothalamus in the brainstem in modulating rhythm oscillations in the medial septum and should therefore be considered as generators of theta instead(Bland et al., 2006). However, electrophysiology studies of sliced brain sections indicate that MSDB excitation of MSDB is alone sufficient togenerate theta (Goutagny et al., 2008).

There exists a structural and functional coupling of the basal forebrain septal region and the hippocampus, hence the designation of the grouping as the septohippocampal system (Bland and Colom, 1993; Colom, 2006). The septohippocampal region comprises of cholinergic, GABAergic, and a recently identified novel population of glutamatergic inputs (Colom et al., 2005; Crutcher et al., 1981; Freund and Antal, 1988; Frotscher and Leranth, 1985; Gulyas et al., 1991; Sotty et al., 2003). It is shown that septohippocampal GABAergic and cholinergic neurones are responsible for theta generation in the hippocampus (Apartis et al., 1998; Wu et al., 2002; Yoder and Pang, 2005). The role of glutamatergic neurones in theta has yet to be validated but it is shown that the local population of glutamatergic neurones in the MSDB form synaptic contacts with the cholinergic/GABAergic projection neurones and may thus play a modulatory role (Hajszan et al., 2004; Manseau et al., 2005).

1.3.5 The septohippocampal system and neurogenesis

Septohippocampal fibres project mainly onto the DG and more modestly to the CA3 region, with sparse connections to CA1 (Crutcher et al., 1981). Electron

microscopy shows that these fibres innervate only neurones, forming a dense network of axosomatic contacts with granule cells and axodendritic contacts with hilar cells within the DG (Chandler and Crutcher, 1983). Retrolabelling with fluorescence dyes further revealed that the hilus of the DG receives connections mostly from the medial septum and significantly from the vertical and diagonal limb of the diagonal band (Yoshida and Oka, 1995).

Given their heavy innervations of the DG, it is not surprising that loss of these septohippocampal neurones affects neurogenesis. Fimbria/fornix lesions decrease progenitor proliferation in the DG (Lai et al., 2003). Excitotoxic lesions to the MS that partially denervates cholinergic and GABAergic efferents reduced survival of newly generated neurones (Van der Borght et al., 2005). Extensive depletion of septohippocampal cholinergic cells by a specific immunotoxin, 192-Saporin, also led to a decline in the number of neurones in the rat hippocampus (Cooper-Kuhn et al., 2004).

1.4 HYPOTHESIS

Running is a potent mediator of adult neurogenesis but the underlying mechanisms remain unknown to date. What is known is that running enhances progenitor proliferation specifically in the dentate gyrus and evokes theta in the hippocampus. It is tempting to speculate that there may be a nexus between running, theta-inducing septohippocampal neurones and adult hippocampal neurogenesis, as it provides a multi-tier link at the different conceptual behavioural, systems and cellular levels. The key question to ask is: what is the exact factor(s) responsible for activity-dependent hippocampal progenitor proliferation?

Growth factors play crucial roles in regulating neurogenesis within the developing and adult brain. However, as receptors of these ligands are widespread in the brain, and different cell populations have differential responses to them, most growth factors display pleiotropic effects, making it difficult to attribute them as unambiguous modulators of cellular processes such as proliferation, differentiation, migration, and survival. Also, most experimental outcomes are derived from external applications such as intracerebroventricular infusions or direct injections into the site of the hippocampus, and may not accurately represent the actual molecules present in the neurogenic milieu. Some groups have proposed that endocrine cytokines and growth factors such as β-endorphins, VEGF and IGF may be involved in modulating the proliferative

effects of running, but it should be noted that these molecules have to cross the blood-brain barrier where they have to negotiate the highly selective tight junctions of endothelial cells in concentrations high enough to elicit a noteworthy effect.

Direct synaptic inputs represent a direct mode of transport of neurogenic factors straight to the cradle of the DG. However so far, none of the known neurotransmitter and neurotrophins anterogradely delivered to DG has a mitogenic effect on neural progenitor cells (Altar and DiStefano, 1998). Initial GABAergic inputs onto Type II progenitors serve to promote neuroneal differentiation rather than progenitor proliferation (Tozuka et al., 2005). Glutamatergic inputs are present only in the later stages of neurogenesis(Ge et al., 2006). Neurotrophins like BDNF and NGF foster differentiation and survival of newly generated neurones rather than expand the pool of precursor cells (Frielingsdorf et al., 2007; Lee et al., 2006; Nygren et al., 2006; Rossi et al., 2006). Also, studies have indicated that expression of these neurotrophic factors do not increase with exercise (Engesser-Cesar et al., 2007). Hence the putative mitogen may be a factor that is neither a conventional neurotransmitter nor neurotrophin, but can similarly be conveyed by afferents to the hippocampal neurogenic hotbed.

A working hypothesis is that the act of physical activity evokes theta and the subsequent release of mitogenic factor(s) from the septohippocampal neurones.

Theta provides a convenient bridge between behavioural and cellular physiology (Burgess and O'Keefe, 2005). The putative mitogen(s) undergoes anterograde transport into the DG neurogenic niche and stimulates the expansion of the local population of transit amplifying progenitors. Other trophic factors and neurotransmitters then act in concert to promote the emergence and survival of new neurones.

“… for a brain to be useful, it should adapt to the outside world. The brain has to be calibrated to the metrics of the environment it lives in, and its internal connections should be modified accordingly. If the statistical features of the environment reflect one particular constellation, the evolving brain should be able to adapt its internal structure so that its dynamics can predict most effectively the consequences of external perturbation forces.”

György Buzsáki, 2006. Rhythms of the Brain*. New York: Oxford University Press,* pp.15.

2. SEPTOHIPPOCAMPAL CHOLINERGIC NEURONES AND RUNNING-MEDIATED NEUROGENESIS

2.1 INTRODUCTION

One of the emerging themes for this project's hypothesis is that septohippocampal neurones involved in theta play a role in running-mediated neurogenesis. The basis for the hypothesis stems from observations that (i) increases in frequency of theta are correlated with increases in the intensity of movement (Bland and Colom, 1993) and that (ii) septohippocampal neurones critical for theta also synapse onto dentate granule cells (Chandler and Crutcher, 1983) found in the neurogenic locality. Here in this study, the role of this group of basal forebrain septal neurones in neurogenesis was investigated.

Although there is still considerable debate over the exact identity of the key pacemaker(s) in locomotion-induced oscillations, it is agreed that both septal cholinergic and GABAergic systems act synergistically to regulate synchronous firing in the hippocampus (Bland and Oddie, 2001; Buzsaki, 2002; Mizuno et al., 1991; Nilsson et al., 1990; Teitelbaum et al., 1975; Yoder and Pang, 2005). The septohippocampal cholinergic afferents provide a tonic excitatory drive for hippocampal theta while GABAergic neuroneal systems phasically modulate theta oscillations via a disinhibitory action . There is also a reciprocal connectivity of hipposeptal neurones onto GABAergic neurones in the medial septum, allowing for theta regulation by excitatory-inhibitory networks (Gulyas et al.,

2003). Recently, a third population of the septohippocampal pathway, with its own distinct firing pattern has been identified (Sotty et al., 2003). These glutamatergic neurones are estimated to form up to a quarter of the septohippocampal projections (Colom et al., 2005), but their role in theta rhythm generation has yet to be determined.

The fibres of the septohippocampal afferent system innervate all regions of the hippocampal formation. The septohippocampal cholinergic cells extensively innervate all types of cells, including pyramidal cells, granule cells and interneurones (Frotscher and Leranth, 1985; Leranth and Frotscher, 1987) whereas GABAergic projections selectively terminate on hippocampal interneurones (Freund and Antal, 1988; Gulyas et al., 1991). The interneurones in the hippocampus are a diverse group of cells that are GABA-containing and establish inhibitory axo-axonic and modulatory axo-dendritic contacts (Freund and Buzsaki, 1996). The large population of principal cells controlled by the local axon arborizations of interneurones allow for global control of hippocampal activity (Gulyas et al., 1991). Indeed, given the extensive contacts of septal cholinergic and GABAergic synaptic terminals onto the hippocampal DG region, directly or indirectly via interneurones, it is not difficult to envisage the likelihood of the distal regulation of neurogenesis by means of neurotransmitter or mitogen transport from the MSDB.

To address an aspect of the hypothesis, it was decided to employ the use of lesioning to eliminate either cholinergic or GABAergic neurones of the septohippocampal pathway. The first few septohippocampal lesioning experiments carried out decades ago were anatomical transections, in which the fimbria/fornix linking the septum to the hippocampus was cut (Green and Arduini, 1954; Petsche et al., 1962). Other lesioning protocols include electrolytic lesioning (Sainsbury and Bland, 1981) and intraventricular excitotoxin infusions such as NMDA, kainic acid, AMPA, ibotenic acid and quisqualic acid (Lee et al., 1994a; Waite et al., 1994a; Waite et al., 1994b; Yoder and Pang, 2005). In both cases, although the fibre tracts were left intact, there was still indiscriminate ablation of various neuroneal bodies at the site of injection.

Most lesioning experiments now employ the use of “molecular neurosurgery”. The underlying basis of molecular surgery is an immunotoxin. The toxin works by targeting cells with the pertinent surface antigens, and destroys them whereupon endocytosis by interfering with their protein translational ability (Wiley and Kline, 2000). Cholinergic neurones in the basal forebrain of rats have been eliminated successfully using 192 Immunoglobulin G-Saporin (192-Ig-SAP) (Apartis et al., 1998; Bassant et al., 1998; Berchtold et al., 2002; Cooper-Kuhn et al., 2004; Lee et al., 1994b; Waite et al., 1994b; Wenk et al., 1994). 192-Ig-SAP is a conjugate of saporin, a toxin derived from soapwort; and a monoclonal antibody of p75 neurotrophin receptor ($p75^{NTR}$). This receptor is predominantly located in cholinergic neurones of the basal forebrain, hence targeting of $p75^{NTR}$ spares

those located elsewhere in the brain, even the adjacent striatum and nucleus accumbens (Nilsson et al., 1990; Waite and Thal, 1996; Waite et al., 1994b).

Incidentally, the degeneration of basal forebrain cholinergic neurones is one of the hallmarks of senile dementia and AD (Roman and Kalaria, 2006; Whitehouse, 1993, 1998; Whitehouse et al., 1983a; Whitehouse et al., 1982; Whitehouse et al., 1983b). The “cholinergic hypothesis of AD" posited more than two decades back expounded that the loss of neurotransmitter acetylcholine in the CNS due to cholinergic neuroneal dysfunction is significantly related to the cognitive symptoms associated with AD and advanced age (Bartus, 2000; Bartus et al., 1982; Flicker et al., 1983). As basal forebrain cholinergic afferents are principal sources of cholinergic fibres to the hippocampus, some groups have proposed that the loss of these neurones affects hippocampal neurogenesis, which in turn lead to a decline in mnemonic deficits (Cooper-Kuhn et al., 2004; Mohapel et al., 2005).

The following experiments in this chapter were carried out using a mouse-specific version of the 192-IgG-SAP toxin: murine p75-Saporin (mup75-SAP). Radioenzymatic assays have shown that when mu p75-SAP was injected into the forebrain, choline acetyltransferase (ChAT) activity was substantially reduced in the basal forebrain and hippocampus (Berger-Sweeney et al., 2001). ChAT is a key enzyme in acetylcholine synthesis and has been used most consistently as a marker of cholinergic neuroneal integrity (Contestabile and Ciani, 2008; Gil-Bea

et al., 2005). Mu p75-SAP injections impair learning and memory, with lesioned mice exhibiting worse performances in the radial-arm water maze (Hunter et al., 2004) and the1-day Morris water maze task (Berger-Sweeney et al., 2001).

There is no specific molecular toxin for GABAergic neurones on the market yet. Until now, the only way to induce loss of GABAergic neurones is through non-specific excitotoxic lesions (Yoder and Pang, 2005). For this study, I have ventured to induce GABAergic loss through the means of another immunotoxin, Ox7-saporin (Advanced Targeting Systems, San Diego, CA). Ox7 is a monoclonal antibody that targets the antigen Thy1.1, widely expressed by adult neurones in rats and mice (Wiley et al., 1989). The use of this particular toxin produced a marked loss in GABAergic cells. This was assayed by parvalbumin (Parv) immunostaining, a marker specific for septal GABAergic neurones (Kiss et al., 1990). However, as Ox7-saporin is a generic suicide transport agent, the elimination of GABAergic cells is tied with corresponding loss of other neurones. Also, the extent of GABA deletion in the MSDB varies, and is not reproducible. The use of kainic acid for leisions was also explored but similar results were encountered: loss of GABA cells coupled with collateral damage to other surrounding neurones. In view of a lack of targeted GABAergic cellular lesion and in order not to obfuscate the results, it was decided that the experiments should solely be based on clear-cut cholinergic lesions. Here, I will attempt to investigate whether (i) septohippocampal cholinergic lesioning has any effect on running-mediated neurogenesis, assessed by the use of S-phase marker BrdU.

2.2 MATERIALS AND METHODS

2.2.1 Animal treatments

Adult female Swiss Albino mice (8-10 weeks) were obtained from the Centre for Animal Resources (CARE), Singapore. The mice were housed in the Animal Holding Unit (AHU), National University of Singapore, under a 12 hr light: 12 hr dark cycle, with *ad libitum* access to food and water. The mice were group housed and allowed to acclimatize to their environment for one week prior to commencement of the experiments. All animal procedures were conducted with approval from the Institutional Animal Care and Use Committee (IACUC), National University of Singapore, and were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" and the "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research", National Research Council, USA.

The mice were anaesthetized with a cocktail of hypnorm and midazolam before undergoing bilateral intracerebroventricular microinjections of of saline or mu p75-SAP (Advanced Targeting Systems, San Diego, CA). Holes were drilled at the following stereotaxic coordinates: AP −1.6 mm, ML ±1.0 mm, and DV −2.4 mm. The dose of the toxin was titrated to determine the dose producing the most effective depletion of the cholinergic cells in the MSDB without compromising the well-being of the mice. A dose of 3.6 µg/µl was selected and injected into each ventricle over the course of 5 min using a 1 µl Hamilton syringe with a 26-gauge

stainless steel needle (SGE Analytical Science, Austin, TX). The syringe was retracted for 0.1 mm before leaving for an additional 5 min in the ventricle. The mice were allowed 10 days to recover, during which they were weighed daily and given glucose saline infusions. Mice exhibiting severe weight loss (<80% of their original weight) were euthanized by anaesthetic overdose.

Both sham lesioned and mu p75-SAP lesioned mice were then randomly assigned to the various treatment groups. For the runners, they were individually housed in cages equipped with a running wheel each. For the non-runners, they were each exposed to an immobilized running wheel to control for the possibility of the running wheel serving as an environmental enrichment source. The mice were left with their running wheels for 12 days. A photo-sensor was used to monitor the distance run by each mouse.

At the end of 12 days of exercise, BrdU (Sigma, St Louis, MO) at a dose of 20 mg/ml dissolved in saline with 0.06 N NaOH and titrated to a pH of 7.4, was injected intraperitoneally at a concentration of 300 mg/kg, a single high but non-toxic dose

2.2.2 Immunohistochemistry

The animals were anaesthetized with an overdose of pentobarbital (Nembutal, Ovation Pharmaceuticals, Deerfield, IL) either at (i) 24 hours after BrdU administration to assess for neural cell proliferation or (ii) 4 weeks later for cell

survival and differentiation. The mice were then transcardially 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 fixative. The basal forebrains of the mice were then sectioned using a vibratome (Vibroslice, World Precision Instruments, Sarasota, FL) at a thickness of 40 μm prior to immunohistochemical assays. For detailed investigation of cell proliferation, the hippocampi of the mice were processed (LeicaTP1020, Leica Microsystems, Wetzlar, Germany), embedded in paraffin, and cut in 6 μm coronal sections on a rotary microtome (Leitz 1512, Leica Microsystems) before mounting onto slides. For investigation of neural differentiation, the hippocampi of the 4 weeks group were sectioned at a thickness of 40 μm using the vibratome and stored in phosphate buffered saline (PBS) at 4°C until use.

For the paraffin sections, the sections were first de-paraffinized with xylene and subsequently rehydrated with descending concentrations of ethanol prior to incubation in 0.3% hydrogen peroxidase to quench endogenous peroxidase activity. PBS was used for all washing. Sections were then pretreated with 4 N HCL (30 min) and trypsin (1 mg/ml in PBS, 10 min, 37˚C) for antigen retrieval. Blocking was carried out using 5% horse serum for 20 min, followed by 30 min of incubation with a mouse monoclonal anti-BrdU antibody (1:200, Neomarkers, Fremont, CA). Sections were then incubated with biotinylated secondary horse anti-mouse antibody for 30 min, and avidin-biotin complex for another 30 min according to the manufacturer's instructions (ABC system, Vector Laboratories,

Burlingame, CA), with nickel intensified diaminobenzadine as a chromogen (Vector Laboratories). The slides were rinsed in tap water, dehydrated with 95% and 100% ethanol before washing with xylene, and mounted.

For the vibratome sections, immunofluoroscence double-labelling was carried out on the free- floating sections. The sections were pretreated with 2 N HCl before blocking in 5% goat serum. The primary antibodies used were rat monoclonal anti-BrdU (1:200, Accurate Chemical, Westbury, NY), mouse monoclonal anti-NeuN (1:200, Chemicon, Temucula, CA) and rabbit polyclonal anti-GFAP (1:400, DakoCytomation, Glostrup, Denmark). The secondary antibodies used were Cy2 goat anti-rat (1: 200, Jackson Immunoresearch West Grove, PA), Alexa-Fluor 594 goat anti-mouse and goat anti-rabbit (1:200, Molecular Probes, Eugene, OR). The sections were mounted with Pro-Long anti-fade reagent (Molecular Probes) before being coverslipped.

To label cholinergic neurones in the basal forebrain sections, goat polyclonal anti-ChAT antibody (Chemicon) was used with biotinylated donkey-anti-goat secondary antibody (1:200, goat ABC staining system, Santa Cruz Biotechnology, Santa Cruz, CA) and nickel-enhanced DAB as chromogen. Random but corresponding samples were taken from the medial septum sections of each of the non-lesioned and lesioned groups to carry out double-immunofluorescence labelling of ChAT and Parv (1:200, Chemicon). The double-

labelling protocol used was similar to that described above, except that the HCl step was omitted.

2.2.3 Microscopy

Basal forebrain sections of each mouse were taken at 3 different intervals, at bregma 1.18 mm, 0.98 mm and 0.74 mm, according to the mouse atlas (Paxinos, 2001a) as representative samples for counting the number of MSDB cholinergic neurones. The images of ChAT-positive cells in the MSDB were captured with a digital camera (Magnafire SP,Optronics, Goleta, CA) under a 20X objective using a BX50 microscope (Olympus, Tokyo, Japan) and counted semi-automatically (Image Pro Plus, Media Cybernetics Inc., Silver Spring, MD, USA).

For the paraffin sections, BrdU-labelled cells from one-in-five serial sections (at least 30 µm apart) throughout the rostro-caudal extent of the dentate gyrus were viewed through a 40 X objective using the BX50 microscope. Digital images were captured for the purpose of counting (Magnafire SP, Optronics). For the 4 weeks group, one-in-five sections double-labelled with either BrdU-NeuN or BrdU-GFAP were analyzed using a laser scanning confocal microscope (LSM 510, Carl Zeiss, Göttingen, Germany) under 400 X magnification using sequential illumination with 488 nm and 546 nm wavelength lasers. Colocalization was established by analyzing the overlap between the antigen expressions by orthogonal reconstruction throughout the entire z-stack and in the xy-yz direction (LSM 510, Zeiss).

2.2.4 Quantification of labelled cells

The BrdU-positive cells in the granule cell layer, and their co-expression with GFAP- and NeuN- positive cells, were counted by an investigator blind to the coding (see Acknowledgements). For both the 24 hr and 4 weeks group, the number of BrdU positive cells in one side of the dentate gyrus in a section was pooled and divided by the length of the granule cell layer within that particular dentate gyrus to determine the mean number of BrdU cells per length of dentate gyrus. Sections were taken by sampling at equal intervals from the hippocampus region nearer to the septal end for more consistent BrdU labelling. This reference sample volume was 1000 μm thick. The mean number of BrdU cells per length of dentate gyrus was further divided by the thickness of the section to obtain the average number of labelled cells per traced area. The estimated number of BrdU cells per brain is obtained by multiplying the average number of labelled cells per area by the mean length of the dentate gyri of the sections sampled and the reference sample volume.

2.2.5 Statistical analyses

All statistical analyses were performed using SPSS software version 14.0. Analysis of variance (ANOVA) was performed for all groups, followed by appropriate post-hoc analysis if comparisons were found to be significant. The Levene's test for Equality of Error Variances was applied to all groups to check for homogeneity of variances. Differences were considered to be statistically

significant when $p<0.05$. Data are expressed as means value ± standard error mean.

2.3 RESULTS

2.3.1 Cholinergic lesions in the MSDB are partial but selective

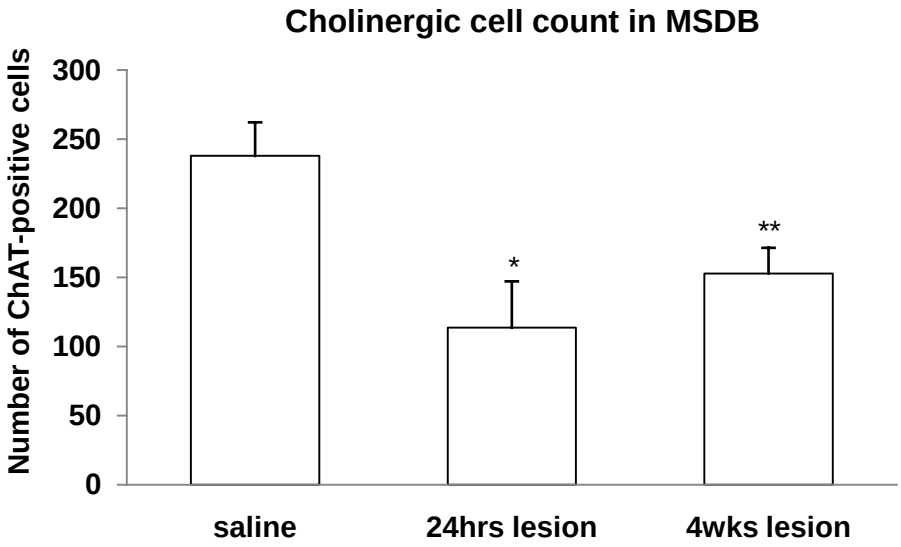
A total of 1 µl was injected into the ventricles of the mice, at a concentration of 3.6 µg/µl that is a trade-off between more complete lesioning and lower mortality rates. The mu p75-SAP lesioned mice had greater weight loss than the saline treated controls after surgery. Due to the adverse side effects of the toxin that included aggressive behaviour and drastic weight loss, 18 out of 28 lesioned mice survived, a 65% survival rate. This is comparable with a 68% survival previously reported (Berger-Sweeney et al., 2001; Hunter et al., 2004).

One-way ANOVA with Dunnett's post-hoc tests indicated that the bilateral injections of the toxin resulted in a significant depletion of cholinergic neurones in the MSDB ($F_{2,19} = 5.63$, $p<0.05$) for both the lesioned groups (FIGURE 2-1i). It should be noted that *24 hours lesion* and *4 weeks lesion* refers to the time-point in which the animal was sacrificed after the single BrdU injection. The number of post-lesion days is 23 days and 50 days respectively. There was no significant difference in the ChAT-positive neurones between runners and non-runners. The slight and non-significant decline in the depletion percentage of cholinergic cells in the 4 weeks group relative to the 24 hr group could be attributed to the drop in efficacy of the toxin due to batch specificity (this is consistent with what the manufactuer has commented on the declining specificity of the immunotoxin on its website at www.atsbio.com). Nonetheless, loss of cholinergic neurones for

both groups was significant, selective and specific. Parv-immunoreactive cells GABAergic neurones in the MSDB (Kiss et al., 1990) were unaffected by the lesions (FIGURE 2-1(ii)).

Cholinergic deafferentation also did not affect the distance ran by the mice. The distance accumulated by each runner daily ranged from 4 km to 25 km, with no difference in the number of revolutions of running wheel covered between the lesioned (mean = 272346 ± 3933) and the non-lesioned group (mean = 246852 ± 2373), showing that lesioning does not impair running ability.

