NOVEL ASTROCYTE-SPECIFIC TRANSGENIC MICE IDENTIFY DISTINCT POPULATIONS OF TRANSIENT AMPLIFYING PROGENITOR CELLS AND LONG-LIVED NEURAL STEM CELLS IN THE SUBGRANULAR ZONE OF THE ADULT MOUSE BRAIN

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DEDICATION

To my parents and husband for their love and support.

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by

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DISSERTATION

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A lack of previous interest in the astrocyte biology field has resulted in a meager number of astrocyte specific molecular markers and practical protocols to study their biology and function. The most common and accepted astrocyte marker is glial fibrillary acidic protein (GFAP), but in normal, healthy adult brains, GFAP expression is limited to the neurogenic regions, white matter tracks,

and pia. This limited expression is not a true representation of astrocyte distribution patterns and does not account for astrocyte heterogeneity. Through a careful screen, cystatin C (CstC) and phospholipase A2 Group VII (PLA2G7) were identified as astrocyte specific molecular markers. To study the role of these genes in astrocyte biology, inducible CreER^{T2} mice were generated. Using the CstC-CreER^{T2} and PLA2G7-CreER^{T2} mouse lines, in concert with the Rosa26YFP reporter line, cell fate tracking experiments were performed in both embryonic and adult time points in tandem with extensive immunohistochemistry. Both astrocyte-specific CreER^{T2} lines demonstrated limited expression in embryos as early as embryonic day 12.5 and extensive expression throughout the entire central nervous system in all adult time points analyzed. In adult animals, both CstC-CreER^{T2} and PLA2G7-CreER^{T2} Rosa26YFP positive cells had strong expression in both neurogenic niches. Using careful quantification and immunohistochemistry colocalization, the dynamics of these transgenic lines were meticulously researched in the subgranular zone (SGZ) of the dentate gyrus. The CstC-CreER^{T2}/Rosa26YFP cells represent a transient amplifying compartment in the SGZ while PLA2G7-CreER^{T2}/Rosa26YFP positive cells comprise a long-lived neural stem cell compartment. Discovery of these astrocyte dynamics in the SGZ complements the literature indicating astrocytes are the true neural stem cells and adds knowledge of new viable markers and cell populations to the field. Results of these studies reveal not only new molecular markers for astrocytes in

both embryonic and adult scenarios, but also help to identify the underlying heterogeneity of astrocyte populations, as well as adult neural stem cells. Future utilization of these inducible transgenic mouse models could provide the potential to study both astrocyte and neural stem cell pathophysiologies.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Loomis, L and Flynn, NE (2006) Determining glucocorticoid receptor activity in American dietary components using S-gal as a novel reporter substrate. *Journal of Undergraduate Chemistry Research*. 3: 111-114.

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

ALS – amyotrophic lateral sclerosis

AMPA – α-aminio-3-hydroxyl-5-methyl-4-isoxazole-propionate

ANOVA – statistical analysis of variance

APC – astrocyte precursor cell

ApoE – apolipoprotein E

ATP – adenosine triphosphate

Aqp4 – aquaporin 4

AraC – cytosine-β-D-arabinofuranoside (anti-mitotic agent)

