

Cyclosporin A Promotes Neural Precursor Cell Survival to Enhance Neurogenesis in the Hippocampus

by

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Institute of Medical Science
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Abstract

Neural precursor cells (NPCs) in the adult mammalian brain show potential in applications of regenerative repair in the CNS. Recent work has shown that Cyclosporin A (CsA), a commonly used immunosuppressive drug, expands the size of the NPC pool in the lateral ventricle subependyma by promoting cell survival, with no concomitant effect on proliferation kinetics. We asked if CsA had similar effects on neural precursors in the neurogenic dentate gyrus within the hippocampus. In vitro, we found that CsA increases the size of the NPC pool. Our in vivo studies reveal an increase in proliferating immature neurons in mice subcutaneously infused with CsA for seven days. A time course study reveals that CsA promotes the survival of newly born NPCs, which can then go on to form new neurons in the DG. Together these findings support the hypothesis that CsA administration promotes neurogenesis and may have implications for tissue repair following injury.

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List of Abbreviations

aCSF	artificial cerebrospinal fluid
BrdU	5-bromo-2-deoxyuridine
CNS	central nervous system
CsA	Cyclosporin A
DG	dentate gyrus
EGF	epidermal growth factor
FBS	fetal bovine serum
FGF2	basic fibroblast growth factor
GCL	granular cell layer
GFAP	glial fibrillary acidic protein
ML	molecular layer
MPTP	mitochondria permeability transition pore
NDS	normal donkey serum
NGS	normal goat serum
NPC	neural precursor cell
NSC	neural stem cell

PBS	phosphate buffered saline
P0	passage 0 (P1,2,3,etc.)
SE	subependyma
SEZ	subependymal zone
SFM	serum-free media
SGZ	subgranular zone
SVZ	subventricular zone

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Chapter 1 – Introduction and Literature Review

1.1 – Stem Cells

Stem cells, by definition, have the ability to differentiate and form all of the cell types in a mature organism. Populations of stem cells exist that can continually replace cells that are lost over the life of the organism. It is due to these properties that stem cells have received increasing attention over the past two decades, especially in regenerative medicine whereby these stem cells can be manipulated to form new tissues as part of a strategy to replace or repair injured or diseased tissues.

From a developmental standpoint, all cell types of an organism arise from a totipotent fertilized egg cell. The first cells to differentiate from a fertilized egg form the trophoectoderm, which is responsible for nurturing the growing embryo and developing into a large portion of the placenta, the inner cell mass, which makes up the actual embryo, and the germ line stem cells (Lodish, Berk et al. 2008). The inner cell mass divides into three germ layers, each responsible for separate cell lineages: (i) the ectoderm that forms the epidermal and neural cells; (ii) the mesoderm that forms the heart, muscle, connective tissue, and blood; and (iii) the endoderm which forms the stomach and organs of the gastrointestinal tract (Lodish, Berk et al. 2008). These three germ layers undergo further divisions that lead to populations of stem cells with restricted potential.

In general terms, the somatic stem cell is the earliest precursor in a cell lineage. Progenitor cells arise from the stem cell and go on to form functionally mature cell types (Weissman 2000). These progenitor cells can be rapidly dividing, termed “transit

amplifying cells”, to greatly increase the final number of mature cells. To summarize, stem cells arise from the totipotent fertilized egg cell to become multipotent tissue specific stem cells. For example, the hematopoietic stem cell located in the bone marrow gives rise to all the other blood cells. Of particular interest in this thesis is the neural stem cell, which is capable of giving rise to the main phenotypes of the nervous system and will be discussed later.

Stem cells undergo cell division and how they go about this process leads to two defining properties. The first is that stem cells have the ability to replace and maintain a population of themselves over the lifespan of the organism. This property is termed self-renewal (van der Kooy and Weiss 2000) and symmetrical cell division is involved in the early expansion of the stem cell pool. The second property is termed multipotentiality in which stem cells can undergo asymmetric division, where one daughter cell retains its stem cell properties while the other cell differentiates into a mature cell type (Lodish, Berk et al. 2008). The decisions of stem cells to undergo symmetric or asymmetric division and the ultimate fate of progenitor cells are not just determined by intrinsic factors within the cell. The external local environment, termed the niche, also regulates cell behavior and development (Scadden 2006). Indeed, the niche plays an important role in not only ensuring the stem cell population is maintained but also in preventing excessive proliferation and this requires a balance of signals that regulate cell survival, proliferation, and death (Scadden 2006).

Neural tissue arises from ectoderm, in which the single cell epithelial lining of the ectoderm forms the neural tube (Alberts, Johnson et al. 2008). Within the neural tube, neuroepithelial cells lining the early ventricle contain stem cells that go on to form the majority of the neurons and glia in the brain (Doetsch 2003).

1.2 - Discovery of adult neurogenesis

Neurogenesis is defined as the process by which undifferentiated neural progenitors generate new nerve cells. It was established as central dogma that this process only occurred during embryonic and neonatal development and that after birth, the adult neuron cell population remained static. Many of the pioneers of neurobiology, such as Santiago Ramon y Cajal, Camillo Golgi, Rudolf Albert von Koelliker, and William His, were interested in the notion of adult neurogenesis. However, studies using the Golgi Silver Chromate technique (Koelliker 1896, Cajal 1899-1904, His 1904) led the pioneers to regard the adult nervous as static and incapable of regeneration. This prevalent view was challenged with the development of DNA labeling of dividing cells using ^3H -thymidine autoradiography in 1960 (Messier and Leblond 1960). Using this technique, Joseph Altman was able to visualize evidence of dividing cells in the young and adult rat brain's neocortex (Altman 1963, Altman 1966), dentate gyrus (Altman and Das 1965), and olfactory bulb (Altman 1969). It was later discovered that these labeled dividing cells in the dentate gyrus and the olfactory bulbs exhibited structural characteristics of neurons using electron microscopy (Kaplan and Hinds 1977, Kaplan and Bell 1984). The idea of a static adult nerve cell population was dealt another blow with studies in canaries showing that the vocal control nuclei of male brains showed increased

neurogenesis during singing season that died off when the season was over (Goldman and Nottebohm 1983, Paton and Nottebohm 1984).

A study done in the rhesus monkey where injection of ^3H -thymidine did not result in the detection of neurogenesis, suggesting that adult neurogenesis did not occur in primates and possibly humans, further clouded the picture and added to the controversy (Rakic 1985). However, the picture of adult neurogenesis became clearer in the 1990s, when Reynolds and Weiss cultured adult mouse brain with growth factors and were able to produce new neurons (Reynolds and Weiss 1992). This report led to renewed enthusiasm in the study of adult neurogenesis and led to the discovery of this process occurring in the adult human brain (Eriksson, Perfilieva et al. 1998). This study by Eriksson et al. involving human cancer patients was a defining point that led to the acceptance of adult neurogenesis as the phenomenon was observed in a highly evolved and complex brain.

1.3 - The Discovery of Neural Stem Cells

At first, the existence of neural stem cells in adults was not proven until 1992 when Reynolds and Weiss published a report describing the isolation and characterization of neural stem cells found in the adult mammalian brain. The in vitro assay allowed researchers to isolate and expand neural stem and progenitor cells. Brain tissue is dissociated into single cells and cultured in serum free media (SFM) containing the growth factors epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). The neural stem cells (NSCs) go on to proliferate and form free-floating, clonally derived cell aggregates called “neurospheres”. Further study in neurospheres

revealed that a single neurosphere is a mixed population of neural stem and progenitor cells with a relative composition of approximately less than 1% stem and more than 99% progenitor cells (Morshead, Benveniste et al. 2002). These neurospheres can be dissociated into single cells and replated in the same culture conditions to form additional secondary neurospheres (Figure 1-1B). Also, these neurospheres can be removed from growth factors and cultured on an adhesive substrate in the presence of differentiation factors such as fetal bovine serum (FBS), leading to the formation of neurons, astrocytes, and oligodendrocytes (Reynolds and Weiss 1992, Weiss, Dunne et al. 1996) (Figure 1-1C). These demonstrate the two cardinal properties of stem cells: stem cell self-renewal and multipotency.

As to whether humans have such cells as seen in rodents, a subsequent study found that neural precursor cells (NPCs) reside in the human hippocampus and can be isolated for study *in vitro* (Roy, Wang et al. 2000). Novel approaches developed in the study of human neurogenesis have also identified NPCs in the human retina that have the ability to form secondary neurospheres, neurons, photoreceptors, and glia, which may enable regenerative repair applications (Mayer, Carter et al. 2005). The presence of these cells in humans opens the door to potential regenerative repair strategies.

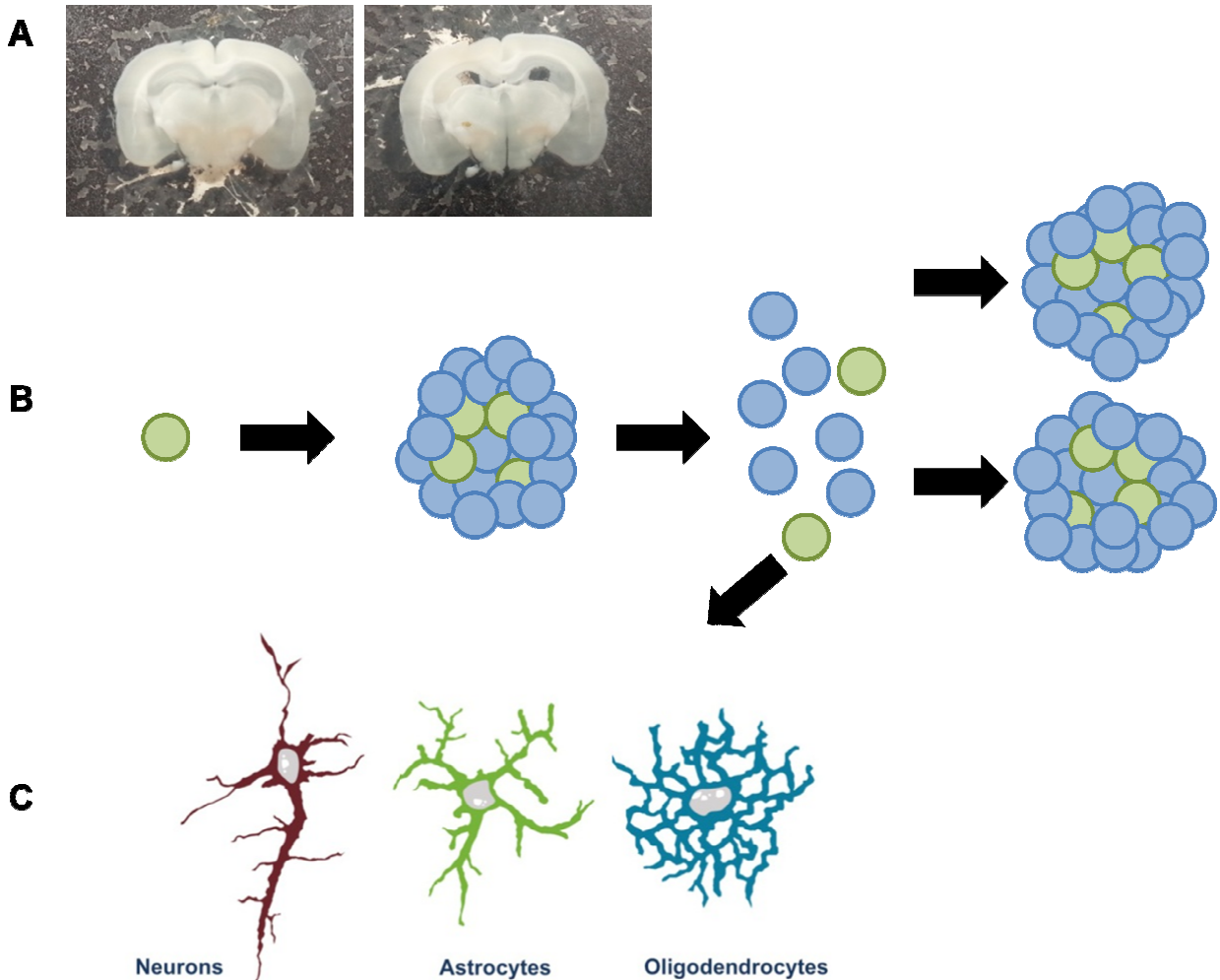


Figure 1-1. The neurosphere assay is used to isolate a population of self-renewing multipotent stem cells from the adult brain. **(A)** Tissue can be isolated from the dentate gyrus by microdissection of brain slices. **(B)** The tissue is dissociated into single cells and grown in serum-free media containing growth factors to form clonally derived neurospheres. **(C)** Neurospheres are able to display multipotentiality by displaying the three main phenotypes of the CNS. Image of phenotypes adapted from (Casarosa, Zasso et al. 2013).

1.4 - Neurogenic regions in the adult brain

Though Reynolds and Weiss isolated stem cells *in vitro*, there remained the question of where exactly these stem cells resided *in vivo*. Subsequent studies have identified the subependymal zone (SEZ) as the region from which Reynolds and Weiss isolated stem cells (Luskin 1993, Morshead, Reynolds et al. 1994, Chiasson, Tropepe et al. 1999). The SEZ is a thin area, two to three cell layers thick, that lines the forebrain lateral ventricles and contains three cell types of the NSC lineage: the NSCs that give rise to transit amplifying precursors and the downstream progenitors, either neuroblasts or glia (Morshead, Reynolds et al. 1994, Doetsch and Alvarez-Buylla 1996). In the SVZ, neural stem cells give rise to precursors that migrate along the rostral migratory stream into the olfactory bulb, where they differentiate and mature into inhibitory interneurons (Doetsch and Alvarez-Buylla 1996). In most mammals, including humans, the SEZ contains the highest population of proliferating cells in the adult brain (Gates, Thomas et al. 1995), with an estimate of 30,000 cells generated bilaterally in the mouse SVZ everyday (Lois and Alvarez-Buylla 1994). However, neurogenesis in the SVZ is not the focus of this thesis, instead focusing on hippocampal neurogenesis.

Another neurogenic region is the dentate gyrus (DG) of the hippocampus (Alvarez-Buylla, Seri et al. 2002). In the DG, new neurons arise from NPCs in the subgranular zone (SGZ) that proliferate and generate neuroblasts that migrate into the overlying granule cell layer where they mature into excitatory neurons (van Praag, Christie et al. 1999, Seri, Garcia-Verdugo et al. 2001). In both neurogenic regions of the brain, many of the newborn cells undergo cell death within their respective lineages. In the DG, 50% of the newborn neurons die under baseline conditions (McDonald and Wojtowicz 2005).

Also, the new cells that differentiate into young neurons represent a very small (less than 0.5%) of the total population of mature neurons but eventually mature in both their functionality and morphology similar to the neurons that had been born during development (Kronenberg, Reuter et al. 2003, Laplagne, Esposito et al. 2006). The process of neurogenesis is described in more detail below.

1.5 - The dentate gyrus and the hippocampus

The dentate gyrus of the hippocampus contains concentric layers comprised of the molecular layer (ML) and the granule cell layer (GCL). The ML contains mostly afferent fibers and dendrites and the GCL is densely packed with the somas of granule cells. These two layers are referred to as the fascia dentate and along with the hilus, which contains mossy cells that receive inputs from granule cells via mossy fibers, make up the dentate gyrus (Blackstad 1958). The dentate gyrus contains mainly excitatory granule projection neurons but there are interneurons as well (Patton and McNaughton 1995). In the hippocampus, there exists neuronal circuitry that is vital to our understanding of hippocampal function. The backbone of this circuitry is the trisynaptic loop consisting of the DG, CA3, and CA1 (Figure 1-2). Impulses enter through the entorhinal cortex (EC) and are propagated by excitatory synapses in DG, CA3 and CA1, back into the EC, completing the circuit (Andersen, Bliss et al. 1971). Co-existing with this excitatory pathway is a rich network of inhibitory interneurons that form feedforward and feedback synaptic connections throughout the hippocampus and within the DG (Gulyas, Hajos et al. 1996).

In the context of hippocampal neurogenesis, integration with the above circuitry is vital for newly born neurons to become functional. Though, the functional significance of adult hippocampal neurogenesis is not fully understood, there is evidence of its role in learning and memory. Studies in rodents correlate adult neurogenesis with learning and memory (Snyder, Hong et al. 2005, Deng, Saxe et al. 2009). Other correlated functions include pattern integration and separation (Aimone, Wiles et al. 2009, Clelland, Choi et al. 2009, Sahay, Scobie et al. 2011), spatial memory (Nilsson, Perfilieva et al. 1999, Snyder, Hong et al. 2005, Luo, Jiang et al. 2007) and contextual learning (Saxe, Battaglia et al. 2006, Wojtowicz, Askew et al. 2008). In rodents, mature adult-born neurons are more likely to become activated in response to learning events over pre-existing neurons that were born during development (Ramirez-Amaya, Marrone et al. 2006, Kee, Teixeira et al. 2007). Thus, adult-born neurons serve an important role in memory encoding and retrieval. Interestingly, ongoing hippocampal neurogenesis can play a role in a decay process that continuously clears memories from the hippocampus (Frankland, Kohler et al. 2013). Frankland et al. suggest that as newborn granule cells integrate into the established hippocampal circuits, they form new input and output connections that may overwrite or redirect existing connections, leading to forgetfulness as a retrieval cue no longer invokes a previously stored pattern. A recent study showed that increasing neurogenesis in adult mice was sufficient to cause the mice to forget and that decreasing neurogenesis during infancy reduced infantile amnesia (Akers, Martinez-Canabal et al. 2014). Thus, in terms of cognitive function, increasing hippocampal neurogenesis is beneficial for learning, but may come at a cost of forgetting and overwriting memories.

Neurogenesis in the hippocampus has been linked to pathological conditions such as epilepsy, stroke, Alzheimer's disease, anxiety disorders, and depression and thus, this process has clinical implications beyond baseline physiological functions. For example, abnormal integration of dentate granule cells is linked to temporal lobe epilepsy, memory dysfunction, and can lead to schizophrenia and autism (Hester and Danzer 2014). Thus, the role of hippocampal neurogenesis extends beyond physiological baseline conditions and further understanding of this process is of strong clinical relevance in a variety of neuropathies.

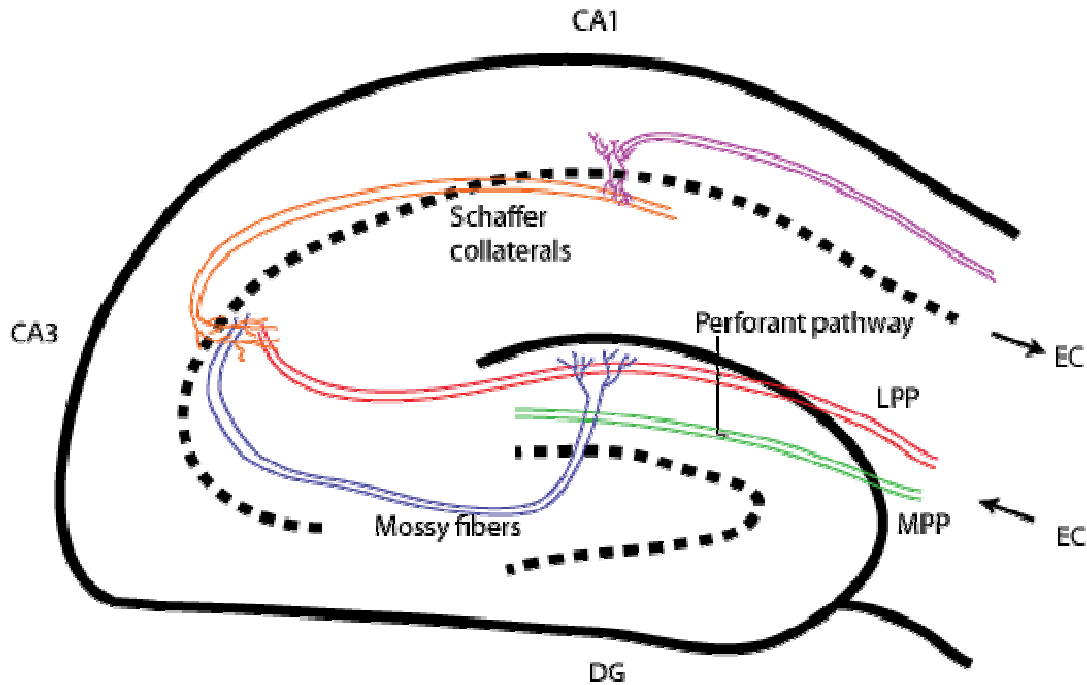


Figure 1-2. The hippocampal neural network. The backbone of this circuitry is the trisynaptic loop consisting of the DG, CA3, and CA1. Impulses enter through the entorhinal cortex (EC) and are propagated by excitatory synapses in DG, CA3 and CA1, back into the EC, completing the circuit. First, impulses from the EC enter the DG via the lateral and medial perforant pathways (LPP and MPP respectively). The DG is connected to CA3 via mossy fibers, CA3 is connected to CA1 via Schaffer collaterals, and CA1 sends projections back into the EC. The CA3 also directly connected to the EC via the LPP.