(i)



(ii)

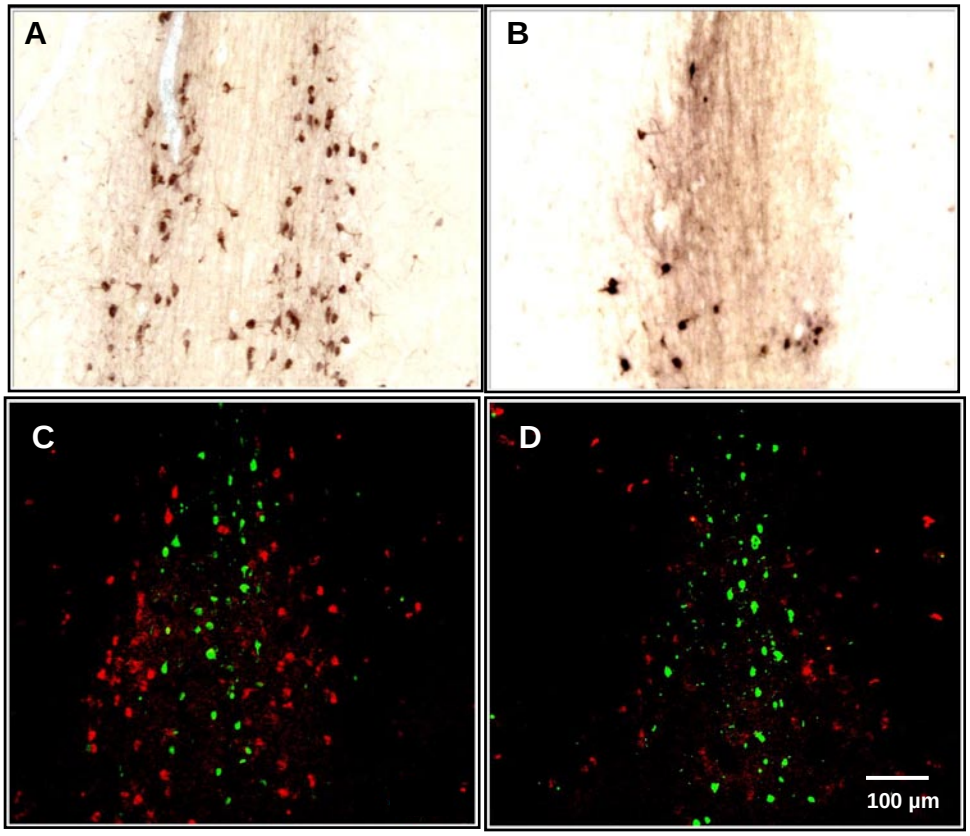
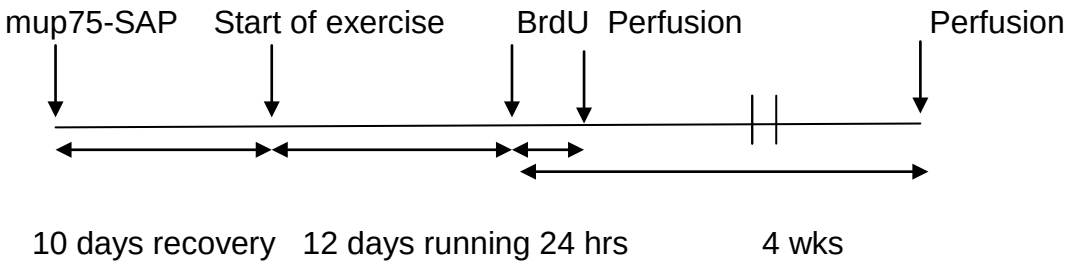


FIGURE 2-1 Effects of mu p75-SAP on cholinergic neurones (i) Mean number of ChAT-positive cells in the MSDB: 24 hrs control (269 ± 38); 24 hrs lesioned (113 ± 33); 4 weeks control (194 ± 5); 4 weeks lesioned (152 ± 18). A 52% depletion of ChAT labeled neurones (**p*<0.05) and a 36% depletion (***p*<0.01) was recorded for the 24 hrs group and 4 wks group respectively. (ii) Intracerebroventricular injection of 3.6 µg/µl of mu p75-SAP suffices to deplete most of the cholinergic neurones located on the lateral part of the medial septum. (A) saline control (B) lesion (C) confocal images of control, with double-labelling of ChAT (red) and Parv (green) (D) Parv-positive cells remained intact despite of loss of ChAT-positive cells.

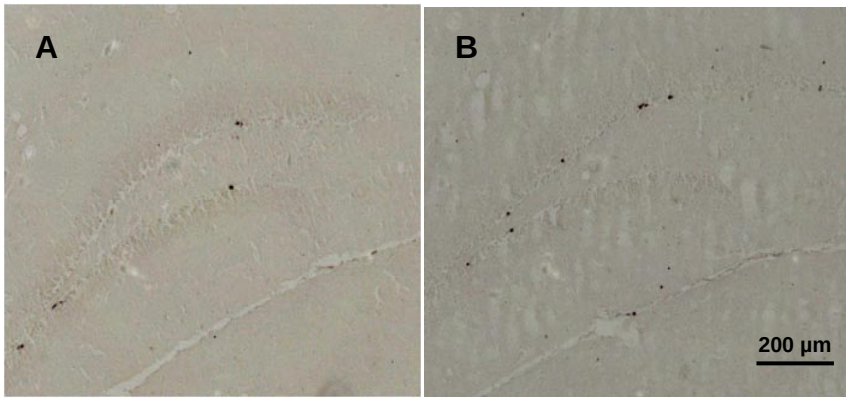
2.3.2 Partial cholinergic lesions do not affect baseline progenitor proliferation but potentiate the running-induced increase

Both non-lesioned and lesioned mice were randomly assigned to running and non-running groups (to be sacrificed 24 hours or 4 weeks after injection of BrdU following 12 days of free access to a running wheel or control exposure to an immobilised running wheel (FIGURE 2-2i), Sections through the hippocampus were immunostained for BrdU (FIGURE 2-2ii). For each brain, systematic sampling of dorsal hippocampal tissue sections from bregma -1.50 mm to -2.50 mm was employed. BrdU-immunopositive cells along the length of the subgranular zone (SGZ) and granule cell layer of the dentate gyrus were counted. Two-way ANOVA showed that running ($F_{1,25}$ = 15.68, p < 0.001) and lesioning ($F_{1,25}$ = 8.88, p < 0.01) each had a very significant effect on the number of BrdU positive cells in the dentate gyrus. There was significant interaction between running and lesioning ($F_{1,25}$ = 5.69, p < 0.05). Post hoc analysis using independent samples two-tailed t-tests revealed that running increases the number of BrdU-labelled cells in unlesioned (t_{12} = -2.19, p < 0.05) and lesioned animals ($t_{7.053}$ = -3.31, p < 0.05). Comparisons between the runners of the lesioned group and control group demonstrated that cholinergic denervation significantly potentiated the running-induced increase in BrdU cells (t_{12} = -2.78, p < 0.05) (FIGURE 2-2iii). There were no differences in mean length of the dentate gyrus in all treatment groups.

(i)


mup75-SAP
Start of exercise
BrdU
Perfusion
Perfusion
10 days recovery
12 days running
24 hrs
4 wks

(ii)


A
B
200 µm

(iii)

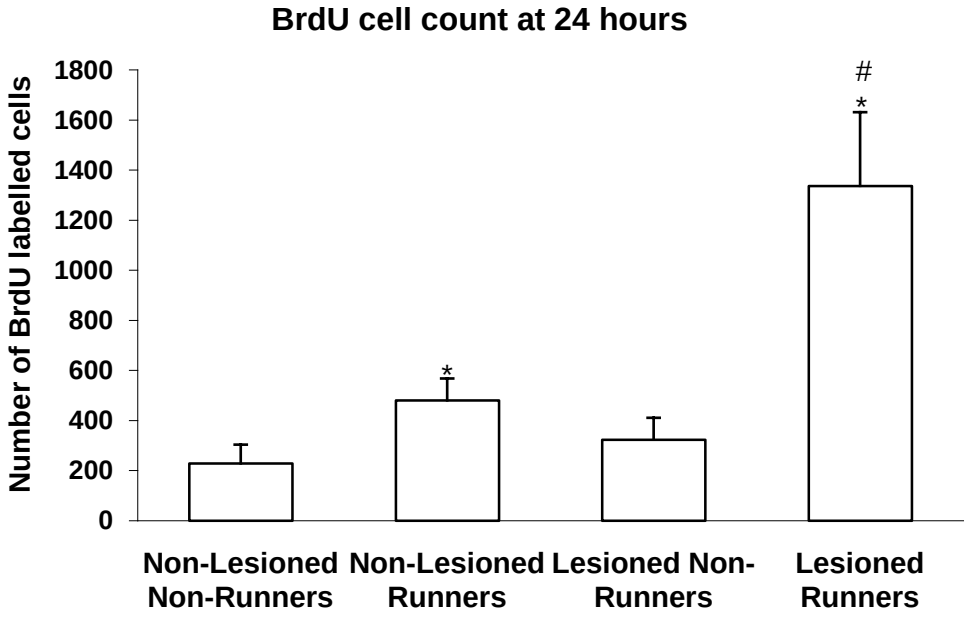

BrdU cell count at 24 hours
Number of BrdU labelled cells
1800
1600
1400
1200
1000
800
600
400
200
0
*
#
*
Non-Lesioned Non-Runners
Non-Lesioned Runners
Lesioned Non-Runners
Lesioned Runners

FIGURE 2- 2 Effects of running on progenitor proliferation of cholinergic lesioned animals (i) Experimental timeline (ii) Immunohistochemistry of BrdU in the dentate gyrus of (A) non-runner and (B) runner. (iii) Number of BrdU-positive cells 24 hrs after BrdU administration in non-lesioned non-runners (n = 7), non-lesioned runners (n = 7), lesioned non-runners (n = 8) and lesioned runners (n = 7). Running increased the number of BrdU-labelled cells in both the unlesioned and lesioned groups (*p < 0.05). Comparisons between the runners of the lesioned group and control group demonstrated that cholinergic denervation significantly potentiated the running-induced increase in BrdU cells (#p < 0.05).

2.3.3 Partial cholinergic lesions do not affect survival of progenitor cells in non-runners but reduce cell survival in runners

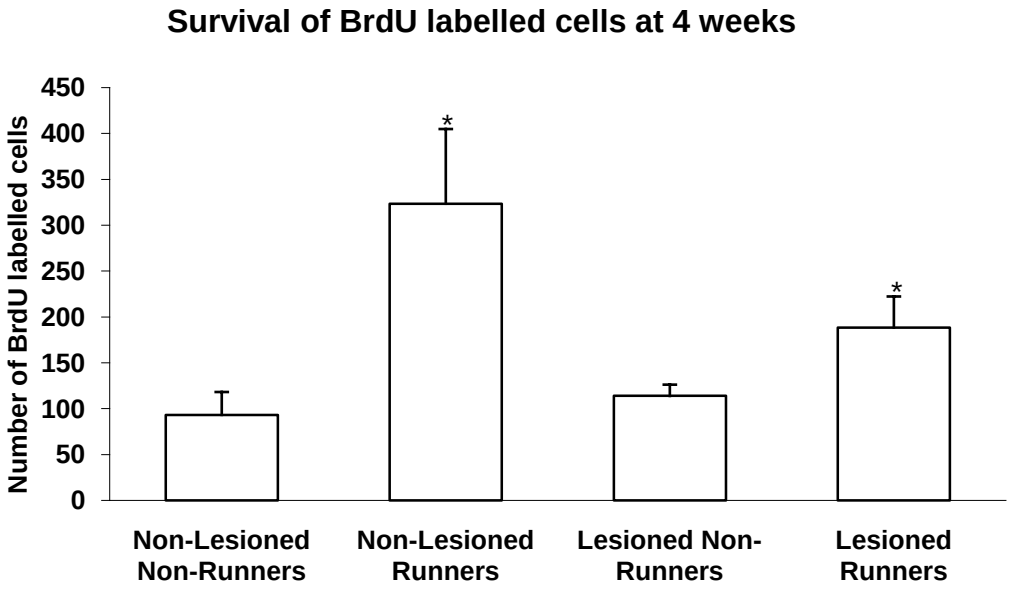
Two-way analysis of variance showed that running has a very significant effect on the survival of BrdU-positive cells after 4 weeks ($F_{1,17} = 15.25$, $p < 0.01$). This was a result of significant increases in BrdU-positive cells in both the unlesioned ($t_6 = -2.70$, $p < 0.05$) and lesioned groups ($t_{11} = -2.20$, $p < 0.05$) (FIGURE 2-3i). There is a trend to show that lesioned runners have less surviving cells than non-lesioned runners.

To analyse the effect of running and lesioning on the numbers of BrdU-labelled cells over time, a three-way ANOVA was performed. Lesioning ($F_{7,42} = 5.91$, $p < 0.05$), running ($F_{7,42} = 13.851$, $p < 0.001$) and time ($F_{7,42} = 20.321$, $p < 0.0001$) significantly influenced the number of BrdU-labelled cells. There was also significant interactions between lesion and time ($F_{3,42} = 5.929$, $p < 0.05$) and between running and time ($F_{3,42} = 7.536$, $p < 0.01$). The three-way interaction between running and lesion over time was significant ($F_{3,42} = 4.22$, $p < 0.05$), hence we carried out further statistical tests to compare the effect of time within the lesion and running groups. For follow-up analysis, an index of cell survival was calculated by dividing the number of BrdU-labelled cells surviving at the 4 weeks time point by the mean number of labelled cells 24 hours after BrdU administration (although it must be cautioned that due to the differences in sampling of brain sections at 24 hours and 4 weeks, the comparisons of timepoints here is not entirely optimal). Two-way ANOVA showed that

significantly decreased the percentages of BrdU positive cells surviving after 4 weeks ($F_{1,17}$ = 12.84, p < 0.01). There was also a significant interaction effect between running and lesioning ($F_{1, 17}$ = 8.41, p < 0.05). Post-hoc two-tailed t-tests revealed that cholinergic lesioning significantly decreased the percentage survival of newborn cells in the dentate gyrus of runners compared to non-runners (t_{11} = 4.62, p < 0.001). The proportion of BrdU cells surviving after 4 weeks was marginally, but not significantly, reduced in lesioned runners (t_3 = 3.11, p = 0.05) (FIGURE 2-3(ii)).

Taken together, the two data sets on survival of progenitors and their progeny suggest that the lack of cholinergic inputs do not affect the survival of progenitor cells, but did not sustain the viability of running-induced progenitor cells beyond a month.

(i)



(ii)

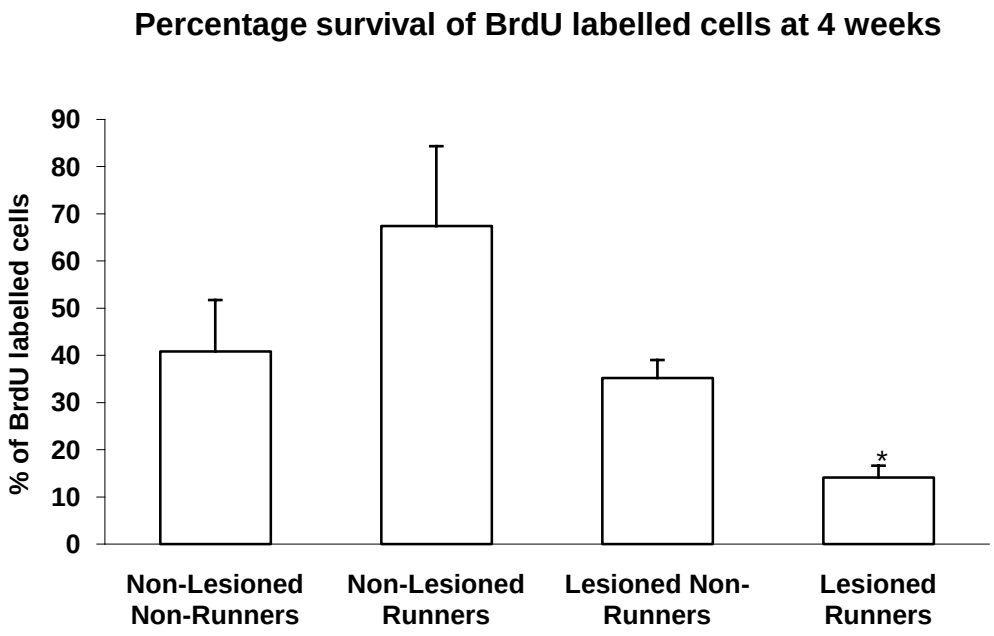


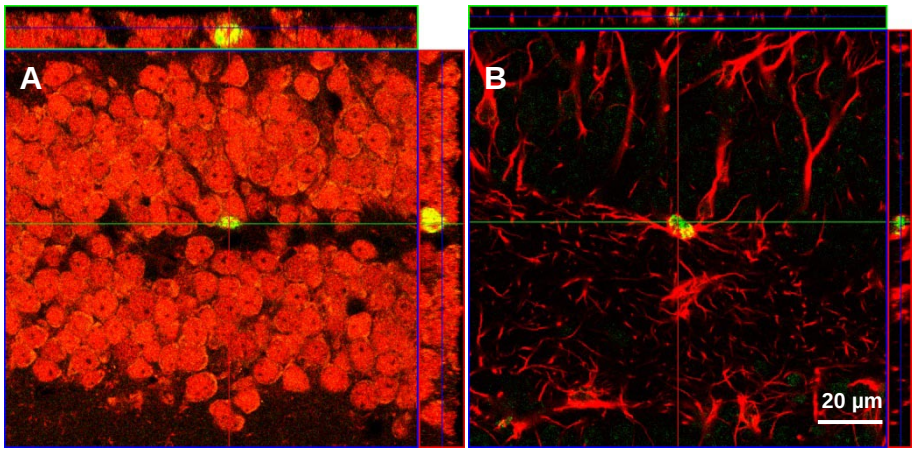
FIGURE 2-3 Effects of running on survival of progenitor cells (i) There was a significant increase in the number of BrdU-positive cells in both the non-lesioned runners (n = 4) and lesioned runners (n = 6), compared to non-lesioned non-runners and lesioned non-runners (n=7), respectively. (**p* < 0.05). (ii) The percentage of BrdU-labelled cells that survive beyond the one month period was significantly lower in cholinergic lesioned runners (**p* < 0.001).

2.3.4 Partial cholinergic lesions do not affect neurogenesis

To determine the phenotype of surviving differentiated newborn cells at the 4 week time point, double immunolabelling was carried out to assay for co-expression of either neuroneal specific nucleus protein (NeuN), a marker for mature neurones, or glial acidic fibrillary protein (GFAP), an astroglial marker, with BrdU labelling in cells within the granule cell layer of the DG (FIGURE 2-4i). Neurogenesis is defined by the number of cells colocalized for BrdU and NeuN. Running had a very significant effect on neurogenesis ($F_{1, 17} = 12.12$, $p < 0.01$). In the sham lesioned group, the runners showed enhanced neurogenesis ($t_6 = -2.54$, $p < 0.05$). Comparisons between runners and non-runners in cholinergic deafferented mice showed that although running was discontinued 4 weeks earlier, the effect of running on neurogenesis was still significant ($t_{11} = -2.111$, 1-tailed t-test $p < 0.05$) (FIGURE 2-4ii). Within the runners, lesioning had no effect on neurogenesis ($F_{3, 17} = 1.126$, $p = 0.286$). Neither lesioning ($F_{3,17} = 2.676$, $p = 0.120$) nor running ($F_{3,17} = 2.379$, $p = 0.141$) affected the percentage of surviving BrdU-labelled cells that differentiated into neurones (Table 2).

Astrogenesis, as determined by the number of colocalized BrdU- and GFAP-positive cells, remained constant despite the various treatments (lesioning: $F_{3,17} = 0.036$, $p = 0.852$; running: $F_{3,17} = 4.136$, $p = 0.189$). Similarly, lesioning ($F_{3,17} = 1.433$, $p = 0.248$) and running ($F_{3,17} = 0.271$, $p = 0.609$) did not affect the proportion of astrocytes (Table 2).

(i)



(ii)

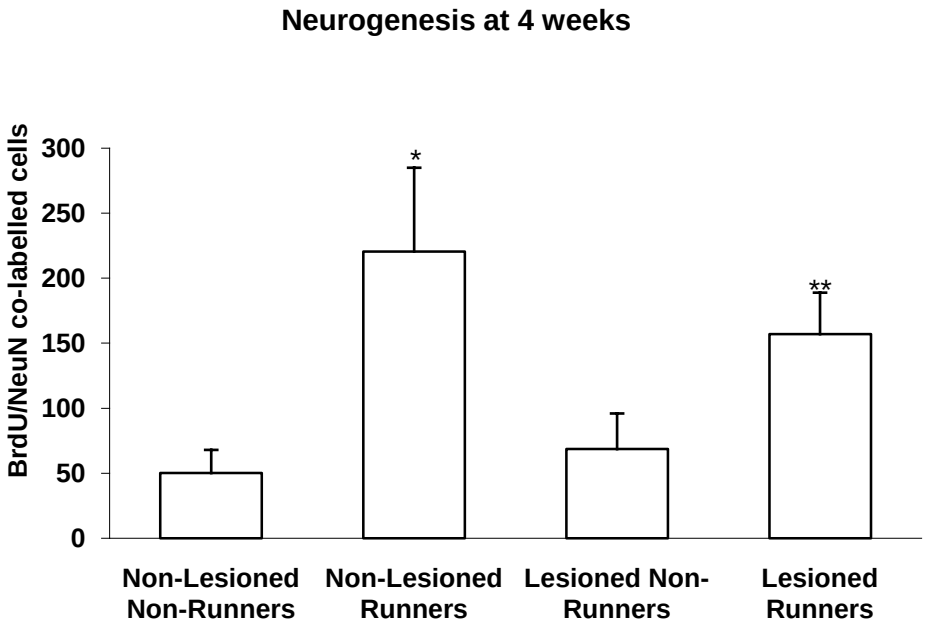


FIGURE 2-4 Effects of running on neurogenesis (i) Confocal images showing z-series reconstruction of a cell double labelled with (A) BrdU *(green)* and NeuN *(red)* and (B) BrdU *(green)* and GFAP *(red)*. (ii) Running increased the neurogenesis significantly in non-lesioned runners (n = 4) compared to non-lesioned non-runners (**p* < 0.05) and marginally in lesioned runners (n = 6) compared to lesioned non-runners (***p* < 0.05, one-tailed).

	Non-lesioned, Non-Runner	Non-lesioned, Runner	Lesioned Non-Runner	Lesioned Runner
Proliferation, 24hrs	228.5(74.9)	480.0(87.4)	323.5(87.1)	1336.8(293.8)
Survival, 4wks	93.3(24.9)	323.5(81.4)	113.9(12.3)	188.4(33.9)
Survival (%)	40.8(10.9)	67.4(16.9)	35.2(3.8)	14.1(2.5)
Neurones	50.1(17.9)	220.6(64.5)	68.6(27.5)	157.0(32.0)
Astrocytes	7.6(4.5)	25.1(6.5)	17.1(5.3)	13.6(3.1)
Neurones (%)	57.4(8.7)	67.4(9.3)	68.1(6.8)	80.3(3.9)
Astrocytes (%)	5.6(2.4)	11.0(5.3)	22.6(8.6)	10.2(3.4)

TABLE 2 Proliferation, survival and phenotypes of BrdU-postiive cells. Data are mean ± sem.

2.4 DISCUSSION

The rat 192-Ig-SAP, the predecessor of mu p75-SAP, was able to eliminate virtually all cholinergic cells in the rat forebrain (Apartis et al., 1998; Bassant et al., 1998; Berchtold et al., 2002; Leanza et al., 1996; Lee et al., 1994b). Compared to the corresponding rat toxin, the mouse toxin was not as potent and could not elicit the same effect. The reduction, nevertheless, is significant, with almost half the cholinergic neuroneal population depleted. The percentage loss in the findings is comparable to other groups (Hunter et al., 2004). That aside, partial lesioning of the cholinergic neurones in the basal forebrain may be more similar to that of patients diagnosed with AD. The targeting of cholinergic forebrain cells by mup75-SAP is also specific, with GABAergic cells in the MSDB remaining intact.

As stated in the first chapter, neurogenesis is governed by a kaleidoscope of mitogenic signals, transmitters and trophic factors. The factors act spatially and temporally to modulate distinct steps in the maturation process of the neurone. Among the physiological factors, running is one of the most robust inducers of neural progenitor cell division (Brown et al., 2003; van Praag et al., 1999b). A similar result is shown here, where running increases BrdU-labelled progenitor cells by two-fold. Cholinergic lesioning has no effect on progenitor proliferation. This is also in line with other studies involving pharmacological manipulation of the cholinergic system (Kaneko et al., 2006; Kotani et al., 2006). Interestingly,

cholinerigic deafferentation potentiated the running-induced effect on proliferation, leading to a three-fold increase over the non-lesioned runners. This may be a result of neuroinflammation, as the immunotoxin injections may pathologically perturb the brain. Activation of microglial cells, the resident macrophages of the CNS, is a hallmark of neuroinflammation and has been shown to be correlated to a rise in cell proliferation (Battista et al., 2006). In studies conducted with mu p75-SAP lesions, activation of CD45-positive microglial cells are simultaneously observed with the reduction of ChAT-positive neurones in the basal forebrain as early as 1 day after surgery (Hunter et al., 2004). However, no similar increase in progenitors in the lesion controls (i.e. non-runners) was detected, so the effect cannot be solely attributed to CNS inflammation. Further assays of expression of inflammatory cytokines (e.g. TGFβ) in the hippocampus could be performed to assess if the marked elevation in cellular genesis in lesioned runners was a result of neuroinflammation.

It is previously reported that on the average, 50% of the newly generated cells die by apoptosis (Biebl et al., 2000). A similar percentage of progenitor loss occurs in this study. In contrast, about two-thirds of the original BrdU-labelled cells in the running group are still able to survive beyond the one-month period. However, no change in the number of surviving cells in the cholinergic deafferented groups was recorded, unlike studies done in rats with 192IgG-SAP lesions (Cooper-Kuhn et al., 2004; Mohapel et al., 2005). The findings were unexpected because studies conducted using cholinergic agonists and

antagonists yielded results which indicated that the transmitter acetylcholine is involved in survival of newborn cells (Kaneko et al., 2006; Kotani et al., 2006). One possibility is that unlike 192-IgG-SAP, the mouse-specific toxin used was not able to completely obliterate all septocholinergic neurones. Another reason for the circumvention in decline of survival could be the accumulation of neurotrophic factors in the hippocampus. Neurotrophins such as NGF, BDNF and NT-3 are synthesized in the hippocampus and undergo retrograde transport to the MSDB where they maintain survival and function of septal neuroneal populations (Schindowski et al., 2008). The selective ablation of basal forebrain cholinergic neurones and the ensuing impaired retrograde transport may lead to accumulation of these neurotrophins in the hippocampus, where they exert a pro-survival effect on the newly generated neurones, and ward off the apoptotic ramifications from the lack of cholinergic input.