B-raf – serine/threonine-protein kinase B-Raf

BAC – bacterial artificial chromosome

BBB – blood brain barrier

BLBP – brain lipid binding protein

BrdU - thymidine analog bromo-deoxyuridine

Ca^{2+} – calcium ion

CNS – central nervous system

CNTF – ciliary neurotrophic factor

CRM – control modulatory region

CstC – Cystatin C

CstC-CreER^{T2}/Rosa26YFP – cystatin C-CreER^{T2}/Rosa26YFP Mice

CstC-CreER^{T2}/DTA BpA – cystatin C-CreER^{T2}/DTA BpA Mice

Cx30 – connexin 30

DAPI – 4'-6-diamidino-2-phenylindole, dihydrochloride

DCX – doublecortin

EGFR – epidermal growth factor receptor

FBS – fetal bovine serum

Fgfr3 – fibroblast growth factor receptor 3

GABA – gamma-aminobutyric acid

GBM – glioblastoma multiforme

GFP – green fluorescent protein

GFAP – glial fibrillary acidic protein

GLAST – sodium-dependent glutamate/aspartate transporter

H&E – hematoxylin and eosin

HCCAA – hereditary cystatin C amyloid angiopathy

iGluR – ionotropic glutamate receptors

IHC – immunohistochemistry

IL6 – interleukin 6

IP – intraperitoneal injection

K^+ – potassium ion

KO – knock-out type mice

KRAS - V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

LIF – leukemia inhibitory factor

mGluR – metabotropic glutamate receptors

Na^+ – sodium ion

NeuN – neuronal nuclei

Nf1 – neurofibromatosis type 1

NMDA – N-methyl-D-aspartate

Olig2 – oligodendrocyte transcription factor 2

PAF – platelet activating factor

PBS – phosphate buffered saline

PFA – paraformaldehyde

Phospho-Akt– p-Akt

PLA2G7 – phospholipase A2 Group VII

PLA2G7-CreER^{T2}/Rosa26YFP – phospholipase A2 Group VII CreER^{T2}/Rosa26YFP Mice

PLA2G7-CreER^{T2}/DTA BpA – phospholipase A2 Group VII CreER^{T2}/DTA BpA Mice

PSA-NCAM – polysialylated neural cell adhesion molecule

RMS – rostral migratory stream

S100β – S100 calcium binding protein B

SEM – standard error of the mean

SGZ – subgranular zone of the dentate gyrus

SVZ – subventricular zone of the lateral ventricle

TAM – tamoxifen

TBS – tris buffered saline

Tlx – orphan nuclear receptor/*tailless*

TMZ – temozolomide

VEGF – vascular endothelial growth factor

CHAPTER ONE

INTRODUCTION: ASTROCYTE AND NEURAL STEM CELL BIOLOGY

Development of the Central Nervous System

The development of the central nervous system (CNS) during embryogenesis and the early post-natal period of an animal's life is maintained through a variety of cellular interactions resulting from the expression of a multitude of genes (Rubenstein and Rakic 1999). The brain parenchyma contains predominantly neurons and glia, the latter of which can be subdivided into two different groups: macroglia and microglia. Microglia consist primarily of macrophages (about 15% of the total number of cells within the CNS) that are capable of phagocytosis and activation of the innate immune system (Barron 1995; Carson, Thrash et al. 2006). The other subset of glial cells are macroglia, which include astrocytes, oligodendrocytes, radial glia, and empendymal cells (Williams, Abney et al. 1985). In total, there are 5-10 times more glial cells within the cerebral cortex than neurons (Nowakowski and Hayes 1999). Overall, the consensus is that the most important cell types in the brain are neurons, astrocytes, and oligodendrocytes. Within this text, astrocytes and their derivatives will be part of the main focus. During embryogenesis, the three main cell types in

the CNS, neurons, astrocytes, and oligodendrocytes, emerge in the murine mouse brain at distinct, but overlapping, time junctures. Neurons are born first as early as embryonic day 9 in the mouse, and their number peaks at about embryonic day 14 and gradually decreases from that point onward until birth. Astrocytes slowly emerge in very small numbers around mid-gestation of E12-13, but truly peak postnatally around the time of birth and slowly diminish about ten days thereafter. Conversely, oligodendrocytes are the true "late bloomers" and begin emerging at late gestation (E15) and continue to be born until a few weeks after birth (Sauvageot and Stiles 2002; Kriegstein and Alvarez-Buylla 2009).

While the most discussed studies, in this text, will be in relation to mouse CNS development, it is important to note the similarities between rodents and primates. Despite the fact total gestational time between rodents and primates varies greatly, the time nature has given for neurodevelopment remains similar between the species. For example, neurons in the dorsal lateral geniculate are born between days E10 and E15 (5 day range) in a mouse and E36 and E43 (7 day range) for a rhesus monkey, even though their gestational periods are drastically different (Rodier 1980). Another similarity is the continued development of the brain after birth in both rodents and primates; postnatal development will be discussed later in this introduction (Nowakowski and Hayes 1999).

During embryogenesis, the development of the CNS is centralized around the neural plate, which is comprised of the neuroectoderm and neural tube (Dudek 2007). The neural tube is comprised of three different dimensions along which it divides: the longitudinal, circumferential/tangential, and radial/laminar dimensions (Nowakowski and Hayes 1999). The neural tube acts as a source for all of the embryonic stem cells needed for the development of the CNS (Ford-Perriss, Abud et al. 2001).

