GENETIC ARCHITECTURE OF NEUROGENESIS IN THE ADULT MOUSE FOREBRAIN: INSIGHTS FROM QUANTITATIVE TRAIT LOCUS ANALYSES

by

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Abstract

Neural stem cells and their precursors, collectively referred to as neural progenitor cells (NPCs), are present in discrete regions of the mature brain, namely the subgranular zone (SGZ) of the dentate gyrus, the subventricular zone (SVZ), and rostral migratory stream (RMS). These NPCs divide and give rise to new neurons in a process called adult neurogenesis. Genetic influence is a major determinant of adult neurogenesis. However, the genetic architecture underlying NPC proliferation and differentiation is poorly understood. My thesis aims to gain insights into the genes regulating NPC proliferation using a phenotypic-driven, genome-wide approach. I first examined nine inbred mouse strains housed in the same condition and across different ages from 60 days (P60) to 2 years. Wide inter-strain differences and negative impact of age on the number of NPCs were observed in the RMS. Genetic background had a significant effect on NPC proliferation and it also differentially influenced the effect of age on this process. The most dramatic inter-strain difference was detected at P60. Heritability estimated ~50% of the differences in NPC numbers were attributed to the genetic variation among the strains. I used quantitative trait locus (QTL) mapping to survey the entire genome for chromosomal segments referred to as QTLs that contribute to the phenotypic differences. Two panels of recombinant inbred strains, AXB/BXAs and BXDs, were employed for QTL mapping. Genetic variation in QTLs on chromosome (Chr) 6 and 11 were significantly associated with the differences in NPC numbers in the RMS. Additional analyses revealed potential interaction of Chr 6 QTL with other loci. These QTLs are hypothesized to harbor genes important for NPC proliferation and downstream experimentation is required to validate the function of these genes. As proof of concept, a candidate gene called Galanin receptor 2 (Galr2) in the Chr 11 QTL was
demonstrated to be a pro-proliferative regulator of NPCs using in vitro techniques manipulating Galr2 expression and Galr2 knockout mice. In summary, I identified novel QTLs underlying NPC proliferation and these loci serve as starting points to identify genes (e.g. Galr2) critical to this process.
Preface

Chapter 1: A portion of subsection 1.2.1.1 describing the effect of nicotine on SVZ cell proliferation was published in Balsevich, G., Poon, A., Goldowitz, D. and Wilking, J. (2014) The effects of pre- and post-natal nicotine exposure and genetic background on the striatum and behavioral phenotypes in the mouse. Behav Brain Res. 266:7-18. I conducted histological analysis on the number of proliferative cells in the SVZ of preadolescent and adult C57BL/6J mice. I generated Figure 9 in this paper and partially contributed to the Results and Discussion sections.

Portions of Chapter 2 have been published in:


I contributed to experimental design and was responsible for all major areas of data collection, data analysis, and manuscript composition. Z. Li, a former postdoctoral fellow in the Goldowitz lab, contributed equally to publication #2 by generating hypotheses, performing data collection, analyzing results, and drafting the manuscript. Dr. G.W. Wolfe, a former member of the Goldowitz lab, provided technical assistance in tissue collection and sectioning. Professors L. Lu and R.W. Williams provided assistance on QTL analyses. Professors N.L. Hayes and R.S. Nowakowski aided in the design of cell cycle analysis. Dr. Goldowitz conceived the studies, aided in all experimental design, and provided critical review of the manuscripts.
Portions of Chapter 3 have been published in:


I contributed to experimental design and was responsible for all major areas of data collection, data analysis, and manuscript composition. Z. Li contributed equally to publication #1 by generating hypotheses, performing data collection, analyzing results, and drafting the manuscript. Dr. G.W. Wolfe provided technical assistance in tissue collection and sectioning. Professors L. Lu and R.W. Williams provided assistance on QTL mapping at the GeneNetwork and data interpretation. Professors N.L. Hayes and R.S. Nowakowski aided in the design of cell cycle analysis. Dr. Goldowitz conceived the studies, aided in all experimental design, and provided critical review of the manuscripts.

Chapter 4 contains unpublished data that is part of a manuscript in preparation. I conceived the study and designed the experiments. I was responsible for data collection, analysis, and manuscript composition.

Mice used in this study were housed, bred, and euthanized in accordance with ethical guidelines. All procedures were approved by the Animal Care Committee at the University of British Columbia (Animal Care Certificate: A12-0190).
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<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
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<tr>
<td>APP</td>
<td>Amyloid Precursor Protein</td>
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<td>β-gal</td>
<td>β-galactosidase</td>
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<td>BLBP</td>
<td>Brain Lipid-Binding Protein</td>
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<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<td>bFGF</td>
<td>Basic Fibroblast Growth Factor</td>
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<td>Cdk</td>
<td>Cyclin-dependent kinase</td>
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<td>Chr</td>
<td>Chromosome</td>
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<td>CV</td>
<td>Cresyl Violet</td>
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<td>Ctx</td>
<td>Cortex</td>
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<td>Dcx</td>
<td>Doublecortin</td>
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<td>DG</td>
<td>Dentate Gyrus</td>
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<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
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<td>FGF2</td>
<td>Fibroblast Growth Factor 2</td>
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<td>GalC</td>
<td>Galactocerebroside</td>
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<td>Galr2</td>
<td>Galanin receptor 2</td>
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<td>GCL</td>
<td>Granule Cell Layer</td>
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<td>GCV</td>
<td>Ganciclovir</td>
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<td>GF</td>
<td>Growth Fraction</td>
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<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
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GFP  Green Fluorescent Protein

HB-EGF  Heparin-Binding Epidermal Growth Factor

HD  Huntington’s Disease

HSD  Honestly Significant Difference

HTT  Huntingtin

IGF-1  Insulin-like Growth Factor-1

LOD  Likelihood of the odds

LRS  Likelihood Ratio Statistic

LSN  Lateral Septal Nucleus

LV  Lateral Ventricle

MAP2  Microtubule-Associated Protein 2

MAPK  Mitogen-Activated Protein Kinases

NeuN  Neuronal nuclear antigen

Nr2e1  Nuclear receptor subfamily 2, group E, member 1

NPY  Neuropeptide Y

NPC  Neural Progenitor Cell

NS  Not Significant

OB  Olfactory Bulb

P  Postnatal day

PD  Parkinson’s Disease

PSA-NCAM  Polysialylated Neuronal Cell Adhesion Molecule
QTL  Quantitative Trait Locus
Rb   Retinoblastoma Protein
RI   Recombinant Inbred
RMS  Rostral Migratory Stream
SD   Sprague-Dawley
SEM  Standard Error of the Mean
Sept9 Septin 9
SGZ  Subgranular Zone
SHR  Spontaneously Hypertensive Rats
shRNA Short hairpin RNA
SORLA Sortilin-related receptor with A-type repeats
Sox2 Sex determining region Y-box 2
Str  Striatum
SVZ  Subventricular zone
T_c  Total cell cycle time
T_s  S-phase time
TGF  Transforming Growth Factor
TK   Thymidine Kinase
VEGF Vascular Endothelial Growth Factor
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To my parents
Chapter 1: Introduction

1.1 Neurogenesis in the adult mammalian brain

1.1.1 Historical overview of adult neurogenesis

Adult neurogenesis, which refers to the continual production of new neurons in the adult brain, is one of the most fascinating neuroscience discoveries. The existence of adult neurogenesis was controversial as it challenged one of the central dogmas of neurogenesis established by Santiago Ramón y Cajal, the father of modern neuroscience, when he wrote, “In the adult centres, the nerve paths are something fixed, ended and immutable. Everything may die, nothing may be regenerated” (Ramón y Cajal, 1928). Joseph Altman and Gopal D. Das published the first histological evidence of adult neurogenesis in the 1960s where they used tritiated $[^3]H$ thymidine, a chemically modified DNA nucleoside that is incorporated into dividing cells to visualize labeled, mitotic cells in the dentate gyrus of the hippocampus (Altman and Das, 1965a) and the subventricular zone adjacent to the anterior lateral ventricles of the adult rat forebrain (Altman and Das, 1965b). Their histological analysis also revealed a continuous stream of mitotic cells from the SVZ to the olfactory bulb (OB) (Altman, 1969). Michael S. Kaplan and his colleagues later confirmed the observations reported by Altman and Das using electron microscopy of mitotic cells labeled with $[^3]H$-thymidine in adult rat and mouse brains (Kaplan and Hinds, 1977; Kaplan and Bell, 1984; Kaplan et al., 1985). Under the electron microscope, morphology of the labeled cells resembled granule cells in the dentate gyrus and olfactory bulb 30 days after the $[^3]H$-thymidine administration (Kaplan and Hinds, 1977). However, these groundbreaking findings contradicted the No-New-Neurons dogma and
were highly controversial. Many questioned the validity of the labeling method as cells undergoing DNA repair may also take up the $[^3\text{H}]-\text{thymidine}$ and the techniques used were inadequate in proving new neurons were generated rather than glia (Gross, 2000). Furthermore, a similar study by Pasko Rakic cast doubt on the existence and relevance of adult neurogenesis in higher order animals (Rakic, 1985). Rakic performed systematic analysis of autoradiographs prepared from rhesus monkeys injected with $[^3\text{H}]$ thymidine and failed to identify any $[^3\text{H}]$ thymidine-positive cells in the adult monkey brain (Rakic, 1985). As a result, the controversy of adult neurogenesis prevailed.

It was the discovery of adult neurogenesis in songbirds that revived the field (Goldman and Nottebohm, 1983). Nottebohm and his colleagues were the first to show newly generated neurons synapse with other neurons in the vocal control nucleus of the songbird. Their data indicated the incorporation of new neurons into existing neural networks (Burd and Nottebohm, 1985). The field started gaining momentum in the 1990s thanks to improvements in labeling methods, cell-type specific markers, and imaging techniques. Gerd Kempermann and colleagues published one of the landmark papers where they introduced Bromodeoxyuridine (BrdU) labeling of mitotic cells in the dentate gyrus of adult mice from different genetic backgrounds (Kempermann et al., 1997a). BrdU is a synthetic thymidine analog that is similar to $[^3\text{H}]-\text{thymidine}$ and gets incorporated into newly synthesized DNA of replicating cells during the S phase. Unlike $[^3\text{H}]-\text{thymidine}$, BrdU can be used in combination with other cell-specific markers, thus allow one to simultaneously examine the proliferative phased and identity of cell (Kempermann et al., 1997a). BrdU labeling was later used to demonstrate neurogenesis in the human (Eriksson et al., 1998) and monkey (Gould et al., 1998; Callaghan et al., 1999). The advancement in methodologies such as focal x-irradiation and treatment with cell cycle arrest
drugs (e.g. temozolomide) allowed alterations of adult neurogenesis in vivo. The application of these techniques further shed light into the functional roles of adult neurogenesis in hippocampal dependent learning and memory (Callaghan et al., 1999; Shors et al., 2001) and olfaction-dependent behaviors (Callaghan et al., 1999; Breton-Provencher et al., 2009; Valley et al., 2009). Substantial progress in the field of adult neurogenesis has been made over the past two decades. Now adult neurogenesis is well-established across different mammalian species and is accepted by the neuroscience community as a source of neuroplasticity in the adult brain.

1.1.2 Major sites of adult neurogenesis

Neurogenesis persists in discrete regions of the adult brain. In adult rodents, neurogenesis occurs predominantly in the dentate gyrus of the hippocampus and the subventricular zone (SVZ) of the forebrain (Cooper-Kuhn et al., 2002; Neumeister et al., 2009). The rostral migratory stream (RMS) is the rostral extension of the SVZ where neural precursor cells migrate toward the OB and differentiate into interneurons (Lois and Alvarez-Buylla, 1994; Kuhn et al., 1996). These brain regions that support the production or recruitment of new neurons are referred to as neurogenic (Gould, 2007). The cellular composition and organization for these neurogenic regions are described in the following subsections.

1.1.2.1 Subgranular zone (SGZ) of dentate gyrus

The SGZ is located at the interface of the hilus and the granule cell layer of the dentate gyrus. Major cellular components of the SGZ neurogenic niche are Type 1, 2a, 2b, and 3 cells. Type 1 cells are radial glial-like astrocytes with triangular shaped soma and apical process extending beyond the granule cell layer and branch diffusely into the molecular layer of the
dentate gyrus (Figure 1.1). Type 1 cells express stem cell markers Nestin and sex determining region Y-box 2 (Sox2) as well as astrocyte marker Glial Fibrillary Acidic Protein (GFAP) and radial glia marker Brain Lipid-Binding Protein (BLBP) (Steiner et al., 2004). Type 1 cells are putative neural stems that can divide asymmetrically to generate either neuronal lineage restricted progenitor cells (Type 2 cells) or glial lineage restricted progenitor cells (Steiner et al., 2004). Type 2a and 2b cells are active proliferating cells that have small soma and short processes (Figure 1.1). Both subtypes express Nestin and are distinguished by the absence (Type 2a) or presence (Type 2b) of Doublecortin (Dcx), an early neuronal marker (Doetsch et al., 2002b; Kronenberg et al., 2003). Type 2 cells give rise to Type 3 cells, which are also referred to as neuroblasts (Figure 1.1). These cells are characterized by rounded soma and they express Dcx and Polysialylated Neuronal Cell Adhesion Molecule (PSA-NCAM), a marker for migrating neurons. Type 3 cells give rise to new neurons, specifically the granule cells in the dentate gyrus. It is estimated that ~3000 new neurons are added daily to the dentate gyrus of adult 4-month old rat brain (Doetsch et al., 2002b; Rao and Shetty, 2004). The maturing granule cells first express calretinin, which is an early neuronal development marker, followed by expression of mature neuron and granule markers, neuronal nuclear antigen (NeuN) and calbindin (Doetsch et al., 2002b; Brandt et al., 2003). The dendrite of the maturing neurons extends into the molecular layer of the dentate gyrus and their axons reach the CA3 subfield of the hippocampus proper (Figure 1.1). It takes the newborn neurons approximately one month to fully mature and exhibit similar electrophysiological properties to the mature granule cells in the dentate gyrus (van Praag et al., 2002; De Marchis and Puche, 2012).
Figure 1.1 Developmental stages during adult hippocampal neurogenesis.
Summary of five developmental stages during adult hippocampal neurogenesis: (1) activation of quiescent radial glia-like cell in the subgranular zone (SGZ); (2) proliferation of non radial precursor and intermediate progenitors; (3) generation of neuroblasts; (4) integration of immature neurons; (5) maturation of adult-born dentate granule cells (based on Ming & Song, 2011, with permission).

1.1.2.2 Subventricular zone (SVZ) and rostral migratory stream (RMS)

The SVZ is the largest neurogenic region in the adult brain. It is located throughout the lateral, medial, and dorsal walls of the lateral ventricles (Alvarez-Buylla et al., 2008). Different types of neural progenitor cells are present in the SVZ, mainly the Type B, C, and A cells. The
Type B cells morphologically resemble radial glia, which are neural stem cells in the developing brain. The type B cells also possess properties of stem cells including the ability to self-renew and give rise to multiple cell types. The Type B cells express stem cell marker Nestin as well as glial markers vimentin and GFAP (García-Verdugo et al., 1998). In terms of lineage progression, type B cells give rise to Type C cells, which are also known as transient amplifying cells that give rise to Type A cells. Type C cells are the largest cells in the SVZ characterized by globular shaped soma and express Nestin as well as proneural protein Mash1 (also referred to achaete-scute complex homolog 1) (Figure 1.2). Whereas, the Type A cells that are also referred to as migrating neuroblasts, have smaller and more elongated soma and express neuronal migration markers Dcx and PSA-NCAM (Doetsch et al., 1997). Electron microscopy revealed that Type A cells aggregate into chains throughout the SVZ and are ensheathed by the Type B cells (Doetsch et al., 1997). Focal cluster of Type C cells are interspaced among the Type A chains (Doetsch et al., 1997). In addition to the type B cells, another cell type called the ependymal cells is also considered to function as adult neural stem cells in the brain (Chojnacki et al., 2009).

Ependymal cells form a single layer separating the SVZ from the lumen of the ventricle. These cells are ciliated and are proposed to set up the cerebrospinal fluid (CSF) flow around the lateral ventricles where chemotactic factors are secreted by the choroid plexus (Sawamoto et al., 2006). The circulation of CSF help establish caudal to rostral gradients of chemotactic factors that direct the migration of SVZ precursors to the RMS (Sawamoto et al., 2006). Lineage tracing of ependymal cells in the adult rodent brain revealed that these cells can give rise to other neural precursors in the SVZ, RMS, and new neurons in the OB (Reynolds and Weiss, 1992; Johansson et al., 1999). Despite the strong evidence of ependymal cell proliferation \textit{in vivo}, these cells failed to self-renew and demonstrate multipotency where they were unable to generate both
neurons and glial cells in culture (Chiasson et al., 1999; Jablonska et al., 2007) (Capela and Temple, 2002). As a result, the identity of these periventricular neural stem cells remains controversial (Chojnacki et al., 2012).

The Type A neuroblasts born in the SVZ migrate rostrally and merge into the RMS, a discrete pathway that leads the neural precursors into the OB. In adult mice, the RMS is approximately 3-5mm in length (Doetsch and Alvarez-Buylla, 1996; Lois et al., 1996) and the neural precursors are reported to migrate at speeds of ~50-100μm/h (Bolteus and Bordey, 2004; Hall et al., 2009; Nieman et al., 2010). In addition to the neuroblasts, the RMS is comprised of astroglia that form a glial tube to encapsulate the migrating cells and prevent these cells dispersing into surrounding tissues. The neuroblasts migrate by sliding along each other in the RMS. Movement of the neuroblasts towards the OB is directed by a combination of intrinsic and environmental cues (Shi et al., 2004; Ghashghaei et al., 2007). The SVZ-derived neuroblasts retain their capability to replicate and these cells actively divide while migrating in the RMS (Menezes et al., 1995; Yu et al., 2000). Over 30,000 neuroblasts are present in the RMS on a daily basis (Lois and Alvarez-Buylla, 1994; Shi et al., 2004), this makes RMS the second largest proliferative zone in the adult brain following the SVZ.

Upon arrival at the OB, the neuroblasts detach from the chains, migrate radially to one of the overlying cell layers and differentiate into either granule or periglomerular interneurons (Luskin, 1993; Shi et al., 2004). The majority of the newborn cells (75-99%) differentiate into granule cells that receive Gamma amino butyric acid (GABA) as neurotransmitter and the remaining 1-25% of new born cells become periglomerular cells that use GABA and/or dopamine as neurotransmitter (Luskin, 1998; Winner et al., 2002; Belluzzi et al., 2003; Carleton et al., 2003). The use of c-Fos, a marker for neuronal activity, showed the new born neurons
respond to odor stimuli (Jin et al., 2001). Retroviral labeling of neural progenitor cells in the SVZ revealed ~50% of the new born neurons died shortly after their arrival in the OB (15-45 days after birth) and the survival of the new born neurons is dependent on sensory input (Tonchev et al., 2005). Trans-synaptic neuronal tracing with green fluorescent protein (GFP) expressing virus showed the survived interneurons become synaptically and functionally integrated into existing OB circuitry (Carlén et al., 2002).

Figure 1.2 Developmental stages during adult SVZ-RMS-OB neurogenesis. Summary of five developmental stages during adult SVZ neurogenesis: (1) activation of radial glia-like cells in the subventricular zone in the lateral ventricle (LV); (2) proliferation of transient amplifying cells; (3) generation of neuroblasts; (4) chain migration of neuroblasts within the rostral migratory stream (RMS) and radial migration of immature neurons in the olfactory bulb (OB); (5) Synaptic integration and maturation of granule cells and periglomerular cells in the OB (based on Ming & Song, 2011, with permission).
1.1.3 Functional implications of adult neurogenesis

1.1.3.1 Functional relevance of adult hippocampal neurogenesis

The continuous generation of new neurons in the dentate gyrus has been implicated in hippocampal-dependent learning and memory. However, the functional roles of this process remain controversial as inconsistent results were reported from different labs. Summaries of findings from different experimental approaches are provided in this section.

One approach to determine whether the newly generated cells are functional is by using electrophysiological techniques that measure the electrical activity of neurons. Van Praag and colleagues were the first group to perform electrophysiological recordings on newborn neurons and demonstrate the neuronal electrophysiological properties of newborn neurons resembled existing mature granule cells in the dentate gyrus (van Praag et al., 2002). Synaptophysin immunohistochemistry further revealed that adult-born neurons received synaptic inputs from other neurons in the circuitry (van Praag et al., 2002). The growth of new neurons was tracked using retroviral labeling with GFP. By 4 weeks, newborn neurons were found to acquire the morphological characteristics of the mature granule cells and were functionally integrated into the pre-existing circuitry (van Praag et al., 2002).

To determine the extent to which newly generated granule cells is involved in hippocampal-dependent behaviors, different methods for blocking neurogenesis and behavioral tests have been used. Dupret et al. (2002) developed a transgenic mouse model that overexpresses pro-apoptotic protein Bax in adult hippocampal NPCs. Bax-induced apoptosis in the NPCs resulted in decreased hippocampal neurogenesis which in turn resulted in a decline in spatial learning and memory performance, as assessed by Morris water maze where mice are
required to learn visual cues placed around the maze and build a spatial representation of their surroundings in order to locate the hidden platform underneath the water surface (Lazic et al., 2004). Revest et al. (2009) used the same transgenic mouse model and showed impairment of hippocampal neurogenesis increased anxiety-related behaviors, as measured by tests such as elevated plus maze and predator exposure test (Revest et al., 2009). Other approaches used to induce apoptosis in the adult neural precursors include focal x-irradiation where x-ray was administered to a restricted fraction of the brain containing hippocampus (Saxe et al., 2006). Ablation of adult neurogenesis can also be achieved using transgenic mice such as GFAP-TK TG mice where GFAP-positive NPCs express thymidine kinase (TK) that is targeted by the antiviral drug ganciclovir (GCV). Upon administration of GCV, cell death is induced in GFAP+ cells (van Praag et al., 1999a). Saxe and colleagues used both focal x-irradiation and genetic ablation strategies to suppress hippocampal neurogenesis. They found the impairment of hippocampal neurogenesis resulted in loss of long-term potentiation, as measured by electrophysiological techniques, and decline in associative learning, as assessed by contextual fear conditioning (van Praag et al., 1999b). They also performed Morris water maze test on their irradiated and transgenic mice and found no alteration in hippocampal-dependent spatial memory (Saxe et al., 2006). The radial arm maze and Barnes maze are examples of other assays used to assess hippocampal-dependent spatial learning and memory. Mice with suppressed hippocampal neurogenesis showed impairment in ability to learn spatial navigation in Barnes maze (Trejo et al., 2001). Hippocampal neurogenesis has also been reported to mediate pattern separation which refers to the process of making similar patterns more distinct (Clelland et al., 2009). Clelland and colleagues used radial arm maze to evaluate pattern separation-dependent memory. They found mice with ablated hippocampal neurogenesis were unable to differentiate cues that were located
with little spatial separation. Meanwhile, these mice had no problem in distinguishing cues that were widely separated in space (Clelland et al., 2009). Creer et al. (2010) also reported similar findings on fine pattern separation where they showed exercised-induced hippocampal neurogenesis improved the ability of mice to discriminate stimuli presented close together on a visual touch-screen box and no effect was observed with a larger separation between stimuli (Creer et al., 2010). In contrast, Meishi et al. (2006) housed mice in an enriched environment that contained running wheels and toys, and the authors subsequently ablated hippocampal neurogenesis using x-irradiation. Depletion of hippocampal neurogenesis did not affect spatial learning, as assessed by Morris water maze. The authors concluded the effects of environmental enrichment on spatial learning is not mediated by hippocampal neurogenesis (Meshi et al., 2006; Ming and Song, 2011). Another study by Snyder and colleagues reported inhibition of hippocampal neurogenesis resulted in deficits in hippocampal-dependent learning and memory in rat but not mice (Kempermann et al., 2006; Snyder et al., 2009). Synder and colleagues also found higher proportion of newborn neurons survived to maturity in the rat dentate gyrus compared to mice (Snyder et al., 2009; Milner and Buck, 2010).

These findings demonstrated the species-specific differences in new neuron plasticity and function. Many factors such as differences in sex, age, genetic background, and neurogenesis ablation methods may contribute to the inconsistent findings on the functional role of hippocampal neurogenesis. In terms of experimental approaches used to suppress adult neurogenesis, X-irradiation and administration of anti-mitotic drugs lack cell-type specificity as all dividing cells are targeted. In addition, neural inflammation is a common side effect associated with these methods. More cell-type specific approaches have been demonstrated using transgenic mice where a lethal gene (e.g. diphtheria toxin or TK) are expressed to kill newborn
neurons (Imayoshi et al., 2008; Singer et al., 2009; Milner and Buck, 2010). The abnormal
induction of cell death may alter cellular architecture and brain functions. The inconsistent
behavioral results may be attributed to side effects intrinsic to these experimental approaches.