There was a very pronounced drop in the percentage survival of newborn cells in the lesioned runners. Cholinergic denervation may not be sufficient to affect baseline survival of cells, but it drastically removed the ability of running-induced newborn cells to sustain its viability. Other groups have recorded that prolonged running could increase the survival of newly generated neurones (Kronenberg et al., 2006), but in this case running was not continued throughout 4 weeks following BrdU injection, and hence the survival-promoting effect of running was not carried over. Also, in many models of pathology, the robust induction of adult neurogenesis appears to be transient and non-specific. Cell proliferation is

increased in animal models of brain trauma such as epileptic seizures and stroke but only a small fraction of them survived longer than one month (Arvidsson et al., 2002; Matsumori et al., 2006). Moreover, the neuroinflammatory response to the cholinergic lesions is suggested to be detrimental to the survival of new neurones (Das and Basu, 2008).

Although running was only carried out for the first 12 days of the experiment, this initial bout of activity led to an increase in the number of neurones generated in the granule cell layer. This corroborates with reports that the fate of the newborn cells is decided early (Kempermann et al., 2003). The underlying mechanism behind an increased tally of running-induced neurones is proposed to be an asymmetrical division of multipotent progenitor cells that gives rise to a daughter cell and a neuroneal precursor (Suh et al., 2007). Lesioning did not alter this rise in numbers of newborn neurones, further implying that cholinergic signaling is not involved in the running-mediated increase in neurogenesis. In addition, the cholinergic system has no effect on the phenotypic fate of progenitor cells in the DG, echoing findings in various studies (Cooper-Kuhn et al., 2004; Kaneko et al., 2006; Kotani et al., 2006; Mohapel et al., 2005; Teitelbaum et al., 1975; Van der Borght et al., 2005) and lending weight to the theory that GABA is the critical neurotransmitter determining the lineage of neural progenitors (Tozuka et al., 2005).

A model based on my current hypothesis is proposed here, for the potentiation of running-induced cellular genesis in cholinergic lesioned animals. The reader is referred to the earlier chapter on neurotransmitters and neurogenesis. Electrophysiological studies have revealed the presence of $GABA_A$ receptor on Type II neural precursors (Wang et al., 2005) and GABAergic synaptic terminals on the same cell types (Tozuka et al., 2005). Initial activity-dependent synaptic inputs, i.e. excitatory GABA, induce depolarization in these Type II cells which promotes neuroneal differentiation (Tozuka et al., 2005) and dendritic development (Ge et al., 2006). This phasic GABA activation of newborn cells may be mediated through stimulation from local interneurones (Farrant and Nusser, 2005). Here, the act of running would result in synchronous network oscillations with co-activation of septohippocampal neurones. The excitatory septocholinergic afferents form contacts with hippocampal pyramidal cells and interneurones, resulting in overall excitatory post-synaptic potential, and release of glutamate and GABA vesicles. With lesioning, there are no longer excitatory inputs from septocholinergic neurones onto the hippocampal cells. Coupled with inhibitory fibres from the septohippocampal GABAergic neurones activated during theta, summation of inhibitory post-synaptic potentials on the inhibitory interneurones may occur which hinders the release of GABA. The corresponding absence of stimulatory GABAergic and glutamatergic currents on the neural precursor cells may circumscribe their differentiation and maturation. Since running spurs the division of Type II progenitors, and cholinergic denervation

conversely hampers their differentiation, the effect of the lesioning on running-mediated progenitor proliferation is magnified.

In summary, the septohippocampal cholinergic system is not required for the generation of progenitors and determination of their lineage. The pro-proliferative actions of running are not thwarted by the lack of cholinergic input from the basal forebrain, though prolonging the viability of newborn neurones may require acetycholine-regulated signalling.

The results here do not shed light on the exact identity of the molecular factor(s) underpinning running-mediated neurogenesis nor do they dispute the proposed theory that the septohippocampal pathway and theta are involved. Studies have indicated that fimbria fornix lesions lead to drastic reduction in proliferation in the DG (Fontana et al., 2006; Lai et al., 2003). Denervation of the entorhinal cortex, another principal input of the hippocampus does not affect DG neural proliferation (Fontana et al., 2006). Since septocholinergic neurones do not directly affect progenitor proliferation, it is possible the septohippocampal GABAergic cells that are stimulated during physical exercise may be involved instead. It is postulated that the act of running activates pacemaker theta oscillations in the MSDB that may (i) trigger the release of mitogenic signals from these septohippocampal afferents, and/or (ii) induce excitatory postsynaptic currents (EPSC) in the hippocampus (by way of the disinhibitory circuit discussed

in Chapter 1), effecting an increase in pro-proliferative signals from hippocampal target cells.

As to the precise identity of mitogen mediating this activity-dependent proliferation, there is a wide array of molecular factors that can fit the bill. I have earlier ruled out the possibility of circulating growth factors as their effects are too widespread in the brain, and need not be activity-dependent. As running only affects neurogenesis in the SGZ and not the SVZ, it is reasonable to theorize that the delivery of this mitogenic signal to the hippocampal neurogenic niche is specific and achieved by the axonal transport system.

One of the plausible candidates that effectuate this running-related response may be the secreted developmental protein sonic hedgeghog (Shh). Firstly exogenous application of Shh elicits a proliferative response in progenitors *in vitro* and *in vivo* (Ericson et al., 1995; Lai et al., 2003; Machold et al., 2003) and fate-mapping indicates that the cells affected are multipotent transient-amplifying adult progenitors (Ahn and Joyner, 2005; Lai et al., 2003). Secondly, the elevation in cell proliferation has been shown to be via the recruitment of the Shh signaling pathway (Banerjee et al., 2005). Thirdly, Shh is capable of undergoing anterograde transport from the retina to the brain (Traiffort et al., 2001), suggesting that it may be transported to other areas in a similar fashion. Fourthly, Shh transcripts are co-expressed with setpal GABAergic ones in the basal forebrain (Traiffort et al., 2001). Naturally, these are mere speculations, and the

next few chapters will be devoted to the exploration of this particular morphogen in the context of running-mediated neurogenesis.

3. SHH EXPRESSION IN THE SEPTOHIPPOCAMPAL SYSTEM

3.1 INTRODUCTION

In the previous chapters, I have narrowed down to Shh as one of the potential contenders for the molecular signals of running-mediated neural progenitor proliferation. In this chapter, I shall attempt to provide a condensed review on the extant literature of this secreted protein, focusing on its functions and signaling mechanisms. I also seek to explore the expression of Shh in the adult forebrain, especially in two localized regions: the hippocampus and MSDB.

3.1.1 Say that again…Sonic hedgehog?

In 1980, Christiane Nusslein-Volhard and Eric Wieschaus used a genome-wide mutational screen to identify genes affecting embryonic development in the fruit fly *Drosophila*, which garnered them a Nobel Prize (Nusslein-Volhard and Wieschaus, 1980; Rubin and Lewis, 2000). One of the gene mutations gave rise to short and stubby embryos with a spiny appearance that resembled a hedgehog. The name of this fuzzy mammal was hence bestowed upon the newly discovered gene. Subsequently, vertebrate homologues of the hedgehog gene were given affiliated names. *Sonic hedgehog* is the most famous among its family members, given its multitude of roles in human development. Its quirky name is inspired by the popular video game character, *Sonic the Hedgehog*. Other less illustrious, but no less important members of the Hedgehog (Hh)

family include Indian and Desert hedgehog, which play related roles in growth, patterning and morphogenesis of different regions in both vertebrates and invertebrates.

3.1.2 Functions of Shh

Shh is best known for its role as a morphogen in development, i.e. the ability to generate different cell types in a distinct spatial order by formation of a concentration gradient. This remarkable signalling peptide has a hand in the earliest stage of organogenesis in the embryo. It is present in key signalling centres (floor plate and notochord) responsible for ventralizing the neural tube, the embryonic precursor of the CNS (Marti et al., 1995; Roelink et al., 1994). In addition, it is widely involved in the patterning and growth of many other organs in the body, among them the gastrointestinal tract, skeletal system, heart, teeth, lungs, prostate, just to name a few (reviewed in (Ingham and McMahon, 2001)). Evidently, it goes beyond the scope of this thesis to provide a comprehensive account of the plethora of developmental processes regulated by Shh.

In the brain itself, Shh directs ventral differentiation in the early stages of development. It induces distinct cell fates, for instance, in the form of dopamine-releasing neurones in the midbrain and the serotonergic neurones in the ventral forebrain (Ericson et al., 1995; Hynes et al., 1995; Ye et al., 1998); and motor neurones and oligodendrocytes in the spinal cord (Alberta et al., 2001; Lu et al., 2000; Perez Villegas et al., 1999; Soula et al., 2001). On top of its role as a

morphogen, Shh also acts as an axon guidance cue (Schnorrer and Dickson, 2004). It functions as a chemoattractant in the floor plate of the spinal cord, signalling to the neurones originating in the roof plate to send axonal projections to the ventral floor plate, where they cross to the other side to generate commissural tracts (Charron et al., 2003). As development proceeds, Shh expression appears in the dorsal regions of the brain, including the cerebellum, neocortex, tectum and hippocampus (Dahmane and Ruiz i Altaba, 1999; Dahmane et al., 2001; Machold et al., 2003; Traiffort et al., 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Now, the role of this capricious signal peptide in the dorsal brain as a potent mitogen is to expand the progenitor cell population to the correct numbers (Dahmane and Ruiz i Altaba, 1999; Dahmane et al., 2001; Wallace, 1999; Wechsler-Reya and Scott, 1999).

Given that imprints of the embryonic brain are preserved in adult neurogenic niches, it is not surprising to detect the actions of Shh well into adulthood. Here, the secreted protein goes beyond its roles in development and acts to promote neural progenitor cell proliferation in both SGZ and SVZ (Ahn and Joyner, 2005; Lai et al., 2003; Machold et al., 2003; Palma et al., 2005). Akin to other signalling systems governing cell proliferation, dysregulation of Shh signalling is implicated in tumorigenesis, such as in the development of medullablastomas and gliomas (Berman et al., 2002; Clement et al., 2007; Dahmane et al., 2001; Stecca and Ruiz i Altaba, 2005).

3.1.3 Shh signalling

To fathom how a single signal can evoke a myriad of responses in a temporally and spatially specific manner, it is important to understand the molecular mechanisms underpinning the processing, movement, reception and propagation of the Shh signal. Many of the mechanistic processes of the hedgehog signalling pathway were elucidated through studies of the common fruit fly. Briefly, the secreted Shh protein binds to its receptor Patched1 (Ptc1), relieving the latter's inhibition on the signal transducer Smoothened (Smo), and resulting in the recruitment of the GLI-Kruppel (Gli) family of transcription factors and the subsequent mediation of cellular responses (FIGURE 3-1).

In order for the Shh ligand to be a functionally active peptide, it has to go through a unique posttranslational process involving dual lipid modification. This step occurs early in the secretory pathway, around entry into the Golgi apparatus (Lee et al., 1994a). The Shh protein is produced as a ~45kDa precursor and first undergoes internal autoproteolytic cleavage to give rise to an active 19kDa N-terminal fragment (Shh-N) and a 25-kDa C-terminal fragment. The N-terminal product of the cleavage then receives a cholesteryl adduct at its carboxy-terminal glycine (Porter et al., 1996a; Porter et al., 1996b). Both autoprocessing steps are mediated by the C-terminal fragment (Jeong and McMahon, 2002; Mann and Beachy, 2004). The second lipid modification, carried out at the extreme amino terminus of the Shh-N peptide, is the covalent addition of fatty acid palmitate (Pepinsky et al., 1998). The palmitolyation reaction is catalysed by Skinny

Hedgehog (Skn) (also known as Sightless, Rasp, Central Missing), a membrane-bound acyltransferase (Chamoun et al., 2001; Lee and Treisman, 2001; Micchelli et al., 2002). It is suggested that the dual-lipid modification increases the potency of the Shh signal (Lee and Treisman, 2001; Taylor et al., 2001). Another suggested function of cholesterol and palmitoyl moieties is to cooperatively target Shh-N to lipid rafts, specialized membrane microdomains acting as assemblies for many cellular functions (Jeong and McMahon, 2002; Mann and Beachy, 2004). In neurones, lipid rafts provide a platform for signal transduction, and are intimately involved in neuroneal development and axonal guidance (Kamiguchi, 2006; Tsui-Pierchala et al., 2002).

The intrinsic lipid motifs are also responsible for the regulated release and trafficking of the active hedgehog peptide. Members of the Hh family are capable of exerting their patterning effects via both short-range and long-range signalling (Ingham and McMahon, 2001; Johnson and Tabin, 1995). Long-range signalling is mediated via a freely diffusible form of Shh-N multimer, which migrates as a complex of ~120kDa (Goetz et al., 2006; Zeng et al., 2001). Both cholesterylation and palmitoylation are required for generation of the multimer, proposed to be the physiologically relevant form of Shh in the morphogenic field (Chen et al., 2004; Goetz et al., 2006; Lewis et al., 2001). Dispatched (Disp), a multi-pass membrane protein structurally similar to the Shh receptor, facilitates Shh transport by assembling the Shh active peptides through cholesterol anchors at Shh-producing cells (Burke et al., 1999; Kawakami et al., 2002; Ma et al., 2002;

Tian et al., 2005) and is thought to package them into soluble multimers for long-distance transport (Zeng et al., 2001).

Other molecules, especially lipoproteins, have been implicated in the transport of Shh. In *Drosophila*, lipoprotein particles are required for hedgehog signalling and are proposed to act as vehicles (Panakova et al., 2005). The receptor for Shh proteins, Ptc1, is likewise a lipoprotein receptor (Callejo et al., 2008). In the neural tube of mammals, a low-density lipoprotein (LDL) receptor, Megalin, is shown to bind and internalize Shh-N (McCarthy et al., 2002). Upon endocytosis, Shh-N in non-ciliated kidney cells either undergoes lysosomal degradation or is trancytosed (Morales et al., 2006). Whether megalin is directly involved in bringing Shh-N ligand to its receptor is yet to be resolved, though various models are proposed (Fisher and Howie, 2006).

Ptc1 is an integral membrane protein with a sterol-sensing domain. It has been shown to reside in caveolin-1 rich lipid rafts (Karpen et al., 2001). Binding of the ligand on this twelve-pass transmembrane receptor disinhibits the signal transducer Smo, a G-protein-coupled- receptor (GPCR). The mechanism by which Ptc1 represses Smo is not well understood but recent studies have demonstrated that it may be via cholesterol derivatives such as vitamin D and oxysterols (Bijlsma et al., 2006; Corcoran and Scott, 2006). Other studies have shown that another membrane-bound glycoprotein, Hedgehog Interacting Protein (Hip), acts in parallel to Ptc1 by binding to Shh-N on responding cells (Chuang et

al., 2003; Chuang and McMahon, 1999). Hip sequesters Shh-N at the cell surface, preventing it from acting on Ptc1, and hence adds another dimension of negative regulation to the signalling pathway (Jeong and McMahon, 2005). Both membrane-associated and soluble forms of Hip have been found in discrete brain areas of adult rodents (Coulombe et al., 2004). It is not known how vertebrate Smo operates, but *Drosophila* studies have indicated that the activation of the Hh signalling pathway induces accumulation of Smo on the cell surface, possibly through progressive phosphorylation by kinases like Protein kinase A (PKA) and Casein kinase 1 (CK1) (Jia et al., 2004).

Emerging evidence in recent years has shown that most of the signal reception of Hh-responding cells is centred on the primary cilia (Corbit et al., 2005; Huangfu and Anderson, 2005; Liu et al., 2005; Rohatgi et al., 2007; Tran et al., 2008; Wang et al., 2006). The cilium is a microtubule-based extension of the plasma membrane, and is evolutionary conserved in all cells from single-cell organisms to higher-order mammals. It is a specialized structure assembled and maintained by intraflagellar transport proteins such as kinesin and dynein that moves cargo to and fro in this unique cytoskelet al structure. Mouse mutants with impaired intraflagellar transport machinery exhibit phenotypes associated with defective Hh signalling (Huangfu and Anderson, 2005; Liu et al., 2005) . Smo is translocated from the primary cilia to the cytoplasm in kidney cells upon Shh-N pathway activation (Corbit et al., 2005). β-arrestins, widely known for their roles in desensitization and clathrin-mediated endocytosis of GPCRs, play a role here

in Shh-pathway actuation by targeting Smo to the cilium via a three-pronged interaction with kinesin anterograde motor proteins (Kovacs et al., 2008; Wilbanks et al., 2004). Ptc1 is similarly localized at primary cilia, particularly at the base of the cilium and in particles along the shaft of the cilium, and inhibits Smo by restricting its trafficking from the cytoplasmic pool (Rohatgi et al., 2007). The cilia is also enriched with cytosolic proteins like Gli2, Gli3 and a negative regulator, Supressor of Fused (Sufu) after response to Shh stimulation (Haycraft et al., 2005). Recent work has indicated that the primary cilia is essential for granule neurone precursor proliferation during perinatal development, leading to the establishment of the adult stem cell population in the DG (Han et al., 2008).

Away from the cell surface and into the heart of the protoplasm, the plot thickens. The three members of the Gli family of zinc-finger transcription factors, namely Gli1, Gli2, Gli3, integrate Shh-N signalling in a combinatorial and cooperative fashion (Ruiz i Altaba et al., 2007). Under basal conditions, Gli3 acts a constitutive repressor, with Gli2 functioning too as a repressor in some developmental contexts (Ruiz i Altaba, 1999; Wang et al., 2000). The repressor role of these two proteins arises from their continual cleavage into C-terminal truncations. Upon Shh-N activation, Smo stabilizes and activates full-length Gli proteins and concurrently impedes the production of Gli repressors. Full-length Gli 1 and Gli 2 proteins have somewhat overlapping functions as activators (Ruiz i Altaba, 1999). Together with nuclear co-activators, Zic proteins (Mizugishi et al., 2001), the Gli activators bind to the same consensus sequences as Gli3, exerting

their actions on downstream target genes as well as a positive feedback on their own expression.

The length of Gli proteins is dictated by post-translational ubiquitination processes. In *Dropsohila*, the same protein kinases PKA and CK1 acting on Smo also confer phosphorylation at multiple sites of Cubitus interuptus (the *Drosophila* equivalent of Gli), resulting in the recruitment of the F-box protein, Slimb (Jia et al., 2002; Jia et al., 2005). The F-box protein is part of the Skp-C1/Cullin1/F-box containing complex (SCF complex), a multi-protein E3 ligase that catalyzes (poly)ubiquitination of proteins, subjecting them to processing and/or degradation by 26S proteasomes (Welchman et al., 2005). Upon Shh signalling activation, the same series of sequential phosphorylations also occur in vertebrate Gli3, leading to the binding of β-TRCP (the vertebrate homologue of Slimb) and subsequent Gli3 pruning (Tempe et al., 2006; Wang and Li, 2006). In the absence of signalling, Gli1 is not truncated but is instead completely destroyed (Huntzicker et al., 2006). Gli2 can either be degraded or processed, underpinning its dual role as an activator and a repressor (Bhatia et al., 2006; Pan et al., 2006).

Gli activity is regulated by a multitude of positive and negative modulators. A nuclear-localized kinase, Dyrk1 and an actin-binding protein, Missing in Metastasis (MIM), both act to enhance Gli1 transcriptional activity (Callahan et al., 2004; Mao et al., 2002). On the flip side, proteins like Sufu and REN impede Gli activators by impeding their transfer into the nucleus (Barnfield et al., 2005;

Ding et al., 1999; Dunaeva et al., 2003; Kogerman et al., 1999; Svard et al., 2006). Rab23, a member of the GTPase family is also involved in affecting the function of Gli, presumably through regulation of intracellular vesicle trafficking (Eggenschwiler et al., 2001; Jeong and McMahon, 2001; Wang et al., 2006). The regulation of ubiquitination is an additional point of control for Gli proteins. Besides the SCF complex, another E3 ligase, Itch, targets Gli1 for proteolysis (Di Marcotullio et al., 2006). The protein interactions between Itch and Gli1 are facilitated by the adaptor protein Numb (Di Marcotullio et al., 2006).

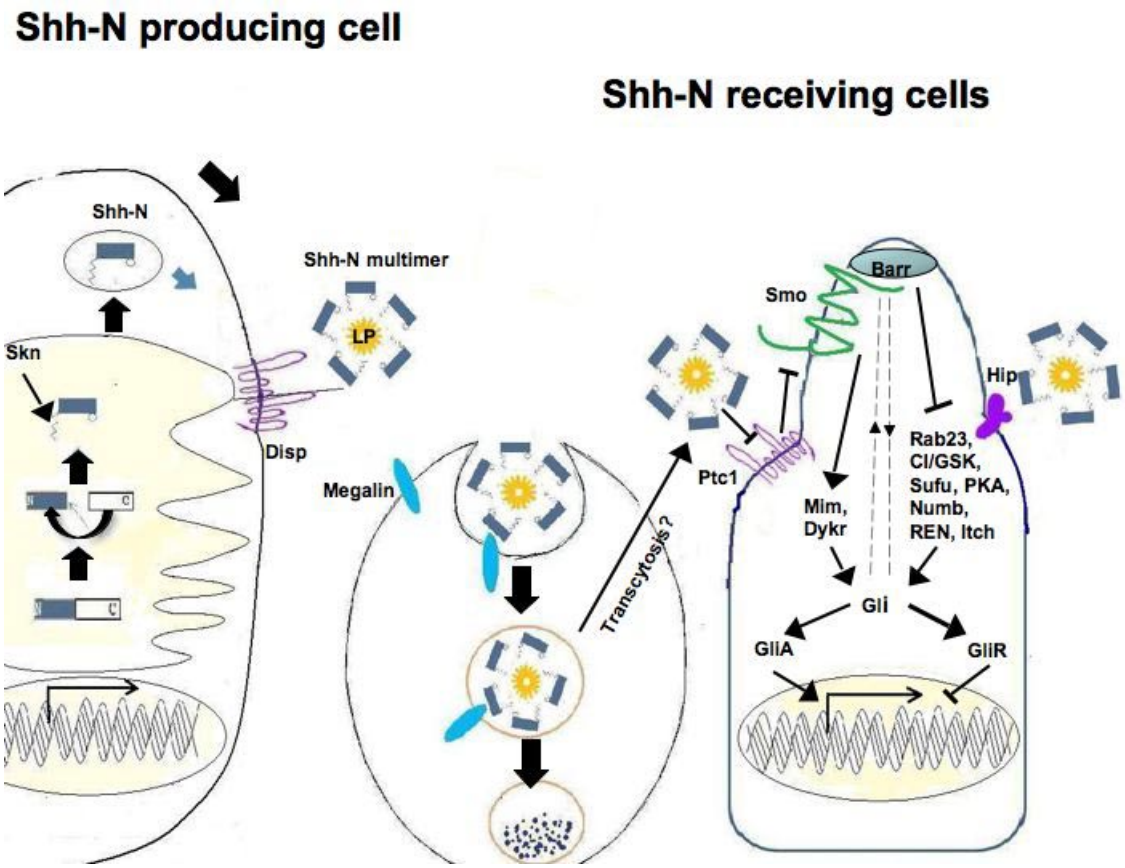


FIGURE 3-1 A schematic diagram on the synthesis, modulation and transduction of Shh activities (adapted from Ruiz i Altaba et al., 2007; Wang et al., 2007)

I have attempted to condense the sheer volume of extant Shh literature into a broad outline, but the reader can appreciate the complexity of its mechanistic processes, ranging from its unusual biosynthesis right down to its control of a bifurcating transcriptional switch. Although research on the molecular mechanisms of the hedgehog signal transduction pathways has expanded exponentially within the last few years, many gaps remain to be filled. For instance, the majority of the experiments conducted on Hh signalling are genetic studies, and are confined to the contexts of embryonic and perinatal development. Are signalling pathways in adult mammals any different? How does Shh switch modes from a morphogen responsible for directing cell fates to that of a mitogen, mobilizing quiescent stem cells to divide and give rise to rapidly dividing daughter cells? How are Shh-N proteins in the adult brain trafficked? Clearly the adult brain is much more complex than its embryonic precursor, with spatially disparate and dynamic signalling networks, and to model the actions of Shh as a function of a concentration gradient will be overly simplistic. Also, most of the current research on Shh revolves around the subcellular level, emphasizing the mechanistic interactions between and within extracellular and intracellular molecules. It would be interesting to observe how behavioural inputs can translate into changes in the Shh transcriptional machinery.

This chapter will be the first of three chapters exploring the role of Shh in neural progenitor proliferation in the adult hippocampus. The series of experiments in

this chapter will centre on the expression of Shh and members of its signal transduction pathway in distinct regions of the adult brain.

In situ hybridization has revealed the presence of Shh and its immediate signal transducers in discrete regions of the adult rat brain (Traiffort et al., 1999; Traiffort et al., 1998). Shh transcripts are present in almost every part of the basal forebrain, including both the vertical and horizontal limbs of the diagonal band, and the lateral wall of forebrain ventricles (Palma and Ruiz i Altaba, 2004; Reilly et al., 2002; Traiffort et al., 1999). Ptc1 expression is also found in basal forebrain cholinergic neurones of postnatal and adult brains (Reilly et al., 2002). Transcripts of both transmembrane receptors Ptc1 and Smo are predominantly found in the DG granule cell area (Banerjee et al., 2005; Traiffort et al., 1999; Traiffort et al., 1998). Smo is also present in the subventricular zone and ventricular ependymal layer of the basal forebrain (Traiffort et al., 1999; Traiffort et al., 1998). Immunohistochemistry studies show that Ptc1 is expressed in the hippocampal formation, especially in the granule cell layer and pyramidal cells of CA1 to CA3 (Lai et al., 2003).

Given the pronounced distribution of Shh transcripts in the DG and the MSDB subregion of the basal forebrain, and its relevance to my project hypothesis, the investigations here will address the protein expression of Shh-N and its downstream effectors in these regions to further probe the identities of Shh-N containing cells.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Female Swiss albino mice, 8-10 weeks old, from CARE (Sembawang, Singapore) were housed in the AHU, at 12:12 light-dark cycle, with *ad libitum* access to food and water. The animals were group-housed for a week to get used to their surroundings prior to experimental manipulation. All experiments were conducted in accordance with Institute Animal Care and Use Committee (IACUC) approved protocols.