Neurulation is the term used for the describing the formation of both the dorsal nerve cord and actual CNS (Schoenwolf and Smith 1990). This is not the first act of embryogenesis, and by the time of its emergence, the rostral and caudal ends have already been determined by paracrine interactions along the longitudinal axis. About half of the caudal end of the neural tube will ultimately develop into the spinal cord, and the rostral end will become the brain (Nowakowski and Hayes 1999). The circumferential differentiation occurring along the neural tube paves the way for the organization of both the motor and sensory functions of the mature CNS (Nowakowski and Hayes 1999). During this phase, the primitive spinal cord is formed and the neural tube closes. Lastly, the radial differentiation causes maturation of the existing structures (Nowakowski and Hayes 1999). These dimensions work together, in tandem, to form a fully functional central nervous system through the birth, migration, and differentiation of many cell types. Several factors help influence this maturation process, an

example of which are members of the fibroblast growth factor family (Ford-Perriss, Abud et al. 2001). All of the factors that induce cell birth, migration, and differentiation will not be discussed within this text, but it is important to note their critical role in this process.

Role of Radial Glia during Embryonic Development of the CNS

After the closure of the neural tube, proliferating cells begin to emerge in areas close to the ventricular zone. In mice, the very first emerging neurons can be observed as early as embryonic day 9 or 10 (Rodier 1980). After dividing in the ventricular zone, neurons reach their final destination in the CNS in one of two currently described ways. One route is through adhesion molecules expressed on the cellular membrane that create a migratory pathway for neural crest cells. The other method – more widely discussed – is that neuronal migration is dependent upon radial glial cells. These radial glia play the role of a cellular guide and allow nascent neurons to crawl along their processes to their final destination (Purves 2004). These two migratory mechanisms occur based on the anatomical location in the developing CNS. Neurons that use radial glia as migration scaffolding are ultimately arranged in layers resulting in the "outside-in" development. These areas include the cerebral cortex, hippocampus, and cerebellum (Nowakowski and Hayes 1999; Purves 2004). Literature also cites

radial glial cells as precursors for neurons within the cerebral cortex (Tamamaki, Nakamura et al. 2001; Anthony, Klein et al. 2004; Jessen 2004).

Common molecular markers for radial glial cells include brain lipid binding protein (BLBP), glial fibrillary acidic protein (GFAP) (only in primates), RC1, RC2, vimentin, Rat-401, and Nestin (Tamamaki, Nakamura et al. 2001; Zhang 2001; Rakic 2003; Anthony, Klein et al. 2004; Anthony, Mason et al. 2005). In mouse embryogenesis, neuroepithelial cells at embryonic day 9 do not stain for any astroglial markers, but by embryonic day 13, these cells stain for astroglial markers and can be described as radial glia (Malatesta, Hack et al. 2003). On a side note, glia originate from distinct parts of the neuroepithelium as compared to neurons, and of these, astrocytes and oligodendrocytes each arise from different precursors. (Wakamatsu 2004). Embryonic day 13 also marks an onslaught of embryonic neurogenesis. Nascent neurons can be found climbing along these radial glial fibers while moving to their final destination where they will detach from the radial glial fibers and differentiate into mature cells (Rakic 2003; Costa and Hedin-Pereira 2010). It is theorized that once these radial glial fibers have completed guiding neurons, they become astrocytes in late gestation and early post-natal time periods (Malatesta, Hack et al. 2003; Rakic 2003).

Post-Natal CNS Development

The developing CNS is still abuzz even after the birth of the animal. In fact, many structures, such as the dentate gyrus of the hippocampus, have yet to be fully formed at birth (Rodier 1980). As previously touched upon, the majority of post-natal activity is formed by the relative population growth of both the astrocytes and oligiodendrocytes, of which the emergence of astrocytes will be the focal point of discussion in this text. Radial glia, a subtype of immature astrocytes that was previously commented on, represent one of the earliest specialized cells and key players within in the developing CNS in the embryonic period. Postnatally, the largest influx of both protoplasmic and fibrous astrocytes is observed towards the end of neurogenesis as radial glia disappear (Rodier 1980; Zhang 2001; Wakamatsu 2004). From this observation, it is a logical conclusion to assume that astrocytes may form from the radial glia after they are finished guiding newly born neurons to their appropriate location within the cortex.