1.6 - The process of neurogenesis: from stem cells to functional neurons

Neurogenesis in the hippocampus is a process that can take four to seven weeks and begins with a dividing precursor giving rise to daughter cells. The daughter cells differentiate into neurons as they migrate away from the site of division and mature over time. Immature neurons begin extending dendrites into the molecular layer while an axon projects toward CA3. Functional synapses are formed and the new neuron becomes integrated into the hippocampal circuit (Figure 1-3) (Kempermann, Gast et al. 2003).

Neural stem cells in the adult SGZ are sometimes termed type-1 cells (Filippov, Kronenberg et al. 2003) and possess a roughly triangular soma with a long apical process extending through the GCL and ML, eventually branching out into many small processes. NSCs express glial fibrillary acidic protein (GFAP), which is a marker for astrocytes, and nestin (Filippov, Kronenberg et al. 2003). The mode of division of NSCs is asymmetrical, where one NSC divides and results in one NSC and a type-2 daughter cell. The type-2 cell expresses nestin and it is termed a transit-amplifying cell because it is able to divide symmetrically multiple times before differentiating into its terminal fate (Filippov, Kronenberg et al. 2003). These type-2 cells appear with dense nuclei and short processes and do not express GFAP (Filippov, Kronenberg et al. 2003). The terminal fate of these cells can vary and the rate at which these cells reach their terminal fate can vary with the environment of the animal (Dranovsky, Picchini et al. 2011). There is evidence that these NSCs in the SGZ can not only give rise to neurons but also glial cells at lower frequencies (Steiner, Kronenberg et al. 2004). However, it is still not known whether these cell types all arise from a single multipotential stem cell

type or from a heterogeneous pool of progenitors with limited potential. Next, transit amplifying cells give rise to more differentiated type-3 cells, which do not express nestin but do express doublecortin (DCX), a protein associated with microtubules (Kempermann et al., 2004). These type-3 cells also have the capacity to proliferate and thus amplify the number of newborn neurons. This stage is reached within 3 days after the initial division event (Kempermann, Jessberger et al. 2004).

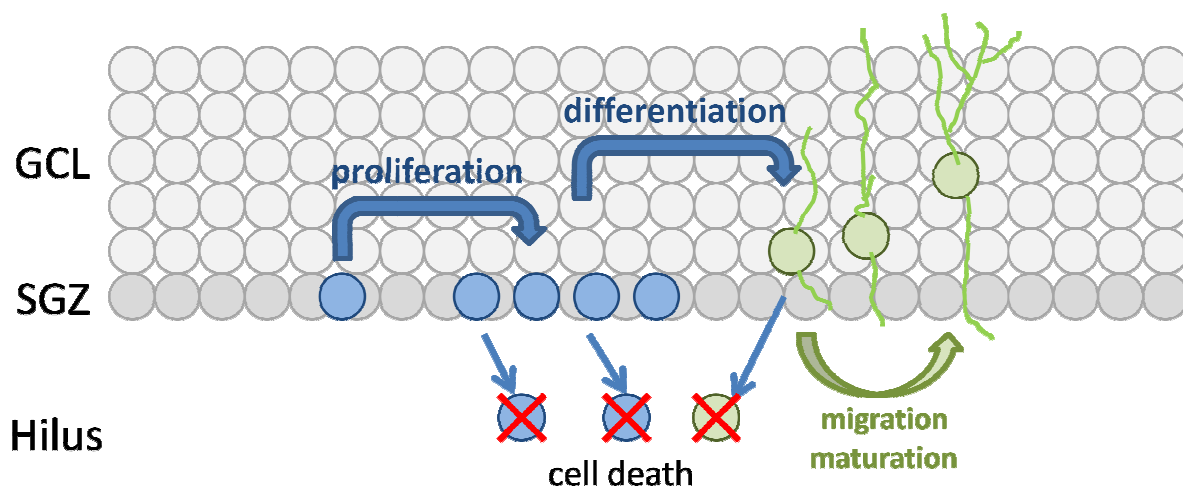


Figure 1-3. The main stages of adult neurogenesis. Neural precursor cells in the subgranular zone (SGZ) proliferate and give rise to daughter cells that either go on to differentiate further into mature neurons or undergo cell death if they are not needed. Surviving cells go on to mature into neurons, which migrate and incorporate themselves in the granular cell layer (GCL). Blue cells represent immature neural precursor cells and green cells represent committed neurons.

The immature neuron stage is postmitotic and characterized by the expression of calretinin, a calcium ion binding protein, and the beginning of NeuN expression (Brandt, Jessberger et al. 2003). Morphologically, the cells each have a rounded or slightly triangular nucleus and a clear apical dendrite accompanied by axonal extension. The axons make contact with CA3 within 3 to 5 days after the initial division event (Hastings and Gould 1999). Dendrites appear within 48 hours and grow about 15 micrometres per day for the first 3 days and nearly double in length at 4 to 5 days (Shapiro, Upadhyaya et al. 2007).

During these initial days of proliferation and amplification, there is a rapid expansion in the number of new cells (Cameron and McKay 2001). However, the population of new cells drops by 50% before stabilizing within one to two weeks (Figure 1-4) (Kempermann, Gast et al. 2003, McDonald and Wojtowicz 2005). This decrease under baseline conditions is possibly due to an inherent selection process in which immature neurons are either chosen to be integrated and function or are eliminated if they remained unused (Biebl, Cooper et al. 2000). This critical period of survival one to two weeks after the initial division event is a period in which interventions such as drug agents can be used to modify the rate of survival above baseline conditions, which can have therapeutic applications that will be discussed in the next section. It may seem that having half the cells die is wasteful; however, the number of new cells produced in the hippocampus is 10000 in young adult male rats (Cameron and McKay 2001) with similar numbers found in humans (Coras, Siebzehnrubl et al. 2010, Knoth, Singec et al. 2010). Compare this number to the total number of granule cells in one rat dentate

gyrus, which is estimated to be one million (Bayer, Yackel et al. 1982), and several thousand cells dying a day may not seem that significant.

Two to three weeks after becoming postmitotic, the new neurons begin expression of calbindin, a calcium ion binding protein that is a marker for all mature granule cells. However, expression of calbindin does not mean the granule cell is fully matured as it takes 4-7 weeks for the new cells to become functionally equivalent to preexisting older granule cells (Biebl, Cooper et al. 2000, van Praag, Schinder et al. 2002). During this period of time, the new neurons must migrate from the SGZ and into the GCL and integrate into the hippocampal circuitry in order to fully mature and become functional (Zhao, Teng et al. 2006). A summary of markers used in the experiments of this thesis has been outlined below (Table 1-1).

Marker	Role
Glial fibrillary acidic protein (GFAP)	Intermediate filament protein expressed in astrocytes (Jacque, Vinner et al. 1978)
β -III-tubulin	Microtubule component expressed only in neurons (Banerjee, Roach et al. 1990)
O4	Antigen expressed on surface of oligodendrocyte precursors (Dubois-Dalcq 1987)
Ki67	Endogenous marker for proliferating cell that is no longer expressed after completing proliferation (Kee, Sivalingam et al. 2002)
Bromodexoyuridine (BrdU)	Thymidine analog that labels S-phase cells and persists in daughter cells (Kee, Sivalingam et al. 2002)
Doublecortin (DCX)	Microtubule-associated protein expressed in immature neurons (Brown, Couillard-Despres et al. 2003)

Table 1-1. Markers used in the experiments of this thesis.

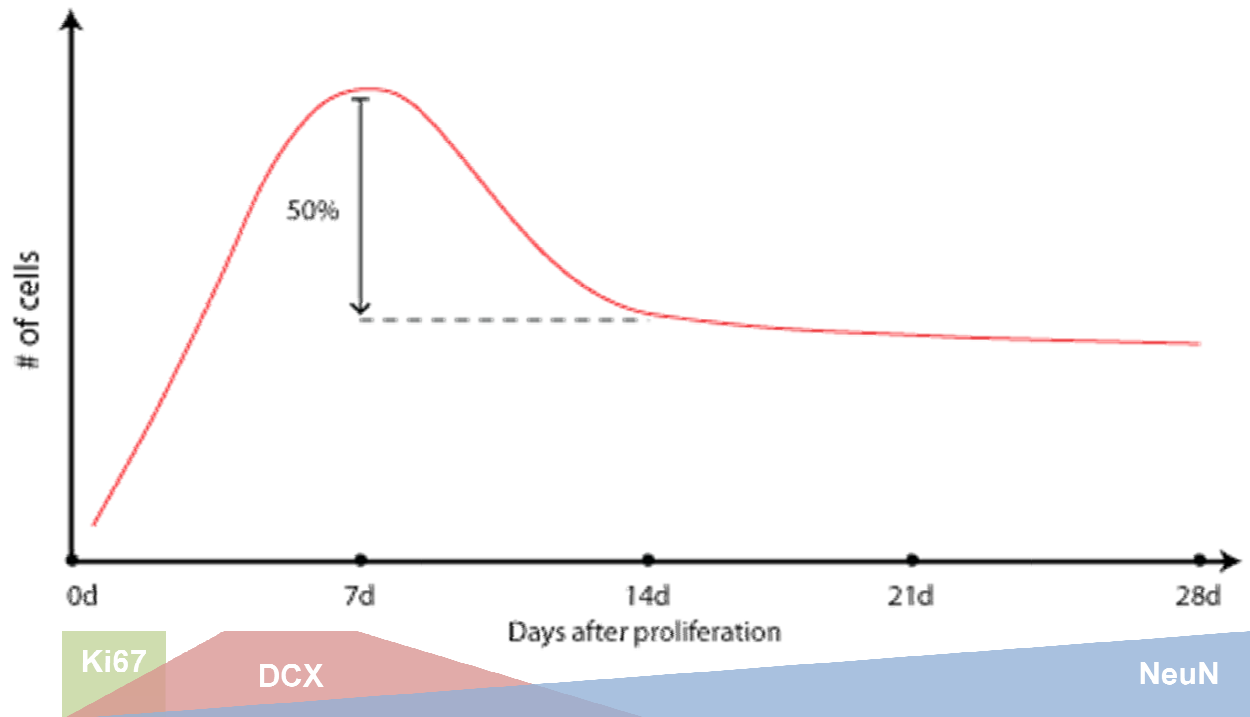


Figure 1-4. Survival curve of newborn cells in the DG and timeline of expression of markers. During the second week after the initial division, approximately half the cells die (Kempermann, Gast et al. 2003, McDonald and Wojtowicz 2005). It is during this critical period that interventions can affect cell survival rate. Ki67 is expressed in proliferating cells. Doublecortin (DCX) is expressed in immature neurons during days 0 to 14, peaking between days 4 and 7. NeuN gradually increases in expression throughout achieves maximum expression after 4 weeks.

1.7 - Hippocampal Neural Stem Cells?

When considering factors that promote endogenous activation of NPCs, one must be cognizant of a number of differences reported in the literature between the NPCs in the two distinct neurogenic regions of the adult brain. Unlike SVZ NPCs, DG colony-

forming cells did not display the properties of multipotency and the ability to self-renew when cultured *in vitro* (Clarke and van der Kooy 2011), unlike what was demonstrated by Reynolds and Weiss (1992) with SVZ neurospheres. Indeed, the vast majority of DG derived colonies produced 100% GFAP+ astrocytes with almost no new neurons forming. Due to their lack of self-renewal and multipotency, this presents the argument that DG colonies are not derived from stem cells. It is possible that the removal of colony-forming cells from their niche *in vivo* is what causes the loss in self-renewal and multipotentiality. Clarke and van der Kooy (2011) investigated this possibility by co-culturing with embryonic brain slices and transplanting adult DG spheres into neonatal mice. They found they could restore the self-renewing properties of the colonies but not their multipotentiality. Thus, they argue that another NPC type in the DG is responsible for the formation of neurons. However, they still could not completely rule out the possible role of the niche due to limitations of recreating the niche environment *in vitro*.

Other studies have suggested that epidermal growth factor (EGF) and basic fibroblast growth factor (FGF2) signaling alone is not sufficient in propagating self-renewing, multipotent cells from the adult rodent hippocampus *in vitro* and that the action of additional signaling pathways such as depolarization, bone morphogenic protein (BMP) signaling, and beta-adrenergic signaling is needed (Bonaguidi, Peng et al. 2008, Walker, White et al. 2008, Jhaveri, Mackay et al. 2010). These studies suggest that self-renewal and multipotency of DG-derived neurospheres cannot be shown using EGF and FGF2 alone, as has been the standard procedure in the neurosphere assay. This suggests that there may be a quiescent pool of stem cells that form neurospheres and they will only do so by activating pathways besides EGF and

FGF2 signaling. It is possible cyclosporine A (CsA) has an effect on this possible separate pool of stem cells. These studies have shown that multipotent stem cells do exist in the hippocampus. In particular, Walker, White et al. (2008) found that culturing in depolarizing levels of potassium chloride generated very large neurospheres over 250µm in diameter containing cells that displayed the cardinal properties of stem cells: self-renewal and multipotentiality. KCl had the opposite effect on the SVZ, generating fewer numbers of spheres, which supports the idea that the spheres generated from the hippocampus are a distinct resident population and not spheres from surrounding regions contaminating the dissection. These conflicting reports on whether the hippocampus contains true stem cells highlight the ongoing controversy in the literature.

1.8 - Potential therapeutic application of adult neural stem cells

The existence of NPCs in the adult brain has lead to much excitement about their potential to repair injuries to the central nervous system (CNS). Neurospheres can be cultured from the hippocampus using the same *in vitro* assay pioneered by Reynolds and Weiss in 1992, with some modifications (Seaberg and van der Kooy 2002, Clarke and van der Kooy 2011). The *in vivo* localization of NPCs and their ability to be isolated *in vitro* lead to two potential cell-based repair strategies: (1) exogenous repair where NPCs are isolated *in vitro* and transplanted into the damaged CNS and (2) endogenous repair which involves activation of resident NPCs to promote tissue repair. Exogenous repair strategies have been performed successfully in other tissue types such as in bone marrow transplantation to treat leukemia (Thomas, Fefer et al. 1977) and in

growing skin to treat burns and ulcers (Mason 2005). There is ongoing research using a combination to decellularized scaffolds or matrixes and tissue-specific stem cells to grow whole organs for organ transplantation and replacement using a patient's own cells, which bypasses the issue of graft rejection. More recently, there have been advances in this area of regenerative medicine as seen in the successful transplantation of tissue manufactured vaginas derived from the patients' own cells, which was necessitated by a congenital defect (Raya-Rivera, Esquiliano et al. 2014). In the context of neurogenesis, the use of exogenous sources of neural stem cells carries advantages in terms of the ability to expand, purify, and modify the cells as needed but this also carries the risks of tissue rejection and possible tumour formation. Exogenous sources of cells also carry challenges in the delivery method and possible surgical complications as many cells that are transplanted do not survive (Cooke, Vulic et al. 2010).

Using endogenous NPCs avoids the above issues and by promoting an individual's own cells to perform repair since resident NPCs have the natural capacity to proliferate, migrate, differentiate, and integrate into the existing neural circuitry. NPCs have been shown to activate in response to injury or disease. For example, epilepsy and stroke can cause NPCs to proliferate and migrate to the site of injury where they differentiate into neurons and glia to repair damage (Arvidsson, Collin et al. 2002, Zhang, Zhang et al. 2004, Jessberger, Zhao et al. 2007). In patients with Alzheimer's disease, Parkinson's disease, or Huntington's disease, NPCs exhibit decreased proliferation and survival of newborn neurons (Winner, Melrose et al. 2011). Thus, understanding and manipulating endogenous NPCs can lead to insights into treating or at least

ameliorating the symptoms of such neurodegenerative diseases. Indeed, a positive correlation between promoting endogenous neurogenesis in the hippocampus and cognitive function recovery has been shown by intraventricular infusion of S100B, a neurotrophic protein secreted by astrocytes, and subsequent cognitive tests using the Morris water maze (Kleindienst, McGinn et al. 2005). More recently, Metformin, a drug used to great type II diabetes, has also been shown to promote neurogenesis in the hippocampus and improve behaviour in the Morris water maze (Wang, Gallagher et al. 2012)

1.9 - Regulating neurogenesis

i. Physical exercise and learning

Physical activity is well-known to have beneficial effects on one's cardiovascular system and overall wellbeing. In addition to this, voluntary exercise has been shown to enhance cognitive function in humans as well as rodents (van Praag, Shubert et al. 2005, Winter, Breitenstein et al. 2007, Creer, Romberg et al. 2010). In humans, exercise has been linked to improved learning (Winter, Breitenstein et al. 2007) and in rodents, improved spatial memory in a Y-maze test has been shown after 14 days of wheel running (Van der Borgh, Havekes et al. 2007). Specifically, exercise increases cell proliferation and neurogenesis in the granular cell layer of the dentate gyrus and can have a neuroprotective effect against traumatic brain injury (Jacotte-Simancas, Costa-Miserachs et al. 2014). Morphologically, exercise enhances the density of mushroom spines and decreases spine motility in newly-born cells (Zhao, Teng et al.

2006). Taken together, these findings support the benefits of exercise in neurogenesis as well as demonstrating how exercise affects the process in both quantitative and qualitative ways.

In addition to physical exercise, mental exercise, or learning, can enhance the survival of hippocampal neurons that are born throughout adulthood. As mentioned earlier, most of these adult-born neurons do not survive past the first few weeks of their life. Studies show that cells that are already born and are present during the learning tasks are more likely to survive than the cells that are in animals that are not undergoing learning exercises. Associative learning tasks such as trace conditioned eyeblink response along with spatial recognition tasks such as the Morris water maze have shown improvements in cell survival (Gould, Beylin et al. 1999, Shors 2008). Other tasks such as social transmission of food preference or skilled reaching tasks can also increase the cell survival rate (Olariu, Cleaver et al. 2005, Wurm, Keiner et al. 2007). It is important to note that the training experience in these tasks is not responsible for the increase in cell survival per se but rather it is the actual learning that takes place during the training that is crucial. The distinction between learning and training has been observed in both trace conditioning and spatial recognition studies. Animals that successfully acquired the conditioned response show an increase in cell survival while animals that do not acquire the condition response do not display such increase (Sisti, Glass et al. 2007, Curlik and Shors 2011). Because the effects of exercise and learning on cell survival are seen in newly born neurons, these new cells have not yet had time to integrate into the existing neural circuitry. Thus, the cell survival effects may be an

indirect result of activity of neighbouring mature neurons that could be releasing various trophic factors and neurotransmitters.

ii. Proliferation and survival factors

One neurotrophic factor that is most commonly mentioned in regard to adult neurogenesis is brain derived neurotrophic factor (BDNF). BDNF is active in the hippocampus, cerebral cortex, and basal forebrain, which are areas vital to learning memory, and higher order thinking (Yamada and Nabeshima 2003). It is also vital for long-term memory (Bekinschtein, Cammarota et al. 2008). BDNF is essential for proper brain development and sensory function and mice born without the ability to make BDNF die soon after birth (Ernfors, Kucera et al. 1995). Increasing BDNF has a positive effect on adult neurogenesis, which was observed with direct intra-hippocampal infusion (Scharfman, Goodman et al. 2005). It has been shown to be released in response to stimuli that promote neurogenesis and is necessary for the effect to be seen (Rossi, Angelucci et al. 2006, Boehme, Gil-Mohapel et al. 2011). Thus, BDNF is an important factor mediating adult neurogenesis.

Another factor of interest is insulin-like growth factor (IGF-1). Like BDNF, IGF-1 expression is also increased as a result of exercise and correlates to an increase in neurogenesis (Nakajima, Ohsawa et al. 2010). IGF-1 is produced primarily in the liver as an endocrine hormone as well as in target tissues in a paracrine fashion. It is produced in response to growth hormone (GH) and thus, IGF-1 mediates systemic body growth and has growth-promoting effects on almost every cell type in the body, including neurons. It has been shown that IGF-1 declines with age and this correlates with a decrease in adult neurogenesis in the hippocampus (Shetty, Hattiangady et al.