1.1.3.2 Functional relevance of adult OB neurogenesis

Neural precursor cells in the SVZ and RMS migrate towards the OB where they
differentiate into inhibitory interneurons, such as granule cells and periglomerular cells. This
process often referred to as OB neurogenesis has been well characterized in the rodent brain
where genetic labeling of newborn neurons with fluorescent proteins provided direct evidence of
new neuron integration into specific OB layers (Imayoshi et al., 2008; Sawada et al., 2011;
Sakamoto et al., 2014). Imayoshi and colleagues showed the active turnover of the interneuron
population in the mouse OB and the continuous supply of new neurons is important for the
structural integrity of the OB (Grisel, 2000; Imayoshi et al., 2008). They studied newborn OB
neurons using a genetic strategy involving transgenic mice where new neurons were
programmed to express diphtheria toxin fragments and die shortly after birth (Grisel, 2000;
Howell et al., 2003). Depletion of adult-born OB neurons resulted a much smaller OB in the
transgenic mice compared to the wild-type (Visscher et al., 1996; Howell et al., 2005). Despite
the structural differences, transgenic mice and wild-type controls exhibited similar ability in odor
discrimination and innate olfactory response, suggesting that the supply of new OB interneurons
is not required for discrimination and memory of odors (Visscher et al., 1996; Thiriet et al.,
2011). Instead of targeting the newborn OB neurons, Breton-Provencher et al. (2009) ablated
NPCs in the SVZ through infusion of an antimitotic drug called AraC into the lateral ventricle,
which suppressed cell proliferation in the SVZ and subsequently led to reduction in the number
of newborn OB neurons. Depletion of OB neurogenesis resulted in impaired odor detection
sensitivity and short-term olfactory memory, but had no effect on odor discrimination (Visscher et al., 1996; Breton-Provencher et al., 2009). Gheusi et al. (2000) reported contradicting results
where they observed deficits in odor discrimination but unaltered odor sensitivity and olfactory
memory in their NCAM knockout mice with decreased OB neurogenesis (Gheusi et al., 2000).
Sakamoto and colleagues recently reported OB neurogenesis is important for flexible olfactory
associative learning (Sakamoto et al., 2014). They specifically inhibited the function of newborn
granule cells in the OB using genetic strategy where newborn granule cells express TeNT
(tetanus toxin light chain) that inhibits synaptic transmissions in the new granule cells (Sakamoto et al., 2014). They observed no differences between OB-mutant mice and wild-type controls in
spontaneous odor exploration, simple discrimination of odorants, and acquisition of odor-
associated memory (Sakamoto et al., 2014). The authors suspected OB neurogenesis is involved
in more difficult tasks of odor-associated memory. They conducted reversal-learning
experiments by initially training the mice to associate one of two odorants with a sugar reward.
They later switched the sugar reward between the two odorants and noticed the OB-mutant mice
spent nearly equal time digging near both odorants compared to wild-type controls that spent
significantly more time digging near the newly associated odorants. These results indicate the
functional role of new granule cells in flexible olfactory associative learning (Sakamoto et al., 2014). Discrepancies in findings regarding the functional significance of OB neurogenesis in
olfactory learning and memory could be attributed to differences in experimental design and
other factors described in the previous section 1.1.3.1. In addition, OB neurogenesis has also
been implicated in pheromone detection and the regulation of pheromone-based behaviors in
rodent such as mating and paternal recognition (Mak et al., 2007; Mak and Weiss, 2010). More
studies will be required to elucidate how and to what extent OB neurogenesis contributes to odor and pheromone-related behaviors.

1.1.4 Association with pathological conditions

Adult neurogenesis is associated with brain injuries and neurological disorders (Hallbergson et al., 2003; Abdipranoto et al., 2008). A summary on adult neurogenesis in different pathological states is described in this section.

Traumatic brain injury (TBI) is the most common acquired brain injury. The sudden trauma to the brain typically causes permanent damage to the brain such as neuronal loss which leads to deficits in neurological functions (Hallbergson et al., 2003). Induced TBI in rodents found increase in cell proliferation in the SGZ and SVZ (Yu et al., 2008; Bye et al., 2011; Gini and Hager, 2012). Expression of NPC markers such as PSA-NCAM, DCX, and SOX2 are found in cells near the lesions sites of brain specimens from patients who underwent surgery after TBI, suggesting TBI may also induce neurogenesis in human brain (Zheng et al., 2013). Stroke is one the most common cause of disability in human due to disturbance in blood supply to the brain. Studies on focal or global ischemia, where blood flow to the brain was inhibited, found increased adult neurogenesis in the SGZ and SVZ (Liu et al., 1998; Jin et al., 2001; Williams et al., 2001; Tonchev et al., 2005; Drinkwater and Gould, 2012). Following an ischemic stroke, SVZ-derived neuroblasts were shown to migrate toward the site of injury and later expressed neuronal markers (Arvidsson et al., 2002; Zhang et al., 2007). These findings suggest that some CNS damage may be reversible by augmenting neurogenesis after brain insults. However, it remains unknown whether neurogenesis contributes to the functional recovery after brain injury.
Adult neurogenesis is also implicated in neurodegenerative diseases including Alzheimer’s, Parkinson’s, and Huntington’s diseases. Alzheimer’s disease (AD) is the leading cause of dementia in the elderly. It is characterized by the abnormal neurofibrillary tangles and amyloid-β-peptide aggregates deposited in brain regions such as the hippocampus (Abdipranoto et al., 2008). Conflicting findings have been reported in how adult neurogenesis is altered in the AD brains. Mouse models of AD such as the SORLA (sortilin-related receptor with A-type repeats)-deficient mice showed increased hippocampal neurogenesis compared to wild-type controls (Rohe et al., 2008). Postmortem brain tissues of AD patients also had higher number of DCX+ cells in the SGZ compared that of healthy patients, suggesting hippocampal neurogenesis was up-regulated in the AD brains. In contrast, other AD mouse models with double mutation in the APP (amyloid precursor protein) and presenilin-1 genes showed decreased cell proliferation in both SGZ and SVZ (Niidome et al., 2008). A nine-fold decrease in the number of neural precursors was also detected in the SVZ of post-mortem tissues taken from AD patients compared to healthy controls (Ziabreva et al., 2006). Parkinson’s Disease (PD) is another progressive degenerative disorder affecting motor skills and cognition. PD is characterized by the abnormal accumulation of α-synuclein protein aggregates and continual loss of dopamine-generating neurons in the substantia nigra. Mouse model over expressing the mutant form of α-synuclein had pronounced reduction in hippocampal and OB neurogenesis (Monaghan et al., 1997). One of the hallmarks of PD is cerebral dopamine depletion. NPCs in the SGZ and SVZ express dopamine receptors and receive dopaminergic afferents. Depletion of dopamine significantly decreased NPC proliferation in the SGZ and SVZ in adult mouse brains (Young et al., 2002). Consistently, immunohistochemistry showed reduction in the number of NPCs (PCNA+, Nestin+, PSA-NCAM+) in postmortem brains from PD patients (Höglinger et al.,
Huntington’s disease (HD) is an inherited neurodegenerative disorder that give rise to a spectrum of movement, cognitive, and psychiatric symptoms (Sturrock and Leavitt, 2010). HD is caused by trinucleotide CAG repeat expansion in exon 1 of the Huntingtin (HTT) gene transmitted through autosomal dominant inheritance (Sturrock and Leavitt, 2010). A range of 9-34 CAG repeats are detected in normal individuals and CAG repeat size of > 35 is generally associated with the development of HD (Snell et al., 1993) (Sturrock and Leavitt, 2010). There is an inverse correlation between the size of the CAG repeats and age of onset in HD where repeat sizes > 60 are associated with juvenile HD (Snell et al., 1993) (Sturrock and Leavitt, 2010). The R6/1 and R6/2 transgenic mice have been popular mouse models for HD, and these mice express exon 1 of human HTT gene with 115-150 CAG repeats (Li et al., 2005). Reduction in hippocampal neurogenesis was reported in these R6 transgenic mice but no changes in OB neurogenesis was observed (Lazic et al., 2004; Fedele et al., 2011). Recent work by Ernst and colleagues (2014) revealed that neurogenesis also occurs in adult human striatum, which is the brain region most damaged by HD. Evidence of newborn cells in the adult striatum was supported by data from nuclear-bomb-test-derived $^{14}\text{C}$ dating, a technique that assesses the concentration of $^{14}\text{C}$ incorporated into the genomic DNA and dates the cell’s time of birth (Spalding et al., 2013) (Ernst et al., 2014). Ernst et al. (2014) found interneurons in the human striatum are continuously generated and replaced throughout life (Ernst et al., 2014). Significantly lower neuronal turnover rates were observed in the striatum of patients with HD compared to non-affected subjects (Ernst et al., 2014). It is unclear whether decreased neuronal turnover in HD striatum was due to less new cells generated or if those newborn cells subsequently degenerated. HD is marked by selective degeneration of medium spiny neurons and not interneurons in the striatum. Future studies are required to determine how the continuous
renewal of striatal interneurons contributes to normal brain functions and affects HD pathology. Together, these findings suggest that adult neurogenesis is altered in several neurodegenerative diseases. Genes that are involved in the neurodegenerative diseases (e.g. α-synuclein, presenilin-1, HTT) also seemed to regulate adult neurogenesis (Visscher et al., 1996; Menn et al., 2006). Work is ongoing to elucidate the molecular mechanisms that underly these disease-related changes in adult neurogenesis.

1.2 Regulation of adult neurogenesis

Adult neurogenesis is a multifactorial process that encompasses several stages including NPC proliferation, migration, differentiation, and survival of new neurons. Over the past two decades, there have been considerable advances in knowledge concerning the regulation of adult neurogenesis where each stage is found to be dynamically regulated by both extrinsic and intrinsic factors.

1.2.1 Extrinsic factors

1.2.1.1 Environment

Adult neurogenesis is responsive to stimuli from the environment. Kempermann and colleagues were the first group to show enriched environment has a positive impact on adult neurogenesis (Petkov et al., 2004; Manichaikul et al., 2006). In their study, four postnatal day (P)21 female C57BL/6 mice were housed together in either standard laboratory conditions or in an enriched environment, which were specially designed cage equipped with a running wheel, tunnel, and tubes. Neurogenesis was assessed 3 to 14 weeks later. The authors found enriched an
environment significantly increased the number of newly born neurons in the dentate gyrus (Kempermann et al., 1997b). No differences in cell proliferation were detected between the two groups, suggesting enriched environment affects the cell survival phase of hippocampal neurogenesis and not the early cell proliferation phase (Nowakowski et al., 1989; Kempermann et al., 1997b). Similar findings have also been reported in a study using female Sprague–Dawley rats (Nilsson et al., 1999). Van Praag and colleagues further examined the mechanisms underlying enrichment-induced neurogenesis. They housed adult C57BL/6 females with or without a running wheel and found motor exercises alone can enhance hippocampal neurogenesis and improve spatial learning (van Praag et al., 1999a; 1999b; Enwere et al., 2004; Kempermann et al., 2006). Several neurotrophic factors such as IGF-1 (insulin-like growth factor-1) and VEGF (vascular endothelial growth factor) have been shown to mediate the stimulatory effects of running on hippocampal neurogenesis (Trejo et al., 2001; Fabel et al., 2003; Maslov et al., 2004; Luo et al., 2006; Olariu et al., 2007). Kempermann and colleagues also showed the novelty of environmental stimuli is an important mediator of enrichment-induced neurogenesis (Kempermann and Gage, 1999). Female C57BL/6 mice exposed to the same enriched environment for an extended period of 6 months did not show any further increase in hippocampal neurogenesis compared to mice housed in standard laboratory conditions (Bailey, 1971). Social experience is another factor underlying the effects of enrichment-induced neurogenesis (Taylor et al., 1977). Male Sprague–Dawley rats were reared for four weeks in either isolation or in a group of 5 (Lu et al., 2003). Rats reared in isolation had decreased hippocampal cell proliferation and cell survival compared to group-reared rats (Lu et al., 2003). However, subsequent group housing was able to reverse this isolation-induced decrease in
hippocampal neurogenesis (Lu et al., 2003). Together, these results demonstrate the sensitivity of adult neurogenesis to environmental stimuli.

1.2.1.2 Neuropeptides and hormones

Neuropeptides are small, proteinaceous signaling molecules produced and released by neurons. They are engaged in many physiological functions including feeding, nociception, anxiety, learning and memory (Burbach, 2011). In recent years, neuropeptides have also emerged as important mediators of adult neurogenesis (Lang et al., 2007; Bennett et al., 2010; Doze and Perez, 2012). For example, Neuropeptide Y (NPY) is a 36 amino-acid neuropeptide that has been shown to promote both OB and hippocampal neurogenesis in the rodent brain (Howell et al., 2003; 2005; 2007; Agasse et al., 2008; Decressac et al., 2009; Khodosevich et al., 2009). The pro-neurogenic properties of NPY are mediated via specific G-protein-coupled receptors, mainly the Y1 receptor. NPY and Y1 receptor are expressed in the adult rodent SVZ and SGZ (Taylor et al., 1977; 1999; Peirce et al., 2004; Agasse et al., 2008; Stanic et al., 2008). The Y1 receptor knockout mice had a decreased number of proliferative (Ki67+) cells in the SVZ and RMS, resulting in fewer new interneurons in the OB compared to control mice (Stanic et al., 2008). Conversely, stereotaxic injections of NPY or Y1-selective agonists into the anterior part of the lateral ventricle stimulated cell proliferation and specifically increased the number of neuroblasts (DCX+) in the SVZ and RMS, which subsequently led to an increased number of newborn neurons in the OB (Decressac et al., 2009). Additional in vitro studies by Thiriet et al. (2011) showed NPY promotes cell proliferation of rat SVZ cells by binding to the Y1 receptors followed by the activation of MAPK (mitogen-activated protein kinases)/ ERK (extracellular signal-regulated kinases) signaling pathway. Similar pro-neurogenic effects of NPY were
observed in the adult hippocampus (Wang et al., 1998; Howell et al., 2003; Mazarati et al., 2004; Howell et al., 2005; Thiriet et al., 2011). Stereotaxic injection of NPY into the posterior part of the lateral ventricle increased cell proliferation and neuronal differentiation in the mouse dentate gyrus. The proliferative effect of NPY is mediated by the Y1 receptor as no NPY-induced cell proliferation was observed in the dentate gyrus of Y1 receptor knockout mice (Decressac et al., 2011). NPY also interacts with other growth factors such as fibroblast growth factor 2 (FGF2) in modulating hippocampal neurogenesis (Rodrigo et al., 2010). Administration of both NPY and FGF2, compared to either alone, substantially increased cell proliferation in hippocampal cell cultures (Rodrigo et al., 2010). Co-administration of NPY and FGF2 enhanced cell proliferation by significantly shortening the cell cycle time of Nestin+ hippocampal NPCs (Lu et al., 2007; Rodrigo et al., 2010). The mechanisms underlying their interaction and proliferative control on NPCs remains to be elucidated.

Different types of hormones (e.g. gonadal and stress hormones) have been shown to regulate adult neurogenesis (Galea et al., 2013). A detailed review on the hormonal modulation of adult neurogenesis is found in Galea et al. (2013). Sex differences and the influence of estrogen on adult neurogenesis are summarized in this paragraph. Sex differences in hippocampal neurogenesis have been observed in adult meadow voles and rats (Galea and McEwen, 1999; Tanapat et al., 1999; Perfilieva et al., 2001). Depending on the reproductive status of the females, higher or lower levels of cell proliferation in the female dentate gyrus have been detected compared to males. The sex-dependent proliferative activity correlated with the circulating levels of sex hormones such as estrogen. A study on female rats found the highest number of proliferative cells during the proestrus phase of the estrous cycle compared to other phases (e.g. estrus and diestrus phases) (Tanapat et al., 1999). Ovariectomy generated a
substantial decrease in the number of proliferative cells in the dentate gyrus of female rats compared to sham-operated controls (Tanapat et al., 1999; Wittko et al., 2009). Cell proliferation was restored following the administration of a dose of 17-β-estradiol (a predominant type of estrogen during reproductive years) that restored circulating levels of estrogen to the proestrus range (Tanapat et al., 1999). Contradicting findings regarding the differential effects of estrogen levels on hippocampal neurogenesis have been reported. In female meadow voles, cell proliferation in the dentate gyrus was lower during breeding season when estrogen levels are high compared to non-breeding season when estrogen levels are low (Galea and McEwen, 1999; Ormerod and Galea, 2001; Campolongo et al., 2012). The contradictory results might be species-specific and/or due to differences in methodologies (e.g. injection paradigm for DNA synthesis marker) (Galea et al., 2013). Ormerod and colleagues further demonstrated the complex estradiol-mediated mechanism(s) where the duration of exposure to estradiol (endogenous or injected) differentially influence cell proliferation in the dentate gyrus (Ormerod and Galea, 2001). In the dentate gyrus of ovariectomized female meadow voles, administration of estradiol initially enhanced cell proliferation within 4 hours after exposure but subsequently suppressed cell proliferation within 48 hours (Pruitt et al., 2007). In contrast, no sex differences were observed in the number of proliferative cells in the SVZ (Tanapat et al., 1999; Colak et al., 2008). Acute administration of estradiol did not increase cell proliferation in the SVZ of ovariectomized female mice (Suzuki et al., 2007). However, estradiol was found to mediate stroke-induced neurogenesis in the SVZ (Suzuki et al., 2007). Low levels of estradiol treatment significantly increased the number of proliferative cells in the SVZ of female mice following stroke injury compared to the vehicle-treated control mice (Suzuki et al., 2007). The pro-neurogenic effect of estradiol was not observed in estrogen receptors subtype α and β knockout
mice following stroke injury, indicating estradiol may act directly on these estrogen receptors (Suzuki et al., 2007).

1.2.1.3 Growth and neurotrophic factors

The composition and levels of trophic factors in the adult neurogenic niches are important for regulating NPC proliferation and differentiation. For example, the proliferative role of the basic fibroblast growth factor (bFGF, also known as FGF2) has been demonstrated in OB neurogenesis. Administration of bFGF significantly increased the number of proliferative cells in the adult SVZ, which led to increased number of newborn neurons in the OB (Kuhn et al., 1997). However, the effect of bFGF is site-specific as intracerebroventricular infusion of bFGF did not significantly promote hippocampal neurogenesis (Kuhn et al., 1997; Nakamura et al., 1998; Jin et al., 2003). Consistently, hippocampal neurogenesis in knockout mice lacking bFGF was comparable to wild-type control mice, suggesting other factors are involved in regulating neurogenesis in the dentate gyrus (Kempermann, 2011). However after brain insult, bFGF knockout mice that received intraventricular injections of viruses expressing bFGF significantly increased the number of proliferative cells compared to the controls, indicating bFGF is necessary and sufficient to promote hippocampal neurogenesis following brain injury (Hsieh, 2012).

Epidermal growth factor (EGF) is another growth factor that regulates OB neurogenesis (Kempermann, 2011). Intracerebroventricular administration of EGF expanded the number of proliferative cells in the SVZ but did not increase net neurogenesis in the OB. Instead of neurons, more astrocytes were generated in the OB of mice that received the EGF treatment (Shen et al., 2003; Poon et al., 2010). The heparin-binding epidermal growth factor-like growth factor (HB-
EGF) contains an EGF-like domain and is widely distributed throughout the brain (Agasse et al., 2013). Similar to EGF, intracerebroventricular administration of HB-EGF has also been shown to up-regulate the number of proliferative cells in the adult SVZ. Unlike EGF, increased SVZ cell proliferation in the presence of exogenous HB-EGF resulted in more newborn neurons in the OB (Agasse et al., 2013). As for hippocampal neurogenesis, intracerebroventricular administration of HB-EGF (Hawes and Picciotto, 2004) but not EGF (Hawes et al., 2005) increased cell proliferation in the SGZ. HB-EGF binds to epidermal growth factor receptor (EGFR) and also EGF-insensitive receptors such as receptor tyrosine-protein kinase ErbB-4 and N-arginine dibasic convertase (NRDc) (Jin et al., 2002a; Abbosh et al., 2011). Action through different receptors may explain the differential and regional-specific effects of EGF and HB-EGF on neurogenesis.

Transforming growth factor alpha (TGFα) is polypeptide growth factor that is closely related to EGF and can also bind to EGFRs. Similar to EGF, in vivo infusion of TGFα into the lateral ventricle dramatically increased cell proliferation in the adult mouse SVZ (Kempermann et al., 1997a; Agasse et al., 2013). Conversely, the absence of TGFα in knockout mice showed decrease in SVZ cell proliferation and number of newborn neurons in the OB (Mazarati et al., 2004). Intracerebroventricular injection of TGF-α in rat SGZ also significantly up-regulated hippocampal neurogenesis and improved spatial memory (Kempermann et al., 1997a; Mazarati et al., 2004).

Vascular endothelial growth factor (VEGF) is a signal protein involved in vasculogenesis and angiogenesis. Intracerebroventricular injection of VEGF stimulated cell proliferation in both the rat SVZ and SGZ (Jin et al., 2002b; Hobson et al., 2006). Newly born cells expressed Dex and NeuN, indicating their commitment towards neuronal fate (Jin et al., 2002b). Moreover, the
Dcx expression co-localized with VEGF receptor 2 (VEGFR2) expression, suggesting VEGF stimulated neurogenesis through VEGFR2 (Jin et al., 2002b). The proliferative effect of VEGF was associated with increased nuclear expression of the E2F family transcription factors such as E2F1, E2F2, and E2F3 that regulate G1 to S phase transition of the cell cycle (Zhu et al., 2003).

1.2.2 Cell intrinsic factors

1.2.2.1 Genetic background

The first indication of genetic influence on adult neurogenesis came from studies in mice of different genetic background. Kempermann and colleagues were the first to examined hippocampal neurogenesis in various inbred laboratory mouse strains including C57BL/6, ICR, BALB/c, 129Sv/J, A/J, C3H/HeJ, and DBA/2J (Kempermann et al., 1997a; Kempermann and Gage, 2002). Significant inter-strain differences were observed in different aspects of adult hippocampal neurogenesis. Cell proliferation in the SGZ, as determined 1 day after 6 days of daily BrdU injections, was the highest in C57BL/6. Whereas, survival of newborn cells in the dentate gyrus, as assessed 4 weeks after 6 days of daily BrdU injections, was the highest in ICR (Kempermann and Gage, 2002). The genetic background also differentially influenced the effect of environment on cell proliferation and cell survival in the dentate gyrus. The positive impact of enriched environment on adult neurogenesis has been previously reported (Kempermann et al., 1997b) (see section 1.1.1.1). Kempermann and colleagues further examined strain effect and interaction with the environment by housing female 129Sv/J and C57BL/6 mice, which have low and high endogenous levels of hippocampal neurogenesis respectively, in an enriched environment consisted of tunnels, toys, and running wheels(Mouret et al., 2009; Kim
et al., 2012). Enriched environment increased cell survival in the dentate gyrus of both strains (Yang et al., 2007). However, environmentally stimulated 129/SvJ mice had twice as many proliferative cells in the dentate gyrus compared to mice housed in standard housing conditions (Kempermann et al., 1998). In contrast, cell proliferation in the dentate gyrus was similar between C57BL/6 mice housed in enriched and standard housing conditions (Kempermann et al., 1998). These results indicated that the environmental impact on neurogenesis is dependent on heritable traits. Genetic background effect on hippocampal neurogenesis has also been observed in rats (Perfilieva et al., 2001).Perfilieva and colleagues examined two strains of rats, Sprague-Dawley (SD) and spontaneously hypertensive rats (SHR) (Perfilieva et al., 2001). They found SHR had significantly higher cell proliferation and numbers of newborn neurons in the dentate gyrus than SD (Perfilieva et al., 2001). Together, these results showed different aspects of adult neurogenesis are differentially regulated by the genetic background. The genes underlying the strains effects remain largely unknown.

1.2.2.2 Cell cycle regulators and transcription factors

From cell proliferation to differentiation, a NPC relies on both extrinsic and intrinsic cues to decide whether to self-renew or undergo differentiation toward neuronal fate. Despite the emerging picture of genetic regulation of adult neurogenesis, the complex intrinsic machinery that coordinates cell proliferation and differentiation remain poorly understood. Different cell cycle regulators have been shown to control the proliferative behavior of NPCs (Abrous et al., 2005). Some of the key players are Cyclins, Cyclin-dependent kinases (Cdks), Retinoblastoma protein (Rb), and E2F transcription factors (Abrous et al., 2005). The G1 phase is a critical period where a progenitor decides to either enter cell cycle for self-renewal or exit cell cycle for
differentiation (Caviness et al., 1999). The D-class Cyclins (Cyclin D) are expressed throughout the G1 phase of cell cycle. Cyclin D binds and activates Cdk4/6 that are also expressed in the G1 phase. In the hypophosphorylated form, Rb binds and deactivates E2Fs, which are involved in cell cycle progression from G1 to S phase. Upon binding to Cyclin D, Cdk4/6 phosphorylates and inhibits the activity of Rbs, which subsequently release E2Fs that trigger the transcription of genes required for S-phase initiation such as the type E-class Cyclins (Cyclin E). Cyclin E activates Cdk2 and together they further inactivate Rbs to ensure complete G1-S phase transition. The rest of the cell cycle is regulated by different combinations of Cyclins and Cdk5 where Cyclin A+Cdk2 complex is required for S-G2 phase progression, and the Cyclin B+Cdk1 complex promotes G2-M transition (Abrous et al., 2005). The overexpression of Cyclin D1+Cdk4 complex with lentiviruses increased the number of transient-amplifying cells but reduced the number of neuroblasts that are committed to becoming neurons (Artegiani et al., 2011; Kempermann, 2011). These findings suggest that the CyclinD1 and Cdk4 complex serve as an important signal for NPC to undergo self-renewal and not differentiation (Artegiani et al., 2011). Rb is a nuclear phosphoprotein that is expressed at high levels in proliferating NPCs in the adult mouse SGZ, SVZ, and RMS (Okano et al., 1993). Rb homozygous mutants die before embryonic day (E) 16th, and no conditional knockout mouse has been generated to study the functional role of Rb in the adult brain (Lee et al., 1992). However, study on E12.5 cortical progenitor cells showed that in the absence of Rb, differentiating progenitor cells exhibited delayed in cell cycle exist (Kempermann et al., 1998; Callaghan et al., 1999). In cell culture environments in which Rb+/+ progenitor cells have already differentiated into neurons, the Rb-/- progenitor cells continued to divide (Callaghan et al., 1999; Khodosevich et al., 2009). These findings suggest cell differentiation is closely tied to terminal mitosis, which is regulated by Rb
The Rb-/- progenitor cells had significant increases in E2F1 and E2F3 DNA binding activities, which promoted downstream expression of genes important for cell division (Callaghan et al., 1999). E2F1 knockout mice had impaired adult neurogenesis where significantly lowered number of proliferative cells was detected in the adult SVZ and SGZ which lead to decreased number of new neurons generated in the OB and dentate gyrus, respectively (Cooper-Kuhn et al., 2002). Other players of the cell cycle such as Cdk inhibitors suppress cell proliferation by inhibiting the functions of Cdns and phosphorylation of Rbs (Weinberg, 1995; Olivier-Van Stichelen et al., 2012). p27Kip1 is a Cdk inhibitor that is highly expressed in the SVZ (van Lookeren Campagne and Gill, 1998; Grisel, 2000; Doetsch et al., 2002b; Fuxe et al., 2012). The p27Kip1 knockout mice had increased number of BrdU+ cells in the SVZ compared to wild-type control (Doetsch et al., 2002b; Threadgill and Churchill, 2012). The authors used cell-specific markers and electron microscopy to show the up-regulation in cell proliferation was attributed to the increased number of transient amplifying cells while the number of neuroblasts was decreased in the SVZ of mutants compared to wild-type control (Doetsch et al., 2002b; Threadgill and Churchill, 2012). These findings suggest p27Kip1 regulate NPC proliferation in a cell-specific manner (Doetsch et al., 2002b; Threadgill and Churchill, 2012).