The change from radial glia to mature astrocytes is also accompanied by a change in cell specific antigen expression, such as loss of RC1, RC2, and Rat-401, as well as expression of a slew of growth factors, including ciliary neurotrophic factor (CNTF), interleukin 6 (IL6), and leukemia inhibitory factor (LIF) (Sauvageot and Stiles 2002; Rakic 2003). One important factor for astrocyte differentiation is basic helix-loop-helix transcription factor,

oligodendrocyte transcription factor 2 (Olig2). Olig2, which is traditionally associated with oligodendrocytes, is also present in radial glia and has a transient expression in maturing astrocytes that is eventually undetectable when they have fully matured (Cai, Chen et al. 2007). While Olig2 is found in developing astrocytes in both gray and white matter, a deficit of Olig2 during development will result in severe loss of white matter astrocytes (Cai, Chen et al. 2007).

To support the theory that cortical astrocytes originate from radial glia, several cell labeling studies have been completed. In one such study, a fluorescent DiI dye was injected into the radial glia of newborn ferrets. The radial glia were traced from birth to 6 weeks using the DiI dye combined with molecular markers GFAP (for mature astrocytes) and vimentin (for radial glia). The findings support the idea that as radial glia disappear (lose vimentin expression), they begin to mature into astrocytes (gain GFAP expression) (Voigt 1989).

Other studies have described complimentary results by utilizing transgenic mice. One such study used Cre-recombinase under the control of a human GFAP promoter. This line labeled radial glia during embryogenesis, which were found to mature into a portion of the astrocyte population (Malatesta, Hack et al. 2003). Many of the transgenic mice used to study the fate of radial glia do not have an inducible method of gene expression. This results in a smearing effect throughout the entire embryonic neurogenesis period by labeling the earliest precursors that

express the gene, which includes those pertaining to neurons as well as astrocytes. This makes pinpointing a certain day to study the gene's effect virtually impossible.

From these experiments, it can be concluded that radial glia form the foundation for the entire development of the brain. They serve as scaffolding for the migration of cells throughout the brain, and later become the precursors to neurons, astrocytes, and oligodendrocytes. In 1999, the Barres laboratory discovered a novel cell that was coined an "astrocyte precursor cell (APC)" (Mi and Barres 1999). The APCs were isolated from rat optic nerves during both late embryogenesis and the early postnatal period. These cells were immunoreactive positive for Pax2 and A2B5, both astrocyte specific markers. APCs in culture differentiated into GFAP positive astrocytes after the addition of LIF and CNTF to the serum free media (Mi and Barres 1999).

Another cell type that could be considered an APC are NG2 (a proteoglycan) expressing cells. *In vivo* studies indicate NG2 positive cells are multipotent and can produce not only protoplasmic astrocytes, but also neurons and oligodendrocytes (Trotter, Karram et al. 2010). However, since NG2 cells do not exclusively produce astrocytes, they may not fully fall under the category of an APC. Approximately 5-10% of glial cells (both oligodendrocytes and astrocytes) in the developing CNS are immunoreactively positive for NG2.

Through the use of an NG2creBAC transgenic mouse, it was revealed that while NG2 cells do generate oligodendrocytes throughout the entire brain and spinal cord, NG2 cells are also responsible for the creation of some portion of protoplasmic astrocytes in both the spinal cord and forebrain (Zhu, Bergles et al. 2008; Zhu, Hill et al. 2008).

Weyhenmeyer's lab also isolated and created an APC cell line called 1H91. These GFAP negative cells were isolated from the mouse cerebellum on embryonic day 16. 1H91 cells differentiate into GFAP positive astrocytes at a rate directly correlated with the local level of epidermal growth factor (Seidman, Teng et al. 1997). The 1H91 cells were not positive for the A2B5 antigen like the APCs described by Barres' group, making them separate and distinct APC populations (Lee, Mayer-Proschel et al. 2000). Other than these examples, there are not many known APCs, although more are believed to exist. As an example, NG2 cells differentiate into only protoplasmic astrocytes and not fibrous astrocytes, providing evidence towards multiple different APCs being responsible for the full astrocyte population in the adult mouse central nervous system.