2005). This decline in neurogenesis can be abated somewhat by infusion of IGF-1 in aged animals, which also reduces age-related cognitive decline (Lichtenwalner, Forbes et al. 2001).

How learning rescues new neurons may be due to gamma-aminobutyric acid (GABA). GABA is a neurotransmitter that is traditionally thought to have an inhibitory effect. Over 20% of all neuronal synapses use GABA as the primary neurotransmitter. However, GABA depolarizes immature adult-born neurons (Overstreet Wadiche, Bromberg et al. 2005) and this depolarization can lead to the formation of new synapses by these new neurons (Ge, Goh et al. 2006). Thus, the act of learning can induce changes in GABAergic activity.

iii. Promoting neurogenesis

Besides promoting the proliferation and survival of NPCs, another strategy involves manipulating and directing a larger proportion of the NPC population to differentiate into the neural lineage. This strategy often involves the manipulation of inherent signaling pathways that dictate whether the cell undergoes self-renewal or differentiation. In the hippocampus, the Wnt/ β -catenin pathway plays a role in many developmental processes such as neuronal maturation, migration, connectivity, and synaptic formation. Using a BAT-gal reporter mouse expressing a β -catenin-activated transgene, it was found that this pathway was specifically active in the granule cell layer in the adult hippocampus (Lie, Colamarino et al. 2005). Wnt signaling has been shown to have different effects on neurogenesis in a stage specific manner (Hirabayashi, Itoh et al. 2004). During early neurogenesis, the Wnt pathway promotes self-renewal and

maintains the NPC pool (Machon, Backman et al. 2007) but in mid to late neurogenesis, it induces progenitors in this stage to further differentiate into neurons (Munji, Choe et al. 2011). Also, adult hippocampal astrocytes express Wnt-3 and this has been shown to induce progenitors to differentiate into neurons (Lie, Colamarino et al. 2005). Going further into Wnt signaling is beyond the scope of this thesis but the complexity, interplay, and temporal differences in the Wnt signaling pathways make them difficult to manipulate for a specific effect, such as only promoting neurogenesis. However, it is important to not discount the role this signaling pathway plays in the adult hippocampus in regard to neurogenesis.

Another study identified the aPKC-CBP pathway as essential for the normal generation of neurons from embryonic NPCs (Wang, Weaver et al. 2010, Wang, Gallagher et al. 2012). By identifying this pathway, it was found that metformin, a widely used diabetes drug, activates this pathway in the liver through AMP kinase (He, Sabet et al. 2009). Interestingly, metformin was shown to have an effect in adult rodents by increasing neurogenesis in the hippocampus through the aPKC-CBP pathway, which subsequently led to an improvement in cognitive performance in the water maze task (Wang, Gallagher et al. 2012).

iv. Pharmacological agents

Many substances have been discovered to have effects on adult neurogenesis. One class in particular that has been extensively researched in the field is the class of antidepressants. Adult hippocampal neurogenesis has been linked to depression (Sahay and Hen 2007). Though it has not been implicated as a primary cause, it may be required for antidepressants to affect one's behavior because the time course of antidepressants coincides with the time course of maturing neurons (Sahay and Hen 2007). Because of this time course, antidepressant treatment requires chronic usage over time courses of months. Nonetheless, many antidepressants, such as tranylcypromine, imipramine, and fluoxetine, have been shown to enhance hippocampal neurogenesis by improving both cell survival and proliferation (Chen, Pandey et al. 2006, Kim, Kim et al. 2013). More recently, because of their observed effects in the hippocampus, antidepressants are being studied to treat Alzheimer's disease, a neurodegenerative disease. Recent findings show that antidepressants can upregulate BDNF, thus promoting neurogenesis, and reduce toxic β -amyloid peptides (Kim, Kim et al. 2013). Though antidepressants are not the focus of this thesis, their potential applications in treating neurodegenerative disease highlight the importance of adult hippocampal neurogenesis.

1.10 - Cyclosporin A: Discovery and characterization

A drug that has been shown to have a positive direct effect on the survival of NPCs is cyclosporine A (CsA). CsA is a small 11-member cyclic polypeptide discovered in the early 1970s in Basel, Switzerland by workers of Sandoz Limited. They were originally

screening for drugs with potential antifungal and/or antibiotic activity (Dreyfuss, Härri et al. 1976). From soil sample analysis, CsA is a metabolic product of *Tolypocladium inflantum* with low toxicity in mice. When administered in mice, it was discovered that CsA selectively suppressed antibody and T-lymphocyte mediated immune responses while sparing haemopoietic tissues (Borel, Feurer et al. 1977). This was viewed as a breakthrough in immunopharmacology because prior to CsA, many agents that were used to suppress the immune system were also used to treat cancer patients, and thus, such agents were cytostatic, cytotoxic, and carried harmful side effects (Borel, Di Padova et al. 1990). CsA can be administered orally or intravenously and is absorbed via the small intestines, albeit incompletely. Once CsA is in the blood circulation, it is bound to erythrocytes and leukocytes, with a stronger preference for the former. CsA is metabolized by the liver and excreted through the bile (Khan 2008).

In the cell, CsA binds to cyclophilin A, a cytosolic receptor. The resulting CsA-cyclophilin A complex then binds to the calmodulin-stimulated protein phosphatase calcineurin. This inhibits calcineurin's ability to perform its serine-threonine phosphatase activity, resulting in the translocation of nuclear factor of activated T cells (NFAT) transcription factors into the nucleus of activated T cells. The NFAT family is involved in the activation of genes responsible for the expression of interleukin-2, interleukin-4, and CD40L. Therefore, by inhibiting NFAT via CsA, production of interleukin by the T-cell is also inhibited (Nomura, Inamura et al. 1994). Because of CsA's T-cell immunosuppressive properties, CsA is currently well established in clinical practice for many T-lymphocyte mediated applications, especially in the area of liver, heart, and kidney transplants to prevent graft rejection (White and Calne 1982). In

addition, the drug is also used in rheumatic, dermatologic, gastrointestinal, and ophthalmologic conditions. For example, CsA has been used to treat psoriasis, pyoderma, gangrenosum, vasculitis, scleroderma, and eczemas (Callen 2001, Berth-Jones 2005). CsA has also been used to treat ulcerative colitis (Hanauer 2004) and reduces symptoms in patients with dry eye disease (Baiza-Duran, Medrano-Palafox et al. 2010).

By blocking calcineurin, other pathways that are not involved in immunosuppression are also affected. One pathway involves neuronal nitric oxide synthase (nNOS). Inhibition of calcineurin keeps nNOS in its inactive desphosphorylated state, which reduces nitric oxide radical production (Heales, Bolanos et al. 1999). Reduced levels of nitric oxide radicals leads to reduced levels of peroxynitrite, which is a reactive oxygen species known to rupture cell membranes and cause cell death (Deng, Thompson et al. 2007). Another pathway involves the Bcl-2 associated death (BAD) protein. BAD promotes apoptosis by disrupting normal mitochondrial function and by binding with and blocking Bcl-xL, a protein with pro-survival functions (Kaminska, Gaweda-Walerych et al. 2004). Calcineurin's role in this pathway is to dephosphorylate BAD, which allows it to translocate to the mitochondria. Thus, survival may be enhanced by blocking calcineurin from dephosphorylating BAD, which prevents BAD from translocating to the mitochondria.

Other cyclophilins have been discovered, such as cyclophilin B, C, D, E, G, H, and 40, with cyclophilin D being involved in neuroprotection (Snyder, Sabatini et al. 1998). Cyclophilin A is the most abundant and has the highest affinity for CsA. Cyclophilin D is of interest due to its containing a mitochondrial targeting pre-sequence that is cleaved

once the protein has translocated into the mitochondrial matrix, where it becomes a component of the mitochondrial permeability transition pore (MPTP). As a component of the MPTP, cyclophilin D is involved in the regulation of the release of molecules from the mitochondrial matrix (Bernardi, Broekemeier et al. 1994). Therefore, CsA can act through cyclophilin D to have an effect on cell survival and cell death as the release of mitochondrial molecules is often the prelude to apoptosis.

The MPTP plays a critical role in ischemic neuronal cell death (Schinzel, Takeuchi et al. 2005). Studies on mice that lack cyclophilin D expression have shown decreased MPTP formation and also that the mice develop smaller infarcts in a stroke model than their cyclophilin D expressing counterparts (Schinzel, Takeuchi et al. 2005). However, there are challenges in manifesting CsA's neuroprotective effects through cyclophilin D in the brain. First, the brain has about a 20-fold greater biodistribution for CsA compared to T-cells (Goldner and Patrick 1996) and thus requires a significantly high and chronic administration of the drug before neuroprotective effects are observed. Such high doses lead to undesired side effects such as nephrotoxicity and hepatotoxicity. Second, CsA has a limited ability to cross the blood brain barrier (BBB). Thus, there is a strong need to isolate the desired neuroprotective actions of CsA from its toxic immunosuppressive effects and several CsA analogs like NIM811, FR901459, and UNIL025 with more specific mechanisms of action under investigation (Hansson, Mattiasson et al. 2004). There has been progress in isolating the mechanisms of CsA. It is recently reported that CsA acts through this MPTP pathway to enhance cell survival in SVZ neurospheres (Sachewsky, Hunt et al. 2014). This was elucidated by using two drug analogs that act through either the calcineurin-independent or calcineurin-

dependent pathway, NIM811 and FK506 respectively. NIM811 was the analog found to have similar effects to CsA on cell survival, thereby showing that CsA acts through the calcineurin-independent MPTP pathway (Figure 1-5).

In regard to NPCs, *in vivo* and *in vitro* assays have demonstrated that CsA enhances NPC survival. In particular, Hunt, Cheng et al. (2010) have showed that when NPCs are exposed to CsA *in vitro*, increased numbers and size of forebrain SVZ-derived neurospheres are seen, with no concomitant effect on their differentiation profile or proliferation kinetics. Hunt, Cheng et al. (2010) also demonstrated that intraventricular or systemic delivery of CsA to adult mice leads to an increase in the number of neurospheres and the number of proliferating NPCs in tissue slices as revealed by bromodeoxyuridine (BrdU) labeling. BrdU is a thymidine analog with a short *in vivo* bioavailability of a few hours that is incorporated during S-phase (Kempermann 2006). Because an experimenter can control the dosage of BrdU, and it only stays for a few hours, it allows one to measure newborn cells over a chosen short period of time. Thus, based on these experiments, CsA has a survival effect both *in vitro* and *in vivo* on adult mice NPCs.

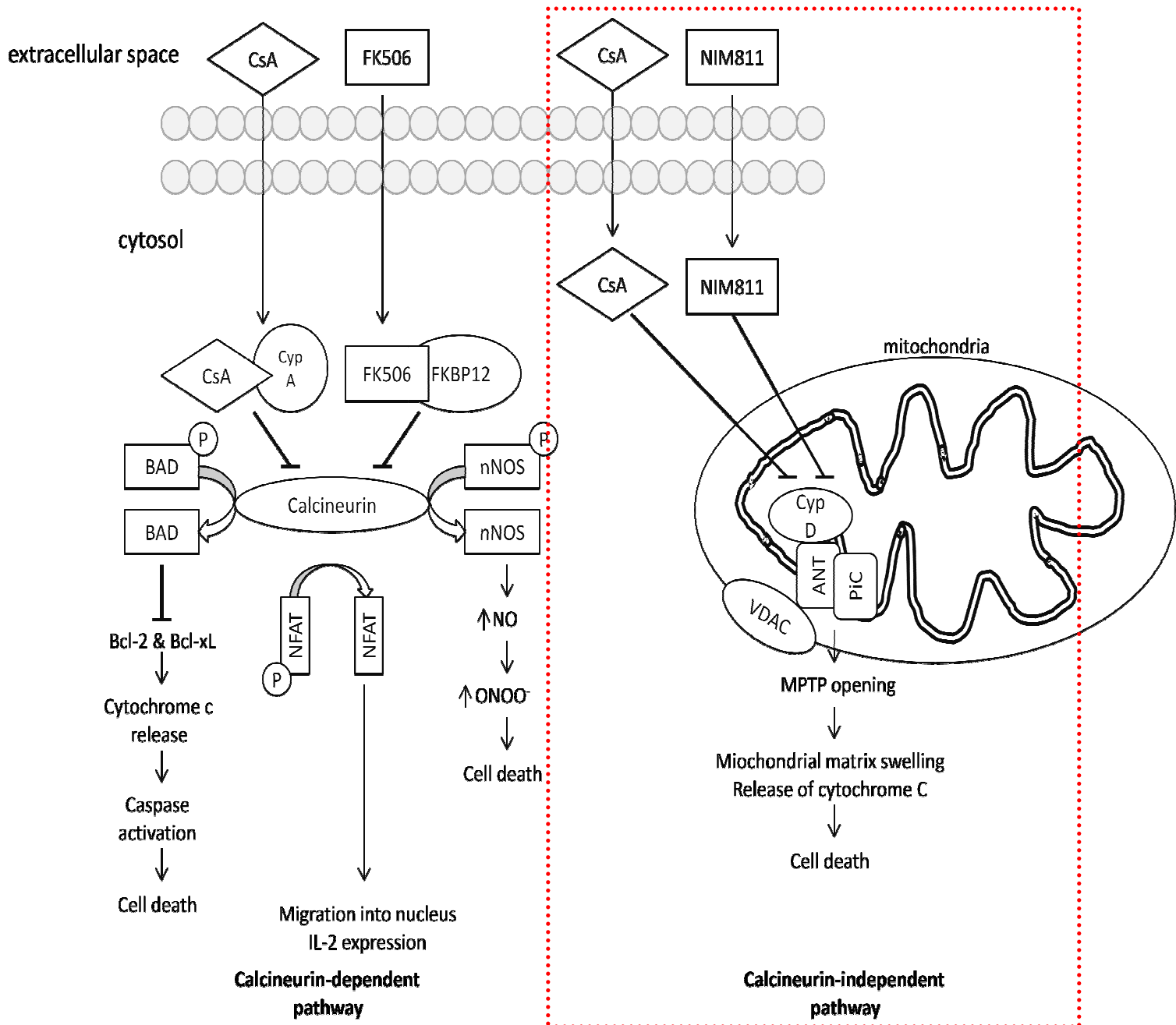


Figure 1-5. The mechanism of action for CsA. CsA acts through the calcineurin-independent pathway (red box) and promotes cell survival by inhibiting the opening of the mitochondria permeability transition pore (MPTP), which is an event that leads to apoptosis. This mechanism was demonstrated in NPCs derived from the subependyma. Thus it is not known if CsA acts through the same mechanism for DG-derived NPCs.

1.11 - Research Hypothesis and Objectives

Given the established role of the hippocampus in learning and memory, enhancing cell survival within this region could potentially enhance cognitive function in the uninjured brain and/or injured brains. We hypothesize that CsA will promote survival of the DG NPCs and subsequently lead to an increase in neurogenesis.

Research Objectives:

- i) *To examine the effects of CsA on neural precursor cells following administration in vitro and in vivo.* We predict that CsA administration will increase the numbers of neurospheres isolated from the adult mouse DG.
- ii) *To examine the effects of CsA on the numbers of progenitors and new born neurons following administration in vivo.* We predict that CsA administration in vivo will increase the numbers of proliferating NPCs and new born neurons in the DG.

Chapter 2 - Materials and Methods

2.1 - Neurosphere assay

Adult DG neurospheres were derived from 2 to 4 month old male CD1 mice (Charles River, Wilmington, MA, www.criver.com). Experimental procedures were performed in accordance with the Guide to Care and Use of Experimental Animals and approved by the Animal Care Committee at the University of Toronto.

Mice were sacrificed by cervical dislocation. Mouse brains were embedded in 2% low-gelling temperature agarose (Sigma-Aldrich, St Louis, MO, www.sigmaaldrich.com) for 20 minutes at 4 °C. The embedded brain was then sectioned on a Vibratome into 300-500µm thick slices. The DG was removed under a dissecting microscope (Zeiss, Jana, Germany, www.zeiss.com). Both the brain slices and the DG tissue was kept in artificial cerebrospinal fluid (aCSF) containing 124 mM NaCl, 5mM KCl, 1.3 mM MgCl₂, 2 mM CaCl₂, 26 mM NaHCO₃, and 10 mM D-glucose. DG tissue was then dissociated using trypsin 13,000U/10mL, hyaluronidase 1,300 U/10mL, and kynurenic acid 1.33mg/10mL in aCSF consisting of a higher concentration of Mg²⁺ (3.2 mM MgCl₂) and lower Ca²⁺ (0.1 mM CaCl₂) (hi-lo aCSF) for 25 minutes at 37 °C in Falcon tubes that were gently rocked during this time. After incubation and rocking, the tissue was spun down in a centrifuge and the trypsin enzyme solution was removed. The tissue was then resuspended in serum free medium (SFM) with 0.67 mg/mL trypsin inhibitor (Roche Diagnostics) to inhibit trypsin enzyme activity. The DG tissue was then triturated using a small bore glass pipette. Cells were washed and resuspended in serum-free media (SFM) supplemented with epidermal growth factor

(EGF) (20 ng/mL; Sigma-Aldrich), basic fibroblast growth factor (bFGF) (10 ng/mL; Sigma-Aldrich), heparin (7.35 ng/mL; Sigma-Aldrich), B27 (1X; Invitrogen), and 1% penicillin/streptomycin (Invitrogen). Cell viability was assayed using Trypan blue exclusion before live cells were plated at clonal density of 10 cells per μ L in uncoated 24-well plates (Nunclon, Rochester, NY, www.nuncbrand.com). A stock solution of cyclosporin A (0.2mg/mL; Bioshop Canada) was made by dissolving solid cyclosporin A in an anhydrous ethyl alcohol:growth factor-supplemented SFM solution in a 1:1 ratio. Subsequently this solution was added to the cultures at various concentrations. A separate 1:1 anhydrous ethyl alcohol:growth factor-supplemented SFM solution was made without CsA for control conditions. Neurospheres were quantified after 7 days of undisturbed culture. Neurospheres of at least 50 μ m in diameter were counted based on previous morphologies reported (Clarke and van der Kooy 2011).

For long term experiments, cell cultures were bulk passaged every seven days in the continued presence or continued absence of 100ng/mL CsA. For example, a cell culture that was exposed to CsA in the primary culture was exposed to CsA in the subsequent first, second, and third passages. Cell cultures that did not receive CsA in the primary culture did not receive any CsA in the first, second, and third passages. In differentiation assays, single neurospheres were picked from each condition and plated onto laminin-coated wells in the presence of 10% FBS for 7d with or without 100 ng/mL CsA, depending on the prior condition in which they were exposed. Hence, spheres grown in the presence of CsA were also differentiated in the presence of CsA and spheres grown without CsA were differentiated without CsA.

For human DG tissue, the protocol was based on the aforementioned protocol for mouse but with some modifications. The dentate gyrus was carefully dissociated from the hippocampus using a blade and samples were brought to the clean lab in ice-cold aCSF and processed within 1 hour after surgery. Tissue was incubated with a solution of trypsin and hyaluronidase at for 45 minutes at 37°C and then treated with DNase (1000 U, Sigma) to digest DNA. The obtained suspension was filtered through a nylon mesh filter (70 µm, Falcon). After centrifugation, the pellet was resuspended in trypsin inhibitor solution and centrifuged again. The cells were then resuspended in SFM containing 20ng/mL of both EGF and bFGF and 7.35ng/mL of heparin. From this solution, cells were plated at clonal density into 24-well plates containing the SFM, mitogens, and CsA at concentrations of 0, 50, 100, 300, 500, and 1000 ng/mL. Half of the media was changed once a week for four weeks. After four weeks, the number of colonies over 50 µm in diameter was quantified.

2.2 - In vivo studies

Adult male CD1 mice (2 to 4 months, 25-30g; Charles River) were immunosuppressed with a daily dose of CsA (15mg/kg/day) or vehicle delivered subcutaneously via an osmotic minipump (Alzet Osmotic Pumps). CsA or vehicle was delivered at a rate of 0.1 µL per hour for 7 days. Animals were housed in the University of Toronto animal facilities and maintained in accordance with the institutional guidelines. Neurospheres from these animals were isolated by the above-described dissection and quantified after 7 days in culture.