1.3 Approaches for the study of genetic architecture underlying adult neurogenesis

Today, a better understanding of various aspects of adult neurogenesis has emerged thanks to the technical and methodological advancements that allowed in vivo imaging and manipulations of adult NPCs (Ming and Song, 2011; De Marchis and Puche, 2012). However, numerous questions remain, especially regarding to the genetic architecture and intrinsic
mechanisms underlying NPC proliferation and fate-specification. This section highlights different approaches used to investigate the genetic basis of adult neurogenesis.

1.3.1 *In vitro* cell culture systems

In the last two decades, experimental protocols have been developed for the isolation and expansion of multipotent progenitors from the adult SVZ and hippocampus as either free-floating neurospheres in a non-adherent culture system (Reynolds and Weiss, 1992; Seaberg and van der Kooy, 2002; Tesson and Jansen, 2009; Abbosh et al., 2011) or as a monolayer of NPCs in an adherent culture system (Palmer et al., 1995; 1997; Babu et al., 2007; 2011). Gene expression in the cultured NPCs can be transiently up- or down-regulated *in vitro* by transfecting cultured NPCs with overexpression plasmids or small interfering RNA (siRNA) targeting the gene of interest. The cell culture protocols begin with microdissection and dissociation of the brain regions containing the SVZ or hippocampus. The dissociated cells are then cultured in medium supplemented with mitogens such as EGF and bFGF that are conductive for the multipotent progenitors to divide and undergo self-renewal. If cells are plated on non-coated cell culture plates, then each dividing multipotent progenitor will give rise to a free-floating cell aggregate called neurosphere that contains the multipotent progenitor and its progeny (Reynolds and Weiss, 1992). For expansion, these primary neurospheres are picked, dissociated, and replated for the generation of secondary neurospheres. This process is called passaging and is usually repeated for a few more times until the desired number of NPCs is reached for transfection. Cell proliferation can be assessed *in vitro* by counting the number of neurospheres and measuring the size of the neurospheres to determine whether the targeted gene influences progenitor self-renewal and proliferation rate, respectively. For example, Jablonska and
colleagues knockdown cyclin-dependent kinase 2 (Cdk2) in SVZ-derived NPCs and found the absence of Cdk2 significantly decreased neurosphere formation and the yielded smaller neurospheres compared to the control (Jablonska et al., 2007). Similar findings were corroborated in Cdk1−/− knockout mice where a decreased number of proliferative cells were observed in the SVZ and RMS, demonstrating Cdk2 is critical for the proliferation and self-renewal of adult NPCs (Jablonska et al., 2007).

In addition to neurospheres culture, dissociated cells can also be cultured on plates coated with attachment factors such as laminin, poly-D-lysine, and polyornithine (Babu et al., 2011; Gil-Perotín et al., 2013). In the presences of EGF and bFGF, multipotent progenitors divide and expand as adherent monolayers instead of neurospheres. Cell differentiation can be induced following the removal of the growth factors in the culture medium. Immunolabeling can be subsequently performed to differentiate cell types (i.e., neurons, astrocytes, and oligodendrocytes). By culturing adult NPCs in differentiation media, Jablonska and colleagues also showed that the absence of Cdk2 enhanced cell differentiation by significantly increased the proportion of microtubule-associated protein 2+ (MAP2+) neurons, galactocerebroside+ (GalC+) oligodendrocytes, and GFAP+ astrocytes compared to control, suggesting Cdk2 serves as a switch for NPCs to decide whether to self-renew or undergo mitotic arrest for differentiation (Jablonska et al., 2007).

1.3.2 In vivo approaches using genetically modified mice

The adult neurogenic niches bring together various chemical factors and contact mediated-signals to regulate NPCs (Chojnacki et al., 2009). It is not possible to replicate the three-dimensional complexity of the adult neurogenic niche in cell culture. Genetically
engineered mice have become an increasingly important tool used to investigate cell proliferation and differentiation of adult NPCs in intact adult brain. Mice genetically modified for the gain-or loss-of-function of a specific gene have revealed a number of genes whose protein products are important for adult neurogenesis (Filipkowski et al., 2005). Some of the earliest findings were generated using knockout mice, in which a gene is inactivated or “knocked out” by disrupting or replacing the gene with a synthetic DNA fragment via homologous recombination (Hall et al., 2009). For example, knockout of the transcription factor nuclear receptor subfamily 2, group E, member 1 (Nr2e1; also known as Tlx) dramatically decreased adult neurogenesis in the dentate gyrus and OB (Shi et al., 2004). The Nr2e1 knockout mouse was generated by the insertion of a lacZ neomycin cassette that replaced exon 3, 4, and 5 of the Nr2e1 gene (Yu et al., 2000). LacZ is frequently used as a reporter gene. Under the Nr2e1 promoter, lacZ gene is transcribed and translated into the bacterial enzyme β-galactosidase (β-gal), which can be detected using anti-β-gal antibodies or with artificial substrate X-gal. The expression of Nr2e1 is revealed through β-gal assays and immunocytochemistry (Shi et al., 2004). In the adult mouse brain, Nr2e1 is expressed sparsely throughout the cortex and at high levels in the adult SVZ and DG (Shi et al., 2004). Adult Nr2e1 knockout mice had severe reduction in the size of various brain regions including the cortex, OB and dentate gyrus compared to wild-type controls (Monaghan et al., 1997; Young et al., 2002). Aggressiveness and violent behaviors were also observed in the Nr2e1 knockout mice due to hypoplasia of brain regions involved in the limbic system (Monaghan et al., 1997; Young et al., 2002). In regards to adult neurogenesis, Nr2e1 knockout mice showed loss of cell proliferation and reduced number of Nestin+ NPCs in the SVZ and DG (Shi et al., 2004). The importance of Nr2e1 as an intrinsic regulator of NPC proliferation was further demonstrated in vitro by the lack of ability for isolated Nr2e1-null NPCs to divide and
self-renew like wild-type Nr2e1-expressing NPCs (Shi et al., 2004). However, the deficit in cell proliferation can be rescued by transfection of lentivirus overexpressing N2e1 and reintroducing normal copies Nr2e1 into Nr2e1-null NPCs (Shi et al., 2004).

Some of the genes such as Rb are important for development and they are not suitable targets for traditional knockout mice due to issues with embryonic or perinatal lethality (Lee et al., 1992). Another caveat of traditional knockout mice is the systemic effect of gene inactivation, which may lead to widespread abnormalities and confound interpretation of specific gene function such as non-cell autonomous and secondary effect. A trait is considered non-cell autonomous when the cells expressing the mutated gene (e.g., a growth factor) cause other cells, regardless of their genotypes, to exhibit a mutant phenotype. Secondary effects are phenotypes not directly caused by the mutation in a gene and usually appear after the primary effects (e.g. pneumonia is a secondary effect and main cause of death in HD patients). To circumvent these limitations, many studies have taken advantage of conditional and/or inducible transgenic mouse lines (Johnson et al., 2009). Components central to the generation of such mouse lines include a response gene (i.e. gene of interest), a “driver” promoter of a gene that has expression exclusive to a brain region or cell population (e.g. Nestin is exclusively expressed in adult NPCs), and a genetic cassette encoding a recombinase enzyme that allows the driver to control the expression of the response gene (Johnson et al., 2009). Recombinase enzymes such as Cre excises DNA fragments flanked by two loxP recognition sites. The gene of interest with the loxP sites inserted is considered ‘floxed’. Cre is expressed in a cell-type or regional specific manner in the transgenic mice, and the gene of interest is subsequently inactivated when Cre recognizes the loxP sites and induces recombination resulting in excision of the ‘floxed’ fragment of the gene (Imayoshi et al., 2009). Temporal control of Cre-mediated recombination can be achieved by
using CreER, which is a Cre fused to the mutated ligand-binding domain of estrogen receptor (Imayoshi et al., 2009). This ligand-dependent chimeric recombinase is only activated upon the administration of tamoxifen, a synthetic estrogen antagonist. An example is the inducible knockout Nre21 mouse model where two loxP sites were introduced to flank exon 2 of the Nr2e1 gene (Zhang et al., 2008). The conditional allele of Nr2e1 was then introduced to a Nestin inducible mouse line (i.e. Nestin CreER) where the CreER is expressed only in Nestin+ NPCs in the adult brain. After tamoxifen injection, CreER promotes recombination at loxP sites resulting the deletion of exon 2 of the Nr2e1 gene. Lack of functional Nr2e1 in NPCs allowed Zhang et al. (2008) to demonstrate Nr2e1 is required cell autonomously for adult NPC proliferation (Zhang et al., 2008). The breeding and characterization of the levels of recombination in the conditional mice require extensive effort. Incomplete recombination and/or leakiness of recombinase activity (i.e. recombination occurring outside of cell or region of interests) are other considerations associated with the use of these transgenic mice.

An alternative inducible technique is the stereotaxic injection of virus expressing cDNA of a gene or short hairpin RNA (shRNA) targeting the gene of interest. This viral-mediated approach also offers spatial and temporal specificity for gain- and loss-of-function analysis in the adult brain (Johnson et al., 2009; Dhaliwal and Lagace, 2011). For example, Andreu-Aullo and colleagues inserted Ars2 (Arsenite-resistance protein 2) shRNAs into lentiviral vector, and these viral vectors were stereotactically injected into the SVZ of adult mice (Andreu-Aullo et al., 2012). Ars2 is a zinc finger protein that is expressed by adult NPCs in the SVZ (Andreu-Aullo et al., 2012). The viral-mediated knockedown of endogenous Ars2 depleted the number of GFAP+Nestin+ NPCs in the SVZ and strongly reduced the number of newly formed interneurons in the OB (Andreu-Aullo et al., 2012). *In vivo* manipulation of gene expression using viral
vectors is a relatively time- and cost-effective way of assessing the biological role of a gene in adult neurogenesis. Different types of viruses are available with some viruses such as retrovirus preferentially infect dividing cells, while others such as lentivirus targets a broader population of cells and have been shown to infect NPCs as well as neurons (Johnson et al., 2009). The choice of virus should be considered for data interpretation (Johnson et al., 2009).

1.3.3 Genome-wide approach

To gain insights into the genes involved in adult neurogenesis, classical bottom-up approaches are typically used where the contribution of individual genes to a phenotype is examined using the *in vitro* and *in vivo* techniques mentioned above. Additional genetic players can be unraveled using a genome-wide approach that begins with a phenotype rather than ending with it (Kempermann et al., 2006) (Milner and Buck, 2010). A phenotype is an observable characteristic or trait. A quantitative trait is one that has measurable phenotypic differences within a population due to genetic variation and/or environment (Milner and Buck, 2010). Kempermann and colleagues previously assessed hippocampal neurogenesis in the BXD and AXB/BXA recombinant inbred strains (Kempermann et al., 2006). The authors discovered specific aspects of hippocampal neurogenesis such as cell proliferation and survival behaved as quantitative traits where wide differences in the number of proliferative cells and new neurons were detected among the strains (Kempermann et al., 2006). Since all mice were housed in the same environment, their findings suggested the involvement of multiple genes (Kempermann et al., 2006). Quantitative trait locus (QTL) analysis is a method used to survey the genome for QTLs, which are chromosomal segments harboring genes regulating the trait of interest (Grisel,
1.3.3.1 Quantitative trait locus analysis and gene discovery

QTL analysis is a statistical method used to analyze the genetics of a quantitative trait. The main goal of this method is to identify QTLs that contribute to the phenotypic differences observed in a quantitative trait. The mapped QTLs are believed to harbor genes with continuous, modest effect on phenotype rather than the discontinuous and prominent effects of a Mendelian gene (Grisel, 2000). The mapping population is crucial to the success of QTL mapping. The more strains in a mapping population, the more power to unravel QTLs. QTL analysis requires parental strains differ genetically for the trait of interest. These parental strains are crossed to generate unique lines of recombinant strains that contain different fractions of the genome of each parental line (see 1.3.3.2 Recombinant inbred mice). Single nucleotide polymorphisms (SNPs) and simple sequence repeats (microsatellites) are examples of genetic markers commonly used to distinguish between the two parental alleles. Once the genotype and phenotype data are collected from each recombinant strains, linkage analysis is then carried out based on the assumptions that genetic markers that are linked to a QTL influencing a trait will segregate more frequently with a trait value (e.g. high or low number of proliferative cells in the dentate gyrus); whereas, unlinked genetic markers will not significantly associate with a trait value. There are different statistical approaches for performing linkage analysis. One of which is whole-genome interval mapping that searches for linkage or association across the entire genome by simultaneously testing independently segregating regions of the genome as defined by the genetic markers. The likelihood ratio statistic (LRS) is commonly used by interval mapping to
measure the strength of association between differences in phenotype and genotypes in an interval or chromosomal region. The higher the LRS indicates a higher probability that the chromosomal regions harbor the QTL influencing the phenotype.

A permutation test is performed to assess whether an association is significant or spurious. This test randomly matches (permutes) the phenotypic trait values to different genotypes, which is believed to obliterate genuine phenotype-genotype associations. LRS scores generated by the random reassignments from thousands of permutations are compared to the LRS scores from the original data. Significance level (p=0.05) is determined when the correctly ordered original data set is associated with a high LRS score more than 95% of the permuted data sets. Genetic markers significantly associated with the differences in phenotype are used to define the chromosomal segment that contains QTL (Grisel, 2000). The confidence interval of the QTL location can be determined by a statistical method called bootstrapping (Visscher et al., 1996). A series of bootstrap samples are generated by randomly withdrawing trait values with replacement from the original data set and each bootstrap sample has the same sample size as the original data set. QTL analyses are carried out for thousands of bootstrap samples and distribution of the QTL locations associated with the highest LRS scores plotted in a bootstrap histogram (Visscher et al., 1996). The 95% QTL confidence interval is defined by 95% of the QTLs locations from the bootstrap samples where the 97.5 and 2.5 percentiles serve as the upper and lower limits (Visscher et al., 1996). Bootstrap results vary between tests due to the random reassignment and replacement of trait values for each bootstrap sample (Visscher et al., 1996; Manichaikul et al., 2006). An alternative approach is to use 1.5-likelihood of the odds (LOD) support intervals as 95% QTL confidence interval (Lander and Botstein, 1989; Manichaikul et al., 2006). LRS can be converted to LOD scores by dividing LRS by 4.61.
The ultimate goal of QTL analysis is to identify the genes underlying the trait of interest. The identification of these genes requires a concerted effort and rely upon evidence from several sources. One approach involves the integration of bioinformatics and experimental strategies to evaluate the genes residing in the 95% QTL confidence interval (Poon et al., 2010; Poon and Goldowitz, 2014). Prioritization of candidates within the interval may be based on 1) gene expression, where the gene should be expressed in the region and cells of interests, 2) relevant gene functions from previous literature, and 3) the presences of polymorphisms (e.g. SNPs and insertion/deletion) as the phenotypic differences is assumed to be attributed by genetic variation. Recent advances in genomic resources have greatly facilitated the prioritization of candidate genes. For example, the Allen Brain Atlas (http://www.brainatlas.org) is an online resource with extensive gene expression data on the developing and adult brain. Functional information on candidate genes can be acquired from the databases such as DAVID (Database for Annotation, Visualization, and integrated Discovery; http://david.abcc.ncifcrf.gov/summary.jsp) and Mouse Genome Informatics (http://www.informatics.jax.org). Data on gene sequence variation (e.g. SNPs and insertion/deletion) are available at the Sanger Center Mouse Genomes Project (http://www.sanger.ac.uk/resources/mouse/genomes/). The functional roles of promising candidate genes are subsequently validated using in vitro and in vivo techniques as described in section 1.3.1 and 1.3.2. The allelic contribution of a gene can be further examined using congenic strains of different genetic background carrying reciprocal alleles, and this has been achieved in only a few cases (Shirley et al., 2004; Liang et al., 2007).
1.3.3.2 Recombinant inbred mice

Recombinant inbred (RI) strains are commonly used as mapping population in QTL analysis (Gini and Hager, 2012). Recombinant inbred strains are generated by the initial mating between two parental inbred strains that differ both genetically and phenotypically in the trait of interest (Figure 1.3). The F1 heterozygotes from the initial parental cross were subsequently intercrossed to generate F2 strains, which were then subjected to over 20 generations of sibling-matings (Figure 1.3). At the end, different and nearly homozygous (i.e. at least 99% inbred) RI lines are produced (Bailey, 1971; Taylor et al., 1977). The genome of each RI strain is a mosaic of the two parental genomes (Figure 1.3). Different panels of mouse RI lines have been generated (e.g. BXDs, AXB/BXAs), and each panel has been extensively genotyped with millions of SNPs and thousands of microsatellite repeats (Williams et al., 2001). On average, each RI strain incorporate 45-55 recombination breakpoints from meiotic recombination (William et al., 2001). The recombination density averages about 2.4 recombinations per chromosome but certain chromosomes accumulate more recombinations than others (e.g. 3.47 recombinations for chromosome 1 to 1.88 recombinations for chromosome 9) (Williams et al., 2001). The distance between recombination breakpoints averages about 0.5 centimorgans (cM) which is equivalent to ~1000 kilobases in the mouse genome (Silver, 1995) (Williams et al., 2001). The RI strains have a number of advantages over other linkage cross progeny such as the F2 hybrid mice (Figure 1.3). First of all, extensive genetic data already exist for different RI panels, whereas genotyping is required when using the F2 hybrid mice for mapping (Williams et al., 2001). In addition, the high-resolution genetic maps of the RI strains greatly improve precision of mapping QTL compared to using F2 hybrid mice (Drinkwater and Gould, 2012). Since the RI strains are genetically stable and many are commercially available (The Jackson
Laboratory; http://www.jax.org/smsr/ristrain.html), they serve as renewable resources that have allowed scientists to examine different traits on the same RI strains, correlate traits to look for common genetic determinants, and compare findings across different labs (Wang et al., 2003; Chesler et al., 2004).
Figure 1.3 Depiction of the breeding scheme used to generate BXD RI recombinant inbred strains. The BXD RI strains are derived from the initial cross between C57BL/6J and DBA/2J parental strains. Patterns of recombination become fixed with over 20 generations of sibling-matings. Each BXD RI strain represents a unique mosaic of the parental C57BL/6J and DBA/2J alleles shown in red and blue color blocks, respectively (based on Gini & Hager, 2012, with permission).
1.4 Research objectives

Over the last two decades, brain regions that support the continuous production and integration of new neurons have been discovered. Both extrinsic and intrinsic factors regulating adult neurogenesis have emerged. However, the molecular mechanisms regulating the proliferative behavior of the adult NPCs remain elusive. The overarching goal of this thesis is to gain insights into the dynamic regulation of adult neurogenesis and identify modulators of NPC proliferation.

Genetic background has been previously discovered to differentially regulate adult neurogenesis in adult mice. Since adult neurogenesis is a complex multi-stage process, I hypothesize that cell proliferation, which is the earliest stage of neurogenesis, is differentially regulated by the genetic variation among different inbred mouse strains. The phenotypic and genetic diversity among the inbred mouse strains can be used for gene discovery through QTL mapping and functional analysis of the candidate genes.

My thesis focused on the SVZ-RMS-OB system, which is the major source of neurogenesis in the adult mouse forebrain. Specific objectives of this thesis are:

1. To determine baseline differences and heritability in the size of the NPC proliferative population among nine different laboratory strains of mice.

2: To identify regions in the genome contributing to the inter-strain differences in the number of NPCs, and prioritize candidate genes in these mapped regions according to their involvement in adult neurogenesis.
3. To investigate the influence of a strong candidate gene, Galanin receptor 2, on cell proliferation in the SVZ-RMS and downstream neuron production in the OB.

Implementation of these objectives should generate sufficient evidence that together make a compelling case for gene discovery and provide additional insights into the genetic architecture of NPC proliferation in the adult mouse forebrain.
Chapter 2: Effect of genetic background on adult neural progenitor cell proliferation: an opportunity to discover genetic modifiers

2.1 Introduction

There have been considerable advances in knowledge concerning the regulation of adult neurogenesis over the past two decades (Johnson et al., 2009; Ming and Song, 2011). Adult neurogenesis is a multifactorial process that encompasses several stages including proliferation, migration, and then the differentiation and survival of new neurons. Each stage is dynamically regulated by both extrinsic and intrinsic factors (Ming and Song, 2011; Khodosevich et al., 2012). Regulation at early stages of neurogenesis, most notably the proliferation of NPCs, is especially complex as a wide range of extracellular factors, stimuli, transcription factors, and epigenetic modifiers have been identified (Kempermann, 2011). To control the proliferative behavior of NPCs, these extrinsic factors must act on an intrinsic system where pro- and anti-proliferative genes are differentially regulated to provide instructions to the NPCs on the appropriate time and frequency to divide. The genetic basis of NPC proliferation, however, is not fully understood.

Previous study by Kempermann and colleagues demonstrated the effect of genetic background on adult hippocampal neurogenesis (Kempermann et al., 1997a; Kempermann and Gage, 2002). They examined seven inbred laboratory mouse strains and found different aspects of adult neurogenesis, especially cell proliferation and survival of newborn cells in the dentate gyrus, were differentially regulated by the genetic background (Kempermann et al., 1997a; Kempermann and Gage, 2002). Adult neurogenesis along the SVZ-RMS-OB is hypothesized to
be under similar influence from the genetic background. Here, I compared baseline cell proliferation in nine inbred laboratory strains held under equivalent housing conditions and at different ages. Cell proliferation was quantified by labeling dividing cells with a single injection of BrdU and subsequent immunodetection of labeled cells. I focused on the RMS, which is the rostral extension of the SVZ and a major site of neurogenesis in the adult rodent brain. The RMS mainly consists of rapidly dividing neuroblasts that are committed to the generation of new interneurons in the OB and it is far more homogenous in terms of cellular composition than the SVZ which also houses proliferating oligodendrocyte precursors in addition to the NPCs (Menn et al., 2006). From inter-strain comparisons and genome-wide association mapping, I showed cell proliferation in the RMS is modulated by the genetic differences present in mouse strains. The background effect is highest in young adults and wanes with advancing age. Strain differences were mapped to several QTLs suggesting the involvement of multiple genetic modifiers regulating NPC proliferation in the adult RMS.

2.2 Materials and methods

2.2.1 Animals

Nine inbred strains, C57BL/6J, FVB/NJ, A/J, BALB/cByJ, C3H/HeJ, CBA/J, DBA/2J, 129S1/SvImJ, and 129X1/SvJ, were used for these experiments. These strains were selected for the following reasons. (1) They are broadly distributed on the mouse phylogenetic tree (Petkov et al., 2004) and are expected to be genetically diverse. (2) All these strains have been haplotyped (National Institute of Environmental Health Sciences/Perlegen), and most of the strains (except for 129X1/SvJ) have been sequenced (Wellcome Trust Sanger Institute—Mouse Genomes...
This genetic information was used to pinpoint the genomic regions associated with the differences in NPC numbers. (3) The nine strains are among the most commonly used mouse strains in research. They are considered priority strains in the Mouse Phenome Database, which currently holds 1092 phenotypic data collected from these inbred strains. This allowed me to examine correlations between my data and other traits deposited at the Mouse Phenome Database. Mice were obtained from the Jackson Laboratory and the National Institutes of Aging. A total of fifty-one 2-3-month-old mice (at least two males and two females for each strain), forty-nine 12-month-old mice (at least two males and two females for each strain), and thirty-nine 18-month-old mice (at least two males and two females for C57BL/6J, A/J, CBA/J, DBA/2J, 129S1/SvImJ, and 129X1/SvJ; females only for the BALB/cByJ, C3H/HeJ, and FVB/NJ) were examined and compared among the nine strains. Additional proliferative data on 7-month and 24-month-old mice (n = 6 and 9, respectively) were collected for the C57BL/6J and DBA/2J inbred strains to study the dynamics of age-related changes in RMS proliferation. All experiments were conducted in accordance with the guidelines of the Canadian Council of Animal Care, and all protocols were approved by the institutional animal care committee.