Historical Overview of Astrocytes

Astrocytes have been studied for over a century. The actual term, "astrocyte," was coined by Michael von Lenhossek in a review written in 1893

(Kettenmann and Ransom 2005). Within that review, Lenhossek also referred to astrocytes as "supportive cells," and the belief that astrocytes were merely a supportive cell within the context of brain physiology was long sustained in the field of neuroscience. Astrocytes earned their name due to the stellate, star-like shapes of these cells when stained by an array of early techniques to study brain histology developed by many historically famous scientists including Golgi and Cajal (Garcia-Lopez, Garcia-Marin et al. 2010). There were several misconceptions concerning both the origin and the role of astrocytes that, due to the lack of interest in the astrocyte biology field, were slow to die.

When the study of neuroglia as a whole was evolving, much of the work was performed in postmortem human tissue. The tissue was not fresh, resulting in break down in the nuclei of glia. This, combined with limited microscope resolution, led scientist Virchow to believe that neuroglia, collectively (including both oligodendrocytes and astrocytes), were a physical syncytium. This physical syncytium was termed the "neurospongium," and its role was to hold together the brain as a physical structural support (Kettenmann and Ransom 2005). Alluding to the idea that astrocytes were typically dismissed as just support cells, the definition of the word glia is derived from a Greek word meaning, "slime and/or glue" (Nedergaard, Ransom et al. 2003). Thus this slime/glue/neurospongium did not provide significant scientific research interest when compared to the elegant neurons that were also visualized in the early stains.

Early in the study of neuroglia (from 1880 to 1920), two types of astrocytes were discovered, depending on location: fibrous glia (white matter) and protoplasmic glia (gray matter). Interestingly, one of the leading scientists of the time, Andriezen, described fibrous glia as arising from the embryonic ectoderm and the protoplasmic glia from the mesoderm. Conversely the "father of neuroscience," Ramon y Cajal, described all neuroglia to have ectodermal origins (Kimelberg 2004). The theory changed again when scientists discovered that these glia were not of ectodermal origin but actually mesodermal in origin. Literature cites both Virchow and Cajal's student, del Rio Hortega describing neuroglia as arising from the mesoderm. The idea that glia created the "neurospongium" was finally dismissed after specific stains for connective tissue failed to support either the ectoderm or mesoderm theories, accompanied by the aid of electron microscopy in the 1950s (Kettenmann and Ransom 2005). Though the concept that astrocytes are not an extension of connective tissue in the brain was finally accepted, it was still widely believed that these cells of glial origin simply existed to fill spaces in between neurons. Subsequent research revealed that astrocytes originate from a much more specialized developing neural tube compartment of the ependymoglia (Abbott, Ronnback et al. 2006). While the simple space filling theory of astrocyte function is no longer the norm, it is still, unfortunately, a belief still held by some in the field.

Golgi was also one of the first to describe the relationship of astrocytes and blood vessels. He accurately hypothesized that astrocytes were responsible for the exchange of nutrients from the capillaries to the neurons (Kettenmann and Ransom 2005; Volterra and Meldolesi 2005). Unfortunately, Cajal had little faith in the idea that glia could be responsible for such a task, as his staining techniques and microscopy provided him a limited view of the astrocyte-capillary observation (Kettenmann and Ransom 2005). Of note, reviews written in the 1960s maintained that astrocytes did not provide nutritional support to neurons based off of Cajal's initial objections to the theory. In fact, it was not until approximately 20 years ago when the scientific community began to accept the nutritional theory (Kettenmann and Ransom 2005).

Due to early misconceptions extrapolated from a lack of refined techniques and analysis, astrocytes were largely dismissed within neuroscience research as "second-class" cells in comparison to neurons. While the study of glial cells is well over a century old, it has only been within the past few decades that the importance of the study of astrocytes has been recognized. Kimbelberg seems to sum and enunciate the feeling towards astrocytes well in the following quote:

> […] this term was applied to cells based on morphology specific staining with Cajal's gold sublimate, and exclusionary definitions such as those cells that were neither neurons, oligodendrocytes, microglia and ependymal cells of the CNS (Kimelberg 2004).