A separate group of mice implanted with CsA or vehicle pumps received three BrdU injections (one every 2 hours at 60 mg/kg) and were killed 2 hours after the last injection. Mice were sacrificed in a carbon dioxide chamber and were perfused transcardially with 4°C sterile 1X phosphate buffered saline (PBS) followed by 4°C 4% (in sterile 1X PBS) paraformaldehyde (PFA). Brains were post-fixed overnight in 4°C 4% PFA before being removed and cryoprotected in 20% sucrose (Sigma-Aldrich) at 4°C for cryoprotection. The brains were then coronally cryosectioned at 18µm at -20°C and mounted on Superfrost slides (Fisher Scientific) for immunohistochemistry. Approximately every 5th section from the hippocampal region was collected on a single glass slide with each slide consisting of at least 5 sections. The hippocampal region typically spanned 5 slides. Another group of animals received seven BrdU injections (once per day at 60mg/kg) prior to having CsA or vehicle pumps inserted subcutaneously that delivered CsA (15mg/kg/day) or vehicle for another seven days. These animals were also perfused and cryosectioned as described above. All animals were housed in the University of Toronto animal facilities and maintained in accordance with institutional guidelines.

2.3 - Immunohistochemistry

Neurospheres were fixed in 4% paraformaldehyde for 20 minutes at room temperature and washed (all washes are performed three times, five minutes per rinse using 1X Stockholm's PBS). Frozen tissue sections were thawed to room temperature and re-hydrated with 1X PBS. Both neurosphere and tissue sections were permeabilized using 0.3% Triton X-100 (Sigma-Aldrich) for 20 minutes, washed, and blocked with either 10% normal goat serum (NGS; Jackson Laboratories) or 10%

normal donkey serum depending on the host animal of the antibodies. Blocking was done for 1 hour at room temperature. Antibodies were diluted 1X Stockholm's PBS plus 10% of the appropriate serum. Fixed neurospheres were incubated with primary antibodies overnight at 4 °C, washed, and incubated with an appropriate secondary antibody for 1 hour at 37 °C, washed, and fluorescently imaged using an Axiovert 200 (Carl Zeiss). Differentiated neurospheres were stained for O4 (mouse monoclonal IgM, 1:1000, R&D Systems), β III-tubulin (rabbit polyclonal IgG, 1:1000, Covance), and GFAP (mouse, 1:500, Sigma). Tissue sections were labeled for Ki67 (rabbit polyclonal IgG, 1:200; Abcam), and DCX (goat polyclonal IgG, 1:200; Santa Cruz Biotechnology). The secondary antibodies used were: Alexa Fluor 647 goat anti-mouse IgG; Alexa Fluor 568 goat anti-mouse IgM; Alexa Fluor 488 goat anti-rabbit IgG; Alexa Fluor donkey anti-goat IgG (all 1:400; Invitrogen). Cell nuclei were counterstained with Hoechst 33342 nuclear stain (0.725 μ g/mL; Invitrogen) for 10 min at 37 °C and washed. For BrdU labeling, brain sections mounted on slides were treated with 1N hydrochloric acid (Sigma-Aldrich) for 20 minutes at 65 °C, washed, and incubated with BrdU antibody (rat monoclonal IgG, 1:100; Abcam) overnight at 4 °C. Slides were washed and incubated with Alexa Fluor 488 goat anti-rat IgG for 2 hours at 37 °C, washed, and then fluorescently imaged.

2.4 - Statistical analysis

Statistical analysis was performed using the Student's *t* test for two-group comparisons and ANOVA for multiple group comparisons (Graphpad Prism software). All data are reported as means $\pm$ SEM unless otherwise stated. **p* < 0.05, ***p* < 0.01, *** *p* < 0.001.

Chapter 3 - Results

3.1 - Cyclosporin A promotes neurosphere numbers but not size

Neural precursor cells can be isolated *in vitro* when cultured in the presence of growth factors where they form free-floating, clonally derived colonies called “neurospheres” (Reynolds and Weiss, 1992). Individual neurospheres are derived from stem cells when plated at clonal densities (Coles-Takabe et al., 2008). Neurospheres can be cultured from the hippocampus using the same *in vitro* assay (Seaberg and van der Kooy, 2002; Clarke and van der Kooy, 2011).

In previous studies by Hunt et al. (2010), CsA was shown to increase both the number and size of spheres in the neurosphere assay. In the neurosphere assay, because we know that neurospheres are comprised of few neural stem cells and mostly downstream neural progenitor cells, the number of neurospheres is indicative of the number of stem cells while the size of the sphere is indicative of the number of progenitor cells. We predict that CsA will have positive effects to the number and size of NPCs derived from the DG using the neurosphere assay, similar to what was observed by Hunt et al. (2010) in the subependyma (SE). Adult primary hippocampal tissue was carefully dissected to ensure that SE NPCs found in the walls of the lateral ventricles were not included in the dissection (Figure 1-1A). Cells were triturated and plated at clonal density (10 cells/uL) in the neurosphere assay in a range of CsA concentrations (range 0 to 1000 ng/ml). The numbers of neurospheres were counted after 7 days *in vitro*. At a concentration of 100ng/mL of CsA, we observed a significant 1.9-fold increase in the number of neurospheres (1.0 ± 0.14 neurospheres vs. $0.54 \pm$

0.086 per 5000 cells; in 100 ng/mL CsA vs. control respectively; $p < 0.05$) (Figure 3-1B). The average size of the neurosphere was unchanged in the presence or absence of CsA (Control 0 ng/mL: $61.3 \pm 12.3 \mu\text{m}$; 50ng/mL: 67.7 ± 11.5 ; 100ng/mL: $66.1 \pm 8.10 \mu\text{m}$; 500ng/mL: $85.7 \pm 10.6 \mu\text{m}$; 1000ng/mL: $71.7 \pm 3.27 \mu\text{m}$; $p > 0.05$) suggesting that the proliferation kinetics of the NPCs forming the neurospheres was not altered (Figure 3-1C).

Another possibility is that because neural stem cells, the founder cells for the neurospheres, likely comprise a much smaller portion of the neurosphere, the size of the sphere may not be sensitive enough to see changes in that fraction. To test this possibility, we passaged neurospheres to examine the effects of CsA on the numbers of neural stem cells within the neurospheres. Since neural stem cells undergo symmetric divisions within a growing clonally derived neurosphere, we predicted that if CsA was enhancing their survival, then we should observe an increase in the number of neurospheres with passaging in the continued presence of CsA. Neurospheres from 0ng/ml and 100ng/ml conditions were collected and bulk passaged in the absence or presence of CsA (100ng/ml) at clonal densities for 3 consecutive passages. We found the number of neurospheres in CsA conditions expanded significantly over control resulting in a 3.46-fold increase in the number of neurospheres at P3 compared to P1 (" 99.5 ± 42.1 N=7" in P3 vs. " 28.7 ± 5.65 N=9" in P1, $*p < 0.05$) (Figure 1-1D). Without CsA, the expansion between P3 to P1 was not significant. Again, we observed no change in the size of the neurospheres at each consecutive passage, suggesting that CsA is promoting symmetric division and survival of neural stem cells and not the downstream progeny.

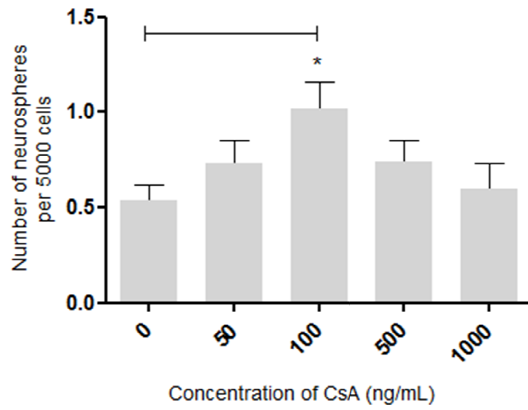
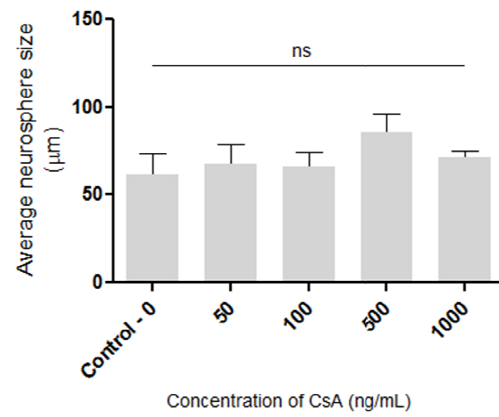
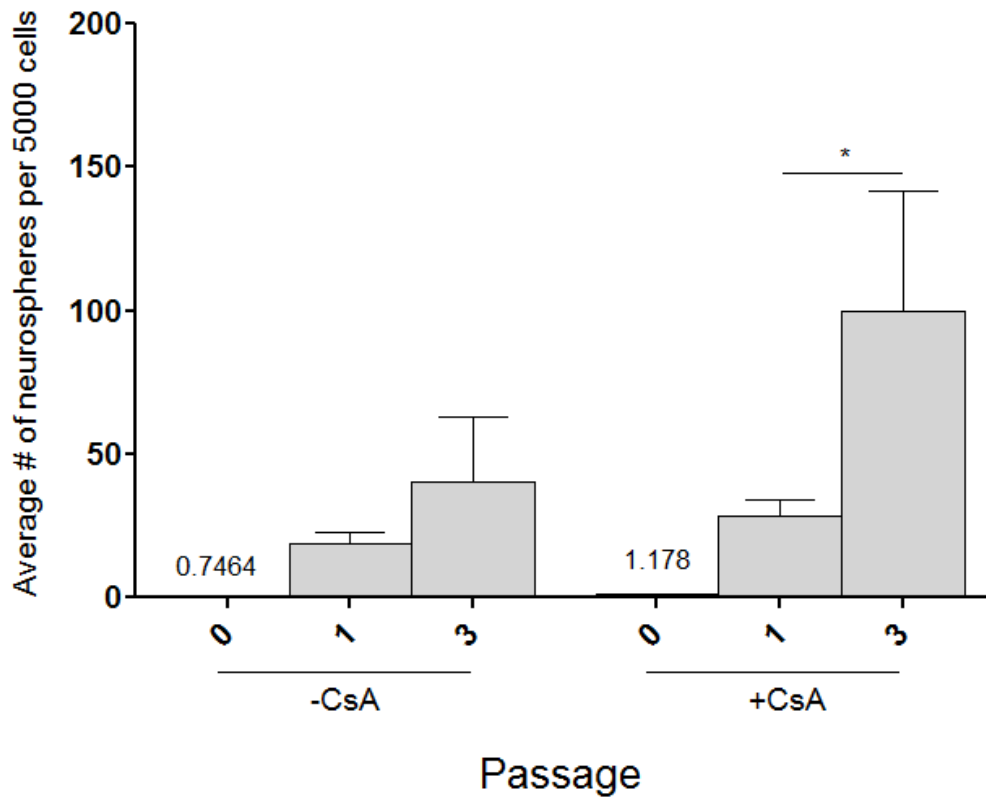
A**B****C****D**

Figure 3-1. Cyclosporin A increased neurosphere numbers but not size. **(A)**

representative images of the dissection of the dentate gyrus from adult mouse brain

tissue slice 500µm in thickness. **(B)** Cyclosporin A was added to dentate gyrus-derived

cells at doses ranging from 0 to 1000 ng/mL, resulting in a 1.9-fold increase in

neurosphere number per 5000 cells plated (1.0 ± 0.14 neurospheres vs. 0.53 ± 0.086

per 5000 cells; in 100 ng/mL CsA vs. control respectively; $F_{4,30}=0.89$, $p < 0.05$, in 1-way

ANOVA, $n=22$, 4 wells per trial). **(C)** Cyclosporin A had no significant effect on the

neurosphere size at any dose ($n=8$ trials). Control 0 ng/mL: 61.3 ± 12.3 µm; 50ng/mL:

67.7 ± 11.5 ; 100ng/mL: 66.1 ± 8.10 µm; 500ng/mL: 85.7 ± 10.6 µm; 1000ng/mL: $71.7 \pm$

3.30 µm; $p > 0.05$ **(D)** Passaging neurospheres grown in (+) CsA or (-) CsA conditions

continuously for 3 passages. Continual passage in 100ng/mL of CsA led to a significant

increase in the average number of neurospheres by passage 3 compared to passage 1

whereas without CsA, there was no significant increase by passage 3 compared to

passage 1. For +CsA, " 99.5 ± 42.1 N=7" in P3 vs. " 28.7 ± 5.6 N=9" in P1, $*p < 0.05$.

For –CsA, " 40.4 ± 22.8 N=8" in P3 vs. " 18.6 ± 4.45 N=10" in P1, $p > 0.05$.

3.2 - Cyclosporin A enriches for neurogenic NPCs

We next sought to determine whether CsA affects the differentiation profile of hippocampal neurospheres. Single neurospheres grown were plated in differentiation conditions containing 10% FBS and SFM in the presence or absence of 100ng/mL of CsA. Spheres that had not yet been passaged but were grown and differentiated in the presence of CsA (“+CsA”) did show a significant increase in the frequency of neurospheres positive for neuron-containing neurospheres, as determined by the expression of the neuronal marker β -III-tubulin, compared to control neurospheres (grown and differentiated in the absence of CsA “-CsA”) (Figure 3-2A). The frequency of glial-only neurospheres was equal to the frequency of spheres positive for β -III-tubulin when grown without CsA (Figure 3-2A, -CsA condition, $p > 0.05$). When neurospheres were passaged three times in the continued presence of 100 ng/mL of CsA, the difference between glial only and β -III-tubulin-containing neurospheres became even more significantly different (Figure 3-2B, +CsA condition, $p < 0.001$). Also, the frequency of glial-only spheres significantly decreased at passage 3 (Glial only in -CsA vs. Glial only in +CsA, figure 3-2B). On the other hand, passaging the spheres in the absence of CsA three times shows no change in the frequencies of glial only and neurogenic spheres (Figure 3-2B, -CsA condition, $p > 0.05$). Taken together, these findings reveal that the continued presence of CsA enhances survival of NPCs that have the capacity to form neurons.

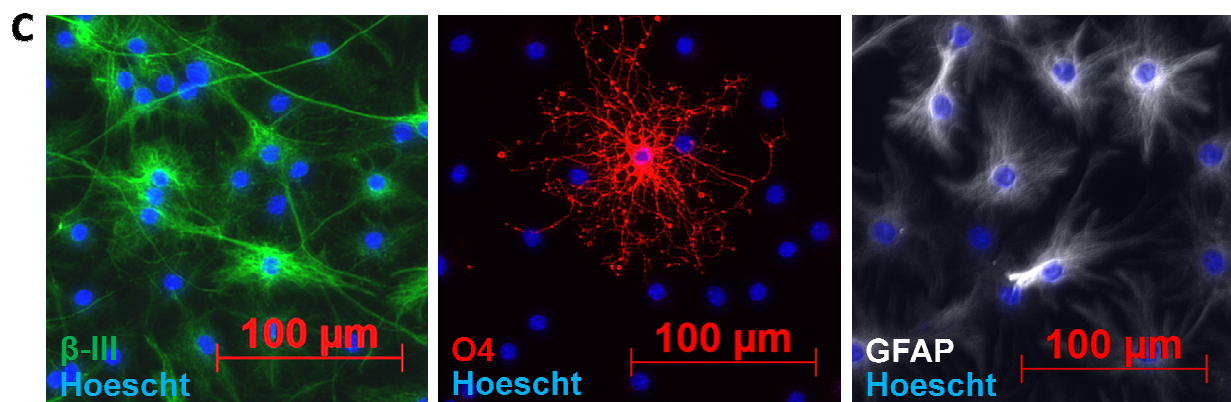
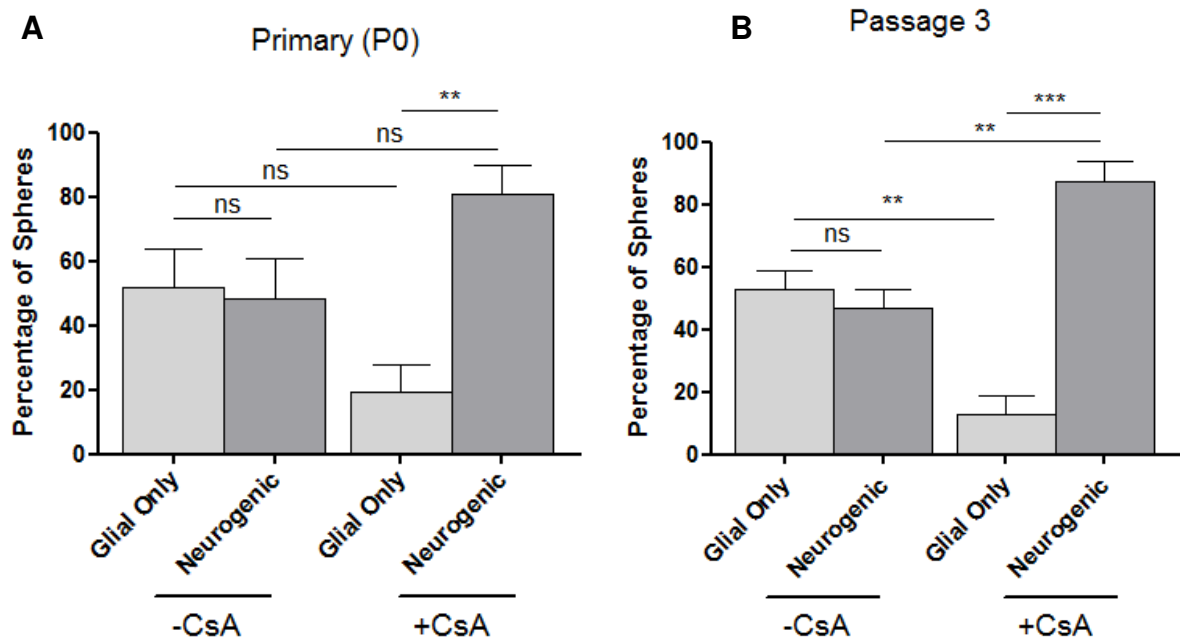


Figure 3-2. Cyclosporin A increases the frequency of neurogenic neurospheres.

(A) At P0, (+)CsA conditions significantly increase the frequency of neurogenic spheres over control (-)CsA conditions. **(B)** At P3, the frequency of neurogenic spheres significantly increases in (+)CsA conditions. **(C)** Representative images of O4(red), β -III-tubulin(green), and GFAP(white) cells all stained with Hoechst to label nuclei (blue).

* $p < 0.05$ *** $p < 0.001$ For P0 experiments, $n=6$ animals per group and for P3, $n=4$ animals per group.

3.3 - Infusion of Cyclosporin A in vivo promotes neurosphere formation

To examine the effects of in vivo administration of CsA on hippocampal NPCs we implanted in CD1 mice with subcutaneous pumps containing either CsA or vehicle control. We predicted that CsA infusion would lead to increased numbers of neurospheres isolated from the DG of CsA-treated mice (Hunt et al, 2010). Mice received systemic CsA or vehicle for seven days, after which the mice were sacrificed and the hippocampus dissected and the cells were plated in the absence of CsA for the neurosphere assay (Figure 3-3A). We observed a significant 1.8-fold increase in the number of neurospheres from mice that received the CsA pump compared to vehicle infused mice (" 2.0 ± 0.18 N=8" CsA pump vs. " 1.1 ± 0.14 N=8" vehicle pump, $**p < 0.01$, Figure 3-3B), similar to the increase seen in SE derived neurospheres following CsA administration in vivo (Hunt et al., 2010). These findings indicate that CsA in vivo can effectively increase the number of stem cells.