3.2.2 RNA extraction and RT-PCR

The mice were overdosed with pentobarbital anaesthetic (Nembutal) and their brains harvested after cervical dislocation. The relevant regions of the brains, i.e, MSDB and hippocampus, together with positive controls cerebellum and the brainstem, were excised and homogenized in ice-cold Trizol (Invitrogen) (50 -100 mg tissue in 1 ml) using a handheld motorized pestle. The upper aqueous phase of RNA was then extracted after addition of chloroform and centrifugation at 12,000 x g. Isopropanol precipitation and 70% ethanol wash were performed before dissolving total RNA in 30 µl of diethyl pyrocarbonate treated water. DNase (Roche) was added to prevent DNA contamination. The quantity and quality of RNA was affirmed by using spectrophotometry.

cDNA was synthesized from 1 μg total RNA, using 20 pmol oligo-dT primers, 3 mM $MgCl_2$, 0.5 mM dNTP, ImProm-II Reaction Buffer and Reverse Transcriptase (Promega, Madison, WI). The components in the PCR mix included 1 μl cDNA sample, 2.5 mM $MgCl_2$, 0.25 mM dNTP, PCR buffer, 0.4 μl (2 units) Taq DNA polymerase (Promega), and 1 μl of each specific primer (Shh: forward 5′-**TCTGTGATGAACCAGTGGCC**-3′, reverse 5′-**GCCACGGAGTTGTCTGCTTT**-3′, product size: 242 bp; Ptc1: forward 5′-**AACAAAAATTCAACCAAACCTC**-3′**,** reverse 5′-**TGTCTTCATTCCAGTTGATTG**-3′, product size: 246 bp; Smo: forward 5′-**GACCACTCCCATAAGGGCTA**-3′, reverse 5′-**GAAGAGGTTGGCCTAGTGGA**-3′, product size: 117 bp; Gli-1: forward 5′-**CTTGAAAACCTCAAGACGCACC**-3′, reverse 5′-**CCATGCACTGTCTTCACGTGTT**-3 ′, product size: 221 bp; Gli-2: forward 5′-**TCACTGAAGGATTCCTGCTCGT**-3′, reverse 5′-**ATCGTCACTTCGGTCAGCTCTG**-3′, product size: 173 bp; Gli-3: forward 5′-**CATTTCCACGGCAACCACA** -3′, reverse 5′- **GCCCACCCGAGCTATAGTTGTT** -3′, product size: 209 bp) (Oligoprobes, Singapore). PCR was carried out with a thermocycler with an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min; and a final extension at 72°C for 5 min. The PCR products were separated on 1.5% agarose gel by electrophoresis and visualized using Chemi-Doc XRS (BioRad, Hercules, CA).

3.2.3 Western blotting

Dissected brain tissues were dounce homogenized using 20 strokes each of the tight-fitting pestle and loose-fitting pestle in buffer (10 mM TRIS, 2 mM EDTA, pH 7.4, with *cOmplete* protease inhibitors, Roche Applied Science), left on ice for 20 min and centrifuged at 13000 x g for 15 min to obtain the supernatant. The protein concentration in the lysate was determined using the Quant-it Protein Assay (Invitrogen). The total cell lysate was stored at -80ºC.

Protein separation was carried out using the Laemmli method. 20-40 µg of protein was denatured with 5X sample buffer containing anionic sodium dodecyl sulphate (SDS) and boiled at 95ºC for 5 min prior to loading onto tris-glycine 7.5%, 14% or 4-15% gradient gels depending on the protein of interest. Electrophoresis was performed on a mini-PROTEAN gel electrophoretic apparatus (Bio-Rad) before blotting onto a nitrocellulose membrane (Bio-Rad). The transfer of proteins and markers from the gel onto the membranes was verified using Coomasie Blue staining and MemPer Kit (Pierce, Rockford, IL), respectively.

To prevent non-specific antibody binding onto membranes, blocking was carried out for an hour (StartingBlock/SuperBlock Blocking Buffer, Pierce) before incubation with primary antibodies overnight on a shaker at 4°C. The various primary antibodies used were goat polyclonal anti-Shh-N (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), goat polyclonal anti-Gli-1 (1: 5000, Abcam,

Cambridge, UK), goat polyclonal anti-Ptc-1 (1:500, Santa Cruz), rabbit polyclonal anti-Smo (1:200, Santa Cruz), and rabbit polyclonal anti-Actin (1:20,000, Sigma-Aldrich, Saint Louis, MO). This was followed by application with the appropriate HRP conjugated- mouse anti-goat and goat anti-rabbit secondary antibodies (1:50,000, Pierce). Tris-buffered saline with 0.1% Tween-20 (TBS-0.05% T-20) was used both as a diluent and as a wash buffer. The chemiluminescent substrate (West Pico) was purchased from Pierce. The bands on the x-ray films were scanned using Chemi-Doc XRS (Bio-Rad).

3.2.4 Immunoprecipitation

Protein G –Sepharose (Sigma) was mixed with Tris-EDTA buffer with protease inhibitor in a 1:3 ratio and spun down for 12,000 x g for 2 min. The supernatant was extracted and washed with buffer four times before resuspension and equilibration for an hour to make up the bead slurry. The stored total cell lysate was thawed and subjected to centrifugation at 14,000 x g for 1 hour. To prevent proteins from binding non-specifically to the beads, the lysate was then pre-cleared by adding 0.1 μl of bead slurry per μl cell lysate, mixed for 30 min with agitation, and spun down to extract the supernatant for immunoprecipitation. 2 μg of Shh-N antibody (Santa Cruz) was added to 100 μg of lysate and incubated for 2 hours. 50 μl of the pre-equilibrated slurry was then added prior to overnight incubation on an inverter. After centrifugation, the supernatant was removed to be kept as negative controls, and the pellet washed with 500 μl of buffer. The washing procedure was repeated 4 times, before a final resuspension in 25 μl of

buffer, and denaturation with 2X sample buffer at 95°C for 5 min. After briefly centrifuging, the supernatant was removed for SDS-PAGE. All steps were performed at 4°C.

Western blot analysis of the immunoprecipitate was performed as above with rat monoclonal IgG2a anti-Shh-N (1 μg, R&D systems Minneapolis, MN) as the primary antibody and goat anti-rat IgG2a (1:2000, Immunology Consultants Laboratory Inc, Newbury, OR) as secondary antibody.

3.2.5 Immunofluorescence

The mice were perfused transcardially with saline and 4% paraformaldehyde, and their brains harvested and postfixed overnight with the same fixative. The brains were then sectioned at a thickness of 40 μm using a vibratome (World Precision Instruments) and stored in phosphate buffered saline (PBS) at 4°C. Free-floating sections were used for all staining. PBS-0.3% Triton X was used as a diluent and PBS used for washing all brain sections. The sections were treated with pH 8.3 sodium citrate for antigen retrieval, before blocking with 10% serum for an hour on an orbital shaker, and incubating overnight at 4°C with the following list of primary antibodies: goat polyclonal anti-Shh-N (1:100, Santa Cruz Biotechnology, Santa Cruz, CA); goat polyclonal anti-Ptc-1 (1:200, Santa Cruz Biotechnology), rabbit polyclonal anti-GFAP (1:500, Dako, Denmark), O4 (mouse monoclonal IgM, 1:200; Chemicon, Temecula, CA), rabbit polyclonal anti-ChAT (,1:200, Chemicon); rabbit polyclonal anti-GAD 65/67 (1:200, Chemicon), mouse

monoclonal anti-Nestin (1:200, Chemicon) and rabbit polyclonal anti-Sox-2 (1:200, Chemicon). The appropriate secondary antibodies were used: 488 Alexa-Fluor Donkey anti-goat secondary antibody, 555 Alexa-Fluor donkey anti-rabbit and 555 donkey anti-mouse, 633 Alexa-Fluor donkey anti-rabbit and 594 donkey anti-mouse IgM (1:400, Molecular Probes, Eugene, OR). Incubation of secondary antibodies was performed at room temperature for an hour. The sections were mounted with ProLong Gold Anti-fade mounting medium (Molecular Probes). Sequential labelling was performed for dual immunohistochemistry.

3.2.6 Cochicine treatment

The animals were pretreated with colchicine in order to intensify perikarya staining. The mice were subjected to anaesthesia with a cocktail blend of 75 mg/kg ketamine and 1 mg/kg medetomidine, and mounted on a stereotaxic frame fitted with a mouse adaptor (Stoelting Co, Woodale, Illinois). The head of the mouse was aligned in a flatskull position by adjusting both the tooth bar and nose clamp, and ensuring that coordinates of lambda and bregma were equal. 1.5 µl of colchicine (Sigma), at a concentration of 6 µg/µl in 0.9 % saline was injected into the lateral ventricles on both sides of the brain at AP -0.22, ML +/- 1.0, DV -2.4 (Paxinos, 2001a) using a 0.5 ml Hamilton syringe. The injection was carried out over 1 min and the needle left in place for 3 min before withdrawing. The mice were kept warm by means of a heated lamp during recovery from anaesthesia and given subcutaneous glucose saline injections to facilitate post-surgical

recovery. Three days after surgery, the mice were sacrificed and their forebrains removed. 30 µm thick coronal sections were cut and stored in 0.1 M PB at 4ºC.

3.2.7 Microscopy

Fluorescent signals were imaged by using an Axioplan 2 imaging fluorescence microscope with standard filter sets attached to a LSM510 META laser scanning microscope (Carl Zeiss, Germany). 488 nm argon, 543 nmHeNe and 633HeNe lasers were used. For closed up images, a z-series of sections with 0.5 µm optical thickness were taken.

3.3 RESULTS

3.3.1 Shh is expressed in the MSDB and hippocampus

RT-PCR was used to assay for the expression of genes involved in Shh signalling (FIGURE 3-2i). Given the prevalence of Shh activity in the cerebellum (Dahmane and Ruiz i Altaba, 1999; Traiffort et al., 1999; Traiffort et al., 1998), the cerebellum was selected as a positive control. Transcripts of Shh, Smo, Ptc and the Gli family of transcription factors (Gli1, Gli2 and Gli3) were all found in both the MSDB and hippocampus. Western blotting was employed to investigate the existence of the protein forms of these genes. The precursor form of Shh (~45 kDa) was detected in both the MSDB and hippocampus, confirming that both regions were sites of synthesis for Shh. The same antibody used to detect the presence of the precursor was unable to pick up the active amino terminal form, Shh-N, indicating that the latter may be found in lesser quantities. Similar to our RT-PCR results, Ptc1 (160 kDa), Smo (87 kDa) and Gli1 (118 kDa) are likewise detected (FIGURE 3-2ii).

In order to ascertain the presence of active Shh-N, an immunoprecipitation assay was run, followed by western blotting analysis. By enriching the Shh fractions, a ~20 kDa band of protein in both MSDB and hippocampal extracts was obtained, confirming the presence of the ligand (FIGURE 3-2iii).

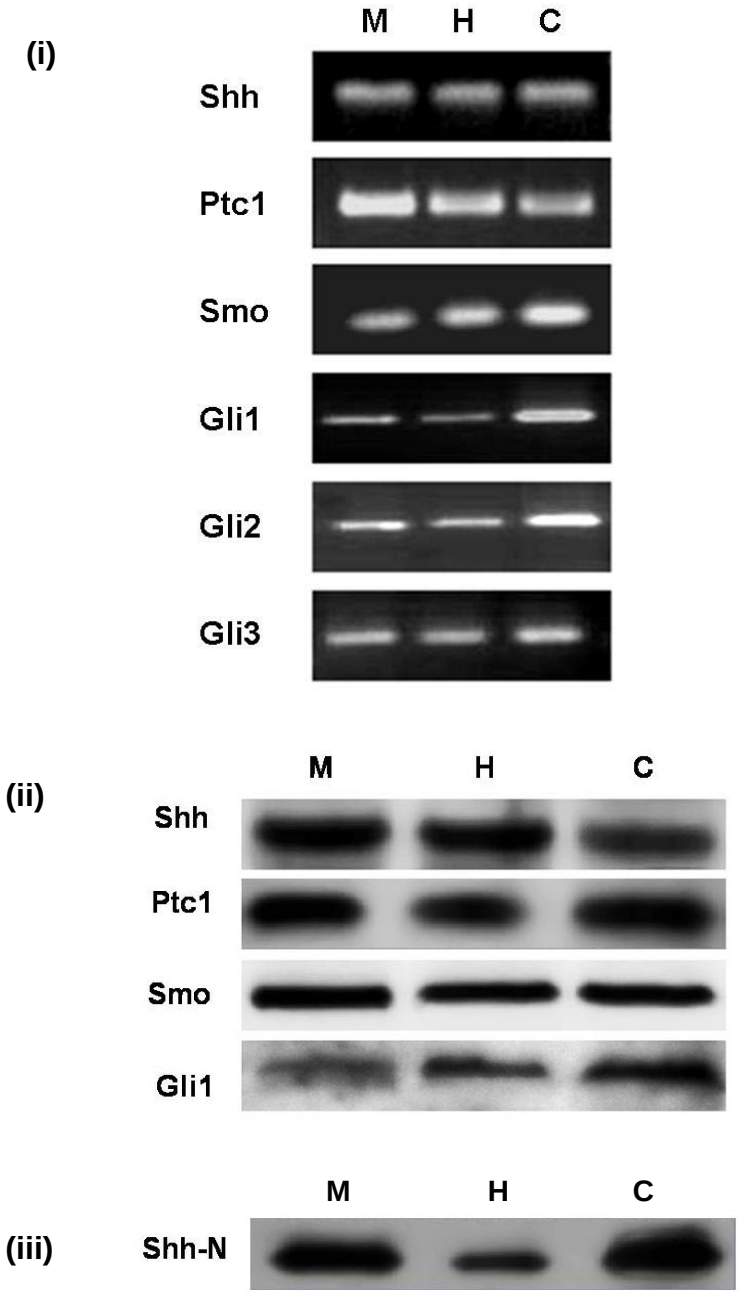


FIGURE 3-2 Expression of Shh and components of its signal transduction pathway in the MSDB and hippocampus (i) RT-PCR analysis of gene expression and (ii) western blots analysis of protein expression show presence of members of Shh signalling cascade in MSDB (**M**), hippocampus (**H**) and positive control cerebellum (**C**). (iii) The active amino-terminal peptide Shh-N can also be found in the MSDB and hippocampus after immunoprecipitation.

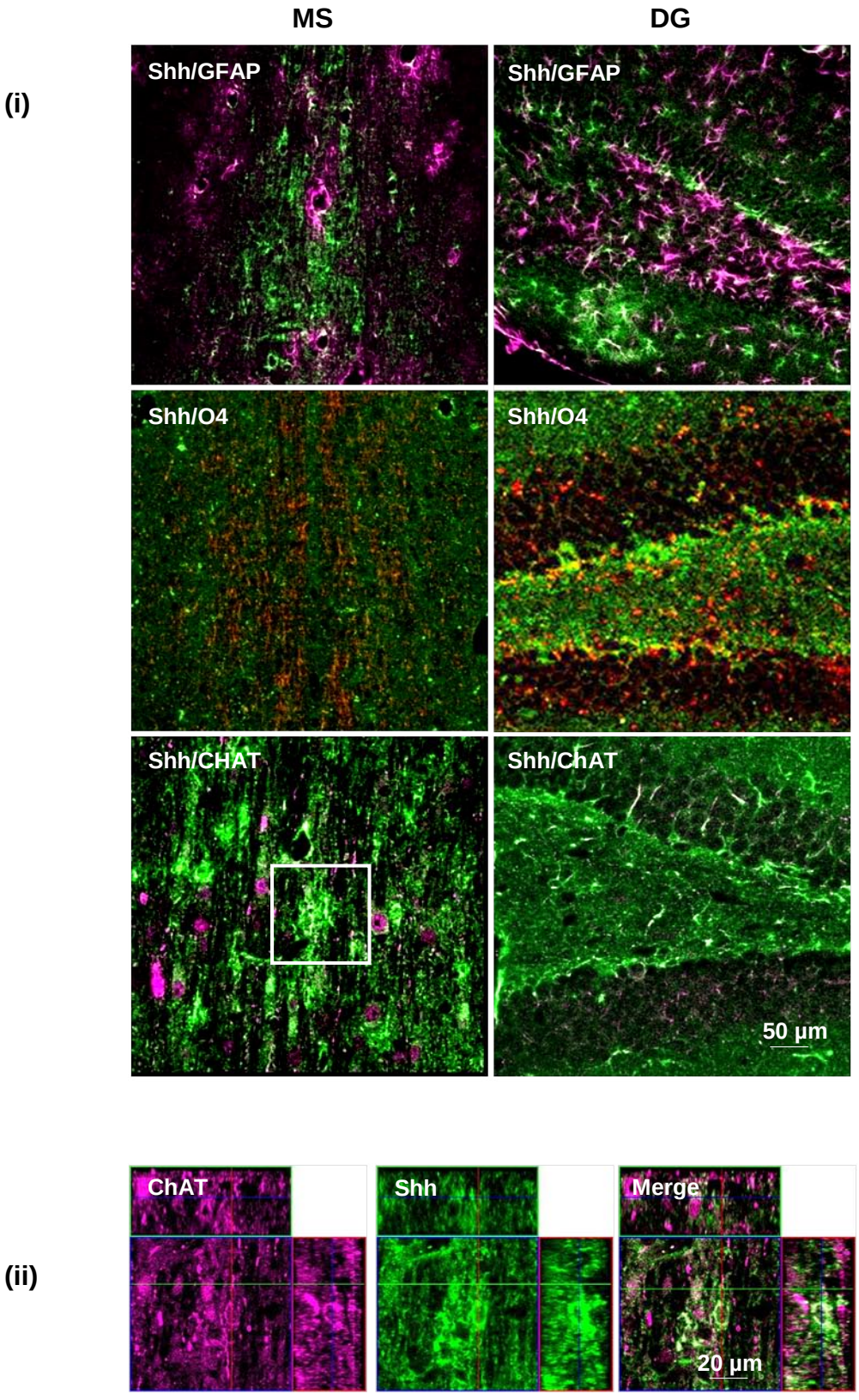
3.3.2 Shh-N is expressed in neuroneal cell bodies in the MSDB and has a punctate profile in the DG

To localize Shh expression in the MSDB and DG, immunohistochemical methods were adopted, making use of an anti-Shh-N antibody to pick up the active form of Shh, and various neuroneal and glial cell antibodies for double immunostaining. The markers used were: ChAT for cholinergic cells, glutamic acid decarboxylase (GAD) for GABAergic cells, GFAP for astrocytes and O4 for oligodendrocytes. To intensify the otherwise weak Shh-N signal, the tissues were pre-treated with heat and sodium citrate for antigen unmasking and bathed in buffer containing Triton-X to permeabilize the cellular membrane (FIGURE 3-3i).

In the MSDB, punctate immunoreactivity for Shh-N was observed in a large population of cell bodies parallel to the longitudinal plane. There was no co-localization of Shh-N with either GFAP- or O4-positive cells, indicating that Shh-N positive cells in the MSDB could be neuroneal in nature. The most intensively stained Shh-N perikarya were seen along the medial septum midline. Although the sizes of the Shh-positive cell bodies varied, with diameters ranging from 10 µm to 30 µm, the shapes were constantly either oval or fusiform. Within the DG, Shh-N-immunoreactive cells were characterized by intense punctation in the hilus. There were also Shh-positive varicosities along the infragranular region of the granule cell layer. In addition, a novel subpopulation of Shh-positive cell bodies in the same locality was identified. Shh-N fibres exist in close proximity with GFAP and O4-positive cells, especially along the SGZ (FIGURE 3-3i).

The ChAT-positve cholinergic cells in the medial septum were mostly medium to large in size (~20 to 30 µm), although clusters of small cells (~10 µm) could be seen. MSDB cholinergic cells were mostly oval or bipolar. A small percentage of colocalization between Shh-N- and ChAT-immunopositive cells could be observed (FIGURE 3-3ii). Presumptive ChAT-positive axon terminals were observed in the DG, although ultrastructural analysis will be required to conclusively establish Shh contacts with cholinergic nerve endings.

In order to enhance the levels GAD in the perikarya, colchicine was injected into the ventricles of animals prior to immunohistochemistry. Colchicine has been reported to enhance the appearance of large sized GABAergic neurones (Onteniente, 1987). In the MSDB, GABAergic cells displayed great variability in size and shape and were more distributed throughout the medial septum compared to ChAT-positive cells. Shh-N staining was intensified concomitantly with the colchicine pre-treatment. Most of the Shh-N cell bodies, especially the medium to large sized ones (15 µm to 25 µm) around the midplane of the MS, co-labelled with GAD. The colchicine-pretreatment ablated Shh-N immunoreactivity in the DG, although it enhanced the cell soma staining of the novel subpopulation of sparse Shh-positive cells in the SGZ. Some of these Shh-immunoreactive cell bodies in the SGZ also colocalized with GAD-positive perikarya (FIGURE 3-3iii).


MS
DG
(i)
Shh/GFAP
Shh/GFAP
Shh/O4
Shh/O4
Shh/CHAT
Shh/ChAT
50 µm
(ii)
ChAT
Shh
Merge
20 µm

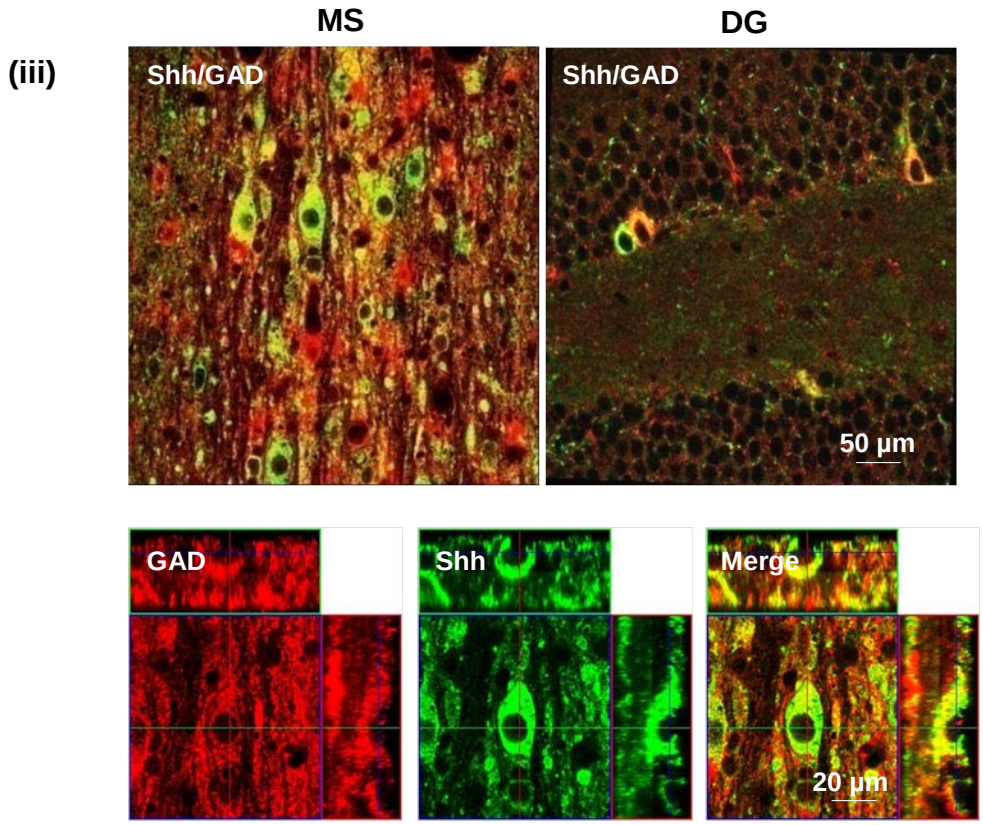


FIGURE 3-3 Localization of Shh-N in the MSDB and DG (i) Confocal images of Shh-N immunopositive cells *(green)* together with GFAP *(magenta)*, O4 *(red)*, and ChAT *(magenta)*. A large heterogeneous population of Shh-N-immunopositive cell bodies were found in the medial septum whereas most of Shh-N-positive presumptive varicosities were found along SGZ and appeared as intense puncta in the hilus. (ii) Higher-power images of boxed area viewed in orthogonal planes showed colocalization of Shh-N with clusters of small ChAT positive neurones (iii) Pre-treatment with colchicine to enhance GAD *(red)* perikarya staining in the MSDB also intensified Shh (green) immunoreactivity. Shh-N immunoreactivity in the DG was reduced. Magnification of boxed area in MSDB showed that Shh-N was colocalized with GAD.

3.3.3 Shh-N is associated with stem cell markers in the DG neurogenic niche

In a bid to further profile Shh-N positive cells, co-staining was performed with several key neural precursor markers namely, Nestin, Sox2 and GFAP. Nestin is an intermediate filament found in Type I and Type II transit amplifying cells (Mignone et al., 2004). Sox2 is a transcription factor for the SRY-related HMG box family, and labels for uncommitted Type I and Type IIa neural precursors (Gubbay et al., 1990; Suh et al., 2007). Besides being an astrocytic marker, GFAP also serves to identify glial-like Type I quiescent neural stem cells (Filippov et al., 2003). Again, a punctate profile of Shh-N could be observed in the dentate hilus, together with fibres along the infragranular region. The anti-Ptc1 antibody extensively labelled the entire volume of granule cells, as well as a few scattered cells in the hilus. Both the likely Shh-N immunoreactive fibres and Ptc-1 immunoreactive cell bodies were in close apposition to the progenitor markers in the DG neurogenic niche (FIGURE 3-4).