Reviews that were written over 20 years ago and yet again in more recent times have described how issues, theories, and hypotheses first described by earlier researchers (previously discussed) over 100 years ago are still being fully researched in labs around world (Somjen 1988; Barres 2008). Sadly, the areas of astrocyte research were not given as much care and attention as other cell counterparts in the nervous system.

Emerging Prospective on the Role of Astrocytes

While astrocytes were traditionally defined as simply GFAP positive cells that play a supportive role in the nervous system, they have recently become star players in neuroscience research and the backbone of brain pathology. It is now widely accepted that astrocytes play a wide variety of roles in the brain to maintain overall homeostasis. Some of these roles include providing neurons with vital nutrients, information processing through neurotransmitter receptors and gap junctions, regulation of blood flow, structural support, and support of adult neurogenesis within neurogenic niches (Volterra and Meldolesi 2005; Chvatal, Anderova et al. 2008; Blackburn, Sargsyan et al. 2009).

Astrocytes and the Microvasculature of the Brain

While Golgi pondered the relationship between astrocytes and blood vessels, his ideas were largely dismissed until the 1980s when more research and evidence pointed towards his original hypothesis (Maxwell, Berliner et al. 1987; Maxwell, Berliner et al. 1989; Volterra and Meldolesi 2005). Many of these early studies simply focused on the theory which asserted astrocytes assisted in maintaining the blood brain barrier (BBB) instead of actually inducing its characteristics, such as the selective passage of substances (Holash and Stewart 1993). The BBB is formed through the interaction between the endothelial cells that make up the capillary walls and the astrocytes in this region. These astrocytes have specialized perivascular endfeet that wrap around the capillaries and regulate them. Astrocytes secrete several different factors that play various roles in maintaining the BBB, some of which are the following: transforming growth factor-β, glial-derived neurotrophic factor, basic fibroblast growth factor, and angiopoietin 1. The BBB phenotype is unique and distinguishes itself from the endotheliums of other tissues; these features are both physical and chemical, and include tight junctions and a variety of brain endothelial transporters which move substances providing neurotrophic support (Abbott, Ronnback et al. 2006).

More macroscopically, astrocytes form what is coined a gliovascular unit. A gliovascular unit is comprised of a single astrocyte, the neurons that are

encompassed by their processes, and the adjacent sections of blood vessels they interact with (Abbott, Ronnback et al. 2006). It was once thought that neurons and their activity were responsible for the vasoactive changes in blood vessel diameter. The "tight coupling between neuronal activity and blood flow" often led to an effect called hyperemia, where the active sections of the brain became engorged with increased blood flow (Zonta, Angulo et al. 2003). There are a variety of ways in which astrocytes control both vasodilation and vasoconstriction of the blood vessels, thereby regulating cerebral blood flow. Vasodilation is caused by calcium influx and the conversion of arachidonic acid to prostaglandin E_2 and epoxyeicosatrienoic acid. Vasoconstriction is a result of arachidonic acid being converted to 20-hydroxyeicosatetraenoic acid (Zonta, Angulo et al. 2003; Gordon, Mulligan et al. 2007). Neuronal activity increases calcium concentrations, which astrocytes uptake through their perivascular endfeet. Through this mechanism, astrocytes are able to stimulate vasodilation, and in a specific reported case, vasodilation occurred 34 ± 6.8 seconds after stimulation (Zonta, Angulo et al. 2003).

Another study by Zonta (2003) provided evidence that astrocytes may possess the intrinsic ability to regulate vascular tone independent of neuronal involvement. Individual astrocytes in cortical slices were stimulated through contact with a patch pipette, and arterioles within a 10-20 μm distance were dilated in 10 of 13 samples. This implies that astrocytes are able to control

vasodilation of the cerebral vasculature without the need for activated neuronal factors, and therefore, that they may play a previously unknown control/regulatory role in hyperemia (Zonta, Angulo et al. 2003). As a single astrocyte can, through all of their thousands of processes, contact up to 100 different neurons, the implications of these discoveries should not be understated (Hamilton and Attwell 2010). The review by Gordon, Mulligan *et al.*, 2007, especially Figure 1 of that text, concisely summarizes the different factors which, in concordance with astrocytes, influence vasculature tone throughout the brain (Gordon, Mulligan et al. 2007).