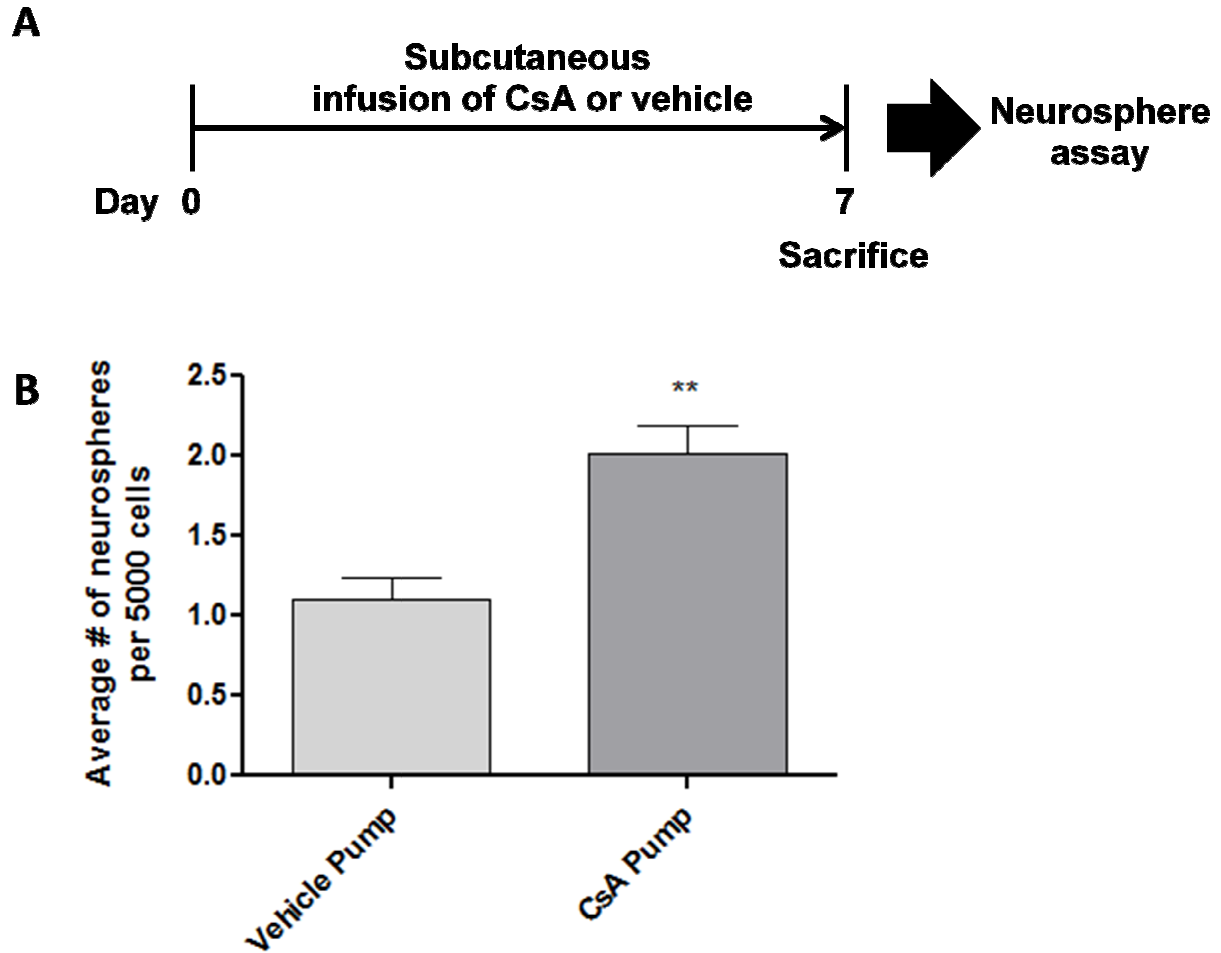


Figure 3-3. Infusion of cyclosporin A in vivo increases neurosphere formation in vitro. (A) Schematic of experiment. Two- to four-month-old adult CD1 male mice were implanted with subcutaneous pumps containing CsA released at a dose of 15 mg/kg/day or vehicle for 7 days. Animals were sacrificed on day 7 to perform the neurosphere assay. Dentate gyrus tissue was digested and cultured for 7 days, after which neurospheres were quantified. **(B)** Animals that received CsA pump produced a significant 1.8-fold increase in the number of neurospheres over control animals (2.0 ± 0.18 spheres per 5000 cells in CsA pump vs. 1.1 ± 0.14 spheres per 5000 cells vehicle pump, $N=8$ for both groups, Figure 3-3B, $**p < 0.01$). $**p < 0.01$

3.4 - Cyclosporin A infusion increases proliferation and neurogenesis in the dentate gyrus

To further examine the behavior of NPCs in the dentate gyrus in the presence of CsA, we performed a number of in vivo assays to examine the proliferation and cell survival within the neurogenic region. First, we examined the number of proliferating cells after 7 days of infusion, a time when we see the increased numbers of neurospheres in vitro. On the day prior to sacrifice, mice received 3 BrdU injections (once every two hours) to label actively dividing cells and they were sacrificed 2 hours after the last injection (Figure 3-4A). We examined the numbers for Ki67 positive cells, a marker for cells that have the capacity to undergo proliferation (Scholzen and Gerdes 2000) as well as the number of BrdU positive cells, a marker of actively dividing cells at the time of injection. We observed significant increases in the numbers of BrdU positive cells (9.8 ± 1.1 N=10 cells per section in CsA-treated animals vs. 5.9 ± 0.96 N=8 cells per section in vehicle control animals, average of 1.7-fold increase, Figure 3-4B) and Ki67 positive cells (9.6 ± 2.9 N=8 cells per section in CsA-treated animals vs. 5.8 ± 2.6 N=6 cells per section in vehicle control animals, with an average 2.3-fold increase, Figure 3-4C) in CsA treated mice relative to controls. These findings are consistent with the in vitro observations that CsA expands the size of the NPC pool.

Since the proliferating cells in the DG are neurogenic, we predicted that CsA administration in vivo would lead to an increase in the number of cells expressing doublecortin (DCX), a marker for immature neurons that is expressed in newborn DG neurons between 1 to 14 days after their generation (Brown et al., 2003). Indeed, we observed a significant 1.6-fold increase in the number of DCX+ cells after 7 days of CsA

infusion (58 ± 8.0 N=3 cells per section for CsA-treated animals and 32 ± 1.3 N=3 cells per section for vehicle control animals, average fold increase of 1.8 ± 0.25 N=3, Figure 3-4D).

If CsA is enhancing cell survival, then we predict that we should observe an increase in the number of newborn neurons in the DG of CsA-treated mice. Previous studies had determined that 50% of the newborn neurons will die between 7 and 14 days after birth (McDonald and Wojtowicz 2005). Because, the period of DCX expression falls within the aforementioned critical period of 7 to 14 days, during which half of the newly born neurons die, we sought to determine whether CsA was improving the survival rates of newly born proliferating neurons above baseline conditions. However, to determine how many of the DCX+ cells arose from proliferating cells that were exposed to the CsA infusion, we prelabeled a cohort of cells by injecting mice with BrdU once daily for seven days followed by CsA or vehicle infusion via subcutaneous pump for another seven days (Figure 3-5A). The CsA infusion was delayed until day 7 of BrdU injections to coincide with the day 7 to 14 period during which half of the newly born neurons die under baseline conditions (Figure 1-4). If CsA was enhancing the survival of newly born neurons, we predicted that the number of cells positive for both BrdU and DCX would be higher for mice that received the CsA infusion. This was indeed observed as mice that received CsA infusion presented a significant 2.05-fold increase in the average number of double-labeled cells positive for both DCX and the BrdU that had been injected for the seven days prior to CsA infusion (16.1 ± 3.25 N=4 double-positive cells per section in CsA-treated animals vs. 7.12 ± 1.53 N=4 double-positive cells per section in vehicle control animals, average fold increase of $2.32 \pm$

0.187 N=4, Figure 3-5B). Together, these findings support the hypothesis that CsA is preventing the death of newborn neurons in the dentate gyrus of the adult mouse.

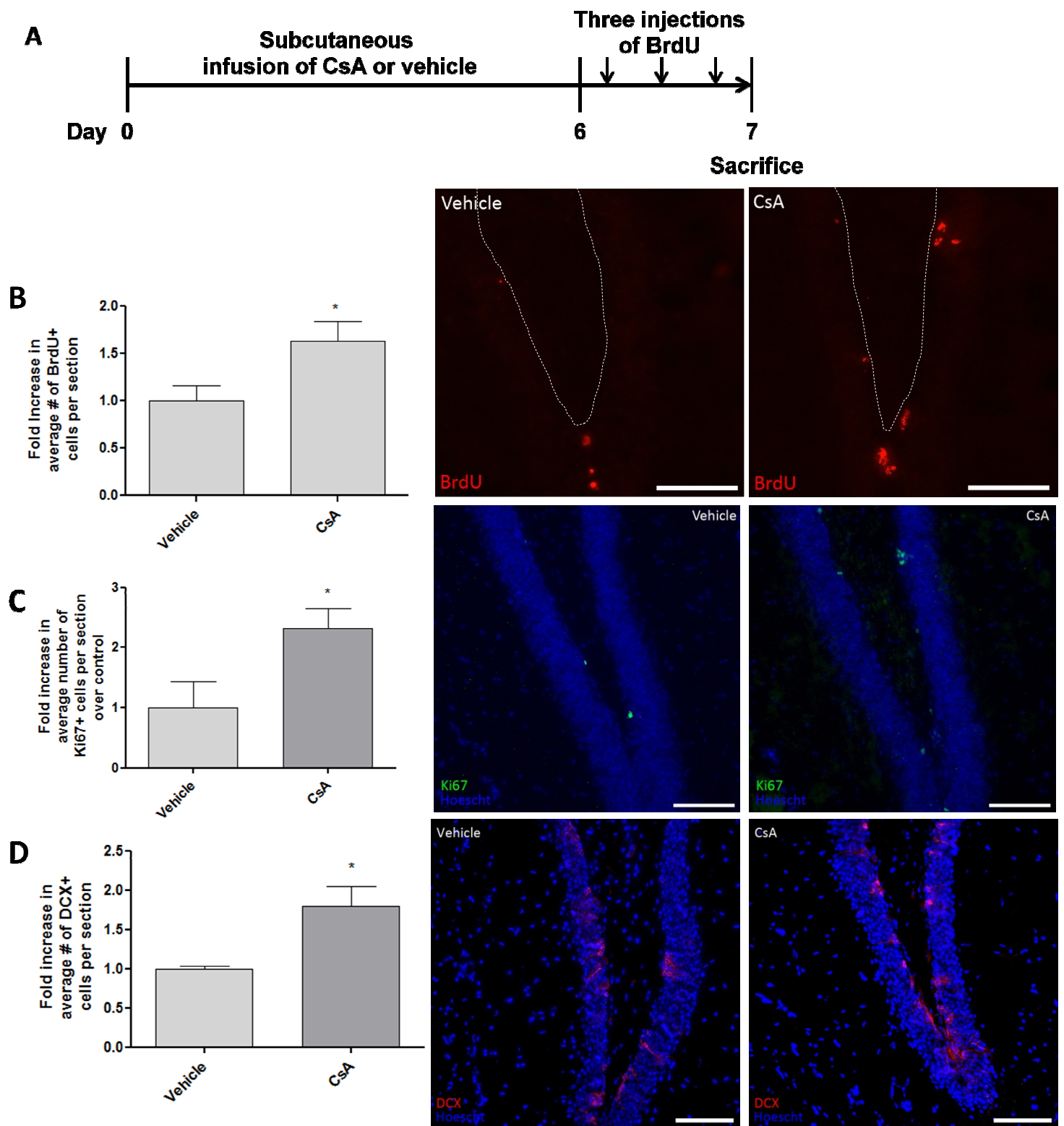


Figure 3-4. Infusion of cyclosporin A in vivo increases BrdU+, Ki67+, and DCX+ cells. **(A)** Schematic of experiment. Two- to four-month-old adult CD1 male mice were implanted with subcutaneous pumps containing CsA released at a dose of 15 mg/kg/day or vehicle for 7 days. Animals were injected 3 times on day 7 with BrdU. Mice were then perfused and cryosectioned for immunohistochemistry. **(B)** Animals that received CsA pumps exhibited a 1.6-fold increase in the number of BrdU+ cells per section over control animals. (9.83 ± 1.13 N=10 cells per section in CsA-treated animals vs. 5.90 ± 0.96 N=8 cells per section in vehicle control animals, average of 1.7-fold increase). Representative images of a dentate gyrus section from a vehicle animal and an animal that received CsA. BrdU+ cells are red. Dotted lines border the area of the dentate gyrus. **(C)** Animals that received CsA pumps exhibited a 2.3-fold increase in the number of Ki67+ cells per section over control animals. (9.61 ± 2.86 N=8 cells per section in CsA-treated animals vs. 5.77 ± 2.55 N=6 cells per section in vehicle control animals, with an average 2.3-fold increase). Representative image of a dentate gyrus section from a vehicle animal and an animal that received CsA. Ki67+ cells are green. Inset of vehicle shows a single Ki67+ cell and inset of CsA shows a cluster of Ki67+ cells. **(D)** Animals that received CsA pumps exhibited a 1.8-fold increase in the number of DCX+ cells per section over control animals. (57.5 ± 8.0 N=3 cells per section for CsA-treated animals and 32.0 ± 1.26 N=3 cells per section for vehicle control animals, average fold increase of 1.80 ± 0.25 N=3). Representative image of a dentate gyrus section from a vehicle animal and an animal that received CsA. DCX+ cells are red. *p < 0.05 **p < 0.01. All scale bars represent 100 μ m.

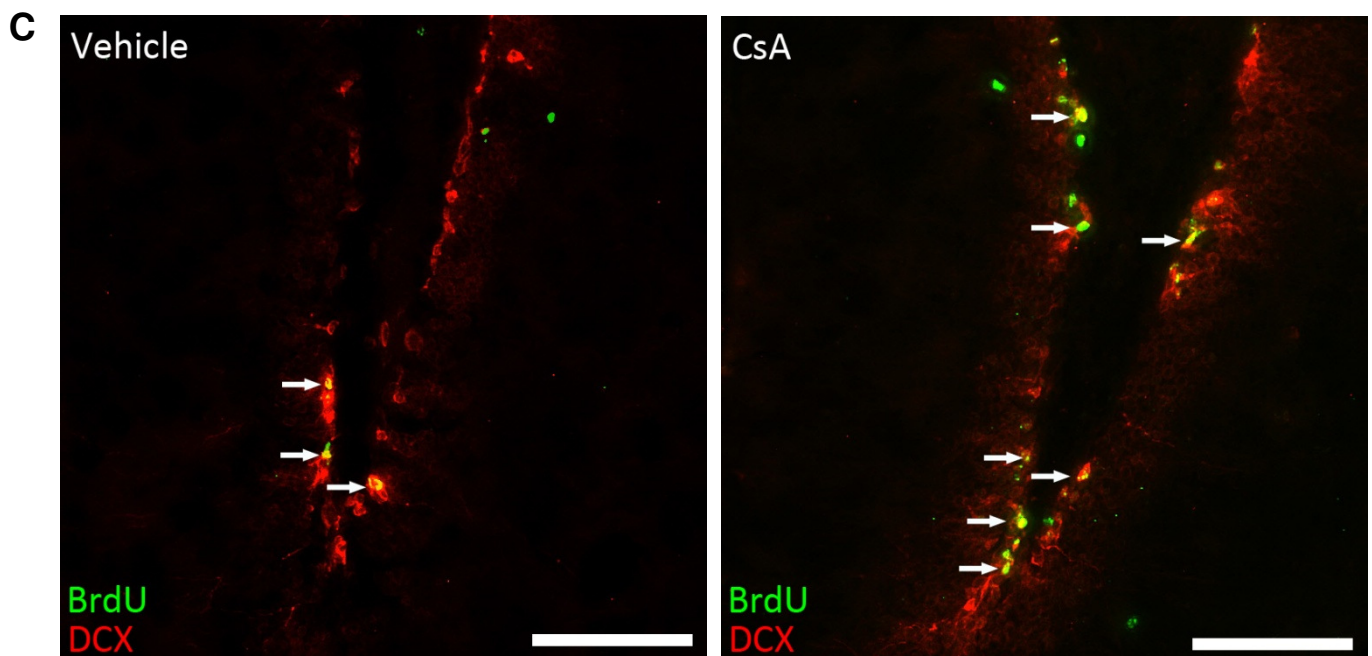
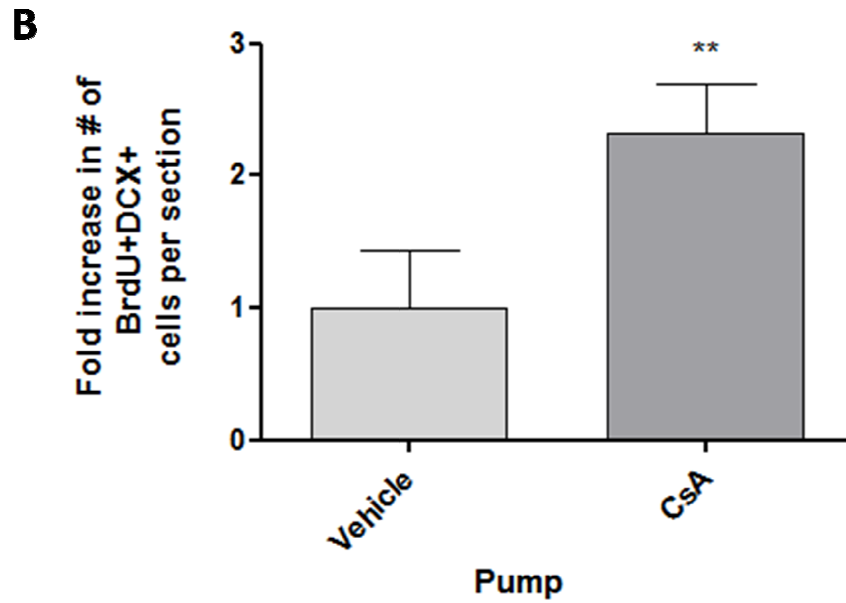
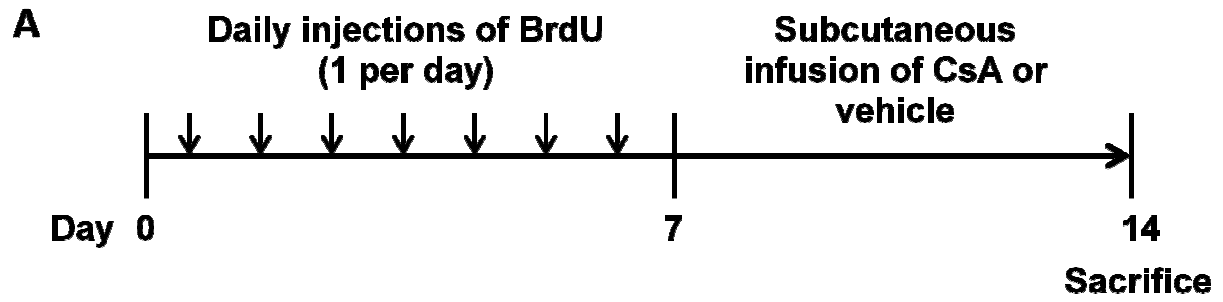


Figure 3-5. Infusion of cyclosporin A in vivo increases the number of DCX+BrdU+ cells. **(A)** Schematic of experiment. Two-month-old adult CD1 male mice received seven daily injections of BrdU and subsequently implanted with subcutaneous pumps containing CsA released at a dose of 15 mg/kg/day or vehicle for 7 days. Mice were then perfused and cryosectioned for immunohistochemistry. **(B)** Animals that received CsA pumps exhibited a 2.3-fold increase in the number of DCX+BrdU+ cells per section over control animals. (16.1 ± 3.2 N=4 double-positive cells per section in CsA-treated animals vs. 7.13 ± 1.53 N=4 double-positive cells per section in vehicle control animals, average fold increase of 2.32 ± 0.19 N=4). **(C)** Representative images of a dentate gyrus section from a vehicle animal and a CsA. BrdU+ cells are green, DCX+ cells are red. Dotted lines border the area of the dentate gyrus. Dashed boxes indicate area that the insets represent. Arrows indicate BrdU+DCX+ cells. * $p < 0.05$. All scale bars represent 100 μm .

3.5 - Cyclosporin A shows an increase in human DG-derived neurospheres

Human DG tissue was obtained from two patients undergoing surgery to treat epilepsy. We performed neurosphere assays and cultured the cells for 4 weeks in the presence of CsA at concentrations ranging from 0 to 1000 ng/mL at clonal density. After 4 weeks, the number of colonies greater than 50 μ m in diameter was quantified. We made the prediction that CsA will have the same effect on human DG-derived NPCs as seen in previous experiments with the mouse, which is to enhance the number of colonies. Preliminary data shows that the optimal effect on human DG-derived colonies is seen at a CsA dose of 300 ng/mL (Figure 3-6). However, attempts to passage the colonies or to differentiate the colonies to determine their characteristics have been unsuccessful and this will require further investigation. Thus, these colonies cannot be termed neurospheres until they demonstrate self-renewal and multipotentiality. Nonetheless, the positive effect CsA has on the number of colonies seen in primary culture at 300ng/mL suggests CsA can enhance the DG NPC population in humans. This further supports the hypothesis that CsA can enhance NPC survival in the DG, which can lead to enhanced neurogenesis. Seeing this effect in human cells is promising in that CsA may have applications beyond immunosuppression.