2.2.2 BrdU labeling and detection

All mice were injected with a single dose of 50 mg BrdU/kg 1 hour before perfusion with acetic acid: 95% ethanol (1:3) as previously described (Poon et al., 2010). The half-life of BrdU in adult rodent brain is ~2 hours (Gilbert et al., 2005). Brains were removed from the skull and processed for paraffin embedding. Each hemisphere was serially sectioned in the sagittal plane at 8 µm and then mounted on Superfrost/Plus slides for anti-BrdU immunohistochemistry. Briefly, sections were deparaffinized, rehydrated, and treated with 1M HCl for 30 minutes at 37 °C.
Slides were incubated with mouse anti-BrdU monoclonal antibody (1:200 dilution; BD Biosciences, Mississauga, ON, Canada) overnight and then with biotinylated horse anti-mouse immunoglobulin (1:200 dilution; Vector Laboratories, Burlingame, CA, USA) for one hour on the next day. BrdU immunoreactivity was subsequently revealed using the VECTASTAIN Elite ABC kit (Vector Laboratories) and 3, 3′-diaminobenzidine (Sigma–Aldrich).

2.2.3 Quantification

To determine the number of BrdU+ cells in the RMS, I first located the RMS by staining every 10th section throughout the left hemisphere with anti-BrdU, and then identified the single sagittal section within the 10-series that had the greatest representation of the RMS for analysis. The linear density of BrdU+ cells per millimeter of RMS length was calculated from a single section that contained the most intact RMS exhibiting the stereotypical trajectory of proliferating cells en route to the OB. BrdU+ cells in the RMS of this optimal section were counted under brightfield illumination and with the aid of a 20× objective (Zeiss 200M Axiovert inverted microscope equipped with Axiovision 4.6 software). RMS length was measured using NIH ImageJ (version 1.42) software. Another counting approach adapted from Lee et al. (2003) was used in which I quantified the number of BrdU-positive cells in every 10th immunostained section (80-µm intervals) throughout the entire medial to lateral extent of the RMS. The total number of BrdU+ RMS cells was calculated for 20 randomly selected animals and this value is highly correlated with the linear density ($R^2 = 0.7744; p < 0.0001$; Supplementary Figure 2.1), thus demonstrating the effectiveness of the single best-section quantification method.
2.2.4 Cumulative BrdU labeling for cell cycle analysis

This experiment was performed by Zhiyou Li, a former postdoctoral fellow in the Goldowitz lab. In brief, BrdU was administered to a new batch of 2–3-month-old male C57BL/6J and A/J mice (5 mg/mL BrdU in 0.9% NaCl and 0.007 N NaOH; 50 mg/kg body weight) every 2 h for a total period of 10 h to ensure that every dividing cell entering the S-phase has the chance to be labeled. Mice were anesthetized with Avertin and perfused transcardially at 0.5, 2.5, 4.5, 6.5, 8.5 and 10.5 h after the first BrdU injection. Sixty animals were used for the cell cycle analysis (five A/Js and five C57BL/6Js for each time point). Consecutive sections were cut at 8-µm thickness, stained with anti-BrdU and counterstained with Cresyl Violet (CV). The labeling index (LI), which is ratio of BrdU+ cells to the total RMS cell population at a given time (t), was determined in brains obtained from mice killed at t = 0.5, 2.5, 4.5, 6.5, 8.5 and 10.5 h after the first BrdU injection. As the RMS is a long, compact cellular architecture, the total cell population was estimated by selecting four representative segments along the course of each RMS (two from the vertical arm, one from the RMS elbow and one from the horizontal arm depicted in Supplementary Figure 2.2). Cell density was determined by the number of cells in these segments divided by the area of these segments. RMS lengths and areas were measured using AnalySIS Opti Version 3.3.776 software (Soft Image System). The density was then multiplied by the total RMS area to estimate the total cells in an RMS. Once the LIs at every time point were calculated for each genotype, the average LI (y-axis) was plotted against the time after the first BrdU injection (x-axis). The equation, \( LI_0 = GF \times Ts/Tc \) was used to calculate the length of the S phase (Ts) and the length of the cell cycle (Tc) (Nowakowski et al., 1989) where \( LI_0 \) is the labeling index at the time of the first BrdU administration (t = 0) and is equivalent to the y-intercept of the graph. Growth fraction (GF) is the proliferating proportion of
the total RMS population and it is equivalent to the maximum LI plotted in the graph where all proliferating cells in the RMS are assumed to be labeled by BrdU at least once (GF = LIt; t ≥ Tc − Ts). Ts and Tc were subsequently calculated using a non-linear least squares fit to the labeling index curve (Nowakowski et al., 1989).

2.2.5 3-Dimensional reconstruction of the RMS and its total cell population

Three mice from each genotype used in the cell cycle analysis were also used for full reconstruction and estimation of RMS cell population size for A/J and C57BL/6J. These studies were performed by Zhiyou Li. RMS was reconstructed using the NeuroLucida and Neuroexplorer software (version 4, MicroBrightField, Inc.). Total cell number was calculated for the entire RMS using the density and volume measurements described above. The total cell numbers were rough estimates because these counts may be inflated due to the inclusion of double cell counts.

2.2.6 Quantitative trait locus analysis

Genome-wide interval mapping of inter-strain differences in RMS proliferation was performed using WebQTL, a module of GeneNetwork (http://www.genenetwork.org/webqtl/main.py), which is an online database that contains genotype information on more than 360 mouse strains including the nine inbred strains being investigated in this study. The nine classical inbred strains are part of the Mouse Diversity Panel genotyped for 140,000 SNPs across the genome (Ghazalpour et al., 2012). The likelihood ratio statistic (LRS) was computed to assess genotype–phenotype associations and identify QTLs, which refer to genomic regions that contain one or more sequence variants that modulate a
phenotypic trait. A permutation test was used to evaluate the statistical significance of association. The significant and suggestive threshold represents the approximate LRS value that corresponds to a genome-wide \( p \) value of 0.05 and 0.63, respectively. LRS scores of the mapped QTLs were converted to the likelihood of the odds (LOD) scores by dividing LRS by 4.61. The confidence limits of each QTL were defined by the 1.5 LOD support interval.

2.2.7 Statistical analysis

Data were analyzed using the JMP10 statistical software (SAS Institute, Cary, NC, USA). One-way analysis of variance was performed to test whether the number of proliferative cells in the RMS varied as a function of strain. Tukey's honestly significant difference (HSD) \textit{post-hoc} tests were employed after ANOVA to determine specific strain differences in each age group. Trends of the age-related decline were assessed by segmented linear regression analysis in which the independent variable, age, is partitioned into intervals based on the proliferative data collected at different time points. General linear modeling was used to determine the contribution of strain, age, sex, and interactions on RMS proliferation. Analyses were considered significant at \( p \leq 0.05 \).

2.3 Results

2.3.1 Strain-specific differences in the number of proliferative cells in the adult RMS

The adult RMS is composed largely of proliferative neuroblasts that are destined to give rise to specific interneurons in the OB. Proliferative activity in the RMS was determined an hour after a single BrdU injection. The distribution of 1-h-labeled BrdU cells is highly localized in the
RMS, which begins at the rostral tip of the lateral ventricle and terminates at the caudal end of the olfactory bulb (Figure 2.1). The shape of the RMS is defined by CV stain (data not shown). The nuclei of BrdU+ cells in the RMS are uniformly labeled and not in a punctate pattern, which is associated with DNA repair (Figure 2.1 B). Significant inter-strain differences in the number of proliferative cells were observed among the nine inbred strains at 2-3 months of age ($F_{8,42} = 3.8136, p = 0.0019$) (Figure 2.2). C57BL/6J had the lowest number of proliferative cells in the RMS than the other strains. The effect of sex on the number of proliferative cells was also examined as near equal numbers of males and females were collected for each strain. No significant sex differences were detected (females: $73 \pm 4.23$ cells/mm vs. males: $70 \pm 3.33$ cells/mm; $t_{49} = 0.64, p = 0.5241$).
Figure 2.1 Distribution of one hour- labeled BrdU+ cells in the adult mouse brain. 
In this study, every 10th sagittal section (8 µm thick) taken medial to lateral of the left hemisphere was subjected to anti-BrdU immunohistochemistry. Stained section shown in (A) contains the most complete RMS out of all the sections surveyed from an exemplary A/J mouse brain (2 months old). (B) A higher magnification image of BrdU+ cells in the RMS. (C) Schematic representation of the stained sagittal section in (A) highlights the location of the RMS relative to the dentate gyrus (DG), lateral ventricle (LV), and olfactory bulb (OB).
Figure 2.2 Cell proliferation in the RMS of nine inbred mouse strains at 2-3 months of age.
Strain information is provided on the x-axis, and the y-axis depicts the mean number of bromodeoxyuridine (BrdU+) cells per millimeter RMS length ± standard error of the mean (SEM). Analysis of variance was performed to test whether the number of BrdU+ cells in the RMS varied as a function of strain. Post hoc Tukey HSD tests were employed to determine specific strain differences (†, p < 0.05 when compared with C57BL/6J). The sample size per strain is indicated in the bars.
2.3.2 Shape, orientation, and size of the RMS in different inbred mouse strains

Systematic analysis of cell proliferation in the RMS of the nine inbred strains showed A/J had 1.6 times more number of BrdU+ cells in the RMS compared to C57BL/6J (Figure 2.2). The RMS from the C57BL/6J and A/J mice was reconstructed from serial sagittal sections to compare their three-dimensional course and to determine the total numbers of RMS cells in each strain. Immunohistological staining and imaging analysis performed by Dr. Zhiyou Li revealed that the general configuration of the RMS in both strains was similar. Schematic 3D illustration of the A/J and C57BL/6J are shown in Figure 2.3 B and C, respectively. Moreover, A/J had approximately 40% more cells in the RMS than C57BL/6J (A/J = 52659 ± 535 and C57BL/6J = 37130 ± 731; Figure 2.3 B and C), which is similar to the 38.5% differences in the number of BrdU+ cells observed between the two inbred strains (Figure 2.2).
Figure 2.3 Shape, orientation, and size of the RMS of A/J and C57BL/6J.

(A) Schematic 3D illustration of the RMS relative to other brain structures including the ventricular system (V), hippocampus (Hipp), and the olfactory bulb (OB). Shapes of Hipp, V and OB are adapted and modified, with permission, from Bock et al. (2006). Top-forward views of the 3D RMS structure in the A/J (B) and C57BL/6J (C) brains were reconstructed from serial sagittal sections. Parentheses in B and C indicate the total number of RMS cells (±SEM) for each strain (n = 3 per group).

2.3.3 Cell cycle lengths of adult neural progenitor cells from different mouse strains

At the cellular level, I sought to determine if the differences in BrdU-labeled cells between A/J and C57BL/6J were due to differences in cell cycle parameters as explored in the
dentate gyrus by Hayes & Nowakowski (2002). First, I determined the LI at each time point under study for both parental strains (Figure 2.4). There was an initial increase of LI with lengthening BrdU exposure time, indicative of a constantly dividing cell population. For both strains, the LI reached a plateau of \( \sim 0.2 \), suggesting that the actively dividing populations in the RMS accounts for approximately 20% of the total RMS cell population. Using the total RMS cell numbers described in Figure 2.3 and a GF value (i.e. the proportion of proliferating cells to the total number of cells in the population) of 0.2, I estimated that the total numbers of actively dividing cells in the RMS were 10531 \( \pm \) 107 and 7426 \( \pm \) 146 for A/J and C57BL/6J, respectively. Moreover, the quantitative analysis of the LI curves showed that there were no significant differences in the cell cycle parameters of the two RMS populations. The ratio of \( T_s/T_c \) was similar (\( \sim 0.57 \)), indicating that the relative length of the S-phase (\( T_s \)) to the whole cell cycle (\( T_c \)) was the same for the two strains. The length of the cell cycle for the proliferative populations in the RMS ranged from 10.5 h (A/J) to 14.5 h (C57BL/6J), and these values overlapped with the cell cycle length for the proliferative population in the dentate gyrus (12–14 h) and were also within the 8–18 h range of cell cycle lengths detected in progenitor cells lining the ventricular cavity of the developing cerebral neocortex (Hayes & Nowakowski, 2002; Takahashi et al., 1995). Although the lengths of cell cycle and S-phase for the proliferative population in A/J RMS appeared to be shorter than the lengths detected in the C57BL/6J RMS, such differences did not reach statistical significance. Therefore, the differences in the number of BrdU-labeled cells in the RMS of the two strains reflected differences in the actual number of proliferative cells, and was not due to differences in cell cycle or S-phase lengths. In line with this conclusion, the proliferative population size in the A/J RMS was \( \sim 40\% \) larger than C57BL/6J RMS.
Figure 2.4 Cell cycle analysis in the RMS of adult A/J and C57BL/6J mice.
Graphs of labeling indices from the counts of BrdU-labeled and non-BrdU-labeled cells after a series of BrdU injections into adult A/J and C57BL/6J mice. The squares represent the average labeling index (LI) (±SEM) (y-axis), for each time point (x-axis). The line extending from each square is the least squares fit. The growth fraction (GF), length of the cell cycle (Tc in hours) and length of the S-phase (Ts in hours) for each genotype were estimated from the equations provided in the graphs. Differences in the cell cycle parameters were observed between the two strains with the A/J RMS cells (A) having shorter Ts and Tc than C57BL/6J RMS cells (B). Although suggestive, these differences were not statistically significant.
2.3.4 Strain comparison in cell proliferation across different ages

It is currently unknown whether the prominent genetic background effect observed in young adults persists into later life. In addition to examining cell proliferation in young adult mice (i.e. 2-3 months of age), I also systematically quantified the number of proliferative cells in the RMS of older adults at 12 and 18 months of age following one-hour pulse of BrdU. Mice from all 9 inbred strains exhibited considerable age-dependent decline in the number of proliferative cells in the RMS (Figure 2.5). An average of 50% and 70% decrease in the number of BrdU+ cells was observed in 12- and 18-month-old mice, respectively, compared with their young–adult counterparts (2-3 month old)(Figure 2.2). Significant inter-strain differences in the number of proliferative cells were observed at 2-3 months (F8,42 = 3.8136, p = 0.0019) and 12 months of age (F8,40 = 4.3052, p = 0.0008) but not at 18 months of age (F8,30 = 1.8459, p = 0.10702). The extent of variation among the strains is not preserved across different ages. Post hoc–specific mean comparisons detected significantly higher number of proliferative cells in 129X1/SvJ than C57BL/6J at 2-3 months of age (89 ± 8.66 and 43 ± 5.48 cells/mm, respectively, p = 0.0003)(Figure 2.2). This 2-fold strain difference was not detected at 12 months of age. Instead, the number of dividing RMS cells in 12-month-old 129X1/SvJ was the lowest among the 9 inbred strains and was 45% less than FVB/NJ that exhibited the highest number of proliferative cells in the RMS (p = 0.0052)(Figure 2.5). Representative sagittal sections presented in Figure 2.6 demonstrate the observed age and genetic background effects on the number of BrdU+ cells in the RMS at 3, 12, and 18 months of age (Figure 2.6).

The dynamics of age-related changes in the RMS proliferative population were further examined in two inbred strains, C57BL/6J and DBA/2J, where additional proliferative data were collected at 7 and 24 months of age (Figure 2.7). In the extended proliferative profiles, highly
significant main effects for strain ($F_{1,43} = 43.2925, p < 0.0001$) and age ($F_{4,40} = 56.6486, p < 0.0001$) were detected (Figure 2.7). In addition, significant interaction effect was obtained for strain × age ($F_{4,40} = 5.9523, p = 0.0009$) as demonstrated by the differential dynamics in the age-dependent decline observed between the two strains (Figure 2.7). Trends of age-related decline were assessed by segmented linear regression analysis. DBA/2J exhibited a steeper decline in the number of proliferative cells in the RMS during the 3- to 7-month period (regression slope is $-5.58, p = 0.004$) compared with that of C57BL/6J (regression slope is $-1.33, p = 0.5478$). The number of proliferative cells further plummeted during the 12- to 18-month period for both C57BL/6J and DBA/2J (regression slopes are $-3.77$ and $-2.7560$, respectively, $p < 0.001$). From 18 to 24 months of age, the number of proliferative cells appeared to level off for both strains.
Figure 2.5 Cell proliferation in the RMS of nine inbred mouse strains at 12 and 18 months of age.

Strain information is provided on the x-axis, and the y-axis depicts the mean number of BrdU+ cells per millimeter RMS length ± SEM. The sample size per strain is indicated in the bars. Analysis of variance was performed to test whether the number of proliferative cells in the RMS varied as a function of strain. (A) Significant inter-strain differences in the number of BrdU+ cells per millimeter RMS length were detected at 12 months of age (*, p < 0.05 when compared with 129X1/SvJ; **, p < 0.01 when compared with CBA/J). At 18 months of age (B), no significant inter-strain differences were detected.
Figure 2.6 Representative sagittal sections demonstrating the age-related decline in the number of BrdU+ cells in the RMS.
Photomicrographs of the RMS from two inbred strains, 129X1/SvJ and C57BL/6J, at 3 (A), 12 (B), and 18 months of age (C). Arrows indicate the operational delineation of the start and end point of RMS. Abbreviation: LV, lateral ventricle. Scale bar = 200 μm.
Figure 2.7 Dynamics of age-related changes in the number of proliferative cells in the RMS of the C57BL/6J and DBA/2J inbred strains from 3 to 24 months of age.

The number of BrdU+ cells per millimeter of RMS length ± SEM is on the y-axis and age in months is shown on the x-axis. A comparison of the linear regression slopes revealed that the rate of decline with age is not the same for the two strains especially during the 3- to 7-months period where DBA/2J exhibited a steeper decline in the number of BrdU+ cells (regression slope is $-5.58$, $p = 0.004^*$) compared with that of C57BL/6J (regression slope is $-1.33$, $p = 0.5478$).
2.3.5 Heritability of neural progenitor cell proliferation and genotype-phenotype association

Heritability of cell proliferation in the adult RMS was determined by the ratio of inter-strain variance over the total variance, which includes both inter- and intra-strain variance (Kempermann et al., 2006). Heritability of cell proliferation at 3 months of age was 0.58, indicating that half of the variation in proliferation is accounted for by the genetic differences present among the strains. Whereas, heritability of cell proliferation at 12 months and 18 months of age was estimated 0.37 and 0.15, respectively. The genetic basis of cell proliferation in the RMS was further examined through QTL analyses of proliferative data at 3 and 12 months of age where significant inter-strain differences were observed. The LRS (y-axis in Fig. 2.8) was computed to measure the strength of linkage between differences in NPC proliferation and genetic variants present among the 9 inbred strains. The genetic diversity among the strains is captured by polymorphic markers (e.g. SNPs and microsatellite markers) distributed across the entire genome (x-axis in Figure 2.8). Genome-wide significance ($p = 0.05$) and suggestive ($p = 0.63$) levels for assessing the confidence of the linkage statistics were estimated by a permutation test ($n = 2000$). The chromosomal region with an LRS score above the genome-wide significance threshold is referred to as a significant QTL, whereas a chromosomal region with LRS score above the genome-wide–suggestive threshold but below the significance level is referred to as a suggestive QTL. At 3 months of age, several suggestive QTLs on multiple chromosomes were identified (Figure 2.8-A). In contrast, the QTL mapping of the 12-month-old data revealed only a few loci that are approaching the suggestive threshold (Figure 2.8-B). The phenotype–genotype association detected at 3 months of age reflects the high heritability of 0.58 estimated from the inter-strain differences observed at 3 months of age. Whereas, heritability of cell proliferation at
12 months of age was 0.37, which indicates that a lesser proportion of the inter-strain variation was attributed to genetic differences, hence the weaker genome-wide association.

Figure 2.8 Genome-wide QTL analysis of inter-strain variation in RMS cell proliferation.
Whole-genome interval mapping for QTLs associated with the differences in the number of BrdU+ cells per millimeter RMS length observed at 3 (A) and 12 months of age (B). The x-axis of each graph represents the chromosomes (1–19 and X), and the blue y-axis on the left depicts the likelihood ratio statistic (LRS). The red and gray horizontal lines across the LRS plot correspond to genome-wide $p$ values of 0.05 and 0.63, respectively. (A) Whole-genome interval mapping of 3-month-old proliferation data revealed several suggestive QTLs ($p \leq 0.63$). In contrast, no significant or suggestive QTL was detected from the mapping of the 12-month-old proliferation data (B).
2.4 Discussion

In this study, I demonstrated that cell proliferation in the RMS is differentially regulated by the genetic background of mice. I provided the first comparative study examining cell proliferation in RMS of nine inbred strains. Significant strain differences in the number of proliferative RMS cells were observed in young adult brains with C57BL/6J having the lowest number of proliferative (BrdU+) cells. The shape and orientation of the RMS were similar among the A/J and C57BL/6J strains, indicating that the observed inter-strain differences reflect differences in the cell number rather than an artifact of the structure. My longitudinal study of the aging RMS from 2 to 24 months of age further showed that the genetic background effect observed in young adults does not persists into later life. Age is a negative regulator of NPC proliferation, and this age effect is demonstrated by the substantial ∼70% decrease in the number of BrdU-labeled cells in the RMS of elderly mice (18- to 24-months old) compared with young adult mice (2-3 months old). A similar decline in the number of proliferative NPCs and decrease in the number of newborn neurons have been reported in the SVZ (Enwere et al., 2004) and the OB (Luo et al., 2006), respectively. This suggests that age uniformly affects neurogenesis in the SVZ–RMS–OB system.

The fact that significantly fewer aged cells were labeled with BrdU than young cells suggests that fewer aged cells are actively dividing in the RMS. Previous studies using a similar BrdU-labeling technique have reported that the age-related decline in BrdU+ cell number reflects a decrease in the actual number of NPCs (Maslov et al., 2004) and is not because of the lengthening of cell cycle (Olariu et al., 2007) or an increase in cells undergoing apoptosis (Mirich et al., 2002; Enwere et al., 2004). Moreover, BrdU availability in the mature brain is not affected by age (Leuner et al., 2007). Taken together, these results lead us to conclude that the
number of proliferative NPCs in the RMS decreases with age. This age-related decline is in agreement with in vitro findings from Stoll et al. (Stoll et al., 2011b) where they observed significantly fewer numbers of dividing NPCs in the aged forebrain cell cultures compared with young cultures. Despite the lowered NPC numbers in the aged cultures, proliferative NPCs still retained their regenerative potential where the aged NPCs were expanded in the presence of specific growth factors (e.g., epidermal growth factor and fibroblast growth factor) and gave rise to mature neurons in the absence of these growth factors (Molofsky et al., 2006; Ahlenius et al., 2009; Stoll et al., 2011a). These findings suggest that the regenerative response of aged NPCs may be therapeutically augmented to compensate for the lost neurons in disease or injured brain.

Characterization of normal NPC aging and the elucidation of factors regulating NPC proliferation in the aging brain are important steps toward the development of such therapy.

The current understanding of the molecular changes in NPCs associated with the age-related decline in neurogenesis is sparse. To gain insights into the genetic factors involved, quantitative data on NPC proliferation can be exploited to map loci and genes that are responsible for the age-related changes in the proliferative potential of the NPC population. In this study, I performed genome-wide association mapping for NPC proliferation using genotype data available at GeneNetwork. I discovered several suggestive QTLs modulating NPC proliferation at 3 months of age but found no significant or suggestive QTLs at 12 months of age. These mapping results indicate the complex, multigenic regulation of NPC and also highlight the shift in genetic contribution to NPC proliferation with advancing age. The striking inter-strain differences in NPC proliferation observed in the young adults indicate the presence of genetic modifiers functioning as pro- or anti-proliferative modulators of NPCs. However, the genetic contribution from strain background is not maintained with advancing age because heritability of
NPC proliferation decreases with age and no strain differences were observed in the elderly mice. This suggests that the function of these pro- or anti-proliferative genes is either repressed or amplified, respectively, during aging. Because of the limited number of sampled strains ($n = 9$), my QTL mapping resolution may be low and population stratification among the strains may inflate the association statistics (Bennett et al., 2010). Therefore, it was not feasible to nominate candidate genes based solely on the nine inbred strains. Instead, I took advantage of large panels of recombinant inbred strains in the next chapter to improve mapping power and resolution.
Chapter 3: Identification of quantitative trait loci and candidate genes that modulate adult neural progenitor cell proliferation

3.1 Introduction

It has also been previously shown that adult neurogenesis is significantly dependent on the genetic background, and the genetic diversity among inbred strains can serve as a reservoir for gene discovery (Kempermann et al., 2006; Poon and Goldowitz, 2013). Marked inter-strain differences in NPC proliferation were detected in the RMS of nine inbred strains at 2 months of age where mice of C57BL/6J background exhibited the lowest number of BrdU+ cells in the RMS and other strains such as A/J and DBA/2J had ~1.6 fold higher BrdU+ RMS cells compared to C57BL/6J. In this chapter, an even wider inter-strain difference was observed among the RI strains (AXB/BXA and BXDs), suggesting the involvement of multiple loci and genes. The large RI sample size and phenotypic diversity greatly improved QTL mapping resolution and precision. Significant QTLs on Chr 6 and Chr 11 were identified to modulate NPC proliferation in the RMS. Regions on Chr 14 and Chr 18 were also found to work additively with the significant Chr 6 in regulating the number of NPCs in the RMS. Functional annotation and gene expression analyses highlighted a subset of candidate genes in the mapped QTL regions. Common features shared by these candidate genes include expression in the RMS, functional implication in cell proliferation/cell cycle progression, and participation in signaling pathways important for neurogenesis. Together, these findings provide insights into the dynamic interplay of genetic loci and underlying genes that may modulate NPC proliferation in the adult brain.
3.2 Materials and methods

3.2.1 Animals

A set of 27 AXB/BXA RI strains (118 mice) was obtained from The Jackson Laboratory (Bar Harbor, ME, USA). BXD RI strains were obtained from two different sources. BXD 1–42 RI strains were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). BXD 43–103 RI strains were provided by Dr. Robert W. Williams and Dr. Lu Lu (University of Tennessee Health Science Center, Memphis, TN, USA). Mice were housed at the University of Tennessee Animal Facility in a pathogen-free, ~23.5°C, and 45–50 humidity environment on a 12 h light-12 h dark cycle. A total of 61 BXD RI strains and 265 mice were used in this study. At least one male and one female per strain were examined. Mice studied were between 50–85 days old. All experiments were conducted according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA) and the Canadian Council of Animal Care. Approval was obtained from the Institutional Animal Care and Use Committee at the University of Tennessee Health Science Center and the Animal Care Committee at the University of British Columbia on all our animal protocols. All mice were injected with a single dose of 50 mg BrdU/kg 1 hour before perfusion with acetic acid: 95% ethanol (1:3) as previously described (Chapter 2, Sub-section 2.2.2).