Synaptic Homeostasis and Neuronal Health

In addition to the supplying nutrients to neurons through their contact with blood vessels, astrocytes also impact neuronal health at the synaptic level. Within the brain, there is a milieu of cells, blood vessels, and neurotransmitters. Excessive amounts neurotransmitters, in particular glutamate, left to mingle around neurons can have devastating effects on neuronal health. Glutamate represents the most common excitatory amino acid transmitter and is located in vesicles at the endplate of the presynaptic cell (Hansson and Ronnback 1995). Astrocytes and neurons utilize the glutamate-glutamine cycle to regulate levels of glutamate. In this cycle, astrocytes maintain homeostasis through uptake of excess glutamate in the synaptic junction. Within the astrocytes, glutamine

synthetase catalyzes the conversion of glutamate to glutamine. Glutamine is synaptically inactive and is released back to the synapse and reuptaken by the neurons with the aid of glutamine transporters, where it is again converted back to glutamate (Hamilton and Attwell 2010). This cycle keeps the intracellular concentration of glutamate in astrocytes low, especially when compared to neurons. Through the use of antibody labeling and electron microscopy, it was shown that glutamate concentrations were 6 times higher in neurons than in astrocytes (Bramham, Torp et al. 1990).

Neurons (and astrocytes) have two different types of glutamate receptors: ionotropic receptors (iGluR) and metabotropic receptors (mGluR). Upon binding of glutamate, iGluR, which are ligand-gated cation channels, allow the passage of sodium (Na^+), potassium (K^+) and, in some instances, calcium (Ca^{2+}) ions. These cations cause a depolarization and an action potential in postsynaptic neurons which results in activation of the following agonists: α-aminio-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and N-methyl-D-aspartate (NMDA) (Hansson and Ronnback 1990). Conversely, mGluRs regulate the resting potential of the postsynaptic cell. Since these receptors are multi-functional in that they can either increase or decrease the resting potential, they have a much wider range of effect on biochemical pathways than iGluRs. Biochemical signaling cascades and second messengers are kicked into motion after glutamate

extracellularly binds to mGluRs and causes phosphorylation of intracellular G proteins (Hansson and Ronnback 1995).

As astrocytes are the most numerous cell type in the brain, and a single cell has thousands of membranous processes that interact with numerous other cells, literally thousands of synapses can be encountered and influenced by one astrocyte (Blackburn, Sargsyan et al. 2009; Hamilton and Attwell 2010). All of the synapses encompassed by an astrocyte are commonly described as "synaptic islands," (Halassa, Fellin et al. 2007; Blackburn, Sargsyan et al. 2009). Within the control of these synaptic islands, gap junctions, and blood vessels, astrocytes are able to modulate the concentration of several different neurotransmitters (i.e. glutamate) and cations throughout the entire brain (Blackburn, Sargsyan et al. 2009). Thus, astrocytes are far more interactive than simply providing synaptic "housekeeping." Their interaction in this is so great that a new term, the tripartite synapse, has evolved. It defines a synapse not only as the pre and post synaptic endplates, but also includes the astrocytic contribution to that system (Haydon 2001; Haydon and Carmignoto 2006; Blackburn, Sargsyan et al. 2009).

While the idea that astrocytes are responsible for neurotransmitter reuptake is generally accepted, the ability of astrocytes to exocytose gliotransmitters (and the mechanism of how it is done) is currently being investigated. Gliotransmitters are similar to neurotransmitters, but are released

from astrocytes instead of neurons. Gliotransmitters will simultaneously act on both pre and postsynaptic neurons, the former of which either activates or inhibits presynaptic neurotransmitter release (Newman 2003; Barres 2008). There are three main gliotransmitters described throughout the literature: glutamate, adenosine triphosphate (ATP), and D-serine (Haydon 2001; Newman 2003; Halassa, Fellin et al. 2007; Hamilton and Attwell 2010). Other less investigated gliotransmitters include homocysteic acid, taurine, atrial natriuretic factor, and tumor necrosis factor-α (Halassa, Fellin et al. 2007). For both glutamate and D-serine, their release from the astrocytes is dependent upon the intracellular concentrations of calcium ions (the evidence for calcium dependence in ATP is currently unclear) (Halassa, Fellin et al. 2007). After an astrocyte is stimulated through either calcium influx or otherwise, the gliotransmitters each have a wide array of targets and functions (Zhang, Wang et al. 2003; Fellin, Pascual et al. 2004; Panatier, Theodosis et al. 2006). And since gap junctions connect the intracellular fluid between neighboring astrocytes, an influx of calcium in one astrocyte can cause a wave of calcium oscillations in the entire astrocyte network. These waves of calcium ions can cause synaptic activity through all connected areas.