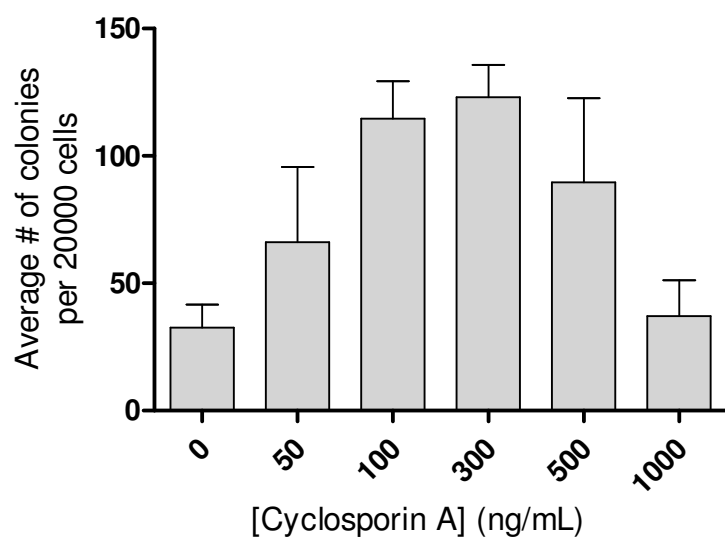


Figure 3-6. Human DG-derived tissue cells trend toward forming more colonies in CsA at 300ng/mL. n=2. Error bars represent standard deviation.

Chapter 4 - Discussion

In this study, we examined the behaviour of NPCs in response to CsA using both in vitro and in vivo models. The in vitro and in vivo studies show that CsA effectively increases the size of the NPC population. Given that SVZ neurospheres are comprised of approximately 1% stem cells and 99% progenitors, the significant change in the numbers of neurospheres accompanied by no change in size of the neurospheres suggest that CsA effects in the dentate gyrus are specific to precursors that have more stem cell-like characteristics rather than downstream progenitors. The increase in the number of neurospheres in subsequent passages suggest that CsA has a direct effect on NPC survival given that subsequent passages do not have the cell types present in primary culture conditions.

When considering the characteristics of NPCs in the DG, a number of differences have been reported in the literature. For Clarke and van der Kooy (2011), DG colony-forming cells did not display the properties of multipotency and the ability to self-renew when cultured in vitro, unlike what was demonstrated by Reynolds and Weiss (1992) with SVZ neurospheres. Indeed, they observed that the vast majority of DG derived colonies produced 100% GFAP+ astrocytes with almost no new neurons forming. The first report of isolating DG spheres with stem cell properties was from rat hippocampus (Palmer, Takahashi et al. 1997). A subsequent report showed that rat and mouse hippocampus often contain contaminating NPCs from SVZ regions (Seaberg and van der Kooy 2002) because the surrounding regions give rise to passageable and multipotent spheres, including the third and posterior LV and the hippocampal arch, which is a collapsed embryonic ventricular zone in the dorsal hippocampus. However,

this study used the same methodology as Clarke and van der Kooy (2011), which used careful dissection of thin brain slices, eliminating the problem of contamination. Clarke and van der Kooy (2011) also reported that the DG spheres could not be passaged unless the spheres were co-cultured with embryonic brain slices, which restored self-renewal but not multipotentiality. Interestingly, by passaging in the presence of embryonic slices, the numbers of spheres were within the ranges observed in our studies with CsA conditions. They argue that another NPC type in the DG is responsible for the formation of neurons based on their co-culture studies. They were able to detect long-term self-renewing multipotential colonies in the DG primordium region at E14 but not at any later time points, suggesting that these cells either migrate away from the region or differentiate such that they lose these self-renewing multipotential properties. However, our findings show that spheres derived from the DG have the capacity to self-renew, given that we were able to perform three passages, and demonstrate multipotentiality, as shown by the presence of GFAP+, β III-tubulin+, and O4+ spheres. The notion that another NPC type in the DG is responsible for the formation of neurons is of interest as based on our findings, it is possible that CsA is specifically enhancing the population of NPCs that have the capacity to give rise to neurons since we see an increase in the frequency of multipotential spheres grown in the presence of CsA.

Mechanistically, it was recently demonstrated that the pro-survival effects of CsA are mediated by the inhibition of the mitochondrial permeability transition (MPT) pore formation (Sachewsky, Hunt et al. 2014). In this study by Sachewsky et al. (2014), there were two pathways through which CsA can act to promote NPC survival (Figure 1-

5). The first pathway was calcineurin-dependent whereby CsA binds to cyclophilin A. The resulting drug-receptor complex binds and inhibits calcineurin, which prevents transcription of interleukin 2 (IL-2), a cytokine responsible for T-cell propagation. Survival is promoted through this pathway because blocking calcineurin also inhibits dephosphorylation of nitric oxide synthase (nNOS) and Bcl-2 Associated Death Promoter (BAD). This pathway was investigated using the calcineurin inhibitor FK506 and nNOS knockout mice. The second pathway did not involve calcineurin and instead involved CsA binding to cyclophilin D, which ultimately blocks MPT pore formation. This MPT pore is involved in apoptosis in which the formation of this pore results in several mitochondrial changes such as: increased inner membrane permeability to ions, disruption of membrane potentials, loss of ion homeostasis, impairment of ATP synthesis, and mitochondrial swelling, which ultimately lead to rupture, cytochrome c release, and ultimately the initiation of apoptosis (Baines et al., 2005). This pathway was investigated using an analogue of CsA, NIM811. Sachewsky et al. (2014) found that the pro-survival effects could be accounted for by NIM811 alone. Interestingly, NIM811 led to functional recovery in a model of stroke, an effect previously observed following CsA administration. Thus, it is possible that effects observed in these studies on hippocampal NPCs are due to the aforementioned mechanism that inhibits apoptosis.

Our in vitro and in vivo studies show an increase in multipotent neurogenic neurosphere formation in the presence of CsA. Our findings are consistent with the idea that CsA is enhancing the survival of DG NPCs and these cells proliferate to give rise to newly born neurons. We cannot rule out the possibility that the DG contains a

population of latent NPCs which are activated by CsA, promoting their proliferation. Indeed, recent studies have suggested that the neurogenic SE of the forebrain contains a population of quiescent neural stem cells (Codega, Silva-Vargas et al. 2014). A study by Walker et al. (2008) observed a 3-fold increase in the number of neurospheres generated from adult mouse DG following neural excitation using depolarizing levels of potassium chloride. Interestingly, the neural excitation resulted in the increase in the number of neurospheres that gave rise to neurons in vitro. We have demonstrated that the mechanism by which CsA promotes the survival of SE NPCs is through binding with cyclophilin D and inhibition of mitochondrial pore formation, thereby blocking apoptosis. We propose a similar mechanism for NPC survival in the DG. However, further investigation is needed to rule out the activation of a latent population of DG NPCs.

Our subsequent in vivo studies show an increase in BrdU+ and Ki67+ cells. Stem cells can undergo asymmetric division, where the stem cell divides self-renews and produces a downstream precursor cell, or divide symmetrically, where the stem cell divides into two copies of itself. It is possible CsA is enhancing the survival of proliferating hippocampal NPCs, which is manifested in the increase in BrdU+ and Ki67+ cells. A neurogenic precursor may divide symmetrically and one of them may die under baseline conditions but is otherwise spared in the presence of CsA, allowing both cells to go on to form new neurons. An alternative possibility is that the DG contains resident NPCs that are normally latent and CsA is activating these NPCs. A study by Walker et al. (2008) observed a 3-fold increase in the number of neurospheres generated from adult mouse hippocampus through neural excitation by using depolarizing levels of potassium chloride. The neural excitation also resulted in the

increase in the number of neurospheres that gave rise to neurons. It is possible CsA may be having a similar effect of exciting a latent population of NPCs that can give rise to neurons. Thus, there are several possible reasons why there is an increase in neurospheres in vitro, proliferation markers in vivo, and neuronal markers in vivo.

Our preliminary finding of a possible effect CsA has on primary human DG tissue is promising and lends support to possible applications of CsA in regenerative repair strategies. There are two main approaches for using stem cells in repairing the CNS: (1) exogenous strategies that involve the transplantation of NPCs and (2) endogenous strategies that aim to activate resident NPCs to promote self-repair. Given that it is already an FDA-approved drug used as an immunosuppressant in organ transplantation, it may be of interest to examine individuals who suffered a stroke that had damaged the hippocampus and had a transplant. If they were administered CsA, it may be of interest to examine if their cognitive function improved following the transplant. More importantly, the finding that CsA promotes NPC survival through a mechanism that does not involve immunosuppression can lead to the development of NPC-based therapies for neurological disease.

The finding that CsA enhances neurogenesis in the adult hippocampus could have important implications for neural repair following injury. The hippocampus plays an important role in spatial recognition, learning, and memory (Corkin 2002, Augustinack, van der Kouwe et al. 2014). Studies have shown that impairing the process of neurogenesis impairs these behaviours (Corkin 2002, Zhao, Ueba et al. 2003), highlighting the importance of ongoing neurogenesis in these tasks (Kee, Teixeira et al. 2007, Akers, Martinez-Canabal et al. 2014). One study showed that a 3-

fold increase in adult mouse neurogenesis through exercise led to significant improvement in the Morris water maze spatial recognition task compared to uninjured controls (van Praag, Christie et al. 1999). A positive correlation between promoting neurogenesis in the hippocampus and cognitive function recovery has been shown by intraventricular infusion of S100B, a neurotrophic protein secreted by astrocytes, and subsequent cognitive tests using the Morris water maze (Kleindienst et al, 2005). More recently, Metformin, a drug used to treat type II diabetes, has also been shown to promote neurogenesis in the hippocampus and improve behaviour in the Morris water maze (Wang et al., 2012). Another recent study showed a correlation between neurogenesis and cognitive function in the presence of prolactin. Mice that were deficient in prolactin exhibited decreased neurosphere formation *in vitro* and decreased BrdU+ cells in the DG *in vivo* (Walker et al., 2012). Prolactin-null mice also displayed poorer performance in cognitive tasks such as the Morris water maze and this deficiency was rescued by infusion of prolactin into the hippocampus (Walker et al., 2012). Our studies show a 2-fold increase in newborn neurons in the presence of CsA suggesting that *in vivo* administration may be beneficial to promote functional recovery following injury. Indeed, there is evidence that CsA can aid in the recovery after ischemic injury in the cortex by promoting endogenous NPC migration to the site of injury followed by regenerative repair in the lesion site (Erlandsson et al., 2011). CsA promotes neurogenesis, which suggests that it may serve as a good candidate molecule to enhance self-repair of the injured brain.

Besides repair of the injured brain, there is a natural process that can result in a decline in neurogenesis without any prior injury. This natural process is aging and the

decline in hippocampal neurogenesis with age is significant. In 21 month old rats, the number of newly added neurons represented only 10% of the amount seen in younger rats of 6 months of age (Kuhn, Dickinson-Anson et al. 1996). In follow-up studies, Kuhn and others showed that the number of newly generated neurons steadily declines with age. This age-related decline in hippocampal neurogenesis has been observed in other species from rodents to humans (Amrein, Isler et al. 2011). The reason for this decline may be due to a decline in the population of neural stem cells. If this is the case, perhaps CsA can aid in delaying the decline by promoting the survival rate of new cells. An alternative reason for the age-related decline in hippocampal neurogenesis can be due to gradual changes to the stem cell niche over the lifespan of the organism that ultimately causes the stem cells to enter quiescence. If this is the case, restoring the original youthful environment, such as through pharmacological intervention, that will keep the stem cells from entering quiescence is a possible solution. Although the mechanism of CsA has been elucidated in the SVZ, we have not determined that CsA acts through the same mechanism in the DG and it is possible that CsA can not only promote survival of NPCs but also activate those that have already gone quiescent.

The concern with age-related hippocampal neurogenesis is not with just the decline but whether the decline results in a decrease in cognitive function. Compared to the SVZ and its role of neurogenesis in olfaction, the functions performed by the hippocampus in cognition are crucial in how we define who and where we are. However, the decrease in hippocampal neurogenesis with age is compensated by the proportional decrease in cell apoptosis, leading to a steady state number of neurons (Cameron and McKay 1999). Moreover, there has been no clear conclusion through

depletion and knockdown studies of the effect of lowered neurogenesis on cognition (Deng, Aimone et al. 2010). Conclusions that may be drawn involving depletion and knockdown treatments are limited due to the possible confounding effects from additional damage and inflammation that come as a result of these harsh treatments. Surprisingly, using a cyclin D2 knockout mouse model, where there is no hippocampal neurogenesis, mice did not exhibit any effect on their learning or anxiety levels (Jedynak, Jaholkowski et al. 2012). However, these mice did exhibit deficiencies in learning-independent activities such as nest construction, exploration, and motor activities. Thus, this suggests that learning is not dependent nor is it affected by the decline in the creation of new neuronal circuits in the course of adult neurogenesis. Therefore, the correlation of age-related decline in hippocampal neurogenesis to cognitive function is currently unclear in the literature as there is evidence that in humans, the aged brain simply activates different areas of the brain compared to the young brain when performing the same cognitive tests (Burgmans, van Boxtel et al. 2010). This suggests that adult neurogenesis over one's lifespan is simply the brain remodeling and consolidating and additional neurogenesis is not necessarily beneficial. Thus the idea of increasing neurogenesis through CsA to compensate for the age-related decline may not yield a clear cognitive benefit. Nonetheless, this does not rule out that CsA and the mechanisms underlying it that promote neurogenesis may be of importance in older individuals who suffer an injury in the brain. As older individuals have decreased neurogenesis, and thus decreased repair capacity, increasing endogenous activation of NPCs through CsA and other drugs is a possible regenerative repair strategy to aid in the functional recovery of the individual.

Chapter 5 - Future Directions

5.1 - Further assessing CsA's effect on survival and proliferation of DG NPCs

In this study, we observed that CsA increases the number of neurospheres but not the average size in vitro. While these measures do suggest that the survival of the colony forming precursor cell is enhanced and that the proliferation of downstream progenitors is unaffected, these measures do not give a complete quantitative picture of the cell division or cell death, measures that can attest to proliferation and survival, occurring within an individual neurosphere.

One possible experiment is live-cell imaging in which a neurosphere is dissociated and cells are placed in a culture chamber and imaged for a few days in the presence or absence of CsA. If cell proliferation kinetics are unchanged, then the number of cell division events that occur over time should be the same for both groups. If cell survival is enhanced by CsA, then a greater proportion of cells should survive over the time period of live imaging with fewer cell death events observed. Though this technique was successfully employed by Hunt et al. (2010), a caveat is that using the current methodology, the number of DG neurospheres derived from an animal is much less than the number derived from the SVZ. Thus, generating sufficient numbers of neurospheres for live cell imaging can be a challenge.

An alternative to assessing cell proliferation is through a pulse exposure of neurospheres to BrdU. Neurospheres will be grown in the presence or absence of CsA and then exposed with a short-term pulse of BrdU. Actively dividing cells in a neurospheres will incorporate the BrdU, which can then later be quantified.

Neurospheres pulsed with BrdU will be dissociated and the number of BrdU+ cells can be quantified. If CsA has no effect on proliferation kinetics, then the number of BrdU+ cells should be relatively the same between control and CsA-treated neurospheres. However, to gain insight into survival effects, a measure of cell death is needed. One such technique is terminal deoxy-nucleotidyl transferase dUTP nick-end labeling (TUNEL). If cell survival is enhanced by CsA, neurospheres grown in CsA should exhibit decreased TUNEL+ cells compared to control. Thus, these two assays of BrdU pulse labeling and TUNEL can give insight into the cell proliferation kinetics and survival of neurospheres.

5.2 - Investigating the mechanisms behind enhanced NPC survival

As Sachewky et al. (2014) have determined, the pathway responsible for the survival effect seen by Hunt et al. (2010) in SVZ neurospheres is the calcineurin-independent pathway whereby mitochondrial pore transition is blocked (Figure 1-5). This was determined using two analogues that each activates a calcineurin-dependent or calcineurin-independent pathway, FK506 and NIM811 respectively. It is possible CsA enhances the survival of NPCs from the DG through the same pathway and thus, it is worth using these analogues to determine the pathway involved. Also, NIM811 is a non-immunosuppressive derivative of CsA, which makes it an appealing molecule for regenerative repair strategies in the CNS where immunosuppression is not desired.

5.3 - Using cyclosporin A to promote cognitive function after injury

Given the role of the hippocampus in learning, memory, and spatial recognition, injuries to this region of the brain can have severe debilitating effects. A prominent case

involving the hippocampus is H.M.'s case. Following a traumatic head injury, H.M. suffered from seizures that greatly reduced his quality of life. Turning to Dr. William Scoville for surgical treatment, Dr. Scoville removed two-thirds of his hippocampus was removed to treat H.M.'s seizures (Corkin 2002). As a result, H.M.'s seizures stopped but he suffered from anterograde amnesia and had difficulty in performing certain spatial tasks (Augustinack, van der Kouwe et al. 2014). By observing H.M., we have gained insight into the important roles the hippocampus can play and how debilitating it can be for people to have this region injured.

Besides physical trauma, the hippocampus can be injured through ischemic stroke, encephalitis, and through neurodegenerative disease such as Alzheimer's. In particular, CsA has been reported to act as a neuroprotective agent against stroke (Osman, Lulic et al. 2011). Cerebrovascular stroke is when the blood supply to the neural tissue of the brain is interrupted. Stroke is second only to ischemic heart disease as the most common cause of death (Marsh and Keyrouz 2010). There is currently no medical therapy that restores neural function and protects against ischemic damage. Acute stroke treatment focuses on revascularization with a lack of clinical data on neuroprotective agents (Adams, del Zoppo et al. 2007). Stroke can occur in two types: ischemic and hemorrhagic (Thrift, Dewey et al. 2001). The majority of stroke cases are of the ischemic variant where decreased blood flow leads to neuronal damage. Irreversible damage occurs in the centre of the ischemia while the surrounding tissue only temporarily impaired (Donnan, Fisher et al. 2008). In studies performed in rats and mice with middle cerebral artery occlusion (MCAo) stroke models, the MPT pore formation has been implicated in triggering cell death (Osman, Lulic et al. 2011).

Therefore, CsA, with its known mechanism in inhibiting MPT pore formation, may be employed to prevent neuronal cell death, which can lead to improvements in the quality of life of patients and restore cognitive function.

Because endogenous NPCs exist in the DG of the hippocampus, using CsA to enhance the survival of these NPCs and promote neurogenesis is a possible repair strategy in response to injury. Current studies in animals employ the Morris water maze to study spatial learning and memory (D'Hooge and De Deyn 2001). This navigation task requires the animal to search a large circular pool to find an invisible platform that allows the animal to escape the water. Surrounding the pool are visual cues to help guide the animal and aid it in spatially recognizing and learning the location of the hidden platform in subsequent trials (Figure 5-1). The time it takes for the animal to escape on to the platform is the main measure of this task, with shorter times in repeated trials indicating that the animal is learning and remembering the location of the platform.

Given the above information, it is possible to study the effects of CsA on hippocampal function in uninjured and injured animals. We have already observed that uninjured animals that receive CsA exhibit increased neurogenesis *in vivo*. We predict that the animals that receive CsA may also perform better in the Morris water maze task compared to control animals. For an injury model, endothelin-1 (ET-1) injection can be used to simulate a stroke in the hippocampus, which has been done previously to impair learning and memory (McDonald, Craig et al. 2008). This model is advantageous in that it can be used to cause specific localized damage to the hippocampus without significantly affecting other regions of the brain. Following ET-1 administration, CsA can

be given to animals post-stroke to enhance NPC repair and regeneration. We predict that injured animals that receive CsA during their recovery period will perform better in the Morris water maze compared to injured animals that received nothing. If these hypotheses are true, then CsA will be a promising drug to be used in endogenous regenerative repair strategies following brain injury or disease.