3.2.2 Anti-BrdU immunohistochemistry and quantification

Brain sections from the recombinant inbred strains were sectioned, stained, and quantified as previously described (Chapter 2, Sub-sections 2.2.2 & 2.2.3).
3.2.3 Quantitative trait locus analysis

Cell proliferation data collected from the AXB/BXA and BXD RI strains was deposited into the GeneNetwork, which is an open-access online database that contains detailed genotype information of each RI strain. Genome-wide interval mapping of QTLs regulating NPC proliferation was performed using WebQTL at the GeneNetwork and strains were combined for mapping as recommended. The likelihood ratio statistic (LRS) was computed to assess the strength of genotype-phenotype association of the genome scans. Permutation test of 2000 permutations was computed to establish the significance and suggestive thresholds where the LRS values corresponded to a genome-wide $p$ value of 0.05 and 0.63, respectively. A significant QTL is referred to as a chromosomal region with LRS score equal or above the genome-wide significant level ($p = 0.05$). A suggestive QTL is a region of the chromosome with LRS score equal or above the genome-wide suggestive level ($p = 0.63$). LRS scores of the mapped QTLs were converted to the likelihood of the odds (LOD) scores by dividing LRS by 4.61. The confidence limits of each QTL were defined by the 1.5 LOD support interval. An additive allele effect estimates the change in average phenotype that would be produced by substituting a single allele from one parent (e.g., A/J) with that from another parent (e.g., C67BL/6J). It is half of the difference between the mean of RI strains that are homozygous for one parental allele compared to the mean of RI strains that are homozygous for the other parental allele. Epistasis is the non-linear interaction between two loci where the combined effect of two loci is different than the addition of individual effects from each locus.
3.2.4 Candidate gene analysis

An integration of bioinformatics strategies and gene expression data were employed to evaluate the underlying genes in the mapped QTL intervals. The genetic variation structure within identified QTL regions were examined using the single-nucleotide polymorphism (SNP) and insertion/deletion (indel) data available at the GeneNetwork SNP browser (genenetwork.org/webqtl/snpBrowser.py). The numbers of SNPs and indels that are associated with each candidate gene, and ones that differ between the two parental inbred strains (e.g. DBA/2 J and C57BL/6 J) were determined. Sequencing data released by the Mouse Genomes Project (http://www.sanger.ac.uk/resources/mouse/genomes) was used to confirm the presences of SNPs and indels in each of the candidate gene. The expression of each candidate gene in the adult brain is visualized using Allen Brain Atlas (http://www.brain-map.org). Microarray data on laser-microdissected NPCs in the RMS (Khodosevich et al., 2009) was used to determine the presence/absence and transcript level of a candidate gene in the RMS. Candidate gene were further assessed on their associated Gene Ontoloy (GO) terms and pathways using information available at the Database for Annotation, Visualization, and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov) and the QTLMiner module of the GeneNetwork (http://www.genenetwork.org/webqtl/main.py?FormID=qtminer). Genes were prioritized according to the following criteria: 1) polymorphic (i.e. associated with ≥ 1 SNPs and/or Indels), 2) expression in the adult neurogenic sites including the RMS, and 3) associated with protein functions in GO terms such as neurogenesis, cell proliferation, and cell cycle progression. Genes were ranked by the numbers of criteria they met (e.g. 0, 1, 2, or 3).
3.2.5 Statistical analysis

One-way analysis of variance (ANOVA) was performed to detect significant inter-strain differences in the number of proliferative cells in the RMS (JMP 10 statistical software, SAS Institute, Cary, NC, USA). Tukey’s HSD post-hoc tests were subsequently employed to determine significant differences between two RI strains. General linear modeling was used to determine the contribution of possible confounding covariates including age, sex, body weight, and strain epoch effects and their interaction effects on RMS proliferation. Residuals from regression fitting these variables for RMS linear density were obtained and subsequently used to adjust for the mean RMS linear density per strain (Poon et al., 2010). Analyses that yielded $P \leq 0.05$ were considered significant. Heritability was estimated in a broad sense where I calculated the ratio of variance that is accounted for by the differences between strains over the total variance, which includes both between-strain variance and within-strain variance (Kempermann et al., 2006).

3.3 Results

3.3.1 Two panels of recombinant inbred strains exhibited wide differences in RMS cell proliferation

Inter-strain differences in the number of BrdU+ proliferating cells in the RMS of parental inbred strains (i.e. C57BL/6J, A/J, DBA/2J) indicate that these strains have an inherent difference in proliferative capability in the adult RMS. To determine the genetic basis for this difference, I first examined the 27 BXA/AXB RI strains generated from parental A/J and C57BL/6J mice. As an assay, I used the numbers of BrdU+ cells as determined from a single
injection of BrdU given one hour prior to sacrifice. Systematic quantitative analysis of cell proliferation in the AXB/BXAs revealed a wide range of differences in the number of BrdU+ cells in the RMS (Figure 3.1-A and Figure 3.2). Strain averages were normally distributed and significant inter-strain differences in the linear density (BrdU+/mm of RMS length) was detected ($F_{28,117} = 3.52; p < 0.0001$). There is a three-fold difference between the minimum and the maximum linear density measured from the RI strains and this range extends beyond the differences observed between the parental strains. Heritability of proliferation in the RMS was estimated to be 0.53, which suggests half of the variation in cell proliferation is accounted for by allelic variation. In order to fully appreciate the complexity of this process, I further explored a separate genetic reference panel called the BXDs. The BXDs is one of the largest murine mapping panels which consists of 80 unique BXD RI strains and the BXD panel is three times the size of the AXB/BXA panel. A substantial range of cell proliferation in the RMS, as detected by the uptake of BrdU, was also identified in 61 BXD RI strains, with a 3-fold difference among the strains (Figure 3.1-B). This range extends beyond the differences detected between the two parental strains, C57BL/6 J and DBA/2 J (Figure 3.1-B). Significant inter-strain differences in RMS linear density was detected ($F_{60,204} = 4.77, p<0.0001$), and heritability of cell proliferation in the RMS is 0.58.

There is no significant sex effect ($t(263) = 0.82, p = 0.4111$; females = $63.97 \pm 1.66$; males = $65.83 \pm 1.55$) or body weight effect ($R^2 = 0.004; p = 0.29$) on the number of proliferative cells in the RMS. I also examined the batch effect as the BXD panel has three epoch substructures: i) BXD 1–30 generated by Benjamin A. Taylor in the 1970s (Taylor et al., 1977), ii) BXD 33–42 generated by Benjamin A. Taylor in the late 1990s (Taylor et al., 1999), and iii)
BXD 43–103 generated by Peirce and colleagues in the early 2000s (Peirce et al., 2004). No significant batch effect on RMS linear density was detected ($F_{2, 262} = 0.44; p = 0.65$).
Figure 3.1 Quantification of the number of proliferating cells in the RMS of two separate panels of RI strains.

(A) RMS linear density (i.e. mean number of BrdU + cells per mm length of RMS ± SEM) of 27 AXB/BXA RI strains (white bars) and their parental strains, C57BL/6 J (red bar), and A/J (blue bar). (B) Mean linear density of 61 BXD RI strains (white bars) and their parental strains, C57BL/6 J (red bar), and DBA/2 J (green bar). The sample size per strain is indicated in the bars.
Figure 3.2 Representative sagittal sections of BrdU-labeled RMS in four separate lines of AXB/BXA adult mice given a single pulse of BrdU for one hour.
These photomicrographs document the varying phenotypes seen in this set of recombinant inbred mice with some lines having abundant proliferating BrdU+ cells in RMS (A), some lines have little BrdU+ cells in RMS (B) whereas others have intermediate numbers of BrdU+ cells (C and D). Arrows indicate the start and end point of RMS. LV, lateral ventricle; scale bar = 200 µm
3.3.2 Significant QTLs associated with the inter-strain differences in RMS cell proliferation

To gain insights into the complex genetics regulating adult NPC proliferation, I performed QTL analysis for the differences in RMS linear density among the RI strains. The AXB/BXA RI and BXD strains consist of unique combinations of haplotypes inherited from the parental strains, which make these RI strains useful for mapping complex/quantitative traits and uncovering chromosomal regions that are responsible for the phenotypic differences. Using the online tool WebQTL (http://www.gnenetwork.org/), I first mapped linear density in the RMS of AXB/BXA RI strains (Figure 3.3) and detected a highly significant QTL on the distal end of Chr 11 (Figure 3.3). This significant QTL has a 1.5-Mb-wide peak that is centered at 116.75 Mb on Chr 11 as defined by the 1.5- LOD support confidence interval. From marker regression analysis, markers D11Mit103 and gnf11.125.992 located in the Chr 11 QTL are significantly associated with trait variation (Figure 3.3-D). The genotypes at these markers revealed that having a C57BL/6J (B) allele in the Chr 11 QTL region is associated with a ~15 BrdU+ cells/mm increase in linear density compared with having an A/J (A) allele ($F_{1,24} = 28.7$, $p < 0.0001$). This finding is contrary to my expectation of having the A allele associated with high trait values and may be attributed to transgressive segregation with other additive or epistatic loci.

Because age has previously been found to influence adult neurogenesis (Tanapat et al., 1999; Poon and Goldowitz, 2013), I regressed the RMS linear density for each animal against age and calculated the average residuals per strain. QTL mapping of variation in the adjusted RMS linear densities generated a whole-genome scan LRS plot that resembled the plot produced from mapping with the unadjusted trait data (Figure 3.3). A prominent QTL is mapped to the distal end of Chr 11 and the genetic markers, D11Mit103 and gnf11.125.992 are associated with
the highest LRS scores (Figure 3.3). The B allele in this QTL interval increases trait value by
~24 BrdU+ cells/mm, suggesting that the removal of covariate could unmask an even greater
genetic effect on phenotype. In additional to the Chr 11 QTL, another QTL is seen at the
proximal end of Chr 2 at 25 ± 5Mb (genome-wide $p < 0.63$; LRS = 10.56; LOD = 4.61; Figure
3.3). Strains having a B allele in this Chr 2 QTL interval is associated with an increase in linear
density of ~22 BrdU+ cells/mm compared with strains carrying the A allele (Figure 3.3).
A  Unadjusted RMS linear density

B  Adjusted RMS linear density

C  Chr 11

D  Unadjusted RMS linear density

E  Adjusted RMS linear density

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Figure 3.3 QTL analyses of cell proliferation in the RMS of AXB/BXA RI strains. Whole-genome interval mapping for QTL modulating the unadjusted RMS linear density (A) and for the adjusted RMS linear density (B) for age. The x-axis in the top panel represents the chromosomes (1–19, & X) and the blue y-axis on the left depicts the likelihood ratio statistic (LRS). The gray and red horizontal lines across the LRS plot mark the genome-wide suggestive ($p < 0.63$) and significant ($p < 0.05$) thresholds, respectively. The highest LRS peak is located at the distal end of Chr 11. (C) LRS plot for the entire Chr 11. The green and red segments represent the average additive effects of A/J ‘A’ alleles and C57BL/6J ‘B’ alleles, respectively, at a genetic marker. The magnitude of the additive effect is shown by the green colored y-axis on the right. (D and E) Screen captures of marker regression reports from mapping the unadjusted (D) and adjusted (E) RMS linear density. Two Chr 11 genetic markers, D11Mit103 and gnf11.125.992, are consistently associated with the highest LRS score.

I also performed QTL analysis for the differences in RMS linear density among the BXD RI strains. I discovered a highly significant locus on Chr 6 (76.8-88.8 Mb; $P = 0.05$) and a suggestive locus on Chr 11 (50-58 Mb; $P = 0.63$) that modulate NPC numbers in the RMS (Figure 3.4). Since age had a significant effect on RMS linear density, I regressed the RMS linear density for each animal against age to ensure the observed genotype-phenotype association is not confounded by differences in age. QTL mapping of the adjusted RMS linear density identified the same significant and suggestive QTLs on Chr 6 and Chr 11, respectively (Figure 3.4-B). I used marker regression analysis to estimate the allelic effect sizes of the mapped QTLs. The genotype of SNP and microsatellite markers underlying the Chr 6 QTL revealed that the C57BL/6 J allele is associated with ~19 BrdU + cells/mm increase in RMS cell proliferation compared with having a DBA/2 J allele. Conversely, genetic markers in the Chr 11 QTL region showed that a DBA/2 J allele has an additive effect of ~11 BrdU + cells/mm compared with having a C57BL/6 J allele. These findings suggest the complex genetic modulation of NPC proliferation with the involvement of more than one QTL.
Composite interval analysis revealed secondary intervals on Chr 14 (40.3-49.2 Mb) and Chr 18 (58.2-74.9 Mb) work additively with the Chr 6 QTL in modulating NPC proliferation in the RMS (Figure 3.4). One-way ANOVA confirmed the allelic effects of the Chr 6 ($F_{1,58} = 39.87; p < 0.0001$), Chr 14 ($F_{1,58} = 16.01; P = 0.0002$), and Chr 18 ($F_{1,58} = 12.56; p = 0.0008$) on RMS proliferation. Pair-Scan analysis was performed to assess two-way interaction between pairs of genetic markers from different chromosomes. Once again, I found significant association of markers in Chr 6 QTL with markers in the secondary Chr 14 and Chr 18 intervals (LRS Full $> 40.1; p < 0.01$). To further assess the type of interaction among these loci (i.e. additive or epistatic), the BXD strains were split into groups according to their genotypes at these QTL intervals, and an average RMS linear density was calculated for each group. The plotted RMS linear densities show how different allele combinations at these loci are associated with different levels of RMS linear density (Figure 3.5). The major effect of the Chr 6 QTL is demonstrated where strains carrying the C57BL/6 J allele in the Chr 6 interval are associated with higher RMS linear densities compared to having the DBA/2 J allele, irrespective of the genotypes at the Chr14 or Chr18 QTL intervals. When the genotypes at these secondary QTLs regions are taken into account, having the C57BL/6 J allele at either the Chr 14 (Figure 3.5-A) or Chr 18 intervals (Figure 3.5-B) are associated with even higher RMS linear densities compared to having the DBA/2 J alleles. The C57BL/6 J alleles in the Chr 14 and Chr 18 QTL regions are respectively associated with $\sim 15$ BrdU+ cells/mm and $\sim 10$ BrdU+ cells/mm increase in RMS cell proliferation compared with having DBA/2 J alleles. These findings suggest the major Chr 6 QTL works additively with loci on Chr 14 and 18 when modulating NPC proliferation in the RMS.
A Genome-wide interval mapping of the unadjusted RMS linear density

B Genome-wide interval mapping of the corrected RMS linear density

C Composite interval mapping controlling for the Chr 6 QTL
Figure 3.4 QTL analyses of cell proliferation in the RMS of BXD RI strains.
The x-axis for figures (A-C) represents the chromosomes 1–19, & X (top panel) and their physical maps in megabases (bottom panel). The y-axis and the blue line depict the likelihood ratio statistic (LRS), which indicates the strength of association between genotypes of markers across the genome and the phenotype (i.e. RMS linear density). The light red and gray horizontal lines mark the significant ($p = 0.05$) and suggestive ($p = 0.63$) threshold, respectively. Whole-genome interval mapping of unadjusted RMS linear density (A) and for the adjusted RMS linear density corrected for the effects of age (B) have mapped a significant QTL on Chr 6 (76.8-88.8 Mb) and a suggestive QTL on Chr 11 (50-58 Mb) regulating RMS linear density. (C) Composite interval mapping revealed an additional significant locus on Chr 14 (39–49.5 Mb) and a suggestive locus on Chr 18 (58–86 Mb) that work additively with the Chr 6 QTL in modulating RMS linear density.
Figure 3.5 Allelic effects on cell proliferation in the RMS.
Plots of RMS linear density (i.e. # BrdU+ cells per mm length of RMS) versus allele genotypes at markers associated with the major Chr 6 QTLs and secondary QTLs on Chr 14, and Chr 18. BXD strains were divided into different groups based on their genotypes at markers closest to the QTL peaks on Chr 6, 14, and 18. B6 and D2 represent homozygous alleles of C57BL/6 J and DBA/2 J for the markers. Dots represent group means ± SEM. (A) Effect of genotype on RMS linear density (y-axis) at markers in the Chr 6 QTL (x-axis) and the Chr 14 QTL intervals (B6 and D2 alleles are represented by red and green colour lines, respectively). (B) Effect of genotype on RMS linear density (y-axis) at markers in the Chr 6 QTL (x-axis) and the Chr 18 QTL intervals (B6 and D2 alleles are represented by red and green colour lines, respectively).
3.3.3 Cell proliferation in the SGZ is regulated by a different set of QTLs

I also examined the genetic architecture underlying the proliferative potential of the SGZ progenitors in comparison with the RMS. I assessed cell proliferation in the SGZ one-hour post BrdU injection. The average total number of BrdU+ cells was calculated in the SGZ of 61 BXD RI strains. Similar to what was observed in the RMS, the number of BrdU + cells in the SGZ significantly differs among the BXD RI strains ($F_{60,170} = 2.88, p < 0.0001$) (Figure 3.6). Heritability of cell proliferation in the SGZ is estimated to be 0.5 ($p < 0.0001$). The number of BrdU + cells in the SGZ was 3.5 fold higher in C57BL/6 J compared to DBA/2 J, which was opposite to the parental strain differences observed in the RMS. However this reversal in phenotypic direction was not observed in the BXD RI panel. For example, BXD68 had both high numbers of BrdU + cells in the RMS and SGZ. Whereas, BXD55 had low numbers of BrdU + cells in the RMS and SGZ. The effects of confounding variables on cell proliferation in the SGZ were also examined. Age had a significant effect on the number of BrdU + cells in the SGZ ($R^2 = 0.059, p = 0.0002$). Strain epoch, sex, body weight differences did not significantly influence the number of proliferative cells in the SGZ.

QTL mapping of cell proliferation in the adult SGZ revealed suggestive QTLs on Chr 1 (40-59 Mb), Chr 5 (104.5-119.5 Mb), and Chr 9 (71–83.7 Mb) and no significant QTL was identified (Figure 3.6). QTL mapping of SGZ proliferation corrected for differences in age revealed the same suggestive QTLs on Chr 1, 5, and 9 (Figure 3.6-C). These SGZ QTLs do not correspond to the loci associated with cell proliferation in the RMS and therefore suggest a different set of genetic modulators regulating the number of proliferative cells in the SGZ. This is further supported by the lack of phenotypic correlation between cell proliferation in the SGZ and RMS ($r = 0.14, p = 0.2917, N = 61$ BXD strains).
A  

# of BrdU+ cells in the SGZ  

Strains  

B  Genome-wide interval mapping of unadjusted number of BrdU+ cells in the SGZ  

C  Genome-wide interval mapping of corrected number of BrdU+ cells in the SGZ
**Figure 3.6** Cell proliferation in the SGZ of BXD RI strains and QTL analyses.

(A) The number of BrdU+ cells in the SGZ (± SEM) of 61 BXD RI strains (white bars) and their parental strains, C57BL/6 J (red bar), and DBA/2 J (green bar). The sample size per strain is indicated in the bars. Whole-genome scan LRS plot generated from QTL mapping of the unadjusted SGZ cell proliferation data (B) and the adjusted data corrected for age effects (C). The x-axis represents the chromosomes 1–19, & X (top panel) and their physical maps in megabases (bottom panel). The y-axis and the blue line depict the LRS, which indicates the strength of association between genotypes of markers across the genome and the phenotype. The light red and gray horizontal lines mark the significant ($p = 0.05$) and suggestive ($p = 0.63$) threshold, respectively. Whole-genome interval mapping of unadjusted SGZ proliferation data (B) and the SGZ proliferation data adjusted for age (C) identified no significant QTL but revealed suggestive QTLs on Chr 1 (40–59 Mb), Chr 5 (104.5–119.5 Mb), and Chr 9 (71–83.7 Mb).

### 3.3.4 Mapped loci harbor genes that may serve as modulators of NPC proliferation

I predict the mapped genomic regions harbor genes regulating the number of proliferative NPCs in the RMS. To test my hypothesis, I implemented a combination of bioinformatics tools including QTLminer at GeneNetwork (http://www.genenetwork.org/webqtl/main.py?FormID=qtlminer website) and DAVID (http://david.abcc.ncifcrf.gov website) to obtain information on genes in the QTL regions. I prioritized genes according to 1) the presence of single-nucleotide polymorphisms (SNPs) and/or insertion/deletions (indels), 2) presence of transcripts in other neurogenic regions (e.g., SVZ or SGZ) in the adult mouse brain, and 3) association with gene ontology (GO) terms such as neurogenesis, cell cycle, or proliferation. To be considered as strong candidate genes, the genes must meet the first two criteria. The candidate genes are further ranked by the numbers of criteria they met (e.g. 2, 3). Although I examined cell proliferation in the SGZ, in this section, I focused my candidate gene analyses on QTLs regulating cell proliferation in the RMS.

In the Chr 11 QTL region identified from QTL mapping of the AXB/BXA cell proliferation data, there are a total of 36 genes, 25 known and 11 predicted, residing in this QTL
interval (Table 3.1). Of all the genes examined, four met all candidate gene criteria and are considered priority genes for future analysis. One of them is galanin receptor 2 (Glr2). Glr2 is the receptor for galanin, a neuropeptide involved in mood regulation that is expressed throughout the brain including SVZ, RMS and DG (Ma et al., 2008). Activation of Glr2 through the binding of galanin has been linked to increased hippocampal neurogenesis in the seizure-induced injured brain (Mazarati et al., 2004). Moreover, the activation of Glr2 induces the mitogen-activated protein kinase (MAPK) pathway (Wang et al., 1998). MAPK is a complex signal transduction pathway that promotes cell division and can also be mediated through the binding of other extracellular growth factors (e.g. FGF-2 and EGF) to cell surface receptors (Fgfr2 and Egfr)(Doetsch et al., 2002a; Frinchi et al., 2008). Disregulation of Glr2 has been linked to depression in human and mouse (Lu et al., 2007).

Another gene, sphingosine kinase 1 (Sphk1) also emerged as a strong candidate gene that may control the number of proliferating cells in the RMS. Sphk1 is expressed in adult murine brain and has been implicated in cellular processes including cell proliferation and cell survival (Kohama et al., 1998; Hait et al., 2006). One major role of Sphk1 is to generate Sphingosine-1-phosphate (S1P) from its metabolic precursor sphingosine, and S1P is a lipid second messenger that plays an important role in both vasculogenesis and neurogenesis (Harada et al., 2004; Mizugishi et al., 2005). My pathway analysis using DAVID (http://david.abcc.ncifcrf.gov:8080/) showed Sphk1 is part of the vascular endothelial growth factor (VEGF) signaling pathway that when activated increases proliferation in the SVZ and also modulates migration of the neural progenitors in the RMS (Wittko et al., 2009). Septin 9 (Sept9) and Cyclin-dependent kinase 3 (Cdk3) are two other genes that are worth mentioning because even though they are not directly linked to neurogenesis, they are both cell cycle regulatory genes. Sept9 is involved in the
progression through G1 of the cell cycle and it is highly expressed throughout the adult mouse brain (Gonzalez et al., 2009). By contrast, Cdk3 is expressed at low levels throughout the adult mouse brain and it is required for G1–S transition (Braun et al., 1998).
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<td>Mfsd11</td>
<td>major facilitator superfamily domain containing 11</td>
<td>116.715328</td>
<td>21.623</td>
<td>13</td>
<td>Integral to membrane</td>
</tr>
<tr>
<td>Mgat5b</td>
<td>mannoside acetylgalcosaminytransferase 5, isoenzyme B</td>
<td>116.780176</td>
<td>68.082</td>
<td>161</td>
<td>Alpha-1,6-mannosyl-glycoprotein 6-beta-N-acetylgalcosaminytransferase activity</td>
</tr>
<tr>
<td>2810008D09Rik</td>
<td>RIKEN cDNA 2810008D09 gene</td>
<td>116.893189</td>
<td>2.166</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Sec14li</td>
<td>SEC14-like 1 (S. cerevisiae)</td>
<td>116.976562</td>
<td>43.973</td>
<td>81</td>
<td>–</td>
</tr>
<tr>
<td>Sept9</td>
<td>septin 9</td>
<td>117.01575</td>
<td>162.665</td>
<td>127</td>
<td>GTP binding, nucleotide binding, cell cycle, cell division</td>
</tr>
<tr>
<td>A930024O17Rik</td>
<td>RIKEN cDNA A930024O17 gene</td>
<td>117.245886</td>
<td>0.807</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>OTTMUSG000000003</td>
<td>Gm11733 predicted gene 111733</td>
<td>117.345681</td>
<td>4.647</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>2900041M22Rik</td>
<td>RIKEN cDNA 2900041M22 gene</td>
<td>117.472689</td>
<td>1.422</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>Tnrc6c</td>
<td>trinucleotiderepeat containing 6C</td>
<td>117.515602</td>
<td>109.151</td>
<td>136</td>
<td>Gene silencing by RNA, regulation of translation</td>
</tr>
</tbody>
</table>

The Single Nucleotide Polymorphism (SNP) count indicates the number of SNPs, which differs between the two parental strains, A/J and C57BL/6J. The number of SNPs within 5kb upstream and downstream of each gene is listed. Other types of polymorphisms such as indels and structural variants are also examined but not provided in this table.
In the significant Chr 6 QTL region (76.8-88.8 Mb) identified from QTL mapping of the BXDs, there are 105 genes that have known/predicted functions and four genes met all my criteria (Table 3.2). Two of these genes, transforming growth factor alpha (Tgfa) and minichromosome maintenance deficient 2 mitotin (Mcm2) have been implicated in adult neurogenesis (Tropepe et al., 1997; Pruitt et al., 2007). Pathway analysis showed Tgfa to be involved in the ErbB signalling pathway which regulates diverse biological processes such as proliferation, differentiation, and survival. In the suggestive Chr 11 QTL region (50-58 Mb), 93 genes have known/predicted functions and three genes met my candidate gene criteria (Table 3.2). One of the candidate genes is secreted acidic cysteine rich glycoprotein (Sparc) which has been previously shown to promote cell proliferation in the SGZ of the dentate gyrus (Campolongo et al., 2012). Composite interval analysis also revealed secondary QTLs on Chr 14 and 18 that interact with Chr 6 QTL in regulating cell proliferation in the RMS. In the Chr 14 QTL region (40.3-49.2 Mb), there are 46 genes with known/predicted functions and two of these met all the criteria listed above: cyclin-dependent kinase inhibitor 3 (Cdkn3) and glucosamine-phosphate N-acetyltransferase 1 (Gnpnat1)(Table 3.2). Despite not being directly linked to neurogenesis, both of these genes regulate cell cycle progression (Boehmelt et al., 2000; Nalepa et al., 2013). In the Chr 18 QTL region (58.2-74.9 Mb), there are 101 genes that have known/predicted functions. Five genes met all of my candidate gene criteria (Table 3.2). One of these genes, SMAD family member 4 (Smad4) has been directly implicated in adult neurogenesis (Colak et al., 2008). Pathway analyses revealed the gene calcium/calmodulin-dependent protein kinase II alpha (Camk2a) in Chr 18 QTL is involved in the ErbB signalling pathway, a pathway also shared by Tgfa in the major Chr 6 QTL. Both Camk2a and Smad4 are
also components of the Wnt signaling pathway that regulate adult neurogenesis (Zhang et al., 2010; Wu and Hen, 2013).