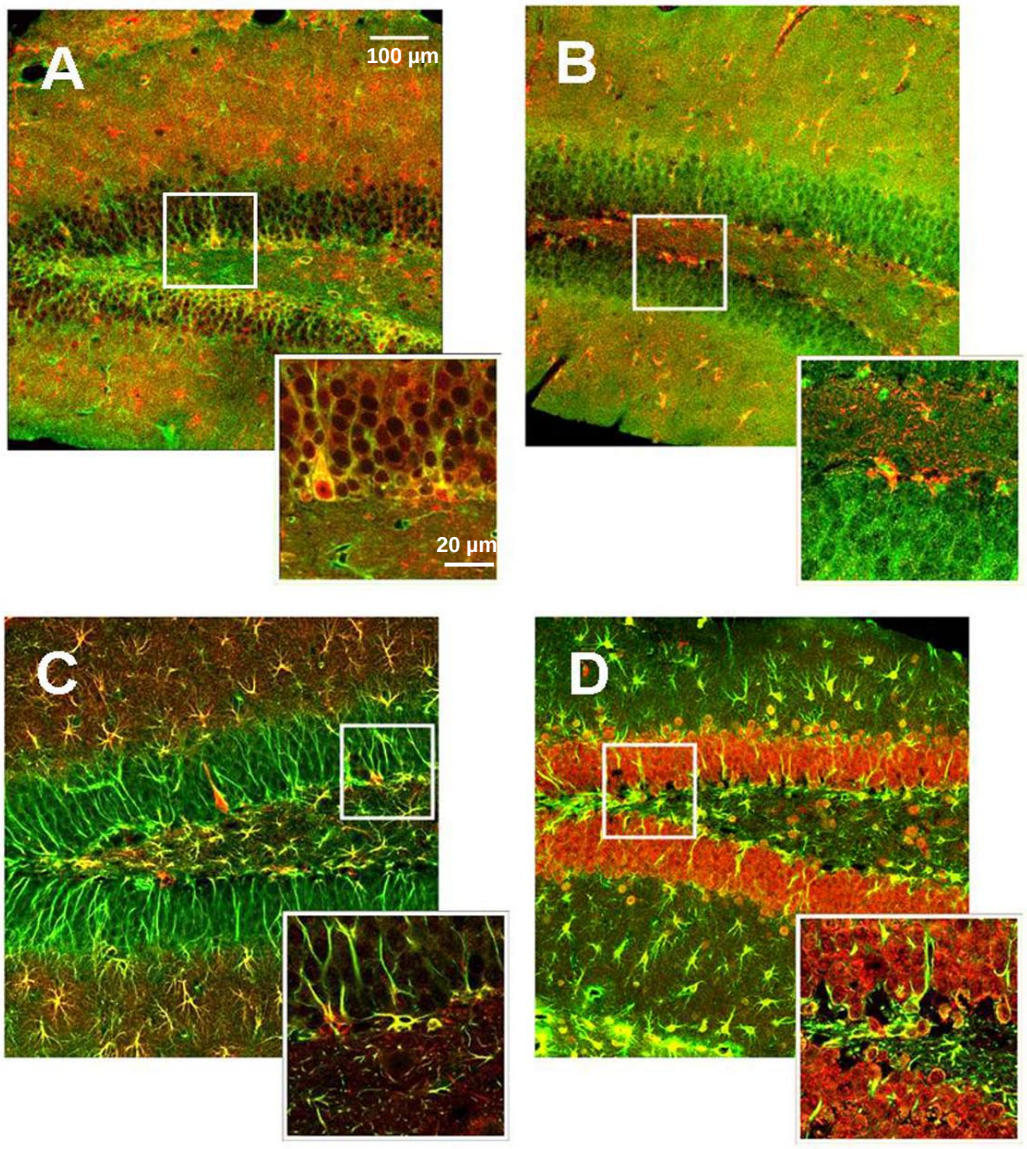


FIGURE 3-4 Expression of Shh and its receptor in the DG neurogenic niche. Confocal microscopy shows that Shh-N immunopositive fibres (*red*) (A-C) were associated with cells expressing markers *(green)* for neural progenitor cells: (A) Nestin (B) Sox2 and (C) GFAP. There is strong presence of receptor Ptc1 (red), throughout the entire granule cell layer (D). GFAP-positive fibres surround some of the Ptc1-labelled soma in the SGZ (*green*).

3.4 DISCUSSION

The transcripts of Shh and components of its signal transduction pathway (i.e. transmembrane receptors Ptc1 and Smo, and the Gli-family of transcription activators and repressors Gli1, Gli2 and Gli3) were all present in the MSDB and hippocampus of the adult brain. The findings agree with previously reported observations using in situ hybridization assays (Traiffort, 1998, 1999) except that the transcript of Shh in the hippocampus was picked up. It was initially thought the result was a false positive, due to the extreme sensitivity of PCR. However, the detection of a ~45 kDa Shh precursor protein in the hippocampus corroborated the presence of Shh-biosynthesizing cells. The lack of post-translational processing could have confined the precursor protein within the secretory pathway of the producing cells within the hippocampus. Furthermore, Ptc1, Smo and Gli1 proteins were detected, verifying that Shh-producing and receiving cells were present in both regions of the brain.

The initial attempts at detecting the active amino-terminal peptide Shh-N in immunoblots were not successful despite rotating among various commercial brands of Shh-N antibodies. This suggests that Shh-N is present in low quantities. A positive result was observed only after prior immunoprecipitation, which enriched the Shh fractions. The low concentrations of Shh-N terminal peptide may be in part due to the potency of the active fragment. It has been

reported that dual lipid modification increases potency over the unprocessed Shh by 30-fold (Pepinsky et al., 1998).

All in all, the mRNA and protein profiles of Shh and constituents of its signalling pathway indicate that Shh signalling is active in the MSDB and hippocampus. Probing the identities of Shh-positive cells may offer clues about accumulation, translocation and activity-dependent regulation of their contents. Hence, the morphology and localization of Shh-expressing cells in the foregoing regions was next investigated. Punctate staining of Shh-N immunoreactive cell bodies was observed in the MSDB. They colocalized, to a certain extent, with two well-characterized cell populations in the MSDB, namely the acetylcholine-synthesizing and GABA-synthesizing cells. Given that a large population of cholinergic and GABAergic cells from the MSDB basal forebrain projects to other regions of the brain such as the hypothalamus, brainstem and hippocampus (Gritti et al., 1994; Gritti et al., 1997; Kohler et al., 1984), it is tempting to speculate on whether the Shh-N peptide from Shh-synthesizing neurones could be co-transported with the neurotransmitters. Alternatively, Shh-releasing cells may exert their effect on neighbouring cells via paracrine signalling. Additional studies are necessary to ascertain their identities as projection neurones and/or local interneurones within the septal circuit.

The immunoreactivity profile of Shh-N in the DG vastly differed from the MSDB. The punctated Shh-N immunoreactivity in the hilus suggests presence of axonal

terminals, axonal boutons or cross-sections of dendrites. The presumptive Shh-N positive fibres along the SGZ could indicate innervation from neurones found in distal regions. Furthermore, subpopulations of individual Shh-N positive cells in the infragranular granule cell layer were identified. Their localization affirms our earlier observations of existence of Shh-producing cells within the hippocampus.

Interesting results were obtained with colchicine injections. The drug has been used widely in many immunohistochemical studies (Dube and Pelletier, 1979). It impedes axoplasmic and dendritic transport by binding to tubulin subunits, hence disrupting the polymerization of microtubule tracts. Colchicine enhanced the levels of GAD and Shh-N expression in the MSDB, but obliterated both GAD and Shh-N immunoreactivity in the DG. The accumulation of Shh-N peptides in the soma of septal Shh-producing cells suggests that the signal peptide may be conveyed by axons to exert is effects elsewhere. The reduction in GAD fibre staining in the DG was expected with the impairment of axonal transport, as a large part of hippocampal GABAergic efferents originates from the septum of basal forebrain (Freund and Antal, 1988; Gulyas et al., 1990). Hence, the elimination of presumptive Shh-N axon terminals in the DG suggests that the source of Shh too may be extrahippocampal.

The population of Shh-N immunopositive cell bodies and nerve endings were in apposition with key markers of neural stem cells in the DG neurogenic niche. The localization profile adds to the growing body of evidence that Shh is involved in

the regulation of adult hippocampal progenitors (Ahn and Joyner, 2005; Lai et al., 2003; Machold et al., 2003). Ptc1, the receptor of Shh, could be found extensively throughout the granule cell layer and in the hilus. The morphology and localization of these cells indicate that these Shh-responsive cells could be neural precursor cells in the SGZ and granule cells (which may include newly matured cells). In development, Shh-N patterns the ventral tube by directing different cell fates at different concentration thresholds (Ericson et al., 1996; Ericson et al., 1995). Hence, Shh-N may be biosynthesized within the DG to act on these nearby cells in a gradient dependent manner. However, it is observed that the numbers of Shh-N positive cells compared to the Ptc1-positve cells in the DG neurogenic niche were few and far between. Going by the sheer numbers of dendrites and axonal terminals in the DG, it is more likely that Shh-N is transported via efferents to mediate its effects on recipient stem cells in the DG.

To summarize the findings in this study, (i) Shh activity is indicated in the MSDB and hippocampus; (ii) A large population of Shh-N synthesizing neurones exist in the MSDB; and (iii) presumptive Shh-N positive nerve endings, together with a novel subpopulation of Shh positive cells are present in the DG, and are closely associated with stem cell markers within the neurogenic niche.

4. ANTEROGRADE TRANSPORT OF SHH IN THE SEPTOHIPPOCAMPAL SYSTEM

4.1 INTRODUCTION

The decision of a stem cell to remain in the quiescent state or self-renew, to proliferate and/or differentiate into a specific lineage is influenced by extracellular cues within its environment. In the embryonic brain, Shh behaves as a “cell-fate switch”, where the cell state changes at critical threshold levels (Ericson et al., 1995). In the postnatal and adult brain, Shh functions to expand the pool of progenitor cells in neurogenic niches (Ahn and Joyner, 2005; Lai et al., 2003; Machold et al., 2003; Palma et al., 2005). It is very much evident that the dynamics of the Shh signals on stem cell function, not unlike other neurogenic factors, hinges on specific time-points and locations. As such, it is worthwhile probing further into the source of Shh signals in the adult DG.

Clues to its mode of delivery can be gleaned from examples of other trophic factors. Wnts are another major family of developmental secreted proteins that share features common to Shh signalling, among them the coupling of lipoprotein receptors and GPCRs in signal transduction. The Wnt3 protein is secreted by the local hippocampal astrocytic population within the DG to act on the expansion of the pool of Type III neuroneal restricted precursors and promote their differentiation into neurones (Lie et al., 2005). On the other hand, the source of trophic factors may be extrahippocampal. The dentate gyrus is the major target

of afferents originating from the septum, supramamillary nuclei, median raphe and the locus ceruleus (reviewed in Leranth and Hajszan, 2007). Neurotransmitters such as GABA, acetylcholine and glutamate have been implicated in the cell differentiation and survival of neural stem cells (Cooper-Kuhn et al., 2004; Kaneko et al., 2006; Kotani et al., 2006; Mohapel et al., 2005; Tashiro et al., 2006; Teitelbaum et al., 1975; Tozuka et al., 2005; Van der Borght et al., 2005), and their delivery to DG is likely to be via extrinsic efferents. This suggests the question: is Shh ferried from another region or is it secreted by local cells within the DG in a paracrine/autocrine manner?

Here, it is postulated that Shh is transported from the MSDB of the basal forebrain to the hippocampus, culminating in the upsurge in progenitor cell proliferation. The hypothesis is based on several pieces of evidence. Firstly, the active form Shh is able to undergo transport in an anterograde manner, as substantiated using pulse-chase autoradiography to show rapid delivery of radiolabelled Shh-N peptides from the adult hamster retina to the superior colliculus by the optic nerve (Traiffort et al., 2001). Secondly, investigations entailing the anterograde transport of horseradish peroxidise (HRP) reveal that the hilus of the dentate gyrus is densely innervated by fibres from the medial septum, particularly at the subgranular zone (Chandler and Crutcher, 1983; Crutcher et al., 1981). Additional electron microscopy work demonstrates that HRP-labelled axons terminate primarily in the hilus, with the remaining ramifying into the granule cell layer and molecular layer. Contacts formed were either

axosomatic or axodendritic (Chandler and Crutcher, 1983). The ultrastructural analysis goes to show that the septohippocampal pathway is capable of dispatching small molecules right to the heart of the germinal centre.

Over and above, the transection of fimbria/fornix, the connection between the septum and hippocampus, drastically reduced adult DG progenitor cells (Lai et al., 2003). Further pharmacological intervention of Shh-N signalling in the hippocampus did not further reduce the number of progenitors (Lai et al., 2003), hinting that Shh-N may be transported from the MSDB to regulate the population of neural stem cells in the DG. Last but not least, and relating back to findings from the preceding chapter, Shh transcripts and precursor proteins are found in the MSDB. Immunohistochemical analyses show intense Shh-immunoreactivity in a large population of cell somata in the MSDB whereas Shh-N positive fibres and synaptic terminals abound in the SGZ and the hilus.

The abovementioned body of evidence collectively implies that MSDB is the site of Shh-producing cells, and the DG recipient of Shh-N peptides from the septohippocampal system. Hence, this second series of the investigations pertaining to Shh will explore the possibility of Shh-N being conveyed from the septum to the DG and address the identities of septohippocampal neurones responsible for transmitting the Shh-N signal.

4.2 MATERIALS AND METHODS

4.2.1 Colchicine treatment and immunohistochemistry

Colchicine was injected into the lateral ventricles of the adult female Swiss albino mice, using the protocol described in Chapter 3. The goat ABC staining kit (Santa Cruz) was used for staining. The relevant sections were pretreated with 0.3% hydrogen peroxidase to quench endogenous peroxidase and blocked for an hour 10% serum prior to incubation with Shh-N primary antibody. The sections were then incubated with biotinylated secondary antibody and the avidin-biotin enzyme before application of the chromogen diaminobenzadine-nickel with hydrogen peroxidase (Vector Laboratories). The slides were rinsed in tap water, dehydrated with 2 times each of 95% ethanol, 100% ethanol and xylene, before mounting. Sections were imaged using a light microscope (BX50, Olympus).

4.2.2 Retrograde tracing

Four adult male Sprague-Dawley rats (280-300 g) were used for immunohistochemical analysis. The rats first underwent surgery for retrograde tracer injections. They were deeply anaesthesized with ketamine xylazine (0.2 ml/100g, Ketamine 75 mg/kg + Xylazine 10 mg/kg), and mounted on a stereotaxic frame (Stoelting Co, Woodale, Illinois). Rectal temperature was automatically maintained at 37.0± 0.5ºC. A flatskull position was achieved by adjusting the nosebar according to bregma and lamdha coordinates. A midline incision was made of the scalp, the periosteum scraped and a hole drilled at the

dentate gyrus of the rats at each of the four coordinates based on the Rat Brain Atlas (Paxinos, 2001b), given below in Table 4-1.

Injection site	AP	ML	DV
1	-3.0	1.5	-3.2
2	-3.0	-1.5	-3.2
3	-4.2	2.2	-3.2
4	-4.2	-2.2	-3.2

TABLE 4 Stereotaxic coordinates of FG injection sites

A 1 µl Hamilton syringe was used to inject 0.1 µl of 4% Fluorogold (FG) (Fluorochrome, LLC, Denver, Colorado) into each drilled hole over 5 min. The syringe was left in place for 2 min, before retracting for 0.1 mm and then leaving in place for a further 15 min. Animals were kept warm during recovery from anaesthesia. The rats were deeply anaethesized 10 to 14 days later and transcardially perfused, the brains removed and postfixed overnight. The brains were cut rostral-caudally from the basal forebrain through the hippocampus at 50 µm thickness using a vibrating microtome, and stored in PBS at 4ºC. Hippocampal sections were counterstained with a neuroneal cell body marker (NeuroTrace® 555/525, Molecular Probes) to determine the site of FG injections. Only septal sections from brains with the correct FG injection site were chosen for immunohistochemical analysis.

4.2.3 Immunohistochemistry

The sections were treated with 0.01M sodium citrate buffer (pH 8.5) for antigen retrieval before blocking with 10% donkey serum. Incubation with goat polyclonal anti-goatShh N (1:100, Santa Cruz Biotechnology, CA) was carried out for 3 days at 4ºC, followed by secondary antibody incubation (488 Alexa-Fluor Donkey anti-goat, 1:400, Molecular Probes, Eugene, OR). To examine colocalization of Shh with cholinergic and GABAergic cell bodies, triple labelling was carried out with rabbit polyclonal anti-ChAT (AB143, 1:500) and mouse monoclonal anti-GAD-67 antibodies (MAB5406, 1:400) (both from Chemicon, Temecula, CA) and the respective secondary antibodies 647 donkey anti-rabbit IgG and 633 goat anti-mouse IgG2a)(1:400, Molecular Probes) to further characterize the neurones.

For immunohistochemistry of glutamatergic neurones, vesicular transporter 1 and vesicular transporter 2 (VGLUT1 and VGLUT2) antibodies were used (1:2000, Chemicon) and probed with Cy2- goat anti-guinea pig IgG (Jackson Immunoresearch, West Grove, PA).

4.2.4 Microscopy and cell counting

The sections were examined in the confocal microscope using argon and HeNe lasers and a 40x objective lens. Excitation for FG is 350-395 nm, and emission is 530-600 nm (361/536). Optical slices were taken at 1 µm intervals through the thickness (z-dimension) of the tissue section.

Systematic random sampling was applied, with one-in-six sections chosen from the MSDB (that corresponds to 1.00 mm to -0.10 mm relative to the bregma in the Paxinos and Watson atlas giving an interval range of 200 μm). To estimate the number of labelled neuroneal cell bodies in the different components of the MSDB, namely the medial septum (MS), vertical limb of diagonal band of Broca (VDB) and both the left and right sides of the horizontal limb of diagonal band of Broca (HDB), the following criteria were devised. Both Shh- and GAD-67-immunoreactive cells were defined by their punctated cytoplasmic staining in which the nuclei of the cells were clearly unlabeled by the dyes. ChAT- and FG-immunoreactive cells were identified by their strong cytoplasmic staining. Any cells that were partially situated at the borders of the confocal image frame were not considered. Guard zones of 5 μm were set for the top and bottom of the confocal images to give an actual dissector height of 30 μm. A counting frame of 230.3 μm by 230.3 μm was adopted. Colocalization of cells was determined by a strong degree of overlap between different fluorophore signals in a reconstructed orthogonal image of at least 35 optical slices. The number of immunoreactive cells in each counting frame was divided by its thickness to give a value based on its volume. The numbers within each subregion were then summed up and their percentages calculated.

4.3 RESULTS

4.3.1 Disrupting axonal transport results in Shh-N accumulation in cell bodies in MSDB and abolishes Shh fibre staining in the DG

To investigate whether the existence of Shh-N in the hippocampus originates mostly from an extrinsic source, mice were pre-treated with colchicine prior to immunohistochemistry. The inhibition of axoplasmic transport led to an increase in perikarya staining for Shh-N in the MSDB concomitantly with a decrease in staining in the DG. The use of a peptide block abolished all positive immunoreactivity, indicating the specificity of the immunostains (FIGURE 4-1).

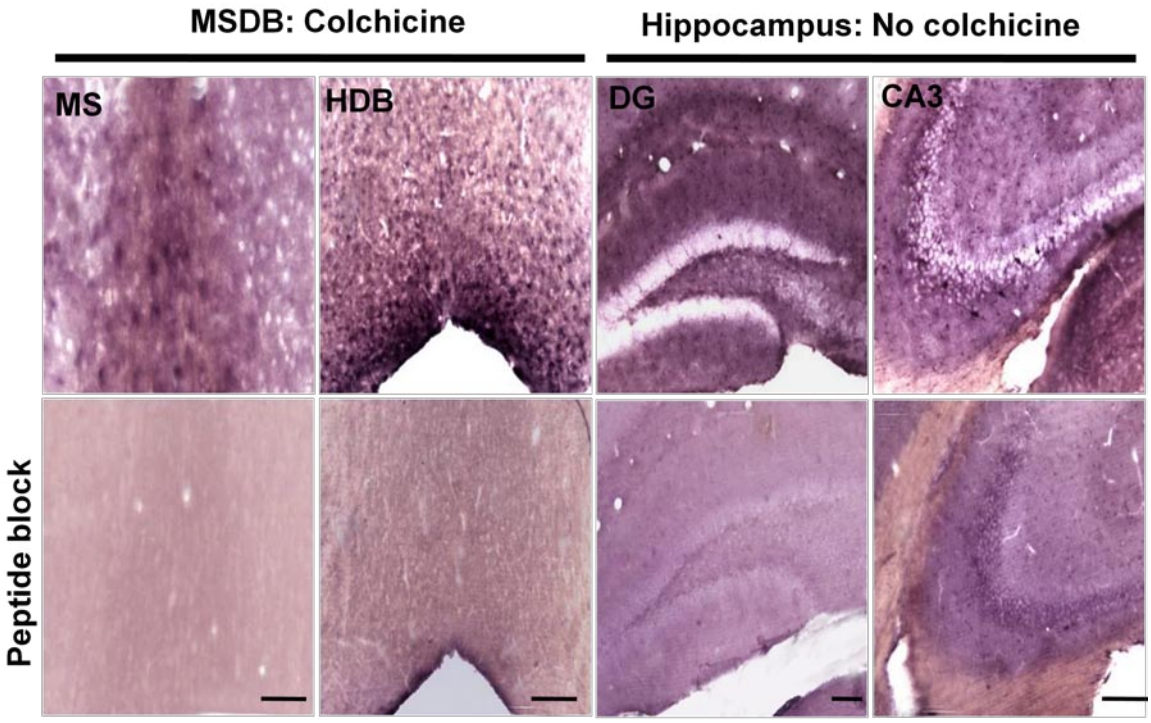


FIGURE 4-1 Effects of colchicine treatment in MSDB and hippocampus Shh-N immunoreactive cells bodies in MSDB and Shh-N positive fibres in hippocampus. Pre-incubation of the primary antibody with peptide block eliminated positive staining. Scale bar: 50 μm

4.3.2 Shh may be transported from the MSDB to the DG

To determine whether Shh positive efferents to the DG are from the MSDB, retrograde labelling was employed. The tracer FG was injected into the rat DG. The specificity of the site of injection was confirmed by counter-labelling with a fluorescent Nissl stain, Neurotrace, before further immunohistochemical analyses on the septal tissues were performed (FIGURE 4-2i). FG immunopositive cell bodies in the MS and vertical diagonal band VDB were distributed mostly along the midline, with a more scattered distribution in the HDB. These cells were of an assortment of shapes (oval, fusiform, polygonal) and sizes, ranging from small to large (~10 μm – 30 μm). Some of the FG-immunoreactive neurones had characteristic puncta in the centre of their cell bodies. Shh-N cells, characterized by their punctated cytoplasmic staining, were found in abundance in the MSDB, usually in clusters markedly along or parallel to the midplane of the MS. They were also varied in size (~10 – 35 μm) and were oval or bipolar.

There are 2 isoforms of GAD in the brain, namely GAD-65 and GAD-67, the latter being significantly higher in the MSDB, and preferentially expressed by the septohippocampal neurones (Castaneda et al., 2005). In the previous experiment, a GAD-65/67 antibody was used for probing GABAergic cells. However, due to the requirement for colchicine pre-treatment that will interfere with retrograde labelling, an anti-GAD-67 antibody was chosen for this set of experiments. The population of GAD- and ChAT-positive cells in the MSDB were of various shapes and sizes. The septohippocampal cholinergic and GABAergic

cell bodies colocalized with Shh-immunoreactive perikarya to a certain extent. The smaller FG/Shh/GAD-67 immunoreactive cells (~10 – 20 μm in diameter) were usually round or oval while the larger cells (~30 μm) were mostly fusiform or multipolar.

A conservative approach was taken for the counting of immunopositive cells. Weakly immunoreactive cell bodies that may be artefacts of non-specific staining were not considered. Hence, the numbers presented in this study should be regarded as minimal percentages of colocalization (FIGURE 4-2ii). About one third of FG-positive cells strongly co-labelled with Shh-N immunopositive cell bodies. About 10% of the FG- and Shh- positive cell bodies also colocalized with ChAT-immunopositive cells bodies in the MS, and about twice the amount in the DB. A larger percentage (30%-50%) of FG and Shh-positive cell bodies colocalize with GAD. As a cautionary note, the data here should be regarded as semi-quantitative as the judging of the signal threshold for cell immunopositivity is subjective.