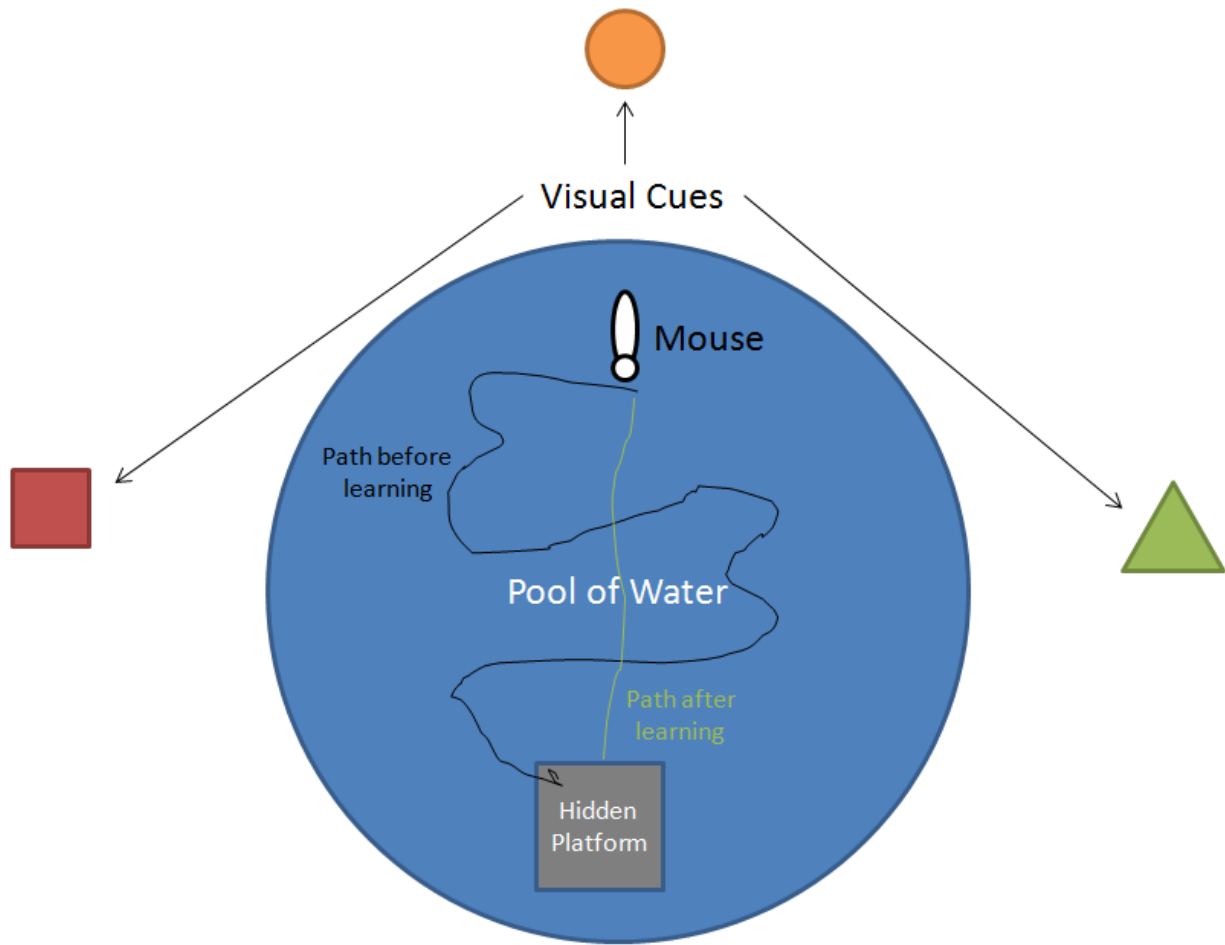


Figure 5-1. Morris water maze. A behavioural task widely used to study spatial learning and memory, the Morris water maze requires a mouse or rat to navigate a circular pool of water to find an invisible platform that allows it to escape the water. Placed around the pool are visual cues to aid the animal in spatial recognition. Animals that learn the location of the hidden platform will take a shorter direct path to the platform. Animals impaired in spatial recognition and learning will have difficulty locating the hidden platform and may continue taking long paths.

Conclusions

Here we show that CsA increases the numbers of neurospheres isolated from the adult mouse DG without affecting their average size. Differentiation studies show that CsA enhances the survival of NPCs that can give rise to neurons. CsA administration in vivo demonstrates that CsA promotes the survival of NPCs and thereby enhancing neurogenesis. Given the established role of the hippocampus in learning and memory, enhancing cell survival within this region could potentially enhance cognitive function in the uninjured brain and/or injured brains.

References

- Adams, H. P., Jr., G. del Zoppo, M. J. Alberts, D. L. Bhatt, L. Brass, A. Furlan, R. L. Grubb, R. T. Higashida, E. C. Jauch, C. Kidwell, P. D. Lyden, L. B. Morgenstern, A. I. Qureshi, R. H. Rosenwasser, P. A. Scott and E. F. Wijdicks (2007). "Guidelines for the early management of adults with ischemic stroke: a guideline from the American Heart Association/American Stroke Association Stroke Council, Clinical Cardiology Council, Cardiovascular Radiology and Intervention Council, and the Atherosclerotic Peripheral Vascular Disease and Quality of Care Outcomes in Research Interdisciplinary Working Groups: The American Academy of Neurology affirms the value of this guideline as an educational tool for neurologists." Circulation **115**(20): e478-534.
- Aimone, J. B., J. Wiles and F. H. Gage (2009). "Computational influence of adult neurogenesis on memory encoding." Neuron **61**(2): 187-202.
- Akers, K. G., A. Martinez-Canabal, L. Restivo, A. P. Yiu, A. De Cristofaro, H. L. Hsiang, A. L. Wheeler, A. Guskjolen, Y. Niibori, H. Shoji, K. Ohira, B. A. Richards, T. Miyakawa, S. A. Josselyn and P. W. Frankland (2014). "Hippocampal neurogenesis regulates forgetting during adulthood and infancy." Science **344**(6184): 598-602.
- Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter, J. Wilson and T. Hunt (2008). Molecular Biology of the Cell. New York, Garland Science, Taylor & Francis Group.
- Altman, J. (1963). "Autoradiographic investigations of cell proliferation in the brains of rats and cats. Postnatal growth and differentiation of the mammalian brain, with implications for a morphological theory of memory." The Anatomical Record **145**(4): 573-591.

- Altman, J. (1966). "Autoradiographic and histological studies of postnatal neurogenesis II. A longitudinal investigation of the kinetics, migration and transformation of cells incorporating tritiated thymidine in infant rats, with special reference to postnatal neurogenesis in some brain regions. ." The Journal of Comparative Neurology **128**(4): 431-474.
- Altman, J. (1969). "Autoradiographic and histological studies of postnatal neurogenesis IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb." The Journal of Comparative Neurology **137**: 433-458.
- Altman, J. and G. Das (1965). "Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats." The Journal of Comparative Neurology **124**(3): 319-335.
- Alvarez-Buylla, A., B. Seri and F. Doetsch (2002). "Identification of neural stem cells in the adult vertebrate brain." Brain Res Bull **57**(6): 751-758.
- Amrein, I., K. Isler and H. P. Lipp (2011). "Comparing adult hippocampal neurogenesis in mammalian species and orders: influence of chronological age and life history stage." Eur J Neurosci **34**(6): 978-987.
- Andersen, P., T. V. Bliss and K. K. Skrede (1971). "Lamellar organization of hippocampal pathways." Exp Brain Res **13**(2): 222-238.
- Arvidsson, A., T. Collin, D. Kirik, Z. Kokaia and O. Lindvall (2002). "Neuronal replacement from endogenous precursors in the adult brain after stroke." Nat Med **8**(9): 963-970.

- Augustinack, J. C., A. J. van der Kouwe, D. H. Salat, T. Benner, A. A. Stevens, J. Annese, B. Fischl, M. P. Frosch and S. Corkin (2014). "H.M.'s contributions to neuroscience: A review and autopsy studies." Hippocampus.
- Baiza-Duran, L., J. Medrano-Palafox, E. Hernandez-Quintela, J. Lozano-Alcazar and O. J. Alaniz-de la (2010). "A comparative clinical trial of the efficacy of two different aqueous solutions of cyclosporine for the treatment of moderate-to-severe dry eye syndrome." Br J Ophthalmol **94**(10): 1312-1315.
- Banerjee, A., M. C. Roach, P. Trcka and R. F. Luduena (1990). "Increased microtubule assembly in bovine brain tubulin lacking the type III isotype of beta-tubulin." J Biol Chem **265**(3): 1794-1799.
- Bayer, S. A., J. W. Yackel and P. S. Puri (1982). "Neurons in the rat dentate gyrus granular layer substantially increase during juvenile and adult life." Science **216**(4548): 890-892.
- Bekinschtein, P., M. Cammarota, C. Katche, L. Slipczuk, J. I. Rossato, A. Goldin, I. Izquierdo and J. H. Medina (2008). "BDNF is essential to promote persistence of long-term memory storage." Proc Natl Acad Sci U S A **105**(7): 2711-2716.
- Bernardi, P., K. M. Broekemeier and D. R. Pfeiffer (1994). "Recent progress on regulation of the mitochondrial permeability transition pore; a cyclosporin-sensitive pore in the inner mitochondrial membrane." J Bioenerg Biomembr **26**(5): 509-517.
- Berth-Jones, J. (2005). "The use of ciclosporin in psoriasis." J Dermatolog Treat **16**(5-6): 258-277.
- Biebl, M., C. M. Cooper, J. Winkler and H. G. Kuhn (2000). "Analysis of neurogenesis and programmed cell death reveals a self-renewing capacity in the adult rat brain." Neurosci Lett **291**(1): 17-20.

- Blackstad, T. W. (1958). "On the termination of some afferents to the hippocampus and fascia dentata; an experimental study in the rat." Acta Anat (Basel) **35**(3): 202-214.
- Boehme, F., J. Gil-Mohapel, A. Cox, A. Patten, E. Giles, P. S. Brocardo and B. R. Christie (2011). "Voluntary exercise induces adult hippocampal neurogenesis and BDNF expression in a rodent model of fetal alcohol spectrum disorders." Eur J Neurosci **33**(10): 1799-1811.
- Bonaguidi, M. A., C. Y. Peng, T. McGuire, G. Falciglia, K. T. Gobeske, C. Czeisler and J. A. Kessler (2008). "Noggin expands neural stem cells in the adult hippocampus." J Neurosci **28**(37): 9194-9204.
- Borel, J. F., F. Di Padova, J. Mason, V. Quesniaux, B. Ryffel and R. Wenger (1990). "Pharmacology of cyclosporine (sandimmune). I. Introduction." Pharmacol Rev **41**(3): 239-242.
- Borel, J. F., C. Feurer, C. Magnee and H. Stahelin (1977). "Effects of the new anti-lymphocytic peptide cyclosporin A in animals." Immunology **32**(6): 1017-1025.
- Brandt, M. D., S. Jessberger, B. Steiner, G. Kronenberg, K. Reuter, A. Bick-Sander, W. von der Behrens and G. Kempermann (2003). "Transient calretinin expression defines early postmitotic step of neuronal differentiation in adult hippocampal neurogenesis of mice." Mol Cell Neurosci **24**(3): 603-613.
- Brown, J. P., S. Couillard-Despres, C. M. Cooper-Kuhn, J. Winkler, L. Aigner and H. G. Kuhn (2003). "Transient expression of doublecortin during adult neurogenesis." J Comp Neurol **467**(1): 1-10.

- Burgmans, S., M. P. van Boxtel, E. F. Vuurman, E. A. Evers and J. Jolles (2010). "Increased neural activation during picture encoding and retrieval in 60-year-olds compared to 20-year-olds." Neuropsychologia **48**(7): 2188-2197.
- Cajal, S. (1899-1904). Texture of the nervous system of man and the vertebrates. Vienna, Springer.
- Callen, J. P. (2001). "Immunosuppressive and cytotoxic drugs in the treatment of rheumatic skin disorders." Semin Cutan Med Surg **20**(1): 58-68.
- Cameron, H. A. and R. D. McKay (1999). "Restoring production of hippocampal neurons in old age." Nat Neurosci **2**(10): 894-897.
- Cameron, H. A. and R. D. McKay (2001). "Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus." J Comp Neurol **435**(4): 406-417.
- Casarosa, S., J. Zasso and L. Conti (2013). Systems for ex-vivo Isolation and Culturing of Neural Stem Cells.
- Chen, H., G. N. Pandey and Y. Dwivedi (2006). "Hippocampal cell proliferation regulation by repeated stress and antidepressants." Neuroreport **17**(9): 863-867.
- Chiasson, B., V. Tropepe, C. Morshead and D. van der Kooy (1999). "Adult mammalian forebrain ependymal and subependymal cells demonstrate proliferative potential, but only subependymal cells have neural stem cell characteristics." The Journal of Neuroscience **19**(11): 4462-4471.
- Clarke, L. and D. van der Kooy (2011). "The adult mouse dentate gyrus contains populations of committed progenitor cells that are distinct from subependymal zone neural stem cells." Stem Cells **29**(9): 1448-1458.

- Clelland, C. D., M. Choi, C. Romberg, G. D. Clemenson, Jr., A. Fragniere, P. Tyers, S. Jessberger, L. M. Saksida, R. A. Barker, F. H. Gage and T. J. Bussey (2009). "A functional role for adult hippocampal neurogenesis in spatial pattern separation." Science **325**(5937): 210-213.
- Codega, P., V. Silva-Vargas, A. Paul, A. R. Maldonado-Soto, A. M. Deleo, E. Pastrana and F. Doetsch (2014). "Prospective identification and purification of quiescent adult neural stem cells from their in vivo niche." Neuron **82**(3): 545-559.
- Cooke, M. J., K. Vulic and M. S. Shoichet (2010). "Design of biomaterials to enhance stem cell survival when transplanted into the damaged central nervous system." Soft Matter **6**(20): 4988-4998.
- Coras, R., F. A. Siebzehnruhl, E. Pauli, H. B. Huttner, M. Njunting, K. Kobow, C. Villmann, E. Hahnen, W. Neuhuber, D. Weigel, M. Buchfelder, H. Stefan, H. Beck, D. A. Steindler and I. Blumcke (2010). "Low proliferation and differentiation capacities of adult hippocampal stem cells correlate with memory dysfunction in humans." Brain **133**(11): 3359-3372.
- Corkin, S. (2002). "What's new with the amnesic patient H.M.?" Nat Rev Neurosci **3**(2): 153-160.
- Creer, D. J., C. Romberg, L. M. Saksida, H. van Praag and T. J. Bussey (2010). "Running enhances spatial pattern separation in mice." Proc Natl Acad Sci U S A **107**(5): 2367-2372.
- Curlik, D. M., 2nd and T. J. Shors (2011). "Learning increases the survival of newborn neurons provided that learning is difficult to achieve and successful." J Cogn Neurosci **23**(9): 2159-2170.

- D'Hooge, R. and P. P. De Deyn (2001). "Applications of the Morris water maze in the study of learning and memory." Brain Res Brain Res Rev **36**(1): 60-90.
- Deng, W., J. B. Aimone and F. H. Gage (2010). "New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory?" Nat Rev Neurosci **11**(5): 339-350.
- Deng, W., M. D. Saxe, I. S. Gallina and F. H. Gage (2009). "Adult-born hippocampal dentate granule cells undergoing maturation modulate learning and memory in the brain." J Neurosci **29**(43): 13532-13542.
- Deng, Y., B. M. Thompson, X. Gao and E. D. Hall (2007). "Temporal relationship of peroxynitrite-induced oxidative damage, calpain-mediated cytoskeletal degradation and neurodegeneration after traumatic brain injury." Exp Neurol **205**(1): 154-165.
- Doetsch, F. (2003). "The glial identity of neural stem cells." Nat Neurosci **6**(11): 1127-1134.
- Doetsch, F. and A. Alvarez-Buylla (1996). "Network on tangential pathways for neuronal migration in the adult mammalian brain." Proc Natl Acad Sci USA **93**(25): 14895-14900.
- Donnan, G. A., M. Fisher, M. Macleod and S. M. Davis (2008). "Stroke." Lancet **371**(9624): 1612-1623.
- Dranovsky, A., A. M. Picchini, T. Moadel, A. C. Sisti, A. Yamada, S. Kimura, E. D. Leonardo and R. Hen (2011). "Experience dictates stem cell fate in the adult hippocampus." Neuron **70**(5): 908-923.
- Dreyfuss, M., E. Härri, H. Hofmann, H. Kobel, W. Pache and H. Tschertter (1976). "Cyclosporin A and C." European journal of applied microbiology and biotechnology **3**(2): 125-133.

- Dubois-Dalcq, M. (1987). "Characterization of a slowly proliferative cell along the oligodendrocyte differentiation pathway." Embo j **6**(9): 2587-2595.
- Eriksson, P., E. Perfilieva, T. Bjork-Eriksson, A. Alborn, C. Nordborg, D. Peterson and F. Gage (1998). "Neurogenesis in the adult human hippocampus." Nat Med **4**: 1313-1317.
- Ernfors, P., J. Kucera, K. F. Lee, J. Loring and R. Jaenisch (1995). "Studies on the physiological role of brain-derived neurotrophic factor and neurotrophin-3 in knockout mice." Int J Dev Biol **39**(5): 799-807.
- Filippov, V., G. Kronenberg, T. Pivneva, K. Reuter, B. Steiner, L. P. Wang, M. Yamaguchi, H. Kettenmann and G. Kempermann (2003). "Subpopulation of nestin-expressing progenitor cells in the adult murine hippocampus shows electrophysiological and morphological characteristics of astrocytes." Mol Cell Neurosci **23**(3): 373-382.
- Frankland, P. W., S. Kohler and S. A. Josselyn (2013). "Hippocampal neurogenesis and forgetting." Trends Neurosci **36**(9): 497-503.
- Gates, M. A., L. B. Thomas, E. M. Howard, E. D. Laywell, B. Sajin, A. Faissner, B. Gotz, J. Silver and D. A. Steindler (1995). "Cell and molecular analysis of the developing and adult mouse subventricular zone of the cerebral hemispheres." J Comp Neurol **361**(2): 249-266.
- Ge, S., E. L. Goh, K. A. Sailor, Y. Kitabatake, G. L. Ming and H. Song (2006). "GABA regulates synaptic integration of newly generated neurons in the adult brain." Nature **439**(7076): 589-593.
- Goldman, S. and F. Nottebohm (1983). "Neuronal production, migration, and differentiation in vocal control nucleus of the adult female canary brain." Proc Natl Acad Sci USA **80**: 2390-2394.

- Goldner, F. M. and J. W. Patrick (1996). "Neuronal localization of the cyclophilin A protein in the adult rat brain." J Comp Neurol **372**(2): 283-293.
- Gould, E., A. Beylin, P. Tanapat, A. Reeves and T. J. Shors (1999). "Learning enhances adult neurogenesis in the hippocampal formation." Nat Neurosci **2**(3): 260-265.
- Gulyas, A. I., N. Hajos and T. F. Freund (1996). "Interneurons containing calretinin are specialized to control other interneurons in the rat hippocampus." J Neurosci **16**(10): 3397-3411.
- Hanauer, S. B. (2004). "Medical therapy for ulcerative colitis 2004." Gastroenterology **126**(6): 1582-1592.
- Hansson, M. J., G. Mattiasson, R. Mansson, J. Karlsson, M. F. Keep, P. Waldmeier, U. T. Ruegg, J. M. Dumont, K. Besseghir and E. Elmer (2004). "The nonimmunosuppressive cyclosporin analogs NIM811 and UNIL025 display nanomolar potencies on permeability transition in brain-derived mitochondria." J Bioenerg Biomembr **36**(4): 407-413.
- Hastings, N. B. and E. Gould (1999). "Rapid extension of axons into the CA3 region by adult-generated granule cells." J Comp Neurol **413**(1): 146-154.
- He, L., A. Sabet, S. Djedjos, R. Miller, X. Sun, M. A. Hussain, S. Radovick and F. E. Wondisford (2009). "Metformin and insulin suppress hepatic gluconeogenesis through phosphorylation of CREB binding protein." Cell **137**(4): 635-646.
- Heales, S. J., J. P. Bolanos, V. C. Stewart, P. S. Brookes, J. M. Land and J. B. Clark (1999). "Nitric oxide, mitochondria and neurological disease." Biochim Biophys Acta **1410**(2): 215-228.

- Hester, M. S. and S. C. Danzer (2014). "Hippocampal granule cell pathology in epilepsy - A possible structural basis for comorbidities of epilepsy?" Epilepsy Behav.
- Hirabayashi, Y., Y. Itoh, H. Tabata, K. Nakajima, T. Akiyama, N. Masuyama and Y. Gotoh (2004). "The Wnt/beta-catenin pathway directs neuronal differentiation of cortical neural precursor cells." Development **131**(12): 2791-2801.
- His, W. (1904). The development of the human brain. Leipzig, Hirzel.
- Hunt, J., A. Cheng, A. Hoyle, E. Jervis and C. M. Morshead (2010). "Cyclosporin A has direct effects on adult neural precursor cells." J Neurosci **30**(8): 2888-2896.
- Jacotte-Simancas, A., D. Costa-Miserachs, M. Coll-Andreu, M. Torras-Garcia, C. Borlongan and I. Portell-Cortes (2014). "Effects of voluntary physical exercise, citicoline, and combined treatment on object recognition memory, neurogenesis and neuroprotection after traumatic brain injury in rats." J Neurotrauma.
- Jacque, C. M., C. Vinner, M. Kujas, M. Raoul, J. Racadot and N. A. Baumann (1978). "Determination of glial fibrillary acidic protein (GFAP) in human brain tumors." J Neurol Sci **35**(1): 147-155.
- Jedynak, P., P. Jaholkowski, G. Wozniak, C. Sandi, L. Kaczmarek and R. K. Filipkowski (2012). "Lack of cyclin D2 impairing adult brain neurogenesis alters hippocampal-dependent behavioral tasks without reducing learning ability." Behav Brain Res **227**(1): 159-166.
- Jessberger, S., C. Zhao, N. Toni, G. D. Clemenson, Jr., Y. Li and F. H. Gage (2007). "Seizure-associated, aberrant neurogenesis in adult rats characterized with retrovirus-mediated cell labeling." J Neurosci **27**(35): 9400-9407.