I further explored the interconnectivity of the candidate genes based on their expression levels in the adult brain. I suspect polymorphisms in these candidate genes are likely to affect gene expression level, similar to the heritable differences in number of RMS proliferative cells observed among the RI strains. Extensive transcriptome data sets are available for BXD RI strains at the GeneNetwork. Because there is no BXD gene expression data specific for the RMS, I queried the BXD whole brain transcriptome data set (UTHSC Mouse BXD Whole Brain RNA Sequence Nov12 RPKM) for the transcript levels of candidate genes identified from QTL mapping of the BXD RI strains (Table 3.2). For many of the candidate genes, considerable variation in gene expression was observed in the brains of different BXD RI strains (Supplementary Figure 3.1). Significant expression co-variation among the candidate genes was also detected and summarized in a network graph (Figure 3.7). Candidate genes that are regulators of cell proliferation (Camk2a, Mcm2, and Smad4) are highly correlated at the transcriptional level. Camk2a is involved in the G1 to S phase transition of the mitotic cell cycle whereas Mcm2 is involved in unwinding of DNA during the S phase of the cell cycle (Afroze et al., 2003; Chen et al., 1999). Smad4 is another candidate gene that regulates cell proliferation and differentiation in the SVZ (Colak et al., 2008). Conditional deletion of Smad4 in the SVZ NPCs resulted in significant decrease in the number of neuroblasts in the SVZ (Colak et al., 2008). Pathway analyses also revealed Camk2a, Smad4, and Gfpt1 are involved in the Wnt signaling pathway. The expression of β-catenin is the downstream target of the canonical Wnt signaling pathway. Gfpt1 encodes a rate-limiting enzyme of the hexosamine biosynthesis pathway, which has been shown to drive the levels of β-catenin and cell proliferation (Olivier-
Van Stichelen et al., 2012). In addition to gene expression covariance, I also detected significant correlation between gene expression and the phenotype (i.e. RMS linear density). The transcriptional differences of candidate genes Anxa4, Ppp2ca, Gnpnat1, and Camk2a are significantly correlated with the phenotypic differences in RMS linear density observed among the BXDs (r = -0.58, r = -0.50, r = -0.28, r = 0.29, respectively; p < 0.03; Supplementary Figure 3.2).
Table 3.2 Strong candidate genes identified in the chromosomes (Chr) 6, 11, 14, and 18 QTL intervals

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Chr</th>
<th>Gene location: start (Mb)</th>
<th>Gene length (kb)</th>
<th>SNPs</th>
<th>Indels</th>
<th>Gene Ontology (GO) annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tgfa</td>
<td>Transforming growth factor alpha</td>
<td>6</td>
<td>86.145</td>
<td>79.742</td>
<td>6</td>
<td>3</td>
<td>Positive regulation of cell division, negative regulation of apoptosis</td>
</tr>
<tr>
<td>Anxa4</td>
<td>Annexin A4</td>
<td>6</td>
<td>86.687</td>
<td>56.745</td>
<td>134</td>
<td>7</td>
<td>Cell growth and survival, cell proliferation, carcinogenesis</td>
</tr>
<tr>
<td>Gfpt1</td>
<td>Glutamine fructose-6-phosphate transaminase 1</td>
<td>6</td>
<td>86.993</td>
<td>49.362</td>
<td>48</td>
<td>1</td>
<td>Amino sugar and nucleotide sugar metabolism, cell regeneration</td>
</tr>
<tr>
<td>Mem2</td>
<td>Minichromosome maintenance deficient 2 mitotin</td>
<td>6</td>
<td>88.833</td>
<td>15.307</td>
<td>3</td>
<td>60</td>
<td>DNA replication initiation, DNA unwinding during replication</td>
</tr>
<tr>
<td>Ppp2ca</td>
<td>Protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform</td>
<td>11</td>
<td>51.912</td>
<td>23.926</td>
<td>1</td>
<td>0</td>
<td>Phosphoprotein phosphatase activity, meiosis, negative control of cell growth and division</td>
</tr>
<tr>
<td>Rad50</td>
<td>RAD50 homolog (S. cerevisiae)</td>
<td>11</td>
<td>53.463</td>
<td>57.801</td>
<td>4</td>
<td>0</td>
<td>DNA repair, homologous recombination, cell cycle</td>
</tr>
<tr>
<td>Sparc</td>
<td>Secreted acidic cysteine rich glycoprotein</td>
<td>11</td>
<td>55.208</td>
<td>25.580</td>
<td>83</td>
<td>5</td>
<td>Response to growth factor stimulus, regulation of cell proliferation</td>
</tr>
<tr>
<td>Cdkn3</td>
<td>Cyclin-dependent kinase inhibitor 3</td>
<td>14</td>
<td>45.692</td>
<td>0.05</td>
<td>3</td>
<td>3</td>
<td>Cell cycle arrest, phosphatase activity</td>
</tr>
<tr>
<td>Gnpnat1</td>
<td>Glucosamine-phosphate N-acetyltransferase 1</td>
<td>14</td>
<td>45.996</td>
<td>12.376</td>
<td>1</td>
<td>0</td>
<td>Amino sugar and nucleotide sugar metabolism, actin dynamics, cell cycle progression</td>
</tr>
<tr>
<td>Camk2a</td>
<td>Calcium/calmodulin-dependent protein kinase II alpha</td>
<td>18</td>
<td>61.085</td>
<td>62.521</td>
<td>0</td>
<td>219</td>
<td>G1/S transition of mitotic cell cycle, neuronal synaptic plasticity</td>
</tr>
<tr>
<td>Seh1l</td>
<td>SEH1-like (S. cerevisiae)</td>
<td>18</td>
<td>67.935</td>
<td>17.718</td>
<td>4</td>
<td>2</td>
<td>Cell division, chromosome segregation, mitosis, cell cycle</td>
</tr>
<tr>
<td>Smad4</td>
<td>Similar to MAD homolog 4 (Drosophila) elA homolog 1 (E. coli)</td>
<td>18</td>
<td>73.799</td>
<td>64.729</td>
<td>160</td>
<td>74</td>
<td>Cell proliferation, tissue morphogenesis</td>
</tr>
<tr>
<td>Elac1</td>
<td></td>
<td>18</td>
<td>73.895</td>
<td>19.442</td>
<td>42</td>
<td>7</td>
<td>tRNA 3'end processing, cell growth and proliferation</td>
</tr>
<tr>
<td>Mapk4</td>
<td>mitogen-activated protein kinase 4</td>
<td>18</td>
<td>74.088</td>
<td>136.463</td>
<td>141</td>
<td>70</td>
<td>Cell cycle, protein amino acid phosphorylation</td>
</tr>
</tbody>
</table>
Figure 3.7 Transcriptional co-expression network graph of candidate genes.
The transcript levels of candidate genes listed in Table 3.2 were extracted from the BXD whole brain RNA sequence data set available at the GeneNetwork. Strong expression co-variations were detected among some of the candidate genes. Strength of correlation between two connected genes is indicated in the legend.
3.4 Discussion

The genetic background has a significant influence on NPC proliferation in the adult mouse forebrain. The numbers of NPCs in the RMS and the SGZ are highly heritable and variable among different inbred strains. The effects of genetic variations on NPC proliferation is likely through a network of genes which will be difficult to reveal from conventional single gene study. Here, I took advantage of the genetic and phenotypic diversity in the AXB/BXA and BXD reference panels, and through genome-wide QTL mapping, I discovered novel genetic loci that likely house modulators of NPC proliferation in the RMS. The Chr 6 and Chr 11 QTLs identified from QTL mapping of the BXD and AXB/BXA RI strains, respectively, are the first significant QTLs to be described for NPC proliferation in adult rodent brain. A closer examination of candidate genes in the mapped loci using public databases on gene ontology, pathways, genetic polymorphisms, and gene expression have led to the identification of a subset of genes associated with adult neurogenesis and/or cell proliferation.

To probe the genetic architecture of a polygenic phenotype as complex as NPC proliferation requires a large genetic reference panel that can provide high resolution and power to identify genes with subtle but significant effects on NPC proliferation. In this study, I utilized the genetic diversity among the AXB/BXA and BXD RI strains as a tool to identify genes and pathways involved in NPC proliferation. My BXD panel of 61 strains is the largest mapping panel employed thus far to study adult neurogenesis, and it is over twice the size the AXB/BXA RI set. In addition, over half of the BXDs in my panel (34 out of 61 strains) were from the more recent UTHSC BXD set, which have approximately twice the number of recombinations per strain compared to the older BXD subset produced by Taylor (http://www.genenetwork.org/mouseCross.html). The large sample size and number of
recombinations present in the RI strains greatly improved the statistical power to map QTLs at high resolution as well as uncover loci-loci interaction. Normal distribution of the RMS linear densities indicates that more than one genetic locus is involved in NPC proliferation. In contrast, if only one gene is involved in regulating RMS cell proliferation, then a bimodal distribution would be observed. Subsequent genome-wide interval mapping provided novel insights into the genetic regions regulating NPC proliferation. The significant QTL on distal Chr 11 was identified to account for ~20% of the inter-strain differences observed among the AXB/BXA RI strains. The significant Chr 6 QTL was identified to account for ~19% of the inter-strain differences observed among the BXD RI strains, and this Chr 6 locus was found to interact additively with two other regions on Chr 14 and Chr 18. Pairwise interaction between Chr 6 QTL and these secondary loci explained ~40% of the inter-strain differences. Genome-wide mapping also revealed a suggestive locus on Chr 11 that accounts for ~11% of the phenotypic variance in the BXD population and it does not directly interact with the Chr 6 QTL. This suggestive Chr 11 QTL does not overlap with the significant distal Chr 11 QTL identified from mapping of the AXB/BXAs. One possible explanation as to why the significant Chr 11 QTL is not replicated in the QTL mapping of the BXDs could be due these two RI panels capturing only a fraction of the genetic variation present in the mouse population. The genetic relationships among the inbred strains were previously examined, and A/J, C56BL/6J, and DBA/2J belong to distinct phylogenetic groups 1, 4, and 6, respectively (Petkov et al., 2004). Therefore, a QTL segregating in the AXB/BXAs, which is generated from the initial mating of parental strain A/J and C57BL/6J, may not be detected in BXDs, which is produced by the initial crossing of DBA/2J and C57BL/6J, and vice versa. Nevertheless the discovery of multiple QTLs, as well as the identification of interacting and non-interacting genetic loci, suggest the genetic network
modulating NPC proliferation is comprised of regulatory pathways and genes of variable interactivity.

To better understand the regulatory pathways modulating NPC proliferation in the RMS, it is important to first identify the molecular players participating in these pathways. It is presumed that the mapped loci harbor genes involved in NPC proliferation. My rather stringent candidate gene analyses revealed several genes in the significant Chr 11 QTL (AXB/BXAs) and Chr 6 QTL (BXDs) regions that are associated with neurogenesis and cell proliferation. Transcriptional covariance and pathway analyses further provided insights into the interconnection among the candidate genes. For example, both the Tgfa gene in the major Chr 6 QTL and the Camk2a gene in the secondary Chr 18 QTL are involved in the ErbB signalling pathway, which regulates diverse biological processes such as proliferation, differentiation, and survival. Tgfa serves as one of the extracellular ligands that bind to EGF receptors and subsequently triggers downstream intracellular ErbB signalling pathway, which is modulated by Camk2a. Camk2a also participates in the Wnt signalling pathway as well as Ppp2ca in the suggestive Chr 11 QTL. Ppp2ca is one of the major Ser/Thr phosphatases, which has been previously implicated as a negative regulator of cell proliferation. Ppp2ca interacts with Axin, a scaffold protein that down-regulates the Wnt signalling pathway by destabilizing β-catenin (Nakamura et al., 1998). These findings highlight the complexity of NPC regulation where genes at different loci converge on the same regulatory pathway and possibly exert their effects in an additive manner. Overlap in genes participating in seemingly independent pathways such as ErbB signalling pathway and Wnt signalling pathways further suggests crosstalk between these pathways. Previous reviews have described the regulation of adult neurogenesis as a fine balance of the different systems of networks (Kempermann, 2011; Hsieh, 2012). The interconnectedness...
between pathways indicates that the perturbation of one may trigger compensatory changes at others (Kempermann, 2011).

In addition to the RMS, I also examined the QTLs modulating cell proliferation in SGZ of the dentate gyrus using my expanded panel of BXDs. The number of proliferative cells in the SGZ differed ~4 fold among the 61 BXD RI strains. A similar fold difference was reported by Kempermann and colleagues where they used Ki67 as a proliferative marker and quantified the number of dividing cells in the SGZ of 29 BXD RI strains (Kempermann et al., 2006). The proliferative differences I detected among the BXDs were mapped to three suggestive QTLs on Chr 1, Chr 5, and Chr 9. Kempermann and colleagues also identified a suggestive Chr 5 QTL from their previous QTL mapping (Kempermann et al., 2006). These loci did not overlap with the QTLs regulating RMS cell proliferation. From these findings I concluded that there are separate sets of genes that differentially modulate the number of NPCs in the RMS and SGZ.

In summary, the rich genetic and phenotypic diversity in the AXB/BXA and BXD RI strains have allowed me to probe the complex networks regulating NPC proliferation in the adult mouse brain. Here, I performed genome-wide scans for QTLs associated with the differences in the number of NPCs among the RI strains. Novel QTLs and significant loci-loci interaction were detected. Using bioinformatics resources, I further identified several candidate genes that may be involved in regulating NPC proliferation and discovered the convergence of these genes on the same/parallel signalling pathways that are known to regulate adult neurogenesis. The combinatorial influence of these regulators on NPC proliferation remains to be elucidated. Nevertheless, my findings offer novel insights into the dynamic interplay of regulatory pathways controlling NPC proliferation and provide a starting point to unravel genes regulating this process.
Chapter 4: Investigation of the galanin receptor 2 (Galr2) as a modulator of neural progenitor cell proliferation

4.1 Introduction

QTL mapping in Chapter 3 identified chromosomal regions that are significantly associated with the inter-strain differences in the number of proliferative NPCs. These regions are hypothesized to harbor genes that regulate NPC proliferation in the adult brain. The identification of regulatory genes requires a concerted effort and relies upon evidence from several sources (Chapter 1; Section 1.3.3.1). Candidate gene analysis prioritized the genes in the mapped regions according to gene expression in the adult mouse brain, presence of polymorphisms, and association with gene ontology terms such as cell proliferation and/or neurogenesis (Chapter 3, Section 3.3.4). Functional tests are required to further prove the involvement of promising candidate genes in NPC proliferation. Here, I examine the role of Galanin receptor 2 (Galr2) gene in NPC proliferation and adult neurogenesis. Galr2 emerged as a strong candidate gene from QTL mapping of the AXB/BXA RI strains and candidate gene analysis (Poon et al., 2010; Chapter 3).

Galr2 encodes a G-protein-coupled receptor that is widely expressed in the rodent brain (Pang et al., 1998; O'Donnell et al., 1999; Hawes and Picciotto, 2004). Upon binding to its ligand, GALR2 induces different intracellular pathways that regulate cell growth, proliferation, or apoptosis depending on the G-protein repertoire in the cell (Wang et al., 1998; Lang et al., 2007). Different effects of Galr2 on cell proliferation have also been reported. For example, overexpression of Galr2 has been shown to promote cell proliferation of rat SGZ-derived NPCs.
and human squamous carcinoma cells in vitro (Abbosh et al., 2011; Banerjee et al., 2011). In contrast, overexpression of Galr2 in rat pheochromocytoma cells, which are neuroendocrine cancer cells of the adrenal medulla, decreased cell proliferation (Tofighi et al., 2008).

Galr2 is a receptor for Galanin, a neuropeptide that is expressed in the adult neurogenic regions and has been previously implicated in different aspects of neurogenesis (Shen et al., 2003; Agasse et al., 2013). A galanin agonist selective for GALR2 was shown to promote neuronal differentiation of NPCs derived from P3 mouse SVZ (Agasse et al., 2013). The same GALR2 agonist was shown to increase proliferation of NPCs derived from the SGZ of P10 rat hippocampi (Abbosh et al., 2011). Administration of selective GALR2 agonists into the hippocampus prevented seizure activity in a rat model of status epilepticus (Mazarati et al., 1998). Prolonged seizures have been shown to increase adult neurogenesis, and it is currently unknown whether induced-neurogenesis is pathophysiological or reparative (Parent and Murphy, 2008). Local infusion of antisense oligonucleotides against Galr2 mRNA into adult hippocampus inhibited the seizure-induced increase in hippocampal neurogenesis (Mazarati et al., 2004).

The effect of Galr2 on SVZ-RMS-OB neurogenesis has not been fully investigated in the adult brain. In this chapter, I confirmed the expression of Galr2 in neurogenic regions in the adult brain and cultured NPCs derived from adult SVZ. I then used both an in vitro aerosphere assay and Galr2−/− mutant mice to examine the role of Galr2 on SVZ-RMS-OB neurogenesis. Overexpression of Galr2 in cultured NPCs derived from the SVZ increased cell proliferation and neurosphere formation in vitro; whereas knockdown of Galr2 decreased cell proliferation and neurosphere formation in vitro. Galr2 knockout mice had significantly fewer numbers of dividing cells in the RMS and newborn neurons in the OB compared to wild-type controls. These findings suggest Galr2 promotes NPC proliferation and OB neurogenesis. The identification of
Galr2 as a modulator of NPC proliferation is a proof of concept of my phenotype-driven, genome-wide approach used to pinpoint chromosomal regions and identify strong candidate genes that may regulate NPC proliferation in the adult mouse brain.

4.2 Materials and methods

4.2.1 Animals

Galr2 null mutant mice (Galr2\(^{+-}\)) were generated from mating heterozygotes Galr2 \(^{+-}\) carrying a copy of the disrupted Galr2 allele. The Galr2 gene was mutated by targeted gene trapping as described previously (Hobson et al., 2006) (Supplementary Figure 4.2). The gene-trap mutation resulted in the absence of Galr2 transcription and this was validated using reverse transcription polymerase chain reaction (RT-PCR) (Supplementary Figure 4.2). The heterozygotes were purchased from the Mutant Mouse Regional Resource Center at UC Davis. These heterozygotes were provided on a mixed C57BL/6J x 129/SvEvBrd background. To increase genetic uniformity and reduce phenotypic variability, Galr2 \(^{+-}\) mice were separately backcrossed to C57BL/6J and 129S1/SvImJ for four generations to acquire ~94% of the backcrossed strain’s genetic background (Silver, 1995). C57BL/6J and 129S1/SvImJ were obtained from the Jackson Laboratory. The absence of full length Galr2 transcript in Galr2\(^{+-}\) mice was confirmed by RT-PCR. Brain tissues were collected from adult mice of either sex at postnatal day 60 (P60). All experiments were conducted according to the Canadian Council of Animal Care. Approval was obtained from the Animal Care Committee at the University of British Columbia on all animal protocols.
4.2.2  Fluorescent immunohistochemistry

Adult A/J mice (P60) were given an overdose of tribromoethanol (Avertin; 125mg/kg of body weight; Sigma-Aldrich) and transcardially perfused with 0.1 M PBS followed by 4% paraformaldehyde. Brains were removed, post-fixed in 4% PFA overnight, and cryoprotected with 30% sucrose in 0.1M PBS. Sagittal sections at 10 µm-thick were serially cut using a rotary microtome (Microm HM 550; Thermo Scientific). Sections were treated with incubation buffer that consisted of 0.1 M PBS, 0.1% Triton X-100, and 10% of the appropriate normal serum. Sections were then incubated with the following primary antibodies: anti-GALR2 (1:100; goat polyclonal; Santa Cruz Biotechnology; sc-16219), anti-Nestin (1:200; mouse monoclonal; Millipore; MAB353), or anti-Ki67 (1:200; rabbit polyclonal; Abcam; ab15580) overnight at 4°C. Sections were washed and incubated with the appropriate secondary antibodies conjugated to different fluorophores (Jackson ImmunoResearch). Slides with the stained section were coverslipped with VECTASHIELD mounting medium with DAPI (Vector Laboratories). Sections were examined with a fluorescent microscope (20X objectives; Zeiss 200M Axiovert inverted microscope) and images were taken with a confocal microscope (60X objective; Olympus FV500 Laser Scanning Confocal Inverted Microscope).

4.2.3  SVZ progenitor cell culturing and siRNA transfection

SVZs from P60 A/J and C57BL/6J males were dissected and dissociated using the Neural Tissue Dissociation Kit containing Trypsin (Miltenyi Biotec; cat. no. 130-093-231). Cell viability and concentration were determined by trypan blue exclusion. The cell suspension was then diluted to 200,000 cells/mL in NeuroCult proliferation medium (Stemcell Technologies; cat. No. 05700 & 05701) containing 20 ng/mL EGF, 10 ng/mL bFGF, and 2 µg/mL of Heparin.
Uncoated 6-well plates were used to generate free-floating neurospheres and each well contained 3 mL of cell suspension. Cultures were incubated at 37°C in a 5% CO2 humidified chamber. At day 7, neurospheres were harvested and dissociated to generate secondary spheres. This passaging of neurosphere cultures was repeated two more times to remove non-proliferative cells from the SVZ. Passaged spheres were dissociated into single cell-suspension for transfection.

RNA-mediated knockdown of Galr2 was carried out by nucleofection of 1x10^6 cells with a pool of 4 pre-designed Galr2-specific siRNAs (100nM; ON-TARGETplus SMART Pool; Thermo Scientific Dharmacon) and non-targeting control siRNAs (100nM; ON-TARGETplus SMART Pool; Thermo Scientific Dharmacon). Program A-033 of the Amaxa Nucleofector-II Device (Lonza) was selected to deliver siRNAs into the cells. The tranfection efficiency is ~80% estimated by transfecting cells with eGFP plasmids and counting the number of GFP+ cells over the total number of transfected cells 48 hours post-nucleofection. The anti-Galr2 siRNA silencing efficiency is ~80% estimated using real-time RT-PCR (TaqMan-based detection method) from cells harvested 24-72 hours post-transfection. Galr2 expression levels were normalized to 18S rRNA and β-actin. The number of neurospheres was counted from each group under brightfield illumination at day 7 post-transfection. The diameter of the neurospheres was measured using NIH ImageJ software (http://rsbweb.nih.gov/ij/).

4.2.4 Galr2 overexpression

Neurospheres were cultured from P60 C57BL/6J males and passaged three times before transfection as described above. Dissociated neurospheres were transfected with 10µg pcDNA-GALR2 (Missouri S & T cDNA Resource Center) or pcDNA (negative control; Invitrogen) using the Amaxa Nucleofector-II system. The pcDNA-GALR2 vector overexpresses human GALR2
cDNA, which shares 85% similarity with the mouse Galr2 cDNA sequence. The number of neurospheres was counted from each group under brightfield illumination at day 7 post-transfection. The diameter of the neurospheres was measured using NIH ImageJ software (http://rsbweb.nih.gov/ij/).