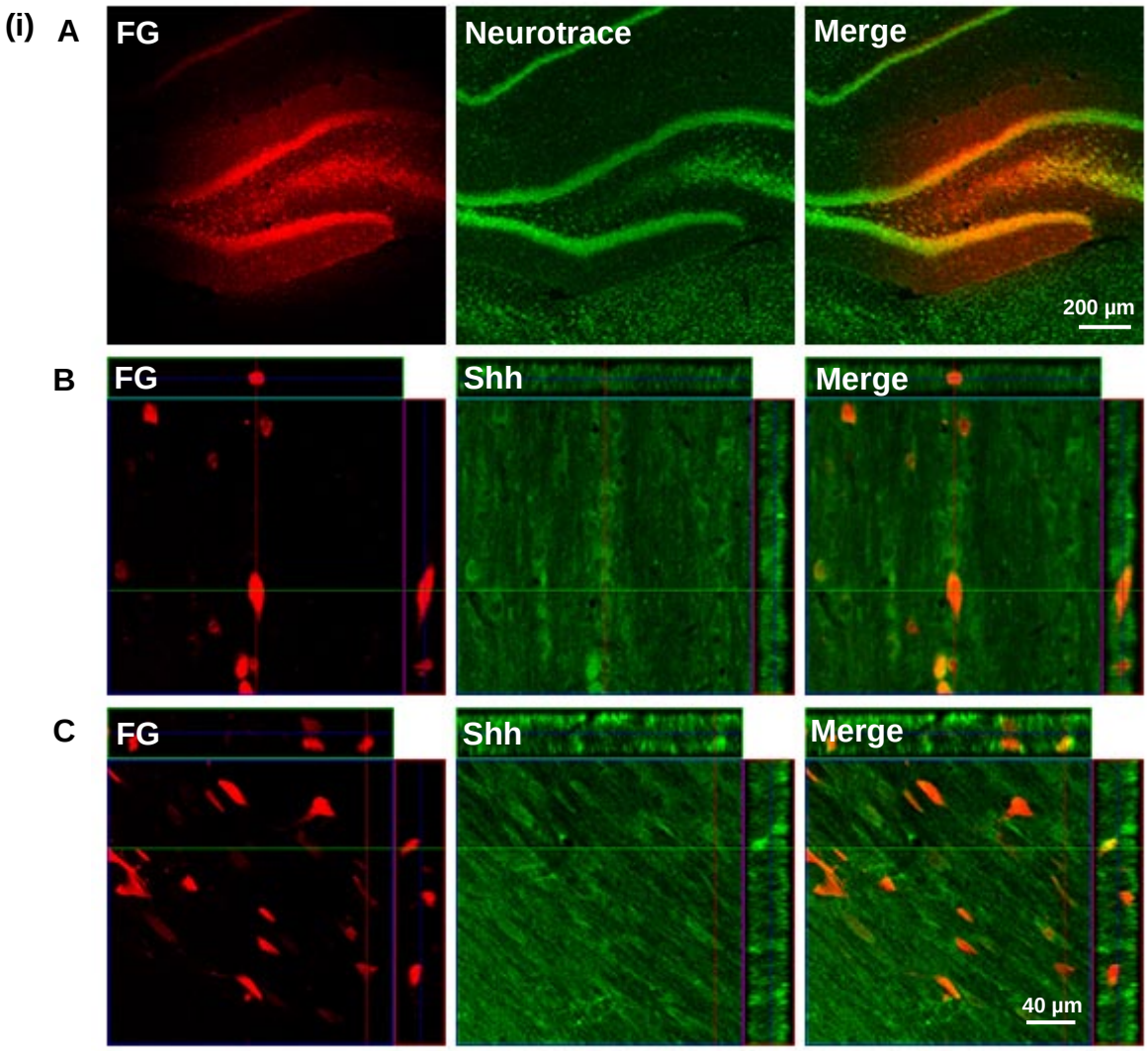


FIGURE 4-2 Retrograde labelling of septohippocampal pathway and co-labelling with Shh in MSDB (i) (A) Retrograde tracer FG was stereotaxically injected into the rat DG, giving a characteristic halo. Confocal z-stacked images of (B) MS and (C) HDB shows some colocalization of FG- and Shh-immunoreactive cell bodies

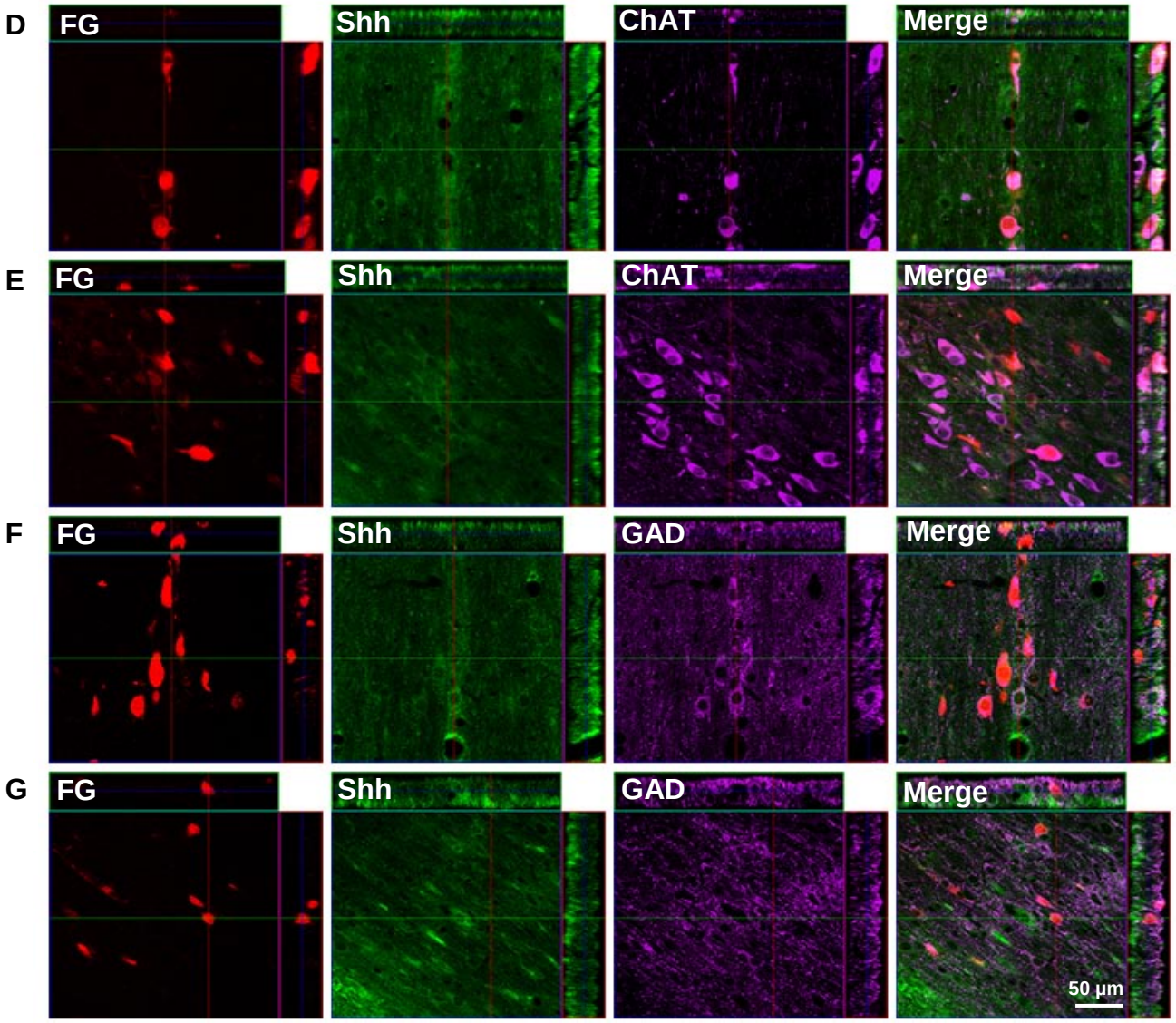
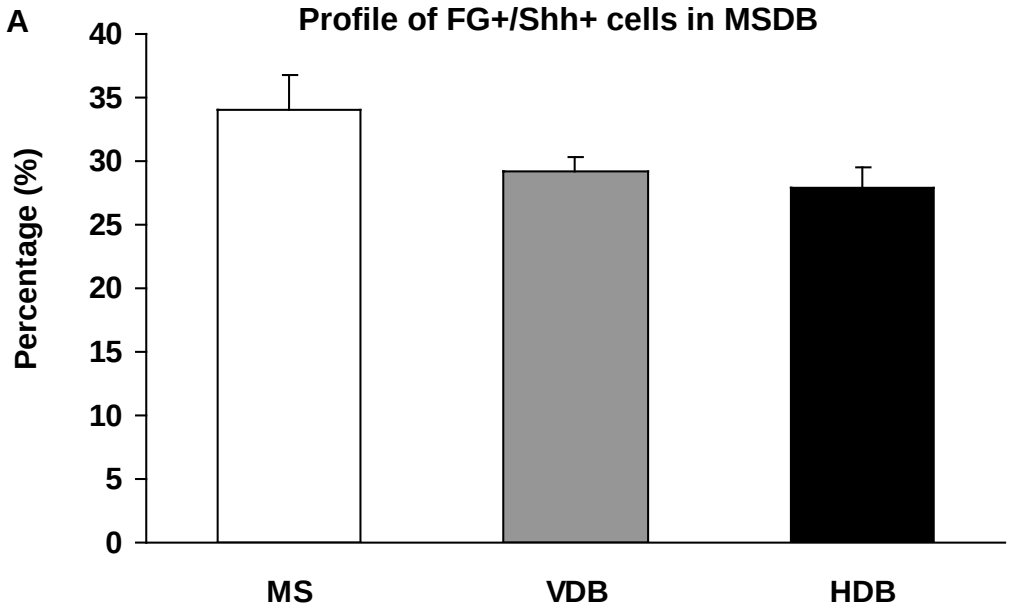
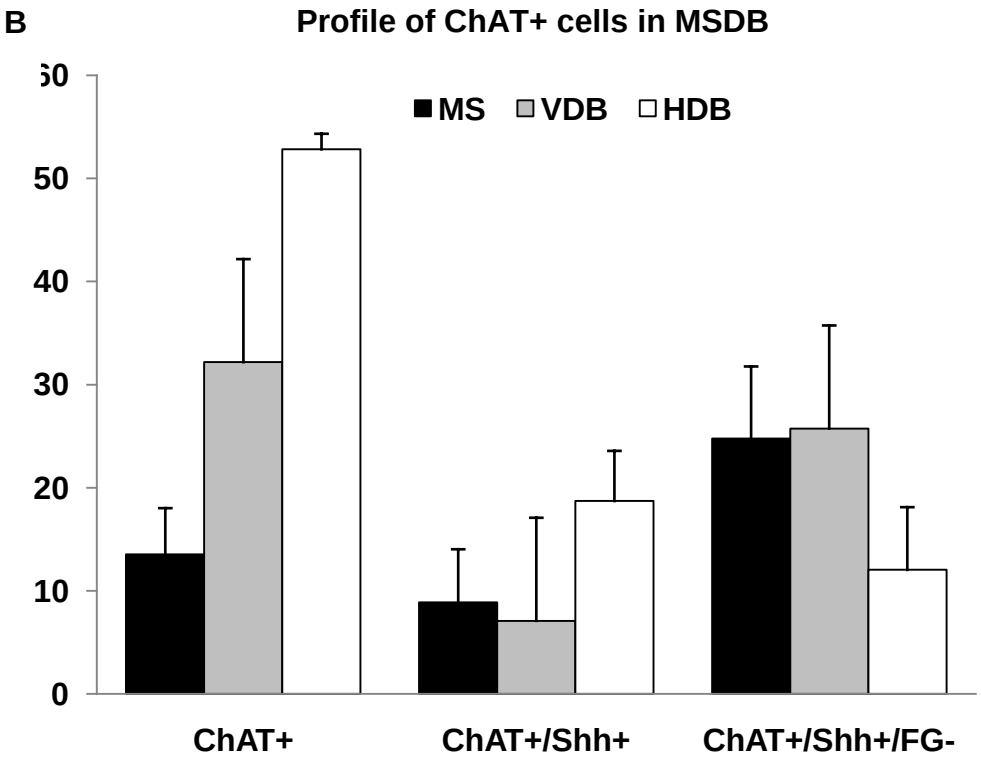


FIGURE 4-2
Retrograde labelling of septohippocampal pathway and co-labelling with Shh in MSDB
(i) Confocal z-stacked images of colocalization of FG- and Shh-immunoreactive cell bodies in (D) MS and (E) HDB. Colocalization of FG-, Shh- and GAD- immunoreactive cell bodies could also be observed in (F) MS and (G) HDB.

(ii)


A
Profile of FG+/Shh+ cells in MSDB
Percentage (%)
40
35
30
25
20
15
10
5
0
MS
VDB
HDB


B
Profile of ChAT+ cells in MSDB
MS
VDB
HDB
50
40
30
20
10
0
ChAT+
ChAT+/Shh+
ChAT+/Shh+/FG-

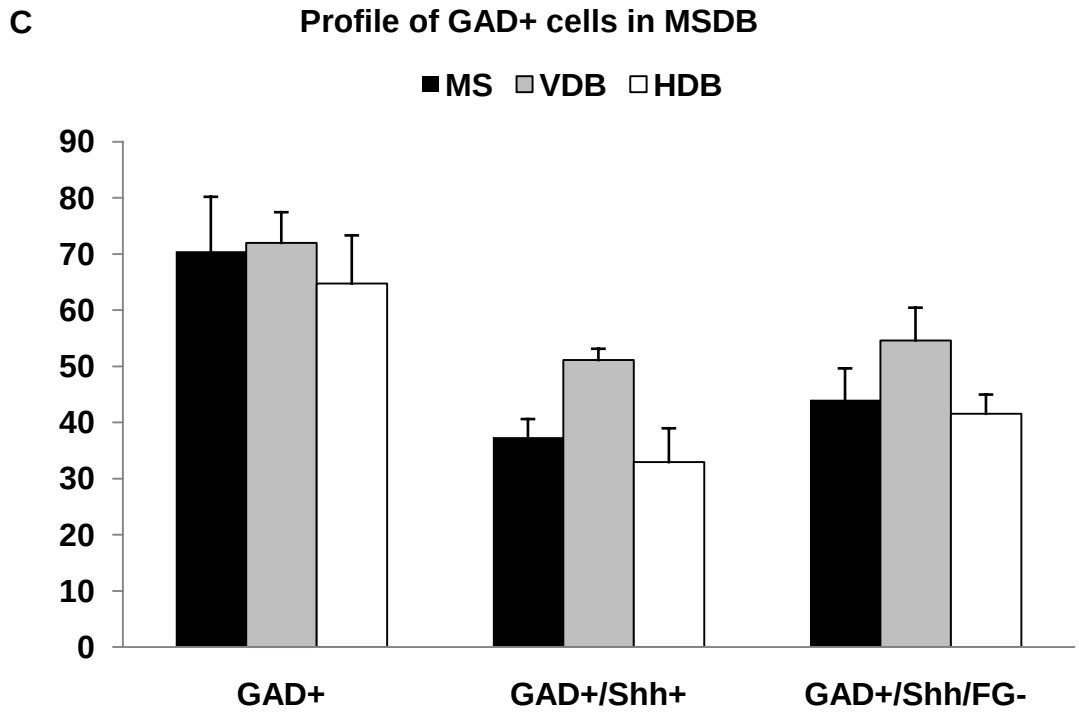


FIGURE 4-2 Retrograde labelling of septohippocampal pathway and co-labelling with Shh in MSDB (ii) Cell profiles of Shh-containing septohippocampal neurones (A) percentage of FG-immunoreactivecells that are Shh-positive (B) bar charts represent, from left to right, percentage of FG-immunoreactive cells that are either ChAT-positive (ChAT+); percentage of FG-immunoreactive cells that are both ChAT- and Shh-positive (ChAT+/Shh+); percentage of non-septohippocampal cells that are ChAT- and Shh-positive (ChAT+/Shh+/FG-) (C) bar charts represent, from left to right, percentage of FG-immunoreactive cells that are either GAD-positive (GAD+); percentage of FG-immunoreactive cells that are both GAD- and Shh-positive (GAD+/Shh+); percentage of non-septohippocampal cells that GAD- and Shh-positive (GAD+/Shh+/FG-).

4.3.3 A subpopulation of Shh-immunoreactive cells in the MSDB are neither cholinergic nor GABAergic

From the previous observations, a fraction of Shh-containing FG cells were neither immunoreactive for ChAT nor GAD67. To ascertain if the population of Shh immunopositive cells in the MSDB were exclusively GABAergic or cholinergic, triple immunohistochemical labelling of the MSDB involving ChAT, Parv and Shh antibodies was performed. Parv, used earlier in Chapter 2, is a specific marker for GABAergic neurones in the MS (Kiss et al., 1990). The GAD antibody could not be used together with the ChAT and Shh-N antibodies due to potential cross-reactivity. The findings indicate a subpopulation of Shh-positive cells were neither cholinergic nor GABAergic (FIGURE 4-3i). This group of Shh-N immunopositive, and ChAT and GAD immunonegative neurones may be either projecting neurones or local circuit neurones within the basal forebrain.

To address the identity of the unknown Shh-positive neurones, glutamatergic neuroneal markers were used, namely vesicular glutamate transporter 1 (VGLUT1) and vesicular glutamate transporter 2 (VGLUT2). The two classes of VGLUTs existed in a complementary fashion in the DG: VGLUT1 was localized to the polymorphic region and molecular layer of the DG, and VGLUT 2 to the supragranular granule cell layer, with decreasing density from the molecular layer towards the hilus. Both VGLUT1 and VGLUT2 immunostaining in the MSDB were barely discernable, especially for VGLUT1. This could be due to the diffuse nature of the vesicles. The outcome was reversed when the animals receive

intracerebroventricular injections of colchicine, which impeded the trafficking of vesicles out from the axon terminals. Intense punctatelly labelled VGLUT1- and VGLUT2- positive cell somata were observed in the longitudinal axis of the mid-plane of the MS, and parallel to the slanted plane of the HDB. The cell bodies were mostly small to medium in size, and were assorted in shapes. When Shh-N was introduced as a dual immunohistochemical marker, it was observed that a few of the Shh-N positive cell bodies colocalize with VGLUT1, and all of them colocalize with VGLUT2 (FIGURE 4-2ii).

Co-immunostaining of VGLUT2 with FG and Shh-N was employed next. Pre-treatment with colchicine was omitted as it would impair retrograde transport of FG. The weakly-immunopositive VGLUT2 cell soma in the MSDB colocalized with FG and Shh-N to a large extent. Quantitation of these colocalized cell bodies was not carried out though, due to the faint immunostaining of VGLUT2 (FIGURE 4-3iii).

To determine if VGLUT-positive cell bodies in the MSDB contained other neurotransmitters, triple labelling of VGLUT-containing neurones with ChAT and Parv in colchicine- treated animals was also carried out. High-power magnified images show that Parv-immunoreactive cells have likely VGLUT1 varicosities on them, possibly indicative of axosomatic contacts. Neither VGLUT1 nor Parv immunostaining overlapped with that of ChAT. Reconstructed z-stack images of

a triple-labelled single cell revealed likely axosomatic associations of VGLUT2 varicosities on a Parv- and ChAT-positive cell (FIGURE 4-2iv).

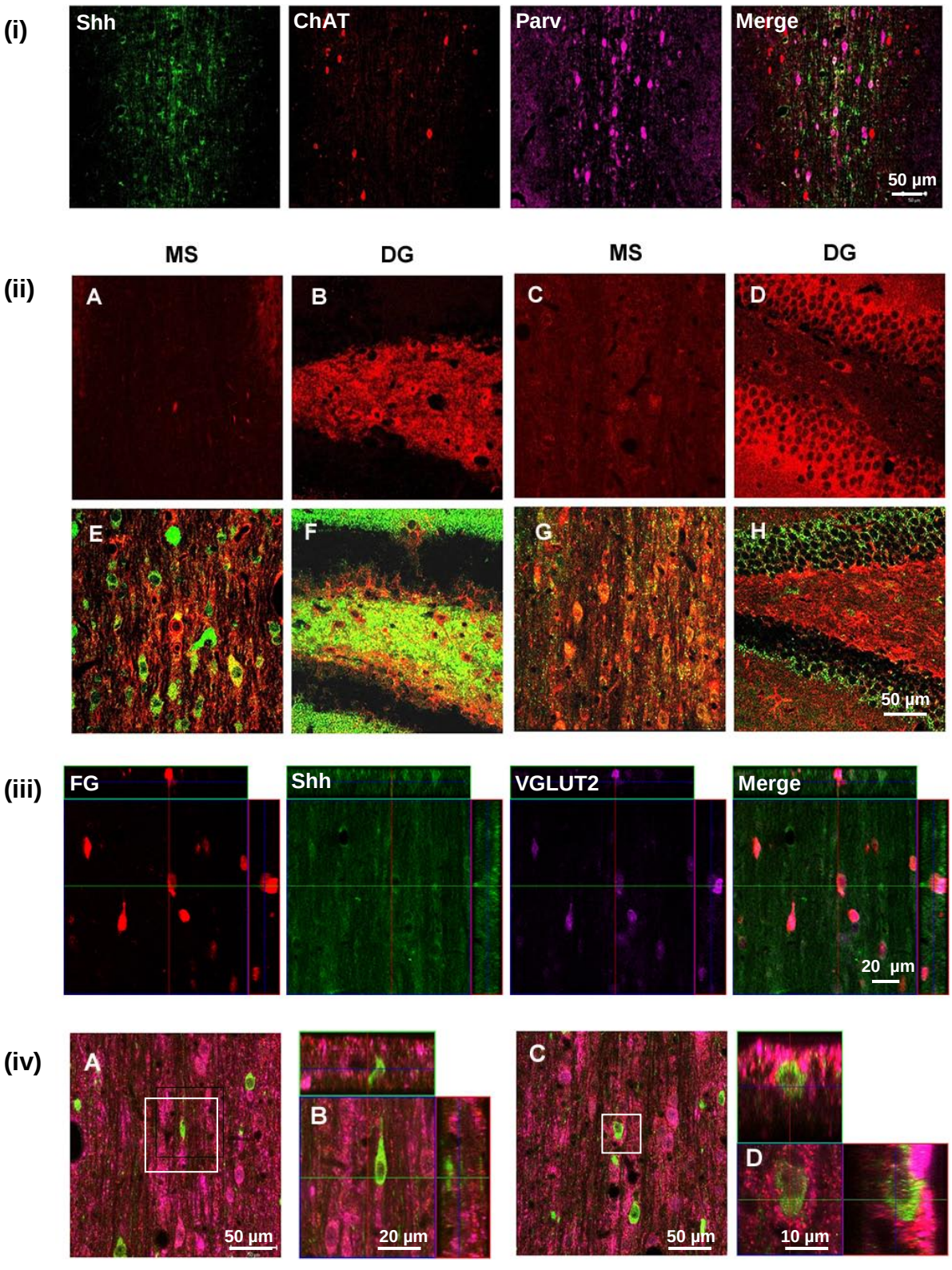

(i)
Shh
ChAT
Parv
Merge
50 µm
(ii)
MS
DG
MS
DG
A
B
C
D
E
F
G
H
50 µm
(iii)
FG
Shh
VGLUT2
Merge
20 µm
(iv)
A
B
C
D
50 µm
20 µm
50 µm
10 µm

FIGURE 4-3 Immunohistochemistry of VGLUT1 and VGLUT2 in septohippocampal pathway (i) Confocal images of clusters of neurones in the MSDB subjected to triple immunohistochemistry show that not all Shh-positive cells are ChAT- or Parv-positive. (ii) Neither (A) VGLUT1 nor (C) VGLUT2 cell soma staining is visible without colchicine, although (B) VGLUT1 staining can be observed in the dentate hilus and (D) VGLUT2 in the granule cell layer. Shh-immunoreactive cells (red) colocalize with (E) a few VGLUT1 and (G) VGLUT2 positive cells in colchicine-treated animals (green). Shh-N positive fibres are also in close proximity to (F) VGLUT1 and (H) VGLUT2- immunoreactive varicosities. (iii) Confocal stacked images show some colocalization of FG with Shh and VGLUT2 cell bodies. (iv) Triple labelling of (A) VGLUT1 and (C) VGLUT2 *(red)* with Parv *(Magenta)* and ChAT *(green)* (B) and (D) shows zoomed in orthogonal images of boxed areas. (B) VGLUT1 immunoreactive varicosities on Parv-positive cells. (D) A ChAT- and Parv- positive cell located along the MS midline is surrounded by VGLUT2 immunopositive varicosities.

4.4 DISCUSSION

The MSDB is anatomically interconnected with the hippocampus, and functionally synchronizes its electrical activity (Bland and Bland, 1986; Freund and Antal, 1988; Lee et al., 1994b; Yoder and Pang, 2005). Within the DG entity, the septal cholinergic efferents synapse onto both principal cells (granule cells) and non-principal cells (interneurones), whereas the septal GABAergic neurones project exclusively onto interneurones in the granular cell layer and the dentate hilus (Freund and Antal, 1988; Freund and Buzsaki, 1996; Frotscher and Leranth, 1985). Apart from the delivery of conventional neurotransmitters, the key findings in this study demonstrated that septohippocampal neurones are capable of transmitting Shh-N peptides to the DG.

The disruption of axoplasmic transport greatly elevated Shh-N protein staining in the cell bodies found in the MSDB, but depleted Shh-N staining within the DG. Subsequent retrograde tracing experiments verified that Shh-N is dispatched by septohippocampal neurones. About a third of the MSDB projections to the DG contain Shh-N. Within the subregions MS, VDB and HDB, the Shh-N septohippocampal neurones are significantly GABAergic. The slightly higher percentage of Shh-N septocholinergic neurones in the HDB is on par with observations that the number of cholinergic neurones in the particular subregion is twice as much as the GABAergic neurones (Brashear et al., 1986). The high population of Shh-expressing GABAergic cell bodies in the MSDB corroborated

previous double in situ hybridization observations of Shh and GAD riboprobes in the basal forebrain (Traiffort et al., 2001).

The existence of noncholinergic, non-GABAergic septal neurones has recently been reported (Sotty et al., 2003). These cluster-firing neurones expressed transcripts solely for either VGLUT1 or VGLUT2. These classes of vesicular glutamate transporters, recently discovered, are definitive markers of glutamate neurones (Bellocchio et al., 2000; Helmuth, 2000; Rothstein, 2000; Takamori et al., 2000). The next part of the experiments was aimed at addressing if the Shh-N secreting septohippocampal cells were glutamatergic cells.

Within the DG itself, there was a high density of VGLUT1 immunostaining in the dentate hilus, and VGLUT2 staining in the supragranular layer, in agreement with previous studies (Kaneko and Fujiyama, 2002; Kaneko et al., 2002). In the MSDB, owing to their diffuse nature, VGLUT immunoreactive cell bodies were detectable only with axoplasmic disruption. Inhibition of axonal transport revealed colocalization of a few VGLUT1 positive cell bodies with Shh-N producing cells. Remarkably, there was extensive co-labelling of Shh-N with VGLUT2 in the septal cell bodies. These septal Shh-N glutamatergic cells may be either local circuit neurones (Hajszan et al., 2004; Halasy et al., 2004) or septohippocampal projecting neurones (Colom et al., 2005). The retrograde labelling experiments showed that these novel Shh-N glutamatergic neurones projected to the DG, although the quantity could not be strictly ascertained. It was possible that the

septohippocampal neurones could co-transmit both glutamate and GABA, as higher powered micrographs showed presumptive VGLUT1- and VGLUT2-immunopositive varicosities enveloping Parv-containing cells, suggesting possible glutamatergic innervation onto Shh-containing GABAergic cells. Further electron microscopic work should be employed in order to establish the exact identities of the axosomatic contacts.