- Jhaveri, D. J., E. W. Mackay, A. S. Hamlin, S. V. Marathe, L. S. Nandam, V. A. Vaidya and P. F. Bartlett (2010). "Norepinephrine directly activates adult hippocampal precursors via beta3-adrenergic receptors." J Neurosci **30**(7): 2795-2806.
- Kaminska, B., K. Gaweda-Walerych and M. Zawadzka (2004). "Molecular mechanisms of neuroprotective action of immunosuppressants--facts and hypotheses." J Cell Mol Med **8**(1): 45-58.
- Kaplan, M. and D. Bell (1984). "Mitotic neuroblasts in the 9-day-old and 11-month-old rodent hippocampus." The Journal of Neuroscience **4**: 1429-1441.
- Kaplan, M. and J. Hinds (1977). "Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs." Science **197**(4308): 1092-1094.
- Kee, N., S. Sivalingam, R. Boonstra and J. M. Wojtowicz (2002). "The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis." J Neurosci Methods **115**(1): 97-105.
- Kee, N., C. M. Teixeira, A. H. Wang and P. W. Frankland (2007). "Preferential incorporation of adult-generated granule cells into spatial memory networks in the dentate gyrus." Nat Neurosci **10**(3): 355-362.
- Kempermann, G. (2006). Technical Notes. Adult Neurogenesis: Stem Cells and Neuronal Development in the Adult Brain. New York, Oxford University Press: 195.
- Kempermann, G., D. Gast, G. Kronenberg, M. Yamaguchi and F. H. Gage (2003). "Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice." Development **130**(2): 391-399.
- Kempermann, G., S. Jessberger, B. Steiner and G. Kronenberg (2004). "Milestones of neuronal development in the adult hippocampus." Trends Neurosci **27**(8): 447-452.
- Khan, M. (2008). Immunopharmacology. Omaha, NE, Science Media, LLC.

- Kim, H. J., W. Kim and S. Y. Kong (2013). "Antidepressants for neuro-regeneration: from depression to Alzheimer's disease." Arch Pharm Res **36**(11): 1279-1290.
- Kleindienst, A., M. J. McGinn, H. B. Harvey, R. J. Colello, R. J. Hamm and M. R. Bullock (2005). "Enhanced hippocampal neurogenesis by intraventricular S100B infusion is associated with improved cognitive recovery after traumatic brain injury." J Neurotrauma **22**(6): 645-655.
- Knoth, R., I. Singec, M. Ditter, G. Pantazis, P. Capetian, R. P. Meyer, V. Horvat, B. Volk and G. Kempermann (2010). "Murine features of neurogenesis in the human hippocampus across the lifespan from 0 to 100 years." PLoS One **5**(1): e8809.
- Koelliker, A. (1896). Handbook of human histology. Leipzig, Engelmann.
- Kronenberg, G., K. Reuter, B. Steiner, M. D. Brandt, S. Jessberger, M. Yamaguchi and G. Kempermann (2003). "Subpopulations of proliferating cells of the adult hippocampus respond differently to physiologic neurogenic stimuli." J Comp Neurol **467**(4): 455-463.
- Kuhn, H. G., H. Dickinson-Anson and F. H. Gage (1996). "Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation." J Neurosci **16**(6): 2027-2033.
- Laplagne, D. A., M. S. Esposito, V. C. Piatti, N. A. Morgenstern, C. Zhao, H. van Praag, F. H. Gage and A. F. Schinder (2006). "Functional convergence of neurons generated in the developing and adult hippocampus." PLoS Biol **4**(12): e409.
- Lichtenwalner, R. J., M. E. Forbes, S. A. Bennett, C. D. Lynch, W. E. Sonntag and D. R. Riddle (2001). "Intracerebroventricular infusion of insulin-like growth factor-I ameliorates

- the age-related decline in hippocampal neurogenesis." Neuroscience **107**(4): 603-613.
- Lie, D. C., S. A. Colamarino, H. J. Song, L. Desire, H. Mira, A. Consiglio, E. S. Lein, S. Jessberger, H. Lansford, A. R. Dearie and F. H. Gage (2005). "Wnt signalling regulates adult hippocampal neurogenesis." Nature **437**(7063): 1370-1375.
- Lodish, H., A. Berk, C. Kaiser, M. Krieger, M. Scott, A. Bretscher, H. Ploegh and P. Matsudaira (2008). Molecular Cell Biology. New York, W.H. Freeman and Company.
- Lois, C. and A. Alvarez-Buylla (1994). "Long-distance neuronal migration in the adult mammalian brain." Science **264**(5162): 1145-1148.
- Luo, C. X., J. Jiang, Q. G. Zhou, X. J. Zhu, W. Wang, Z. J. Zhang, X. Han and D. Y. Zhu (2007). "Voluntary exercise-induced neurogenesis in the postischemic dentate gyrus is associated with spatial memory recovery from stroke." J Neurosci Res **85**(8): 1637-1646.
- Luskin, M. (1993). "Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone." Neuron **11**(1): 173-189.
- Machon, O., M. Backman, O. Machonova, Z. Kozmik, T. Vacik, L. Andersen and S. Krauss (2007). "A dynamic gradient of Wnt signaling controls initiation of neurogenesis in the mammalian cortex and cellular specification in the hippocampus." Dev Biol **311**(1): 223-237.
- Marsh, J. D. and S. G. Keyrouz (2010). "Stroke prevention and treatment." J Am Coll Cardiol **56**(9): 683-691.
- Mason, C. (2005). "Tissue engineering skin: a paradigm shift in wound care." Med Device Technol **16**(10): 32-33.

- Mayer, E. J., D. A. Carter, Y. Ren, E. H. Hughes, C. M. Rice, C. A. Halfpenny, N. J. Scolding and A. D. Dick (2005). "Neural progenitor cells from postmortem adult human retina." Br J Ophthalmol **89**(1): 102-106.
- McDonald, H. Y. and J. M. Wojtowicz (2005). "Dynamics of neurogenesis in the dentate gyrus of adult rats." Neurosci Lett **385**(1): 70-75.
- McDonald, R. J., L. A. Craig and N. S. Hong (2008). "Enhanced cell death in hippocampus and emergence of cognitive impairments following a localized mini-stroke in hippocampus if preceded by a previous episode of acute stress." Eur J Neurosci **27**(8): 2197-2209.
- Messier, B. and C. Leblond (1960). "Cell proliferation and migration as revealed by radioautography after injection of thymidine- H^3 into male rats and mice." Am J Anat **106**: 247-285.
- Morshead, C., P. Benveniste, N. Iscove and D. van der Kooy (2002). "Hematopoietic competence is a rare property of neural stem cells that may depend on genetic and epigenetic alterations." Nature Medicine **8**: 268-273.
- Morshead, C., B. Reynolds, C. Craig, M. Mcburney, W. Staines, Morassutti, S. Weiss and D. van der Kooy (1994). "Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells." Neuron **13**(5): 1071-1082.
- Munji, R. N., Y. Choe, G. Li, J. A. Siegenthaler and S. J. Pleasure (2011). "Wnt signaling regulates neuronal differentiation of cortical intermediate progenitors." J Neurosci **31**(5): 1676-1687.
- Nakajima, S., I. Ohsawa, S. Ohta, M. Ohno and T. Mikami (2010). "Regular voluntary exercise cures stress-induced impairment of cognitive function and cell proliferation

- accompanied by increases in cerebral IGF-1 and GST activity in mice." Behav Brain Res **211**(2): 178-184.
- Nilsson, M., E. Perfilieva, U. Johansson, O. Orwar and P. S. Eriksson (1999). "Enriched environment increases neurogenesis in the adult rat dentate gyrus and improves spatial memory." J Neurobiol **39**(4): 569-578.
- Nomura, T., T. Inamura and K. L. Black (1994). "Intracarotid infusion of bradykinin selectively increases blood-tumor permeability in 9L and C6 brain tumors." Brain Res **659**(1-2): 62-66.
- Olariu, A., K. M. Cleaver, L. E. Shore, M. D. Brewer and H. A. Cameron (2005). "A natural form of learning can increase and decrease the survival of new neurons in the dentate gyrus." Hippocampus **15**(6): 750-762.
- Osman, M. M., D. Lulic, L. Glover, C. E. Stahl, T. Lau, H. van Loveren and C. V. Borlongan (2011). "Cyclosporine-A as a neuroprotective agent against stroke: its translation from laboratory research to clinical application." Neuropeptides **45**(6): 359-368.
- Overstreet Wadiche, L., D. A. Bromberg, A. L. Bensen and G. L. Westbrook (2005). "GABAergic signaling to newborn neurons in dentate gyrus." J Neurophysiol **94**(6): 4528-4532.
- Palmer, T. D., J. Takahashi and F. H. Gage (1997). "The adult rat hippocampus contains primordial neural stem cells." Mol Cell Neurosci **8**(6): 389-404.
- Paton, J. and F. Nottebohm (1984). "Neurons generated in the adult brain are recruited into functional circuits." Science(225): 1046-1048.
- Patton, P. E. and B. McNaughton (1995). "Connection matrix of the hippocampal formation: I. The dentate gyrus." Hippocampus **5**(4): 245-286.

- Rakic, P. (1985). "Limits of neurogenesis in primates." Science **227**: 1054-1056.
- Ramirez-Amaya, V., D. F. Marrone, F. H. Gage, P. F. Worley and C. A. Barnes (2006).
 "Integration of new neurons into functional neural networks." J Neurosci **26**(47):
 12237-12241.
- Raya-Rivera, A. M., D. Esquiliano, R. Fierro-Pastrana, E. Lopez-Bayghen, P. Valencia, R.
 Ordorica-Flores, S. Soker, J. J. Yoo and A. Atala (2014). "Tissue-engineered
 autologous vaginal organs in patients: a pilot cohort study." Lancet.
- Reynolds, B. and S. Weiss (1992). "Generation of neurons and astrocytes from isolated cells of
 the adult mammalian central nervous system." Science **255**: 1707-1710.
- Rossi, C., A. Angelucci, L. Costantin, C. Braschi, M. Mazzantini, F. Babbini, M. E. Fabbri, L.
 Tessarollo, L. Maffei, N. Berardi and M. Caleo (2006). "Brain-derived neurotrophic
 factor (BDNF) is required for the enhancement of hippocampal neurogenesis
 following environmental enrichment." Eur J Neurosci **24**(7): 1850-1856.
- Roy, N. S., S. Wang, L. Jiang, J. Kang, A. Benraiss, C. Harrison-Restelli, R. A. Fraser, W. T.
 Couldwell, A. Kawaguchi, H. Okano, M. Nedergaard and S. A. Goldman (2000). "In
 vitro neurogenesis by progenitor cells isolated from the adult human hippocampus."
Nat Med **6**(3): 271-277.
- Sachewsky, N., J. Hunt, M. J. Cooke, A. Azimi, T. Zarin, C. Miu, M. S. Shoichet and C. M.
 Morshead (2014). "Cyclosporin A enhances neural precursor cell survival in mice
 through a calcineurin-independent pathway." Dis Model Mech **7**(8): 953-961.
- Sahay, A. and R. Hen (2007). "Adult hippocampal neurogenesis in depression." Nat Neurosci
10(9): 1110-1115.

- Sahay, A., K. N. Scobie, A. S. Hill, C. M. O'Carroll, M. A. Kheirbek, N. S. Burghardt, A. A. Fenton, A. Dranovsky and R. Hen (2011). "Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation." Nature **472**(7344): 466-470.
- Saxe, M. D., F. Battaglia, J. W. Wang, G. Malleret, D. J. David, J. E. Monckton, A. D. Garcia, M. V. Sofroniew, E. R. Kandel, L. Santarelli, R. Hen and M. R. Drew (2006). "Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus." Proc Natl Acad Sci U S A **103**(46): 17501-17506.
- Scadden, D. T. (2006). "The stem-cell niche as an entity of action." Nature **441**(7097): 1075-1079.
- Scharfman, H., J. Goodman, A. Macleod, S. Phani, C. Antonelli and S. Croll (2005). "Increased neurogenesis and the ectopic granule cells after intrahippocampal BDNF infusion in adult rats." Exp Neurol **192**(2): 348-356.
- Schinzl, A. C., O. Takeuchi, Z. Huang, J. K. Fisher, Z. Zhou, J. Rubens, C. Hetz, N. N. Danial, M. A. Moskowitz and S. J. Korsmeyer (2005). "Cyclophilin D is a component of mitochondrial permeability transition and mediates neuronal cell death after focal cerebral ischemia." Proc Natl Acad Sci U S A **102**(34): 12005-12010.
- Scholzen, T. and J. Gerdes (2000). "The Ki-67 protein: from the known and the unknown." J Cell Physiol **182**(3): 311-322.
- Seaberg, R. M. and D. van der Kooy (2002). "Adult rodent neurogenic regions: the ventricular subependyma contains neural stem cells, but the dentate gyrus contains restricted progenitors." J Neurosci **22**(5): 1784-1793.

- Seri, B., J. M. Garcia-Verdugo, B. S. McEwen and A. Alvarez-Buylla (2001). "Astrocytes give rise to new neurons in the adult mammalian hippocampus." J Neurosci **21**(18): 7153-7160.
- Shapiro, L. A., P. Upadhyaya and C. E. Ribak (2007). "Spatiotemporal profile of dendritic outgrowth from newly born granule cells in the adult rat dentate gyrus." Brain Res **1149**: 30-37.
- Shetty, A. K., B. Hattiangady and G. A. Shetty (2005). "Stem/progenitor cell proliferation factors FGF-2, IGF-1, and VEGF exhibit early decline during the course of aging in the hippocampus: role of astrocytes." Glia **51**(3): 173-186.
- Shors, T. J. (2008). "From stem cells to grandmother cells: how neurogenesis relates to learning and memory." Cell Stem Cell **3**(3): 253-258.
- Sisti, H. M., A. L. Glass and T. J. Shors (2007). "Neurogenesis and the spacing effect: learning over time enhances memory and the survival of new neurons." Learn Mem **14**(5): 368-375.
- Snyder, J. S., N. S. Hong, R. J. McDonald and J. M. Wojtowicz (2005). "A role for adult neurogenesis in spatial long-term memory." Neuroscience **130**(4): 843-852.
- Snyder, S. H., D. M. Sabatini, M. M. Lai, J. P. Steiner, G. S. Hamilton and P. D. Suzdak (1998). "Neural actions of immunophilin ligands." Trends Pharmacol Sci **19**(1): 21-26.
- Steiner, B., G. Kronenberg, S. Jessberger, M. D. Brandt, K. Reuter and G. Kempermann (2004). "Differential regulation of gliogenesis in the context of adult hippocampal neurogenesis in mice." Glia **46**(1): 41-52.
- Thomas, E. D., A. Fefer, C. D. Buckner and R. Storb (1977). "Current status of bone marrow transplantation for aplastic anemia and acute leukemia." Blood **49**(5): 671-681.

- Thrift, A. G., H. M. Dewey, R. A. Macdonell, J. J. McNeil and G. A. Donnan (2001). "Incidence of the major stroke subtypes: initial findings from the North East Melbourne stroke incidence study (NEMESIS)." Stroke **32**(8): 1732-1738.
- Van der Borght, K., R. Havekes, T. Bos, B. J. Eggen and E. A. Van der Zee (2007). "Exercise improves memory acquisition and retrieval in the Y-maze task: relationship with hippocampal neurogenesis." Behav Neurosci **121**(2): 324-334.
- van der Kooy, D. and S. Weiss (2000). "Why stem cells?" Science **287**(5457): 1439-1441.
- van Praag, H., B. R. Christie, T. J. Sejnowski and F. H. Gage (1999). "Running enhances neurogenesis, learning, and long-term potentiation in mice." Proc Natl Acad Sci U S A **96**(23): 13427-13431.
- van Praag, H., A. F. Schinder, B. R. Christie, N. Toni, T. D. Palmer and F. H. Gage (2002). "Functional neurogenesis in the adult hippocampus." Nature **415**(6875): 1030-1034.
- van Praag, H., T. Shubert, C. Zhao and F. H. Gage (2005). "Exercise enhances learning and hippocampal neurogenesis in aged mice." J Neurosci **25**(38): 8680-8685.
- Walker, T. L., A. White, D. M. Black, R. H. Wallace, P. Sah and P. F. Bartlett (2008). "Latent stem and progenitor cells in the hippocampus are activated by neural excitation." J Neurosci **28**(20): 5240-5247.
- Wang, J., D. Gallagher, L. M. DeVito, G. I. Cancino, D. Tsui, L. He, G. M. Keller, P. W. Frankland, D. R. Kaplan and F. D. Miller (2012). "Metformin activates an atypical PKC-CBP pathway to promote neurogenesis and enhance spatial memory formation." Cell Stem Cell **11**(1): 23-35.
- Wang, J., I. C. Weaver, A. Gauthier-Fisher, H. Wang, L. He, J. Yeomans, F. Wondisford, D. R. Kaplan and F. D. Miller (2010). "CBP histone acetyltransferase activity regulates

- embryonic neural differentiation in the normal and Rubinstein-Taybi syndrome brain." Dev Cell **18**(1): 114-125.
- Weiss, S., C. Dunne, J. Hewson, C. Wohl, M. Wheatley, A. Peterson and B. Reynolds (1996). "Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis." The Journal of Neuroscience **16**(23): 7599-7609.
- Weissman, I. L. (2000). "Stem cells: units of development, units of regeneration, and units in evolution." Cell **100**(1): 157-168.
- White, D. J. and R. Y. Calne (1982). "The use of Cyclosporin A immunosuppression in organ grafting." Immunol Rev **65**: 115-131.
- Winner, B., H. L. Melrose, C. Zhao, K. M. Hinkle, M. Yue, C. Kent, A. T. Braithwaite, S. Ogholikhan, R. Aigner, J. Winkler, M. J. Farrer and F. H. Gage (2011). "Adult neurogenesis and neurite outgrowth are impaired in LRRK2 G2019S mice." Neurobiol Dis **41**(3): 706-716.
- Winter, B., C. Breitenstein, F. C. Mooren, K. Voelker, M. Fobker, A. Lechtermann, K. Krueger, A. Fromme, C. Korsukewitz, A. Floel and S. Knecht (2007). "High impact running improves learning." Neurobiol Learn Mem **87**(4): 597-609.
- Wojtowicz, J. M., M. L. Askew and G. Winocur (2008). "The effects of running and of inhibiting adult neurogenesis on learning and memory in rats." Eur J Neurosci **27**(6): 1494-1502.
- Wurm, F., S. Keiner, A. Kunze, O. W. Witte and C. Redeker (2007). "Effects of skilled forelimb training on hippocampal neurogenesis and spatial learning after focal cortical infarcts in the adult rat brain." Stroke **38**(10): 2833-2840.

Yamada, K. and T. Nabeshima (2003). "Brain-derived neurotrophic factor/TrkB signaling in memory processes." J Pharmacol Sci **91**(4): 267-270.

Zhang, R., Z. Zhang, C. Zhang, L. Zhang, A. Robin, Y. Wang, M. Lu and M. Chopp (2004). "Stroke transiently increases subventricular zone cell division from asymmetric to symmetric and increases neuronal differentiation in the adult rat." J Neurosci **24**(25): 5810-5815.

Zhao, C., E. M. Teng, R. G. Summers, Jr., G. L. Ming and F. H. Gage (2006). "Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus." J Neurosci **26**(1): 3-11.

Zhao, X., T. Ueba, B. R. Christie, B. Barkho, M. J. McConnell, K. Nakashima, E. S. Lein, B. D. Eadie, A. R. Willhoite, A. R. Muotri, R. G. Summers, J. Chun, K. F. Lee and F. H. Gage (2003). "Mice lacking methyl-CpG binding protein 1 have deficits in adult neurogenesis and hippocampal function." Proc Natl Acad Sci U S A **100**(11): 6777-6782.