4.2.5 BrdU labeling and quantification

BrdU was used to assess NPC proliferation in the adult Galr2<sup>−/−</sup> mice and their wild-type littermates (P60). All mice received a single intraperitoneal injection of BrdU (50mg per kg of body weight). One-hour post injection, mice were perfused with a solution of 95% alcohol/acetic acid (3:1). Brains were collected and embedded in paraffin. Brains were sectioned sagittally at 8 µm and every 10th section was mounted. Sections were subjected to anti-BrdU immunohistochemistry as previously described (Poon et al., 2010). In brief, sections were treated with 1N HCL for 30 min at 37°C to denature DNA, incubated with mouse primary anti-BrdU monoclonal antibody (1:200; BD Biosciences) overnight at room temperature, and then incubated with secondary biotinylated horse anti-mouse IgG (1:200, Vector Laboratories) for 1 hour at room temperature. BrdU immunoreactivity was subsequently revealed through the use of VECTASTAIN Elite ABC kit (Vector Laboratories) and 3,3’-diaminobenzidine (DAB; Sigma-Aldrich). The number of BrdU-positive (+) cells in the RMS was quantified as previously described (Chapter 2, Section 2.2.3). Briefly, serial sections containing the RMS in the left hemisphere were examined. The total number of BrdU + cells was counted throughout the entire RMS under brightfield illumination (20X objective; Zeiss 200M Axiovert inverted microscope). RMS linear density (i.e. the number of BrdU+ cells per mm length of RMS) was determined as previously described (Chapter 2, Section 2.2.3). Mice used for analysis of BrdU-labeling in the
RMS were also used to examine the proliferative activities in the SGZ of the hippocampal dentate gyrus. I quantified BrdU+ cells in the SGZ, which is located at the interface between hilus and the granule cell layer of the dentate gyrus. Counts began at the first appearance of the dentate hilus and dentate granule cell layer, and then continued for every tenth sagittal section, throughout the dorsal hippocampus and stopped where the dorsal and ventral components of the hippocampus merge. Data are expressed as the total number of BrdU+ cells ± SEM.

A cumulative BrdU labeling protocol was adopted from Rochefort and Lledo (2005) to investigate newborn cell survival in the adult OB. In brief, four BrdU injections repeated every two hours were administered to P60 Galr2-/- mice and their wild-type littermates. A four-week survival period was given for the BrdU-labeled NPCs to migrate to the OB. Mice were perfused with the same acid-alcohol mix as described above and brains were embedded in paraffin. Coronal sections were serially cut with a rotary microtome at 8 µm. Every 10th section was mounted and processed for anti-BrdU immunohistochemistry. To determine the density of new cells in the OB, the number of BrdU+ cells were counted in the granular cell layer (GCL) of the OB with a 20X objective (Zeiss 200M Axiovert inverted microscope). Area of the GCL was measured by NIH ImageJ software. Volumes between measured areas were determined by multiplying area by the distance between sections. Summation of these sectional volumes was used to compute total volume (mm³) and the number of BrdU+ cells per GCL volume (mm³) was calculated.
4.2.6 Statistical analysis

Significant differences ($p < 0.05$) among multiple groups were determined by one-way analysis of variance (ANOVA) followed by Tukey's HSD post-hoc tests to provide information on which group means are significantly different from each other (JMP 11 statistical software; SAS Institute). Unpaired student $t$ test was performed to determine the statistical significance ($p < 0.05$) of differences between the Galr2$^{+/+}$ and Galr2$^{-/-}$ mice (JMP 11 statistical software; SAS Institute).

4.3 Results

4.3.1 Galr2 is expressed in adult neurogenic sites and NPCs

To determine whether Galr2 plays a role in adult neurogenesis, I first performed immunohistochemistry and studied the expression pattern of GALR2 in the adult A/J brain (Figure 4.1). High levels of GALR2 in the SVZ (Figure 4.1 A, B, D) and low levels in RMS (Figure 4.1 A, C), OB (Figure 4.1 C), and SGZ (Supplementary Figure 4.1) were detected. Partial co-localization of GALR2 and Nestin, which is an intermediate filament protein commonly used to identify NPCs, was observed in the SVZ (Figure A, B, D).
Figure 4.1 Immunodetection of GALR2 in the adult mouse SVZ-RMS-OB.
Expression of GALR2 (green) and Nestin (red) in the dorsal SVZ and RMS (A), ventral SVZ (B), RMS arm in the OB (C) at 20X objective. (D) Confocal imaging of adult SVZ at 100X objective. (E) Negative controls without primary antibodies but with same secondary antibodies used in A-D. Dashed regions delineate the SVZ and RMS. Ctx: Cortex; LV: Lateral Ventricle; LSN: Lateral Septal Nucleus Str: Striatum.

High expression of GALR2 in the adult SVZ suggests that SVZ neural stem cells may express high levels of GALR2. However, the high cell density in the SVZ made it difficult to discern individual cells. To confirm GALR2 expression in the adult neural stem cells, SVZs were dissected from adult A/Js. SVZs were dissociated and cells were cultured in vitro. The cell culture condition favoured cell proliferation and expansion of multipotent neural stem cells. After three passages, cells derived from the SVZ were stained positive for GALR2 (Figure 4.2 A, B), as well as Ki-67, a cell proliferation marker (Figure 4.2 C).

In addition to protein expression, Galr2 transcript levels were also examined in different inbred mouse strains using the transcriptome data at the GeneNetwork (http://www.genenetwork.org/webqtl/main.py). Inter-strain variability of Galr2 expression was detected in the adult hippocampus, and these inter-strain differences positively correlated with the inter-strain differences in NPC proliferation (Figure 4.2 D). Significant differences in Galr2 transcript levels were also detected between A/J and C57BL/6J SVZ-derived neurospheres (Figure 4.2 E). Galr2 transcript level in the SVZ-derived neurospheres positively correlated with the number of primary SVZ-derived neurospheres generated from adult A/J and C57BL/6J SVZs (Figure 4.2 F). The number of primary SVZ-derived neurospheres served as an indicator of the number of multipotent neural stem cells in the adult SVZ. The association of Galr2 expression with the numbers of proliferative cells in the RMS (Figure 4.2 D) and the numbers of primary
SVZ-derived neurospheres (Figure 4.2 F) suggest Galr2 might play a role in regulating NPC proliferation.
Figure 4.2 Expression of Galr2 in cultured NPCs and inter-strain differences in Galr2 transcript levels. (A-C) Immunodetection of GALR2 in cultured NPCs derived from adult A/J SVZ. GALR2 (red) is expressed in intact neurosphere (A). Primary neurospheres were dissociated and plated in wells coated with poly-D-lysine and laminin. (B) NPCs in adherent cell culture were stained with anti-GALR2 antibody (red). (C) NPCs in adherent cell culture were stained with anti-Ki67 antibody (green). (D) Galr2 is differentially expressed in the hippocampi of the nine inbred mouse strains (y-axis; data from the GeneNetwork). Strain differences in Galr2 transcript levels are positively correlated with the strain differences in the number of BrdU+ cells per mm RMS length (x-axis). (E) Relative levels of Galr2 detected by real-time RT-PCR in A/J and C57BL/6J SVZ-derived neurospheres. Expression levels were normalized for 18S rRNA and β-actin. (F) The number of primary neurospheres generated per 500,000-plated SVZ cells from adult A/J and C57BL/6J mice.

4.3.2 Galr2 modulated NPC proliferation and rate of neurosphere formation in vitro

The role of Galr2 on NPC proliferation was first examined in vitro. Neurospheres were derived from adult SVZ cells from A/J and C57BL/6J brains. SVZ-derived neurospheres were passaged three times before transfection with siRNAs against Galr2. For both A/J and C57BL/6J NPCs, transient knockdown of Galr2 resulted in significant decreases in the number of neurospheres compared to the non-targeting control siRNAs group at Day 7 in culture (Figure 4.3). Furthermore, a ~80% depletion of endogenous Galr2, as determined by RT-PCR, significantly reduced the size of the neurospheres with respect to the non-targeting control siRNAs group (anti-Galr2 siRNAs: 70.8 ± 3.5µm vs. non-targeting control siRNAs: 103.3 ± 3.3µm, p< 0.0001). Conversely, the overexpression of human GALR2 increased neurosphere numbers by 31% compared to the empty pcDNA vector control (Figure 4.4). Overexpression of GALR2 also yielded significantly larger neurospheres compared to the empty pcDNA vector control with an average sphere diameter of 130 ± 1.34µm and 99 ± 3.67µm, respectively (p=0.0014). The numbers of neurospheres generated in no-siRNA and no-vector negative controls were similar to the numbers of neurospheres quantified in the non-targeting control and pcDNA empty vector control, respectively (data not shown), suggesting my nucleofection...
techniques did not influence the rate of neurosphere self-renewal. Together, these findings suggest *Galr2* is a pro-proliferative gene that promotes NPC proliferation.
Figure 4.3 Transient knockdown of Galr2 decreased the rate of neurosphere formation in vitro.
(A) Quantification of A/J SVZ-derived neurospheres generation at day 7 post-nucleofection of siRNAs against Galr2 or non-targeting control siRNAs. The bar graphs (means ± SEM) show the number of neurospheres formed per 1 x 10^5 plated SVZ cells. (B) Quantification of C57BL/6J SVZ-derived neurospheres generation at day 7 post-nucleofection of siRNAs against Galr2 or non-targeting control siRNAs. The bar graphs (means ± SEM) show the number of neurospheres formed per 1 x 10^5 plated SVZ cells. (C) Representative photomicrograph of neurospheres in the non-targeting control siRNAs condition (scale bars= 200µm). (D) Representative photomicrograph of neurospheres in the anti-Galr2 siRNAs condition (scale bars= 200µm).
Figure 4.4 GALR2 overexpression increased the rate of neurosphere formation in vitro.  
(A) Quantification of C57BL/6J SVZ-derived neurospheres generation at day 7 post-nucleofection of human GALR2 overexpressing vector or pcDNA empty vector. The bar graphs (means ± SEM) show the number of neurospheres formed per $1 \times 10^5$ plated SVZ cells. (B) Representative photomicrograph of neurospheres in the pcDNA empty vector condition (scale bars= 500µm). (C) Representative photomicrograph of neurospheres in the pcDNA-GALR2 overexpressing vector condition (scale bars= 500µm).

4.3.3 The numbers of NPCs and newborn neurons are decreased in Galr2−/− mice

4.3.3.1 Effects of Galr2 on SVZ-RMS-OB neurogenesis

Findings from the neurosphere assays suggest Galr2 regulates NPC proliferation. I examined the proliferative role of Galr2 in vivo using BrdU to assess cell proliferation in Galr2−/−
mice and their wild-type Galr2\(^{+/+}\) littermates. Anti-BrdU immunohistochemistry revealed Galr2\(^{-/-}\) mice had \(~30\%\) less BrdU+ cells in the RMS compared to Galr2\(^{+/+}\) mice (Fig. 4.5 A-D). No differences were observed in brain weights and body weights of the Galr2\(^{-/-}\) mice compared to their wild-type Galr\(^{+/+}\) littermates (data not shown). No significant sex differences were detected in Galr2\(^{+/+}\) (females: 52.49 \(\pm\) 6.04 BrdU+ cells/mm RMS length vs. males: 55.54 \(\pm\) 3.23 BrdU+ cells/mm RMS length, \(p=0.6698\)) and Galr2\(^{+/+}\) mice (females: 67.83 \(\pm\) 4.57 BrdU+ cells/mm RMS length vs. males: 77.66 \(\pm\) 6.52 BrdU+ cells/mm RMS length, \(p=0.5716\)). The reduction in the number of NPCs in the Galr2\(^{-/-}\) mice was also reflected in vitro. Primary neurospheres generated from adult Galr2\(^{-/-}\) SVZs were 1.8 fold less than the number of neurospheres generated from the adult Galr2\(^{+/+}\) SVZs (Figure 4.5 E), indicating a significantly smaller multipotent NPC population in the SVZs of Galr2\(^{-/-}\) mice compared to Galr2\(^{+/+}\) mice. Knockdown of Galr2 in the wild-type Galr2\(^{+/+}\)-derived NPCs resulted in a significantly fewer number of neurospheres compared to non-targeting control siRNAs group (\(p=0.0271\); Supplementary Figure 4.3). Whereas, in vitro knockdown of Galr2 in the Galr2\(^{-/-}\)- derived NPCs where Galr2 expression is absent showed no difference from the non-targeting control siRNAs group (Supplementary Figure 4.3). This confirms the specificity of the RNAi-mediated knockdown of Galr2.

To determine whether reduced numbers of NPCs in the Galr2\(^{-/-}\) SVZ-RMS results in less newborn neurons in the OB, I employed a cumulative BrdU+ labeling protocol to assess the proportion BrdU-labeled NPCs that migrated and differentiated in the OB. Consistent with previous findings reported by Mouret et al. (Mouret et al., 2009), \(~90\%\) of the BrdU+ cells were found in the granule cell layer (GCL) of the OB after a 4-week survival period. These newly generated neurons labeled with BrdU also expressed the neuronal maker NeuN (data not shown). A significant 21\% decrease in the number of newly generated neurons was detected in the GCL.
of the Galr2\(^{-/-}\) compared to their wild-type Galr2\(^{+/+}\) littermates \((p=0.0358)\) (Figure 4.5 F). This suggests Galr2 affects net OB neurogenesis where absence of Galr2 down-regulates NPC proliferation in the SVZ-RMS, which in turn resulted in less newly generated granule cells in the OB.
Figure 4.5 Reduced SVZ-RMS-OB neurogenesis in Galr2<sup>−/−</sup> mutant mice on mixed background.
(A) RMS linear density (i.e. the number of BrdU<sup>+</sup> cells per mm RMS length) ± SEM in the Galr2<sup>−/−</sup> mutant mice (black bar) and their Galr2<sup>+/+</sup> wild-type littermates (white bar). The sample size per strain is indicated in the bars. (B–C) Representative sagittal sections of BrdU-labeled RMS from the Galr2<sup>+/+</sup> wild-type (B) and Galr<sup>−/−</sup> mutant mice (C). LV, lateral ventricle; scale bar = 200µm. (D) Total number of BrdU cells in the RMS ± SEM of the Galr2<sup>+/+</sup> wild-type and Galr2<sup>−/−</sup> mutant mice. (E) The number of primary neurospheres generated per 500,000 plated SVZ cells from the Galr2<sup>+/+</sup> wild-type mice and Galr2<sup>−/−</sup> mutant mice. (F) Number of newborn cells in the OB granule cell layer (GCL) in the Galr2<sup>+/+</sup> wild-type mice and Galr2<sup>−/−</sup> mutant mice. Y-axis represents the mean number of BrdU labeled cells per GCL volume (mm<sup>3</sup>) 4 weeks after last BrdU injection.
4.3.3.2 Effect of Galr2 on cell proliferation in the SGZ

In addition to assessing the role of Galr2 on NPC proliferation in the SVZ-RMS, I also examined the effect of Galr2 on the number of proliferative cells in the SGZ, another prominent site of NPC proliferation in the adult brain. The actively dividing NPC population in the SGZ was labeled using BrdU. Galr2−/− mutant mice had 30% less BrdU+ cells in the SGZ compared to their Galr2+/+ wild-type littermates (Figure 4.6).

Figure 4.6 Decreased cell proliferation in the SGZ of Galr2−/− mutant mice.
Total number of BrdU+ cells in the SGZ ± SEM of the Galr2−/− mutant mice (black bar) and their Galr2+/+ wild-type littermates (white bar). The sample size per strain is indicated in the bars.
4.3.4 The effect of genetic background on Galr2\(^{-/-}\) mutant phenotype

The Galr2\(^{-/-}\) mutant mice was initially generated on a mixed background (see Section 4.2.1), and these genetic backgrounds have been previously shown to differentially modulate adult neurogenesis (Kempermann et al., 1997a; Chapter 2). To determine whether the function of Galr2 is modulated by the genetic background, I transferred the Galr2 mutant allele onto a more homogenous inbred background by separately backcrossing the mutant Galr2 allele to C57BL/6J and 129S1/SvImJ for four generations to acquire ~94% of the backcrossed strain’s genetic background (Silver, 1995). Anti-BrdU immunohistochemistry revealed Galr2\(^{-/-}\) mice on the 129S1/SvImJ background had significantly fewer total number of BrdU+ cells in the RMS compared to their wild-type littermates \((p= 0.0103; \text{Figure 4.7 A})\). In addition to examining the Galr2\(^{-/-}\) mutant and Galr2\(^{+/-}\) wild-type mice, I also quantified the total number of BrdU+ cells in the RMS of Galr2\(^{+/-}\) heterozygous mice. The Galr2\(^{+/-}\) heterozygous mice had ~15% less BrdU+ cells in the RMS compared to the Galr2\(^{+/-}\) wild-type littermates (not significant). Similarly, the Galr2\(^{-/-}\) mutant mice had ~15% less BrdU+ cells in the RMS compared to the Galr2\(^{+/-}\) heterozygous mice (not significant). These trends suggest the additive effect of Galr2 on NPC proliferation, where consistent quantitative change in NPC numbers is associated with the replacement of a mutant Galr2 with a wild-type, functional Galr2 allele. In contrast, Galr2\(^{-/-}\) mutant mice on the C57BL/6J background had similar levels of BrdU+ cells in the RMS compared to their wild-type and heterozygous littermates (Figure 4.7 B). These findings indicate the genetic background mediates the proliferative effect of Galr2.
4.4 Discussion

In this chapter, I showed the expression of Galr2 in regions that support neurogenesis in the adult brain. The functional role of Galr2 in adult neurogenesis is not fully understood. Here, I
demonstrated the proliferative role of Galr2 in adult SVZ-RMS-OB and SGZ using a combination of in vitro and in vivo approaches.

This is the first study to report the effect of Galr2 on adult SVZ-RMS-OB. Others have examined the function of Galr2 on neurogenesis in early postnatal (P1-3) mouse brain when they treated NPCs derived from the SVZ with synthetic galanin-like ligands (Galanin 2-11, also known as AR-M1896) selective for GALR2 (Agasse et al., 2013). The activation of GALR2 by Galanin 2-11 was reported to promote neurogenesis especially during the neuronal differentiation phase where NPCs commit to neural fate (Agasse et al., 2013). The same GALR2 agonist was administered to NPCs derived from P7-10 rat hippocampi (Abbosh et al., 2011). The authors showed that the GALR2 agonist enhances hippocampal NPC proliferation. Additional staining of NPCs with subtype specific markers showed the presence of GALR2 agonists significantly increased the proportion of proliferating neuroblasts (Abbosh et al., 2011). This is in line with my findings where I detected different numbers of BrdU+ cells in the SGZ and RMS of Galr2−/− knockout mice and their wild-type counterparts. A prominent proliferative effect of Galr2 was observed in the RMS, which mainly consists of actively dividing, BrdU+ neuroblasts. The absence of Galr2 in the Galr2 knockout mice had significantly fewer number of BrdU+ neuroblasts compared to wild-type mice. Unlike previous studies using the GALR2 agonist (Galanin 2-11), which also has moderate affinity for another receptor subtype called GALR3, my study specifically targets Galr2 using both in vitro and in vivo molecular approaches. Nevertheless, my findings along with insights from previous pharmacological studies support the pro-neurogenic role of Galr2 in the rodent brain.

The additive proliferative effects of Galr2 was observed from comparing cell proliferation in the RMS of Galr2−/− mice on the 129S1/SvImJ background and their Galr2+/−
heterozygous and Galr2+/+ wild-type littermates where having a normal copy of Galr2 is associated with a ~15% increase in the proportion proliferative cells in the RMS (Figure 4.7). This additive effect size is the same as the QTL effect size estimated from my previous QTL mapping study using the AXB/BXA RI panel (Poon et al., 2010; Chapter 3). From my QTL analysis, RI strains carrying the C57BL/6J allele in the Chr 11 QTL region have higher number of NPCs in the RMS compared to RI strains carrying the A/J allele (Chapter 3). Since the Galr2 gene resides in the Chr 11 QTL interval, I expected to observe a similar C57BL/6J allelic effect when comparing the number of NPCs in the RMS of Galr2 null, heterozygous, and wild-type mice on the C57BL/6J background. Instead, no differences were observed among these three groups suggesting the effect of Galr2 maybe suppressed by other factors in the C57BL/6J brain (Figure 4.7). The presence of extracellular factors mediating the effect of Galr2 is further suggested by the discrepancy between my in vivo and in vitro data. My in vitro studies demonstrated the cell intrinsic effect of Galr2 on NPC proliferation where the knockdown of Galr2 in C57BL/6J derived-NPCs resulted in decreased numbers of neurospheres whereas the overexpression of normal Galr2 resulted in increased numbers of neurospheres (Figure 4.3 & 4.4). The absence of Galr2 in mice on the C57BL/6J background had low levels of NPC numbers similar to their heterozygous and wild-type littermates, indicating the presence of cell-extrinsic factors that may suppress the effect of Galr2 in the C57BL/6J brain. In summary, Galr2 is dependent on the genetic background where other genetic modifier(s) may directly or indirectly interact with Galr2 in regulating the NPC proliferation.

As an integral membrane protein, GALR2 is in contact with the microenvironment, and serves as a molecular messenger of the cell where it plays an important role in transmembrane signal transduction (Lang et al., 2007). Binding to extracellular ligands such as galanin is thought
to trigger a conformational change followed by the activation of G-proteins on the intracellular face of the plasma membrane. GALR2 signals through different G-proteins which subsequently activates multiple intracellular pathways involved in regulating processes such as cell proliferation, differentiation, and survival (Lang et al., 2007). Very little is known about the G-protein repertoire and the GALR2-mediated signaling activities in the adult NPCs. The genetic background effect on GALR2 function further demonstrated the complexity of NPC proliferation in the adult brain and implied interactions of GALR2 with other regulatory factors. I previously explored potential GALR2 interactions with other proteins by using the STRING database (http://string-db.org) for the construction of known and predicted protein-protein interactions (Supplementary Figure 4.4). To determine whether the association detected at the protein level was also conserved at the transcriptional level, I also constructed a transcriptional co-expression network using the adult BXD hippocampi transcriptome data that contains the SGZ (GeneNetwork, http://www.genenetwork.org/webqtl/main.py; Supplementary Figure 4.3). The network graphs revealed that GALR2 is not only linked to galanin but also interacts with other extracellular peptides such as Ghrelin (Ghrl). Ghrl shares overlapping biological functions as galanin including the regulation of feeding behavior, learning and memory (Sun et al., 2004; Diano et al., 2006). Ghrl is also known to activate MAPK activity via G-protein coupled receptor signaling pathway (Delhanty et al., 2006). It is possible that there are other endogenous peptides in the adult brain that serve as GALR2 agonists or antagonists. These network graphs also revealed Galr2 is associated with other G-protein coupled receptors including prostaglandin E receptor 3 (Ptger3), dopamine receptor 2 (Drd2), and somatostatin receptor 4 (Sstr4). Sstr4 shares common signaling pathways as Galr2 such as inhibition of adenylate cyclase and modulation of MAPK via G-protein dependent mechanisms (Patel, 1999; Lang et al., 2007).
previous study by Moreno and colleagues reported the presence of heteromers formed between dopamine receptor 1/5 and galanin receptor 1 (Moreno et al., 2011). These heteromers responded to both dopamine and galanin in modulating cholinergic neurotransmission in the adult hippocampus (Moreno et al., 2011). This discovery prompted me to hypothesize that heteromers can also be formed between GALR2 and the dopamine receptor, DRD2 (or other G-protein coupled receptors) identified from the interaction network (Supplementary Figure 4.3). Future studies will be required to determine the type of interaction (i.e. physical or indirect interaction) and the functional interplay between Galr2 and its interacting partners (e.g. catalyze subsequent reactions together in a pathway or regulate each other transcriptionally/post-transcriptionally). Taken together, these findings support the pro-proliferative role of Galr2 in adult neurogenesis and highlight the complex regulation of this process with the potential interactions between Galr2 and other genetic modifiers.
Chapter 5: Discussion

5.1 Summary and significance of findings

Thanks to technological advances, mounting evidence has accumulated over the last several decades showing that the brain is a dynamic, plastic organ with regenerative capability and potential for self-repair. Persistent division of NPCs and the production of new neurons have been discovered in discrete regions of the adult brain, namely the SVZ-RMS-OB and the SGZ of the hippocampus. Moreover, previous studies have detected increased neurogenesis under pathological conditions and the presence of proliferative NPCs in damaged brain regions (Chapter 1, Section 1.1.4). These findings have raised hopes for brain repair using the NPCs. To develop effective strategies that harness the NPCs as a renewable source for repair, it will be necessary to understand how neurogenesis is regulated in the mature brain. Many external stimuli and endogenous factors influence NPC proliferation and ultimately, instructions for the NPCs will be intrinsically programmed at the genetic level. However, the genes regulating NPC proliferation are not fully elucidated.

The first indication that genes influence adult neurogenesis came from studies on hippocampal neurogenesis in mice of different genetic background where significant inter-strain differences in the number of proliferative cells and newborn neurons were observed among in the dentate gyrus of different laboratory mouse strains (Kempermann et al., 1997a; Kempermann and Gage, 2002). Modifier genes are hypothesized to mediate the genetic background effects on adult neurogenesis. However, very little is known about these modifier genes and how they regulate adult neurogenesis. The overall goal of my thesis was to gain novel insights into the modifier genes, their interactions, and functional roles in regulating NPC proliferation using a
phenotype-driven, genome-wide approach. The basis of this approach stemmed from the wide inter-strain differences in the number of NPCs in the RMS of nine inbred mouse strains (Chapter 2). It is estimated that half of the phenotypic differences can be attributed to the natural genetic variation among the strains (Chapter 2, Section 2.3.5). This prompted me to take advantage of the phenotypic and genetic diversity among the mouse strains for gene discovery. Major findings of this thesis are discussed below.