All in all, the findings here demonstrated that the Shh-N peptides are being anterogradely transported by septal neurones that mainly use GABA as a neurotransmitter, and in some cases, acetycholine and/or glutamate. The observation that septohippocampal neurones can deliver regulatory factors other than conventional neurotransmitters is not an isolated one. A previous study demonstrating the conveyance of hormones by septocholinergic neurones is another case in point. Infusion of oestrogen into the MSDB of oviarectomized rats increased synaptic spine density of the pyramidal cells in the CA1 region, whereas rats treated with 192IgG SAP did not demonstrate any change in spine morphology (Lam and Leranth, 2003).

The mechanisms by which Shh-N exerts its proliferative effect in the hippocampus can hence be drawn, by reconciling the data here with preceding chapters and other studies. Shh-N is synthesized in the MSDB of the basal forebrain, and is subsequently released into the DG predominantly by septohippocampal GABAergic projections. The ligand binds to Ptc1 receptors of

recipient neural precursor cells residing in the SGZ, activating the Shh-Gli signalling cascade, and resulting in the rapid division of Shh-responding cells. This could account for why exclusive elimination of septohippocampal cholinergic neruons did not reduce progenitor proliferation, unlike fimbria/fornix lesions, where all septohippocampal projections(inclusive of cholinergic, GABAergic and glutamatergiccells) were deafferented. In addition, the introduction of a pharmacological inhibitor of Shh-N signalling did not further attenuate the division of proliferating precursors in the hippocampus (Lai et al., 2003).

Further ultrastructural studies are needed to examine the distribution of Ptc1 receptors on recipient cells and the nature of contacts formed by Shh-secreting septohippocampal axons. Also, it would be interesting to determine if Shh-N is co-transmitted with GABA and/or glutamate, or is released in an independent manner. Shh-N has been found to be associated with lipid rafts (Traiffort et al., 2001), hence the dynamics of its trafficking may differ from the standard synaptic vesicle release of neurotransmitters or the fast axonal transport of peptides like endothelin-1 and amyloid precursor protein (Kaether et al., 2000; Lazarov et al., 2002; Stokely et al., 2005; Stokin and Goldstein, 2006). Additional work is also required to determine whether the release of Shh-N from the septohippocampal neurones is constitutive or activity-dependent. Physical activity evokes theta rhythm, which is arguably provoked by septoGABAergic cells (Brazhnik and Fox, 1997, 1999; Lee et al., 1994b; Xu et al., 2004). Running could be a form of activity that induces septohippocampal GABAergic projections to release Shh-N

into the DG. The effects of running on septohippocampal Shh-N signalling in relation to adult hippocampal progenitor proliferation will be investigated in the next chapter.

In summary, this study demonstrates that septohippocampal neurones transport Shh-N into the DG, mainly via GABAergic projecting cells.

5. RUNNING AND SHH SIGNALLING IN THE SEPTOHIPPOCAMPAL PATHWAY

5.1 INTRODUCTION

A large part of Shh research has been carried out at the cellular and molecular level. Indeed, genetic and biochemical analyses have provided a framework for understanding the physiological systems affected by the Shh signalling pathway. This bottom-up strategy works to provide causal explanations at every level, and to show that defects in Shh signalling can account for why certain physiological systems go awry, as in the case of congenital birth defects such as cyclopia (from which legends of one-eyed monsters originate), and certain adult onset brain tumours. However, little has been documented on the effects of physiological inputs on Shh activity. The aim of this chapter is to demonstrate that behavioural forces can influence Shh signalling, in the context of adult progenitor proliferation in the DG.

The Shh network comprises a positive transcriptional feedforward mechanism within a negative signalling feedback loop (Lai et al., 2004). The positive transcriptional response comes from the nuclear localization and activation of full length Gli activators, which regulates their own expression (Huntzicker et al., 2006; Regl et al., 2002) Negative feedback occurs in the form of signal repressor Ptc1 upregulation (Goodrich et al., 1997; Sanchez and Ruiz i Altaba, 2005). Shh itself has also been reported as a transcriptional target (Dahmane et al., 2001).

The levels of Gli1, Ptc1 and Shh expression can hence serve as a metric for pathway activation.

A recurring theme in this dissertation is that running is a most potent inducer of mitotic signal in adult neural precursor cells. A recent fate-mapping study from Fred Gage and his co-workers showed that running causes the proliferation of Sox2-positive cells (Suh et al., 2007). Sox2 is a marker for Type I glial-like stem cells and Type IIa progenitors, both of which are self-renewing and multipotent. Running can also induce the rare division of quiescent Type I cells (Suh et al., 2007). In other words, running can spur a subpopulation of cells in G_0 phase to enter the cell cycle, and running can also increase the rate of cell division of mitotic Type IIa progenitors.

Complimentary studies adopting similar *in vivo* genetic fate-tracing techniques show striking similarities between Shh signalling and running on stem cell behaviour. An earlier study had revealed that Shh stimulates progenitor proliferation in the DG niche (Lai et al., 2003). Ahn and Joyner followed Gli1-expressing cells over time and found that glial-like neural stem cells and their immediate actively dividing progeny respond to Shh signalling (Ahn and Joyner, 2005).

A posteriori, it is envisaged that Shh is the mitogen released during running responsible for the increase in DG progenitor proliferation. The purpose of this

last series of experiments is to uncover if the physiological act of running can regulate Shh activity concomitantly with neurogenesis within the hippocampus.

To inhibit Shh activity, pharmacological antagonism of its signalling pathway was employed. Cyclopamine is a plant alkaloid used to pharmacologically inhibit the signal transduction cascade (Cooper et al., 1998; Incardona et al., 1998). First discovered as a teratogen, it was so named as it causes cyclopia in embryos (Keeler and Binns, 1968). Cyclopamine is a small molecule antagonist that targets Smo (Chen et al., 2002a; Chen et al., 2002b; Taipale et al., 2000). Previously, other groups have injected directly cyclopamine into the brain and assess for its effects on neurogenesis (Banerjee et al., 2005; Lai et al., 2003). Here, cyclopamine was systematically injected to the animals in the experiments here to avoid the collateral damage produced by direct injection into the brain that could serve as a source of confounding variable. The effects of Shh signalling inhibition on running-induced neurognesis (assayed by S-phase marker BrdU) was concurrently assessed together with the downstream transcriptional responses of Shh activation.

5.2 MATERIALS AND METHODS

5.2.1 Running and cyclopamine injections

Adult female swiss mice (8-10 weeks) were randomly assigned to groups housed individually in cages that either had freely moving (runners) or immobilized (non-runners) running wheels attached. A photosensor was used to monitor the daily distance (number of revolutions of the running wheel) covered by each mouse. The runners and non-runners were then equally divided into 2 groups: vehicle-treated and cyclopamine- treated. The vehicle was made up of 45% 2-hydroxylpropyl-β-cyclodextrin (Tokyo Chemical Industry, Japan) in sterile phosphate buffered saline (PBS) (van den Brink, 2001). Cyclopamine (LC laboratories, Woburn, MA) was prepared by dissolving it in the vehicle at a concentration of 1 mg/ml with continual heating at 60 ^{o}C and stirring for 3 hours. The mice received twice daily intraperitoneal injections either the drug orsolvent for 5 days.

5.2.2 BrdU labelling

10 mg/ml BrdU (Sigma, Saint Louis, Missouri) was dissolved in 0.007 M NaOH and saline. Each mouse was given a single intraperitoneal injection of 300 mg/kg of BrdU half an hour before the last cyclopamine/vehicle injection. 24 hours after BrdU administration, the mice were sacrificed, perfused, and their brains stored in 4% paraformaldehyde fixative overnight. A vibratome (World Precision Instruments, Sarasota, FL) was used to section the mouse hippocampus rostral-

caudally at a thickness of 40 µm. For immunohistochemical analysis, the sections were pretreated with 4 N HCl for 20 min and 0.5 mg/ml of trypsin at 37ºC in the antigen retrieval step before blocking with 10% goat serum for an hour. The sections were then incubated with primary antibody mouse monoclonal anti-BrdU (1:100, Neomarkers) overnight at 4ºC, before incubation with secondary antibody Alexa Fluor 555 Goat anti-mouse (1:400, Molecular Probes) for an hour.

For counting of labelled cells, seven random one-in-five sections (160 µm apart) were selected throughout the entire rostral caudal length of the dentate gyrus and analysed by serial confocal scanning with a 40 X objective lens and a laser wavelength of 488nm throughout the depth of the section. An investigator blinded to the experimental conditions conducted the counting (see *Acknowledgements*). The number of BrdU positive cells in both sides of the dentate gyri in a section was pooled and divided by the sum of the traced granule cell layer outlines and the thickness of the section. The mean number of BrdU-labeled cells per µm of dentate gyrus was multiplied by the distance between the first and last section sampled (960 µm) and the mean length of the dentate gyri among the sections to estimate the total number of BrdU positive cells per brain.

5.2.3 Real-time quantitative PCR

The mice used for real-time PCR assays were subjected to similar experimental conditions as above save for BrdU injections (FIGURE 5-2i). At the end of the exercise and cyclopamine treatments, the mice were given an overdose of

anaesthetic and were decapitated after cervical dislocation. The total RNA used in this experiment was extracted from hippocampi of the animals, using the same methods as described for RT-PCR (see *Materials and Methods*, Chapter 3.2) except that RNA was purified using mini-columns in the Purelink^{TM} Micro-to-Midi kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. cDNA was synthesized from oligodT primers.

Real-time Taqman® PCR, utilizing 5'-nuclease chemistry to detect amplification of the target gene during cycling, was performed using the ABI PRISM® 7500 Real-time PCR system (PE Applied Biosystems, CA). The assay was performed in triplicates in a MicroAmp Optical 96-well reaction plate, using the proprietary TaqMan® Universal mastermix (consisting of ROX internal reference dye, uracil-N-glycosylase(UNG), dNTPs, MgCl2 , buffer and AmpliTaq Gold® DNA Polymerase) and predesigned primer/probe sets for Shh, Ptc1 and Gli1 (TaqMan® gene expression assays). A single reaction contains 250 nm of FAM dye-labeled TaqMan® minor groove binder probes and 900 nm each of forward and reverse primers. All reagents were obtained from the same manufacturer (PE Applied Biosystems). Beta-actin (β-Actin) was used as the endogenous control to normalize for differences in quantity of cDNA targets used across all the experimental reactions. The PCR thermal cycling conditions for amplification of cDNA target gene was set as default: 2 min at 50°C for UNG activation, 10 min at 95°C for polymerase activation, and 40 cycles of 15 s at 95°C and 1 min at 60°C for denaturation and annealing respectively.

Data analysis was performed using the ABI PRISM Sequence Detection Software 1.3.1. The baseline, the initial stages of PCR where there is little change in fluorescence signal and the threshold, the magnitude of the signal generated for the amplification plots of target genes Shh, Ptc1 and Gli1, were adjusted manually. The baseline was set before the start of amplification, and the threshold set within the exponential phase of the curve. The threshold cycle (C_T) value, which is the PCR cycle at which fluorescence reaches a significant threshold value above baseline, was obtained for further comparative analysis.

5.2.4 Western blotting

Western blots were carried out as described in Chapter 3.2. The intensity of the protein bands on the x-ray films, given by pixels X mm^2, was quantified using Quantity One software (Bio-Rad).

5.2.5 Statistical analyses

All statistical analyses were performed using SPSS software version 14.0. Either analysis of variance (ANOVA) or 2-way ANOVA was performed, depending on the number of groups tested. This was followed by Dunnett's post-hoc test or student's independent t-tests when comparisons were found to be significant. The Levene's test for Equality of Error Variances was applied to all groups to check for homogeneity of variances. Differences were considered to be statistically significant when $p < 0.05$.

5.3 RESULTS

5.3.1 Shh signalling is involved in running-mediated adult hippocampal progenitor proliferation

To investigate the effects of Shh signalling interference on the division of progenitor cells in the SGZ, a suitable dosage of cyclopamine had to be initially established. The mice were subjected to varying dosages of cyclopamine, at 10 mg/day/kg, 30 mg/kg/day and 100 mg/kg/day to obtain a dose-response curve. Twice daily injections 12 hours apart were given to spread out the volume of the drug injected in a single shot. The 100 mg/kg/day dose proved to be too toxic for the mice, all of which showed a drastic drop in weight by day 2 and had to be euthanized accordingly. More than half the mice in the 30 mg/kg/day group also suffered massive weight loss of more than 20% and had to be euthanized by day 3 of the injections. The remaining mice of the 30 mg/kg/day group and those in the 10mg/kg/day group appeared to be healthy, and injections were carried to their full term for 5 days. On the fourth day of cyclopamine injections, the mice were given a single shot of BrdU injection to label for dividing cells in the SGZ.

Analysis of variance showed that there was a significant difference in the number of BrdU labelled cells among the groups ($F_{2, 13} = 4.134$, $p < 0.05$). Dunnett's post-hoc tests indicated that a dose of 10 mg/kg/day did not affect BrdU-positive cell count, but a higher dose of 30 mg/kg/day significantly reduced the number of

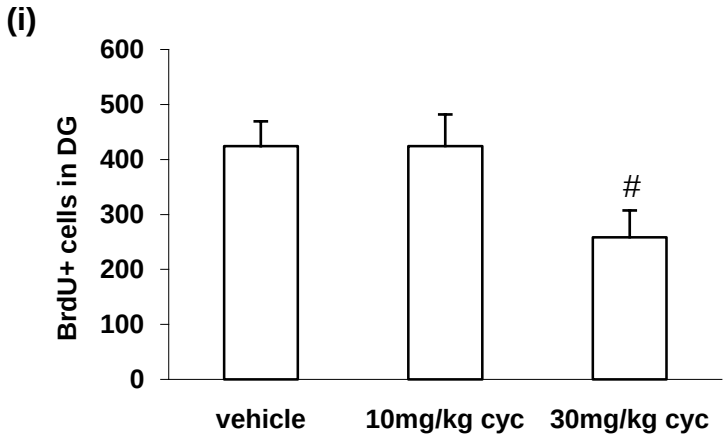
BrdU- labelled cells relative to the vehicle treated controls (p<0.05) (FIGURE 5-1i).

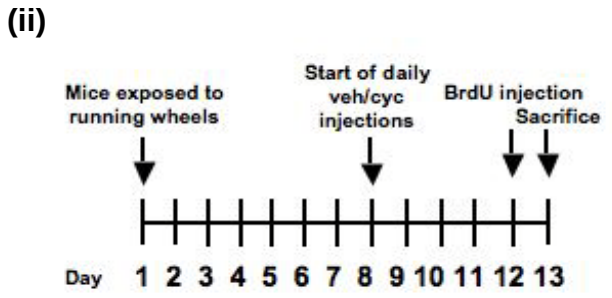
To find out if inhibition of Shh signalling affects running-mediated progenitor proliferation, the mice underwent an experimental protocol that involves physical activity for 12 days followed concurrently by cyclopamine treatment, and BrdU administration. Due to the drastic weight loss sustained by mice given 30 mg/kg/day of cyclopamine, 10 mg/kg/day of cyclopamine was administered to the mice for this part of the experiment. The time frame for the experiments is schematically represented in FIGURE 5-1ii.

Two-way analysis of variance showed that both running ($F_{3,11} = 58.96$, $p < 0.001$) and cyclopamine treatment ($F_{3,11} = 6.425$, $p < 0.05$) had a significant effect on the number of BrdU positive cells in the dentate gyrus. The interaction between cyclopamine and running also significantly affected the number of labelled cells ($F_{3,11} = 14.9$, $p < 0.01$). Post-hoc tests revealed that although running very significantly increases the number of BrdU-positive cells in the vehicle treated groups (2 tailed t-test : $t_6 = 14.7$, $p < 0.001$) and cyclopamine treated groups (1 tailed t-test: $t_5 = 2.567$, $p<0.05$), there was a significant difference between the labelled cell numbers of the 2 groups ($t_{3.744} = 4.0$, $p < 0.05$) (FIGURE 5-1iii).

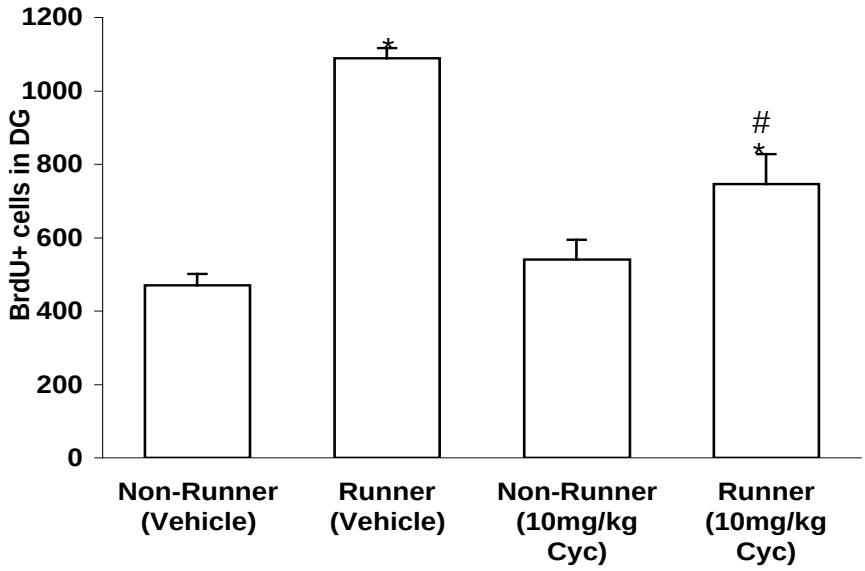
Similar to the previous observations in cholinergic afferented mice in Chapter 2, the administration of cyclopamine did not impair the ability of the mice to run. There was no difference in the distance ran by the mice, regardless of the

varying cyclopamine dosages. Taken together, these results indicate that running increases the number of adult neural progenitors in the DG, but this increase is abrogated by the administration of 10 mg/kg/day of cyclopamine, a dose that is not sufficient to alter baseline progenitor proliferation. In other words, the inhibition in Shh signalling suppresses the running-mediated proliferation of adult progenitor cells.





(iii)



(iv)

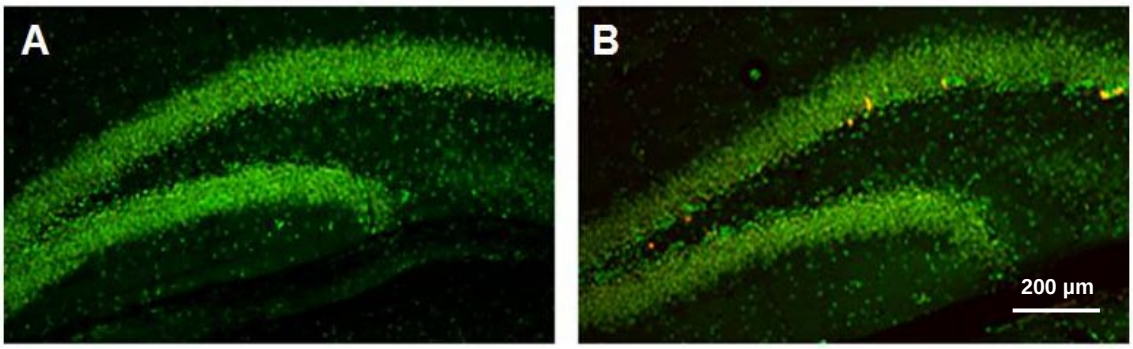


FIGURE 5-1 Effects of Shh inhibition on running-mediated progenitor proliferation (i) A dose of 30 mg/kg/day of cyclopamine (n = 6), but not 10 mg/kg/day (n = 6) reduces baseline progenitor proliferation relative to vehicle controls (n = 7). (ii) Timeline of experiments. The runners had daily access to running wheels for 12 days. (iii) 10 mg/kg/day of cyc is not sufficient to reduce baseline progenitor proliferation in non-runners (n = 3) compared to vehicle-treated controls (n = 4). However, 10mg/kg/day of cyc in runners (n = 4) suppressed the increase in running-induced progenitor cells shown in vehicle-treated non-runners (n = 4). * indicates that running significantly increases the number of BrdU-labelled cells. # indicates a significant drop in BrdU-labelled cells in runners treated with cyclopamine (iv) Photomicrographs of BrdU immuno-reactive cells *(red)* in a (A) runner (10mg/kg cyc)and a (B) runner (vehicle). Granule cells *(green)* are labelled by cell soma marker Neurotrace to show the relative distribution of BrdU-positive progenitors in DG.

5.3.2 Running upregulates Shh transcription in the MSDB in spite of signalling inhibition

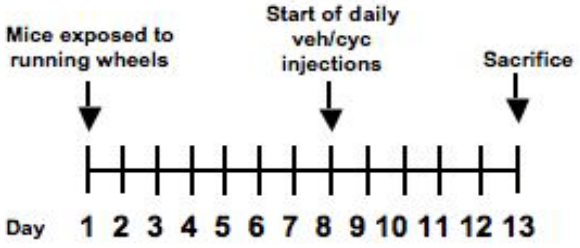
To further explore the interaction between running and Shh signalling, we repeated the experimental conditions i.e. injections of 10 mg/kg/day of cyclopamine on both runners and non-runners, and assayed for the expression of Shh in the MSDB (FIGURE 5-2i). This was achieved by using relative quantification real-time PCR. This technique was employed over other gene expression methods such as northern blot analysis and RNase protection assays for the following benefits: it requires no post-amplification processing, is highly discriminative between almost identical sequences, boasts tremendous sensitivity, and has a large dynamic range (Bustin, 2000; Gentle et al., 2001; Klein et al., 2003; Schmittgen et al., 2000; Wong and Medrano, 2005). Real-time PCR takes advantage of the exponential phase of PCR, an optimal amplification period where the PCR product doubles with every cycle. For data analysis, changes in PCR signals of each Shh-N, Ptc1 and Gli1 gene of the different treatment groups (vehicle-treated runners, and cyclopamine-treated non-runners and runners) were compared to a reference untreated control (vehicle non-runner). The reference is also known as a *calibrator* in real time quantitative PCR terminology. The formulae and calculations of our real-time PCR data are tabulated in FIGURE 5-2ii.

ΔC_T, the adjusted cycle threshold for each target gene (C_T of target gene minus C_T of reference β-actin gene), was used as the dependent variable for our

statistical analysis, as it accurately reflects the direct outcome of the treatment and sample quantity (Yuan et al., 2006). A lower ΔC_T value indicates that the concentration of starting cDNA is higher as it takes less cycles to reach a significant stipulated signal. P-values are derived from testing the null hypothesis that the difference between ΔC_T of the treated samples and calibrator samples (i.e. $\Delta\Delta C_T$) is zero.

Cyclopamine treatment did not cause any changes in mRNA synthesis of Shh precursor protein in the MSDB. Conversely, running produced an increase in mRNA expression ($F_{1,8} = 14.830$, $p < 0.01$). The elevation in Shh mRNA levels was significant in vehicle controls ($t_9 = 2.724$, $p < 0.05$) and cyclopamine-treated animals ($t_9 = 3.724$, $p < 0.01$). Real-time PCR values are tabulated in FIGURE 2i. The ratio of each treatment group over the non-treated vehicle control is calculated using the $2^{-\Delta\Delta CT}$ method (Livak, 2001) for graphic representation in FIGURE 5-2ii.

(i)



(ii)

Target gene	Group	ΔC_T [1] target	$\Delta\Delta C_T$ [2]	Confidence interval [3]	Fold difference [4]
Shh	**VNR** (*n*=5)	8.2117	0.0	(0.370, -0.370)	1.0
	VR (*n*=6)	9.5486	-1.03	(-0.402, -1.652)	2.3
	CNR (*n*=5)	9.4866	-0.06	(0.11, -0.23)	1.0
	CR (*n*=6)	8.9643	-0.58	(0.367, -0.801)	1.5

1. $\mathbf{\Delta C_{T\ target} = \sum(mean\ C_{T\ target\ gene} - mean\ C_{T\ endogenous\ control})/n}$ where the endogenous control is the housekeeping gene β-actin found within the target sample.
2. $\mathbf{\Delta\Delta C_T = \sum(C_{T\ target} - mean\ C_{T\ calibrator})/n}$ where the calibrator is VNR and n is the sample size.
3. **Confidence interval =** $\mathbf{\Delta\Delta C_T \pm ts/\sqrt{n}}$ where t is student's t value at 95% confidence level at n degrees of freedom and s is standard deviation of $\Delta\Delta C_T$
4. $\mathbf{Fold\ difference = \sum 2^{-\Delta\Delta CT}/n}$

(iii)

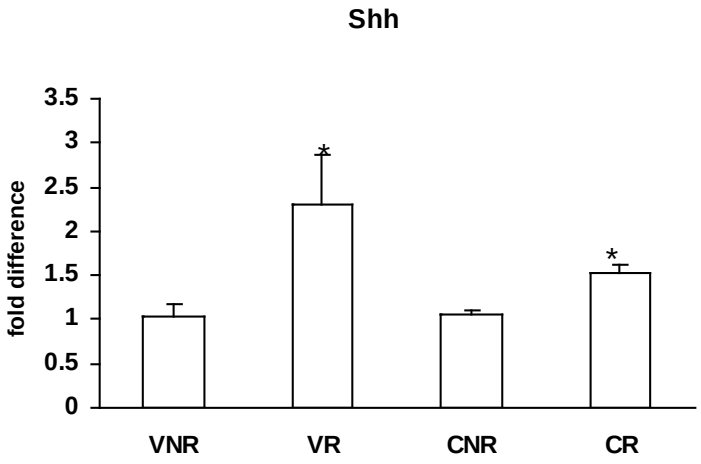


FIGURE 5-2 Effects of running on Shh synthesis in MSDB (i) Experimental timeline. (ii) Confidence interval values of $\Delta\Delta C_T$ (iii) Graphical representation of vehicle runner (VR), cyclopamine-treated non-runner (CNR) and cyclopamine-treated runner (CR); compared to the calibrator, i.e. vehicle non-runner (VNR).The mRNA of Shh increases with running despite cyclopamine treatment. * indicates significant difference between non-runners and runners

5.3.3 Running activates transcriptional responses of the Shh-Gli signalling pathway in the hippocampus

The key recipients of the activated Gli transcription in the hippocampus were quantified. Two-way analysis of variance indicated that running had a very significant effect on the mRNA expression of Shh ($F_{3,19} = 8.992$, $p < 0.01$), Ptc1 ($F_{3, 19} = 28.548$, $p < 0.001$), and Gli1 ($F_{3,18} = 163.028$, $p < 0.0001$). Post-hoc tests revealed that running significantly increases Ptc1 and Gli1 expression in untreated runners ($t_{10} = 2.050$, $p < 0.05$; $t_{10} = 11.388$, $p < 0.001$) and Shh, Ptc1 and Gli1 expression in cyclopamine treated runners ($t_9=3.256$, $p<0.01$; $t_9 = 6.643$, $p<0.001$; $t_{4.966} = 7.037$, $p < 0.001$). The cyclopamine treatment also causes an appreciable decrease in Gli-1 mRNA of runners ($t_9 = 1.991$, $p < 0.05$), showing that inhibition of Shh signalling affects the running-induced expression of Gli1 (FIGURE 5-3).

(i)

Target gene	Group	$\Delta C_{T\ target}$[1]	$\Delta\Delta C_T$[2]	Confidence interval[3]	Fold difference[4]
Shh	**VNR** (*n*=6)	10.88	0.00	(-0.597, 0.597)	1.1
	VR (*n*=6)	10.48	-0.40	(-0.737, -0.069)	1.4
	CNR (*n*=5)	11.37	0.49	(0.219, 0.763)	0.7
	CR (*n*=6)	10.47	-0.41	(-0.830, 0.017)	1.4
Ptc1	**VNR** (*n*=6)	5.12	0.00	(-0.325, 0.325)	1.0
	VR (*n*=6)	4.72	-0.41	(-0.647, -0.172)	1.4
	CNR (*n*=5)	5.39	0.26	(0.131, 0.395)	0.8
	CR (*n*=6)	4.48	-0.65	(-0.877, -0.420)	1.6
Gli1	**VNR** (*n*=6)	11.56	0.00	(-0.618, 0.618)	1.0
	VR (*n*=6)	7.37	-4.19	(-4.616, -3.762)	21.7
	CNR (*n*=5)	11.54	-0.02	(-0.346, 0.307)	1.0
	CR (*n*=6)	8.27	-3.28	(-4.218, -2.351)	11.5

1. $\Delta C_{T\ target} = \sum(\text{mean } C_{T\ target\ gene} - \text{mean } C_{T\ endogenous\ control})/n$ where the endogenous control is the housekeeping gene β-actin found within the target sample.
2. $\Delta\Delta C_T = \sum(C_{T\ target} - \text{mean } C_{T\ calibrator})/n$ where the calibrator is VNR and n is the sample size.
3. **Confidence interval =** $\Delta\Delta C_T \pm ts/\sqrt{n}$ where t is student's t value at 95% confidence level at n degrees of freedom and s is standard deviation of $\Delta\Delta C_T$
4. **Fold difference =** $\sum 2^{-\Delta\Delta CT}/n$

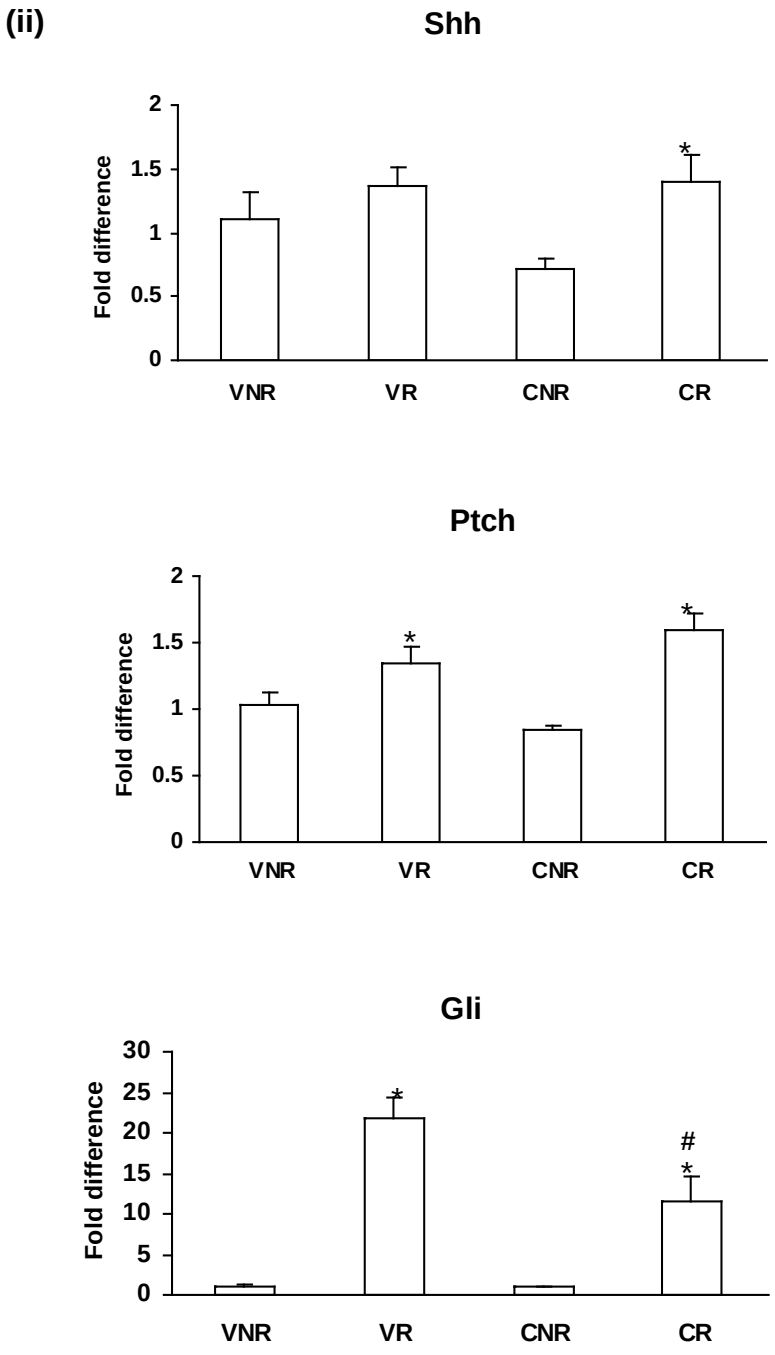


FIGURE 5-3 Effects of running on Shh-Gli transcriptional response (i) Relative quantitative values of real-time PCR on various target genes (iii) Graphs depicting relative amplification values of Shh, Ptc1and Gli 1of different treatment groups * indicates running significantly increases mRNA expression within each treatment group; # indicates that the difference in gene expression levels for cyclopamine and vehicle runners is significant.

5.3.4 Running increases Shh-mediated Gli1 protein expression

The protein expression of Shh, Ptc1, Smo and Gli1 in the hippocampi of mice subjected to the same experimental settings was probed next. Western blot assays showed that the amount of Shh precursor protein and Smo protein remained unchanged despite the varying treatments. Conversely, both running ($F_{1,8}$ = 12.614, p < 0.01) and cyclopamine ($F_{1,8}$ = 248.94, p < 0.0001) treatment affected the protein expression of Gli1 transcription factor. The interaction between cyclopamine and running also altered Gli-1 protein levels ($F_{1,8}$ = 6.205, p < 0.05). Two-tailed independent t-tests revealed that running significantly increases Gli1 expression for non-treated groups (t_2 = 4.995, p < 0.05) while cyclopamine reduced the amount of Gli-1 in both runners ($t_{2.092}$ = 14.932, p < 0.01) and non-runners (t_2 = 8.398, p < 0.05). Running had a significant effect on Ptc-1 expression ($F_{1,8}$ = 11.883, p < 0.01), which further 1- tailed analysis unveiled was significant in cyclopamine-treated animals ($t_{2.038}$ = 3.628, p < 0.05). There was also a strong link between running and cyclopamine ($F_{1,8}$ = 12.089, p < 0.01), in which Ptc1 expression in cyclopamine runners is much higher than its non-treated counterparts (t_4 = 2.806, p < 0.05) (FIGURE 5-4).

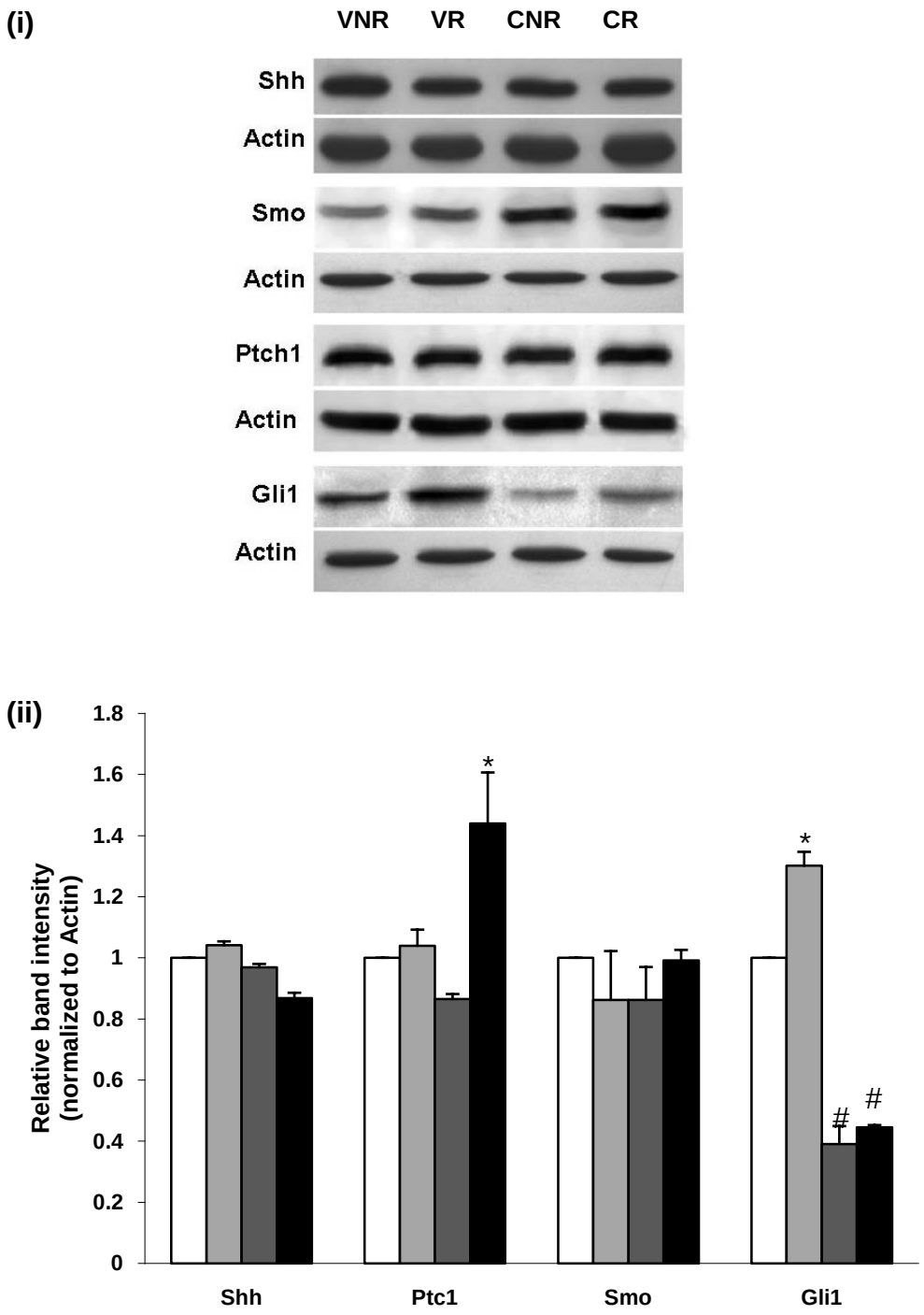


FIGURE 5-4 Effects of running on protein expression levels of Shh signalling cascade. (i) Western blot results of Shh (45 kDa), Ptc1(160 kDa), Smo(87 kDa) and Gli1(118 kDa) in hippocampus. (ii) Graphic representation of mean intensity values of VNR (white bar), VR (light grey bar), CNR (dark grey bar) and CR (black bar), from 3 separate western blots of independently derived, pooled lysates normalized by Actin.* indicates that expression is increased due to running. # indicates suppression of expression by cyclopamine.

5.4 DISCUSSION

The first part of the experiment was to ascertain if Shh signalling can influence progenitor proliferation. Intraperitoneal injections of cyclopamine had proven to be an effective therapeutic against unregulated proliferation of cells in medullablastomas and other tumours by specifically halting deregulated Shh transcription of Gli (Berman et al., 2002; Clement et al., 2007; Sanchez and Ruiz i Altaba, 2005). The results obtained here show that *in vivo* cyclopapmine administration led to a drop in the number of proliferating precursor cells in the SGZ neurogenic niche.

The potential role of Shh signalling in running–mediated neurogenesis was next addressed. Running elevates the number of newborn cells in the SGZ by two-fold, corroborating previous results in Chapter 2. This running-induced increase was stemmed by cyclopamine, at dosages that did not afflict baseline cellular proliferation. This finding draws parallels with another study done in rats, where cyclopamine injection to the hippocampus prevented the electroconvulsive-seizure-mediated increase in proliferation of DG cell progenitors (Banerjee et al., 2005). Both findings indicate the involvement of Shh in activity-dependent neurogenesis.

Running elevates levels of Ptc1 and Gli1 mRNA transcripts in the hippocampus. The increase in Gli1 of runners over non-runners was as much as 20-fold. Unlike both Gli2 and Gli3, which have repressor activities, Gli1 behaves exclusively as a transcriptional activator (Ruiz i Altaba, 1999). Its amplification response is hence a direct function of Shh input on the system. The rise in Gli1 transcripts is reflected in its protein expression level. The increase in Gli1 protein concentration is not as notable as its mRNA expression, indicating post-translational protein modification forces at work. This is likely to be mediated by ubiquitin-dependent processing, which is facilitated by adaptor protein *Numb*, and E3 ubiquitin ligase *Itch*, and culminating in the degradation of Gli1 proteins (Di Marcotullio et al., 2006; Huntzicker et al., 2006). Ubiquitylation represents another checkpoint to prevent excessive activation of Shh-Gli signalling and the ensuing dysregulation of the cell cycle.

Cyclopamine treatment did not affect basal transcript levels of any of the three genes. This is expected, as the drug dosage used did not reduce baseline progenitor proliferation. Nonetheless, the dosage suffices to reduce the running-induced increase of Gli1 transcriptional response by half Cyclopamine also reduced Gli1 protein expression of both non-runners and runners significantly.

Cyclopamine works by binding directly to Smo (Chen et al., 2002a; Chen et al., 2002b; Taipale et al., 2000). To spell out in mechanistic terms, the small molecule antagonist stoichiometrically opposes the activity-dependent

disinhibition of Smo receptor, leading to diminished Smo activity and reduced downstream signal transduction. In this model, the Smo receptor activity is governed by its binding constants and the overall levels of Smo expression within the cell should not change. To support the idea, the results here show that protein expression of Smo remained the same in spite of the divergent signals. Unlike Gli, where responses are based on the flux of Gli protein numbers, Smo activity is mediated by translocation to the cell surface, and its activity suppressed by limiting its trafficking (Jia et al., 2004; Kovacs et al., 2008; Rohatgi et al., 2007; Wilbanks et al., 2004)

Although Ptc1 acts upstream of Smo, its transcription is a recipient of Smo-mediated signal transduction (Goodrich et al., 1997; Sanchez and Ruiz i Altaba, 2005). The upregulation of Ptc1 is to rein in the strong Gli autoregulatory loop, but little is known about how it exerts its suppressor effects. In our findings, running increases the levels of Ptc1 transcripts, once again demonstrating the activity-dependent Shh signalling. Interestingly, Ptc1 expression increases when Smo-activating signals (running) and inhibitory signals (cyclopamine) converge. This suggests that Ptc1 synthesis may be regulated by another pathway other than the canonical Shh pathway. Collectively, the data underlie the complexity of both external and intracellular signal mechanisms within the Shh network.

Gli proteins form the heart of the Shh network by integrating the Shh signal with other intracellular inputs in a temporal and spatial manner(Ruiz i Altaba et al.,

2007). Many groups have argued that Gli1 expression *per se* is the only reliable indicator of an active pathway(Clement et al., 2007; Dahmane et al., 2001; Lee et al., 1997). Going by this argument, the direct Gli1 transcriptional responses in the findings here should be indicative that running activates Shh-Gli signalling cascade, which in turn could regulate the rapid expansion of the pool of adult hipoocampal progenitors.

This present study adds to the growing list of evidence establishing the role of Shh in maintenance and self-renewal of neural stem cells (Ahn and Joyner, 2005; Lai et al., 2003; Machold et al., 2003; Palma et al., 2005). Even so, the molecular events that effectuate the Shh mitogenic response specifically in the adult hippocampus remain undetermined. The Shh-induced transcriptional programs from other cell types may offer clues into the identities of downstream mitogenic molecules. The canonical Shh-Gli signalling cascade triggers critical regulators of cell cycle progression. Key candidates include cyclins D1, D2 that promote quiescent cells to enter the G1 phase of the cell cycle (Mill et al., 2005; Oliver et al., 2003)and transcription factor N-myc, a DNA replication regulator(Hatton et al., 2006; Knoepfler et al., 2002; Knoepfler and Kenney, 2006; Mill et al., 2005; Oliver et al., 2003). Other post-transcriptional targets such as *Deltex* and *Sfrp1* are engaged in cross-regulation of other signalling pathways. This inhibition of Notch and Wnt pathways prevents neuroneal differentiation and hence maintains the pool of progenitor cell in a proliferative state (Jones and Jomary, 2002; Yamamoto et al., 2001). On the other hand, Shh

may also activate independent pathways such as MAPK/ERK and PI3K/Akt pathways (Elia et al., 2007) or act cooperatively with members of the FGF family (Kessaris et al., 2004; Vinothkumar et al., 2008) or NGF (Reilly et al., 2002) in contributing to the rapid expansion of the progenitor cell population. Further work is required in elucidating the candidate molecules that will translate the activity-dependent Shh-signalling cascade into a proliferative signal for adult hippocampal progenitors.

To sum up the findings for this final part of my investigation, (i) running increases transcriptional responses of the Shh-Gli signalling pathway concomitantly with progenitor proliferation in the hippocampus; (ii) blockade of Shh signalling affects the running-induced effects. In other words, running induces Shh signalling in the SGZ and set the wheels of neurogenesis in motion.

6. CONCLUSION

The concept of neurogenic niche was premised on it being a vestige of the embryonic brain, with common cellular factors and signalling pathways embroiled in the formation of newborn cells. Like developmental neurogenesis, adult hippocampal neurogenesis is activity-dependent, and governed by various stimuli, among which is the act of running. Running targets neurogenesis specifically at its early stages. The missing link in the puzzle is the identity and workings of a molecular candidate responsible for transducing this simple physiological input into a cellular signal. The delivery of this mitogen should be specific to the DG hotbed within the hippocampus, hence circulating factors, as put forth by other groups, are not ideal in that they are secreted constitutively and circulate the entire brain.

The mitogen(s) can hail from either a local or distal source. A distal origin is more attractive in this sense, as it incorporates inputs from other parts of the brain to the DG, in an assembly of positive or negative signals from the outer world that translates into net rise/decline of division of precursor cells. There are extensive extrinsic projections to the hippocampus, but the septohippocampal system is arguably the best characterized among them, given its close association with theta rhythm. The oscillations reflect the operative state of the hippocampus in processing incoming signals(Buzsaki, 2002). It is also is evoked during running(Bland and Vanderwolf, 1972; Buzsaki et al., 1983; Kramis et al., 1975;

Skaggs et al., 1996; Teitelbaum et al., 1975; Vanderwolf, 1969), and could provide the link between physiological and cellular systems.

How do my findings fit in the proposed schema of activity-dependent adult hippocampal progenitor proliferation? I will now proceed to recapitulate the salient outcomes of the investigations carried out for this working hypothesis. Firstly, running robustly elevates progenitor proliferation. This running-mediated cell genesis is not affected by the elimination of septohippocampal cholinergic neurones, suggesting that the other known class of neurones present in the septohippocampal system, i.e., the GABAergic neurones, could alternatively be involved

The next part of the thesis focuses on the expression of the putative candidate, Shh (which specifically regulates similar phases of neurogenesis as running, and is co-expressed with septal GABAergic transcripts). A large population of Shh-producing cells exist in the MSDB, and its varicosities are found in abundance in the DG. Cells positive for the Shh receptor envelop the SGZ, and are in close association with neural stem cell markers. This mitogenic factor is transported from the MSDB to the DG, mainly via GABAergic projections.

The last part of the investigations focuses on reconciling physiological inputs with Shh activity. Pharmacological antagonism of Shh signalling reins in the running-induced increase in progenitor proliferation, despite not affecting baseline levels.

Running brings about an increase in Shh production in the MSDB even when signalling is inhibited. Within the hippocampus, running also boosts the transcriptional response of Gli1, which serves as a metric of Shh activity.

This present study is the first of its kind to implicate Shh signalling in the running-induced expansion of the adult progenitor cell pool. It is possible that the theta rhythm evoked by running induces the Shh secreting septohippocampal GABAergic projections to release the mitogen into the DG, where it acts on Shh-responding neural precursors. The findings here may be just one of the pieces of puzzles in delineating the molecular mechanisms underlying running-mediated adult neurogenesis. The intrinsic properties of a stem cell dictates how it will interpret and integrate extracellular cues that will initiate intracellular cascades and activate distinct sets of transcription factors. At any time point, the extrinsic signal acting on the precursor cell may be exclusive, or may act cooperatively with other trophic factors. It is possible that Shh may act in concert with other factors, engaging in cross-talk signalling to increase cellular genesis.

Fully aware that correlation does not imply causation, additional work is required to test this hypothesis further. Work indicated, for instance, includes (i) developing a method to specifically lesion septohippocampal GABAergic neurones and/or complete lesioning of the septohippocampal pathway to assess for possible decline in hippocampal Shh-N and Gli-1 levels (ii) microdialysis assays to detect for the presence of Shh activity in the hippocampus in a running

animal (iii) evoking theta in the MSDB, either through electrical stimulation or the use of pharmacological agonists and antagonists (such as acetylcholinesterase inhibitors and muscarinic agonists) to specifically activate the septohippocampal GABAergic neurones to test for concomitant Shh signalling in the DG, and (iv) ultrastructural studies to establish the nature of contacts between Shh presynaptic terminals and Shh-responding precursor cells.

It is timely, at the end of the dissertation, to once again reflect upon the rationale behind running and its facilitation of cellular plasticity. The functional implications could offer insights into the restricted regenerative capabilites of the adult brain, and also further probe the expanding role of the hippocampus. Cognitive impairments of many neurodegnerative and psychiatric diseases have been increasingly attributed to failing adult hippocampal neurogenesis, such as AD and dementia, depression, and recently, schizophrenia (reviewed in(Kempermann et al., 2008)). It is hoped that the investigations here have shed some light on the molecular mechanisms of adult hippocampal progenitor proliferation, a key step in the development of therapeutics for neurological and neuropsychiatric diseases.

"Every morning in Africa, a gazelle wakes up. It knows it must run faster than the fastest lion or it will be killed. Every morning a lion wakes up. It knows it must outrun the slowest gazelle or it will starve to death. It doesn't matter whether you are a lion or a gazelle; when the sun comes up, you'd better be running."

Herbert Eugene Caen, former San Francisco Chronicle columnist

7. LIST OF PUBLICATIONS

PAPERS

Ho N.F., Han S.P., Dawe G.S. (2009) Effect of running on adult hippocampal neruogenesis in cholinergic lesioned mice. BMC Neuroscience *10*, 57.

PAPERS (submitted/in preparation)

Ho N.F., Dawe G.S. (2009) Sonic hedgehog expression in the septohippocampal system.

Ho N.F., Dawe G.S. (2009) Shh signalling in running-mediated adult hippocampal proliferation.

POSTERS

Ho N.F., Han S.P., Dawe G.S. (28-29 May 2007) Does Shh play a role in running-mediated neurogenesis? The Inaugural Singapore-Taiwan-Hong Kong (CU) Meeting of Pharmacologists, Singapore

Ho N.F., Han S.P., Dawe G.S. (5 to 6 Feb 2007) Shh: a key regulator in running-mediated hippocampal progenitor proliferation? Office of Life Science Conference, Singapore

Ho N.F., Han S.P., Dawe G.S. (14^{th}-18^{th} Oct 2006) Effect of running on neurogenesis in cholinergic lesioned mice. Neuroscience 2006, Atlanta, GA (abstract reproduced in lay-man language for press release)

Ho N.F., Dawe G.S. (4^{th}-6^{th} Nov 2005) Expression of Shh in the Septohippocampal System. Combined Scientific Meeting, Singapore

Ho N.F., Dawe G.S. (3^{rd}-4^{th} Feb 2004) Exercise Promotes Neural Cell Proliferation in the Adult Rodent Brain. The Future of Neurobiology, Singapore

Ho N.F., Dawe G.S. (2^{nd} -3^{rd} October 2003) Exercise Promotes Neural Cell Proliferation in the Adult Rodent Brain. 7^{th} NUS-NUH Annual Scientific Meeting, Singapore

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