**5.1.1 NPC proliferation is a quantitative trait that is influenced by the genetic background and age**

Adult neurogenesis is a multifactorial process and the early stages of neurogenesis, most notably the proliferation of NPCs, are especially complex with many extrinsic and intrinsic factors shown to regulate this process (Kempermann, 2011). The genetic background also has a significant influence on NPC proliferation. In Chapter 2, I demonstrated the genetic background of mice differentially influence the number of proliferative cells in the RMS, which is the rostral extension of the SVZ and a prominent site of NPC proliferation. A prominent genetic background effect is also observed in NPC proliferation in the adult SGZ as well as later stages of adult neurogenesis (e.g. cell survival and differentiation) (Kempermann et al., 1997a; Kempermann and Gage, 2002). Genetic background is also found to differentially influence the effects of other factors such as environment (Kempermann et al., 1998) and age (Chapter 2) on adult neurogenesis. I provided the first comparative study examining cell proliferation in the aging RMS from 2 to 24 months of age. Significant age effect, strain effect, and age × strain interaction effect were detected (Chapter 2, Section 2.3.4). The dynamics of age-related changes in the number of proliferative cells in the RMS of the C57BL/6J and DBA/2J inbred strains were
observed from 2 to 24 months of age. Comparison of the linear regression slopes revealed that the rate of decline with age is not the same for the two strains especially during the 3- to 7-months old period where the DBA/2J RMS exhibited a steeper decline in the number of proliferative cells compared with that of C57BL/6J RMS. Based on these findings, the number of proliferative NPCs in the RMS is defined as a quantitative trait that is regulated by the genetic background and age. I hypothesized the genetic background effect is caused by multiple polymorphic genes with subtle but significant effects on NPC proliferation. To identify these genes, other factors such as age and environment were controlled for the rest of my experiments where young adult mice (~P60) raised in equivalent housing conditions were used.

5.1.2 Multiple loci underlying inter-strain differences suggests the interaction of multiple genes in NPC proliferation

QTL mapping was used to scan the entire mouse genome and pinpoint the chromosomal regions (i.e. QTLs) that harbor genes regulating the number of proliferative NPCs in the RMS. In Chapter 3, two panels of RI strains were employed for QTL mapping (i.e. AXB/BXAs and BXDs). These RI strains were derived from parental inbred strains that exhibited high (i.e. A/J and DBA/2J) and low numbers of NPCs (C57BL/6J). Both RI panels showed wide differences in RMS cell proliferation beyond the inter-strain differences observed between the parental strains, indicating polygenic regulation of NPCs. QTLs were identified at high precision (e.g. 1.5Mb wide interval for the significant Chr 11 QTL) using the high-resolution genetic maps of the RI strains available at the GeneNetwork (http://www.genenetwork.org/webqtl/main.py). The number of proliferative cells in the RMS of the AXB/BXA and BXD RI strains are respectively regulated by major QTLs on distal Chr 11
and Chr 6. These two QTLs are the first significant QTLs to be described for neurogenesis in the adult forebrain and are suspected to harbor genes that regulate NPC proliferation. The large sample size, especially with the BXD RI panel (n=61 strains), has greatly improved mapping power to not only identify a significant Chr 6 QTL regulating cell proliferation but also detect interaction between Chr 6 QTL with other secondary QTLs on Chr 14 and 18. Composite interval and allelic effect analyses revealed the Chr 14 and Chr 18 QTL interact additively with the major Chr 6 QTL. Subsequent candidate gene analyses revealed several genes in these mapped regions that are associated with neurogenesis and cell proliferation (Chapter 3, Section 3.3.4). Some of these genes are involved in pathways previously shown to regulate adult neurogenesis such as the MAPK, VEGF, Wnt, and ErbB signaling pathways. Interconnection among genes from different loci was observed in their shared pathways. For example, both Tgfa in the major Chr 6 QTL and Camk2a in the secondary Chr 18 QTL are involved in the ErbB receptor signaling pathway. Camk2a is also involved in the Wnt signaling pathway. Interconnectivity among the candidate genes is further reflected at the transcriptional level. Transcriptional network graph revealed several candidate genes that are involved in the same biological processes (e.g. cell proliferation) and pathway (e.g. Wnt signaling pathway) are highly correlated at the mRNA level. Candidate genes that are mediators of multiple pathways (e.g. Camk2a) suggest the potential crosstalk between pathways in influencing cell proliferation in the adult brain. Regulation of NPC proliferation is expected to rely on multiple pathways that can sense the regulatory cues from the neurogenic niche and then relay the appropriate instructions back to the NPCs on the appropriate time and frequency to divide. The fine balance between NPC self-renewal and differentiation may be achieved by the selective action of different combinations of genes involved in these regulatory pathways.
5.1.3 *Galr2* is identified as a modulator of NPC proliferation through QTL mapping and functional validation

The mapped QTLs serve as starting points to identify genes regulating NPC proliferation. From my candidate gene analysis, *Galr2* emerged as the most promising candidate in the significant Chr 11 QTL, which was identified from mapping of the AXB/BXA RI strains (Chapter 3, Section 3.3.2). In Chapter 4, several lines of evidence have been generated to support the proliferative role of *Galr2* in adult neurogenesis. First, I showed *Galr2* is expressed in the adult neurogenic regions (Chapter 4, Section 4.3.1). *Galr2* is also differentially expressed in the SVZ-derived NPCs from A/J and C57BL/6J. The expression of *Galr2* is positively correlated with the number of NPCs in the RMS, suggesting the pro-proliferative role of *Galr2* (Chapter 4, Section 4.3.1). Transient knockdown and overexpression of *Galr2* in vitro demonstrated the rate of NPC proliferation is dependent on the expression of *Galr2* (Chapter 4, Section 4.3.2). The proliferative role of *Galr2* is further validated in vivo using *Galr2*−/− knockout mice where the absence of *Galr2* decreased NPC proliferation in the SVZ-RMS and resulted in fewer numbers of newborn cells in GCL of OB (Chapter 4, Section 4.3.3). Since the effect of genetic background on adult neurogenesis is complex and involves multiple loci, *Galr2* is expected to only partially contribute to the genetic regulation of NPC proliferation. I further showed the genetic background differentially influences the effect of *Galr2* on NPC proliferation, suggesting the interaction of Galr2 with other genetic modifiers. The molecular mechanisms by which *Galr2* regulates NPC proliferation is not fully understood. Protein-protein interaction network and transcriptional co-expression networks suggest that, in addition to its known ligand Galanin, GALR2 is linked to other neuropeptides and growth hormones in the microenvironment. GALR2 is also associated with other G-protein coupled receptors that are structurally similar to GALR2.
Previous studies have shown that GALR2 and another galanin receptor subtypes may form heteromers with each other or with other G-protein coupled receptors in the brain (Fuxe et al., 2012). These heteromers may trigger different pathways for galanin and other peptides to simultaneously modulate NPC proliferation and other biological processes in the brain (Fuxe et al., 2012). Together, these data provide evidence that Galr2 acts directly on the NPCs in regulating cell proliferation. The proliferative effect of Galr2 is complex and may be mediated by other extrinsic and/or intrinsic factors.

5.2 Future directions

5.2.1 Individual and combined effects of candidate genes on NPC proliferation

The genetic basis of NPC proliferation likely consists of genes that control the proliferative state of NPCs (e.g. signal NPCs to enter or exit cell cycle) and genes that regulate NPC proliferation (e.g. modify cell cycle length and how frequent the NPCs divide). QTLs identified in my thesis are hypothesized to harbor genes that regulate the number of NPCs in the adult mouse brain. Galr2 is one of the candidate genes in the significant Chr 11 QTL and the functional role of Galr2 on NPC proliferation has been examined in Chapter 4. Galr2 was found to regulate self-renewal of SVZ-derived NPCs and modulate the number proliferating neural precursors in adult RMS. However, the effect of Galr2 was dependent on the genetic background, indicating the presence of other modifiers involved NPC proliferation. Additional modifiers may be discovered through the functional analyses of strong candidate genes in QTLs identified from QTL mapping of the BXD RI strains (Chapter 3, Table 3.2). Methods described in Chapter 4 are designed to provide basic information on whether a candidate gene is expressed...
in the NPCs and regulate NPC proliferation. Further experiments will be required to answer questions regarding the specific functions of the candidate genes. One of which is whether a candidate gene exerts similar or different effects on specific NPC populations in the SVZ, which includes adult neural stem cells, transient amplifying progenitors, and neuroblasts. In Chapter 4, Nestin was the only NPC marker used for co-localization with GALR2, and partial overlap is detected between Nestin and GALR2 expression in the wild-type SVZ (Chapter 4, Figure 4.2). Additional makers such as GFAP, Dlx2, and DCX will be required to determine whether GALR2 is expressed in SVZ astrocytes, transient amplifying progenitors, and neuroblasts. A BrdU label-retaining protocol can be used to identify self-renewing stem cells in the GALR2+ astrocytes (GFAP+) (Colak et al., 2008). For this protocol, BrdU will be added to drinking water for 2 weeks followed by 2 weeks without BrdU to allow labeling of slow-dividing stem cells that retain BrdU (e.g. GFAP+ BrdU+) in contrast to the fast proliferating progenitors that will undergo rounds of cell proliferation and dilute the BrdU in the last 2 weeks where no BrdU is supplied in the drinking water (e.g. Dlx2+ BrdU-). Neuroblasts that leave the cell cycle shortly after the incorporation of BrdU (DCX+ BrdU+) can also be identified using this BrdU-retaining protocol. To identify which NPC population is influenced by Galr2, the proportions of neural stem cells, transient amplifying cells, and neuroblasts will be determined in the adult Galr2-null SVZ-RMS and wild-type SVZ-RMS for comparison. Decreased number of neuroblasts in the RMS was detected in the RMS of Galr2-null compared to wild-type controls (Chapter 4, section 4.3.3.1). Based on the progression of cell types within the adult SVZ, I hypothesize that the reduction in neuroblasts number may be attributable to 1) decreased number of cycling neural stem cells (GFAP+ Ki67+), 2) no changes in the number of proliferative neural stem cells, but these stem cells gave rise to less transient amplifying progenitors (Dlx2+ DCX- Ki67+), 3)
transient amplifying progenitors (Dlx2+) gave rise to less number of neuroblasts (DCX+), or 4) reduced neuroblast self-renewal resulting in less neuroblasts. To test these hypotheses, Galr2 can be deleted in specific populations of adult NPCs using conditional knockout mice. For example, conditional deletion of Galr2 in SVZ astrocytes/stem cells can be achieved using GFAP-CreERT2 or astrocyte-specific glutamate transporter (GLAST)-CreERT2 transgenic mice that are crossed to mice containing loxP-flanked Galr2 gene. Tamoxifen-induced Cre-mediated recombination is expected to result in deletion of a critical segment of the Galr2 gene, which is flanked by the loxP sites. A more sophisticated intersectional genetic strategy based on dual recombination involving both the Cre (e.g. Nestin-Cre) and Flp recombinases (e.g. Dcx-Flpe) can be used to target specific neural precursors (e.g. Nestin+ DCX+ neuroblasts). Alternatively, transgenic mice can be designed to overexpress Galr2 in the NPCs to see if overexpression of Galr2 increases cell proliferation in specific NPC populations. Cell transplantation experiments can be used to verify whether the gene acts in a cell autonomous manner (i.e. the gene is acting on the NPCs and directly influences cell proliferation). For example, if Galr2 is shown to influence the number GFAP+ astrocytes in the SVZ, then GFAP-eGFP mice can be designed to express either wild-type Galr2 or the deleted form of Galr2. GFP+ astrocytes in the adult SVZ can be isolated using FACS and subsequently transplanted into the adult SVZ of wild-type and Galr2-null mice. A previous study using this protocol has shown the transplanted GFP+ cells gave rise to DCX+ neuroblasts in the SVZ-RMS-OB a week after transplantation (Colak et al., 2008). If Galr2 regulates GFAP+ NPC proliferation in a cell-autonomous fashion, then the transplantation of wild-type Galr2+/+ GFP+ cells into the SVZ of Galr2-null mice will give rise to more DCX+ neuroblasts compared Galr2-null mice that received the Galr2−/− GFP+ cells. Conversely, transplantation of Galr2−/− GFP+ cells into the wild-type SVZ should give rise to less
DCX+ neuroblasts compared to the transplantation of Galr2+/+ GFP+ cells into the wild-type SVZ.

In addition to study the contribution of individual candidate genes, future studies are required to delineate the relationships amongst genes and their combined effects on NPC proliferation. QTL analyses suggested the Chr 6 QTL interacts with the Chr 18 QTL in regulating NPC proliferation. Interconnectivity of candidate genes in these QTL was further suggested by pathway and transcriptional covariance analyses (Chapter 3, Section 3.3.4). Future experiments are required to examine the relationships among the genes. Based on my findings and literature review, I hypothesize Tgfa (a Chr 6 QTL gene) and Camk2a (a Chr 18 QTL gene) regulate NPC proliferation via the ErbB receptor signaling pathway. Tgfa encodes a growth factor that is a ligand for the epidermal growth factor receptor, ErbB-1 (Galvez-Contreras et al., 2013). Tgfa mRNA has been detected in the adult striatum, which is adjacent to the SVZ-RMS, as well as the adult OB and DG (Wilcox and Derynck, 1988). ErbB-1 and Camk2a are also expressed in the adult SVZ and RMS (Allen Brain Atlas, http://www.brain-map.org). Upon binding of TGFα to its receptors, the ErbB receptor signaling pathway is activated, which in turn triggers several intracellular cascades that regulate cell proliferation, migration, and differentiation (Galvez-Contreras et al., 2013). The Camk2a encodes a calcium calmodulin-dependent protein kinase that is one of the downstream signaling components of the ErbB receptor signaling pathway. Many cell cycle regulators are cellular targets of CAMK2a including plasma membrane Ca2+-ATPase-4 (Atp2b4) that represses G1-S transition of mitotic cell cycle, and CAMK2a reinforces this Atp2b4-mediated repression of cell cycle progression (Afroze et al., 2003; Yuan et al., 2007). In contrast, Tgfa is known for its pro-proliferative role on NPC proliferation (Tropepe et al., 1997). A ~40% decrease in the number BrdU+ cells has been
detected in the dorsolateral corner of the adult SVZ of Tgfa-null mice compared to wild-type controls (Tropepe et al., 1997). The activation of ErbB receptor signaling pathway via TGFα has also been shown to increase the expression of Mcm2, which is another candidate gene in the Chr 6 QTL (Macleod et al., 2005). Mcm2 encodes a key component of the DNA replication complex during S phase of the cell cycle (Chen et al., 1999). The mRNA expression of Mcm2 is also negatively correlated with Camk2a in the BXD brains (r = -0.76; Figure 3.7), suggesting opposite functions of these two genes where Mcm2 promotes and Camk2a inhibits cell cycle progression. Future experiments will be required to confirm the hierarchic interactions among these candidate genes. Tropepe and colleagues showed the number of constitutively proliferating NPCs in the adult SVZ is decreased in the absence of Tgfa, suggesting these NPCs are responsive to TGFα (Tropepe et al., 1997). To determine whether the ErbB receptor signaling pathway is activated in the presence of TGFα, adult mice would first receive an intracerebroventricular infusion of TGFα and their brains would be immunostained for ErbB-1 and its phosphorylated form (pErbB-1). If many pErbB-1 positive nuclei are detected along the SVZ, this would suggest active ErbB receptor signaling pathway in this zone. Antibodies against pErbB-1 can be combined with NPC specific markers to determine which NPC population is responsive to TGFα. In situ hybridization data from Allen Brain Atlas showed the mRNA expression of downstream ErbB receptor signaling components such as phospholipase C, gamma 1 and 2, Camk2a, and Mcm2 (a predicted cellular target) are present in the SVZ-RMS, and SGZ. The transcript and protein levels of these genes can be confirmed using real-time RT-PCR and western blot in cultured NPCs treated with TGFα or laser micro-dissected SVZ-RMS from brains infused with TGFα. Since Tgfa has been shown to promote NPC proliferation and cell cycle progression (Tropepe et al., 1997; Wang et al., 2012), the activation of the ErbB receptor
signaling pathway by *Tgfa* is predicted to decrease *Camk2a* expression and increase *Mcm2* expression. To test whether *Camk2a* influence *Mcm2* expression, cultured NPCs can be treated with siRNA against *Camk2a* or *Camk2a* overexpression plasmids to see if *Mcm2* expression and NPC proliferation are altered. A combination of genetic crosses can be performed to gain insights into the genetic interactions *in vivo*. For example, if crossing the *Tgfa* overexpressing mice with *Camk2a*-null mice results in more number of NPCs than the two single mutants, then this confirm the pro- and anti-proliferative roles of *Tgfa* and *Camk2a*, respectively. Alternatively, *Tgfa* overexpressing mice can be crossed to *Camk2a* overexpressing mice. If the double mutant results in 1) less NPCs compared to the *Tgfa* overexpressing single mutant, and 2) a similar number of NPCs compared to the *Camk2a* overexpressing single mutant, then these results would suggest that *Tgfa* and *Camk2a* participate in the same genetic pathway and CAMK2a is a downstream signaling component.

5.2.2 **Relevance to human adult neurogenesis**

Emerging studies have shown neurogenesis occurs in the adult human brain and new neurons are continually added to specific brain regions including the hippocampus and striatum (Spalding et al., 2013; Ernst et al., 2014). However, very little is known about the regulation of adult human neurogenesis. Comparative genomics using the Ensembl genome browser ([http://uswest.ensembl.org/index.html](http://uswest.ensembl.org/index.html)) showed the QTLs identified in this study (e.g. mouse Chr 6 and Chr 11) are syntenic with regions in the human genome (e.g. human Chr 2 and 17, respectively). Pairwise alignments showed sequence conservation (> 80% similarity) of candidate genes in the mouse and human genomes. Genetic variation analysis identified several polymorphisms in the human version of the candidate genes and some of the SNPs positions are
conserved between the human and mouse genes (http://uswest.ensembl.org/index.html). It is possible that gene functions and pathways regulating NPC proliferation in mice are conserved in human. The regulatory effects of genes on human NPC proliferation can be tested in vitro by transfecting cultured human NPCs with siRNAs or overexpression plasmids targeting a gene of interest. Previous studies have demonstrated human NPCs can be isolated and cultured in vitro (Johansson et al., 1999; Kukekov et al., 1999). Similar to mouse NPCs, human NPCs can proliferate and form neurospheres in non-adherent cell cultures. Cultured human NPCs also differentiated into neurons and astrocytes, demonstrating the multipotency of these cultured NPCs (Johansson et al., 1999; Kukekov et al., 1999). To conduct knockdown and overexpression experiments, a large number of NPCs are usually required for transfection, and it may be challenging to have an ongoing supply of human brain tissues as the source of NPCs. Recent studies showed NPCs could be generated in vitro via reprogramming of somatic cells such as fibroblasts to NPCs (Kim et al., 2011; Lujan et al., 2012; Zhou and Tripathi, 2012; Wapinski et al., 2013) or directed differentiation of pluripotent stem cells to NPCs (Yu et al., 2014). Yu and colleagues were the first group to demonstrate the in vitro generation of hippocampal NPCs and granule cells from human pluripotent stem cells (Yu et al., 2014). Transplantation of induced hippocampal NPCs into the dentate gyrus of P10 mice gave rise to functional granule neurons in the dentate gyrus (Yu et al., 2014). The induced human NPCs can be an alternative source to investigate the mechanisms underlying human adult neurogenesis. Altered adult neurogenesis has been observed in patients with neurodegenerative diseases (Chapter 1, Section 1.1.4). The study of adult NPCs generated from patients’ fibroblasts may provide insights into the mechanisms underlying these disease-related changes in adult neurogenesis. In addition to disease modeling, induced NPCs may also be used for drug discovery. Continual neuronal loss is
a hallmark of neurodegenerative diseases and decreased NPC proliferation has been observed in the PD and HD brains (Chapter 1, Section 1.1.4). The up-regulation of NPC proliferation and neurogenesis in these disease brains may compensate for cell loss and ameliorate disease symptoms. Pro-proliferative regulators such as \textit{GALR2} and \textit{TGFA} can be targeted \textit{in vitro} (e.g. treat human NPCs with GALR2-agonist and/or TGFα) to see if proliferation is enhanced in the NPCs derived from patients’ fibroblasts. Studies on rodent models for these diseases can provide additional insights into whether enhanced NPC proliferation results in an increased supply of NPCs to the degenerated brain regions, and whether these NPCs can differentiate into specific neurons that are lost in these regions. The significant effect of genetic background on NPC proliferation further suggests the consideration of patient’s genotype when devising therapeutic strategies to effectively boost neurogenesis. Although there is still a long road ahead for the translation of these findings to clinical setting, future studies using human NPCs and the appropriate rodent models should help expand knowledge on factors and mechanisms regulating adult human neurogenesis in normal and pathological conditions.

5.3 Concluding remarks

Dissecting out the different molecular pathways and genes regulating adult neurogenesis is an immense undertaking. Together with the powerful mouse resources and statistical tools, I have demonstrated the feasibility of a phenotype-driven, genome-wide approach with the identification of QTLs that provide starting points to identify genes important for NPC proliferation. The proliferative role of candidate genes can be validated through the molecular approaches employed for \textit{Galr2} as proof of concept. Understanding the molecular players and regulatory networks underlying adult neurogenesis will help identify putative targets
for therapeutic strategies using NPCs to promote neuronal cell regeneration in disease and injured brains.
References


Appendix A: Supplementary figures for Chapter 2

Supplementary Figure 2.1 Correlation between the RMS linear density and total number of proliferating cells in the RMS. RMS linear density (i.e. the number of BrdU + cells per mm length of RMS; x-axis) was determined using the single best-section quantification method, and it is significantly correlated with the total BrdU + cell counts (y-axis) which was determined from surveying every 10th section throughout the medial to lateral extent of the RMS ($p < 0.0001$). Each data point represents count obtained from a randomly selected mouse at 2-3 months of age ($n=20$).
Supplementary Figure 2.2 Schematic sagittal view of an adult mouse brain depicting the general shape and trajectory of the RMS from the subventricular zone of the lateral ventricle (LV) to the olfactory bulb (OB). The RMS is divided into three major components: vertical arm, the elbow, and the horizontal arm. Four RMS representative segments (pink squares) were selected for estimating cell density and size of the proliferative population in the RMS.
Appendix B: Supplementary figures for Chapter 3

![Graph of Mcm2 mRNA level vs Strain]

![Graph of Gpl1 mRNA level vs Strain]

![Graph of Anxa4 mRNA level vs Strain]
Supplementary Figure 3.1 Inter-strain variation in transcript levels of candidate genes in the mouse brain. The BXD whole brain transcriptome sequencing data set (UTHSC Mouse BXD Whole Brain RNA Sequence Nov12 RPKM; www.genenetwork.org) was used to obtain the mRNA levels (y-axis) of candidate genes identified from QTL mapping of the BXD RI strains (x-axis).
Supplementary Figure 3.2 Correlation between the transcript levels of candidate genes and RMS linear density. Inter-strain differences in the expression of Anxa4 (a candidate gene in the significant Chr 6 QTL interval), Ppp2ca (a candidate gene in the suggestive Chr 11 QTL interval), Gnpnat1 (a candidate gene in the suggestive Chr 14 QTL interval), and Camk2a (a candidate gene in the suggestive Chr 18 QTL) were observed in different BXD RI strains. (A) Scatterplot of the Anxa4 transcript levels negatively correlated with the mean RMS linear density. (B) Scatterplot of the Ppp2ca transcript levels negatively correlated with the mean RMS linear density. (C) Scatterplot of the Gnpnat1 transcript levels negatively correlated with the mean RMS linear density. (D) Scatterplot of the Camk2a transcript levels positively correlated with the mean RMS linear density. Each dot represents a BXD strain.
Appendix C: Supplementary figures for Chapter 4

**Supplementary Figure 4.1** Immunodetection of GALR2 (green) and Nestin (red) in the adult mouse dentate gyrus. Dash lines delineate the SGZ.
Supplementary Figure 4.2 Generation and validation of Galr2<sup>-/-</sup> null mutant mice. The Galr2 null mutant mice were generated by Lexicon Genetics (TX, USA). A) The disrupted Galr2 allele consists of a 5.17-kb gene-trapping cassette inserted into the single intron of Galr2 gene. The gene-trapping cassette contains a splice acceptor (SA), a promoterless reporter gene called NEO, and a downstream transcriptional termination sequence (polyadenylation sequence; pA). B) The gene-trap mutation results in an absence of Galr2 gene transcription in the Galr2<sup>-/-</sup> null mutant. The transcription levels of Galr3 and Gapdh were unaffected.
Supplementary Figure 4.3 Knockdown of Galr2 in NPCs derived from SVZ of A) Galr2<sup>+/+</sup> wild-type mice (WT) and B) Galr2<sup>-/-</sup> mutant mice (MUT). Quantification of the number of neurospheres generated at day 7 post-nucleofection of siRNAs against Galr2 or scramble control siRNAs. The bar graphs (means ± SEM) show the number of neurospheres formed per 1 x 10<sup>5</sup> plated cells.
Supplementary Figure 4.4 Visualization of protein and transcript association networks involving Galr2.

(A) Protein-protein interaction network visualized by STRING. Nodes represent proteins and GALR2 is colored in red. The edges delineate predicted functional links and each color represents a type of evidence described in the legend. (B) transcriptional co-expression network graph of Galr2. The transcripts levels of Galr2 and other genes from the protein-protein interaction network were extracted from the BXD hippocampal expression database available at the GeneNetwork. A network graph was then generated showing how the expression of the Galr2 positively or negatively correlated with other genes. Strength of correlation between two connected genes is indicated in the legend.