Glutamate, depending on the context, can be described as both a neurotransmitter and a gliotransmitter. In glial cells, an influx of calcium ions results in a release of glutamate (Haydon 2001). After glutamate has been

exocytosed from the astrocytes, neurons within close proximity begin to experience slow inward currents. These neurons also experience a marked increase in excitability due to the activation of NMDA receptors, as per studies performed in the hippocampus (Haydon and Carmignoto 2006; Hamilton and Attwell 2010). Additionally, after glutamate is released, it can act on the both the mGluR and kainite receptors, thus increasing the amount of transmitter released at the presynaptic level (Halassa, Fellin et al. 2007).

ATP is another common and well known gliotransmitter. The mechanism by which ATP is released from an astrocyte has been studied in the hippocampus and is hypothesized to be due to an increase of GABA (gamma-aminobutyric acid) released from interneurons and an increased intracellular calcium concentration (Hamilton and Attwell 2010). Additionally, other studies demonstrate that phospholipase C is sufficient to evoke ATP release from glia (Haydon 2001). Once released, ATP has multiple targets that include postsynaptic P2X receptors, P2Y receptors, and presynaptic adenosine A1 receptors. After ATP interacts with the P2X receptor, it eventually leads to the AMPA receptors increasing the synaptic transmission. Conversely, ATP can cause synaptic depression when in contact with the adenosine A1 receptors (Halassa, Fellin et al. 2007). On an interesting side note, adenosine created from the release of ATP from astrocytes causes feelings of sleepiness. In genetic mice with astrocytes unable to release ATP, these mice exhibit a reduced sleep

requirement with few of the typical side effects of sleep deprivation (Hamilton and Attwell 2010).

D-serine is expressed in both astrocytes and cortical neurons; consequently there is some debate on the total contribution of D-serine as a gliotransmitter when neurons are also capable of producing this transmitter (Yang, Ge et al. 2003; Hamilton and Attwell 2010). In combination with glutamate (also released from neurons), D-serine behaves as an agonist for NMDA receptors and induces synaptic plasticity by creating a long term potentiation effect (Halassa, Fellin et al. 2007; Barres 2008; Hamilton and Attwell 2010).

As shown, astrocytes play a vital role in both neurotransmitter reuptake and gliotransmitter release, which allows for the regulation of synapses and their effects. Without this, neurons could face detrimental repercussions. Outside of maintaining synaptic homeostasis, astrocytes also provide energy and protective benefits to neurons. For example, the astrocyte-neuron lactate shuttle provides weak and depleted neurons with energy from astrocytic glycogen stores (Blackburn, Sargsyan et al. 2009). This lactate provides neurons with a temporary anaerobic energy source until proper glucose or oxygen levels can be restored, allowing neurons to remain synaptically active and viable (Magistretti 2006). Astrocytes, moreover, provide neurons protection from reactive oxygen and nitrogen species with their high concentrations of anti-oxidants. These

molecules include: vitamin E, ascorbate, and glutathione (Blackburn, Sargsyan et al. 2009).

In summary, astrocytes help to regulate a wide range of neuronal functions that include, but are not limited to, maintenance of the BBB, providing metabolic sustenance to neurons, synaptic plasticity, and neuro-protective benefits through the release of anti-oxidants.

Evidence Supporting the Concept of Astrocyte Heterogeneity

Even on a superficial level, astrocytes exhibit heterogeneity and are organized into two different groups based off of their location: the gray (protoplasmic) and white (fibrous) matter astrocytes. Additionally, the cerebellum contains its own specific type of astrocyte, Bergmann glia (Matyash and Kettenmann 2010). Traditionally, GFAP was considered to be the ultimate marker of astrocytes as a result of studies performed in injured brains where GFAP was found to be upregulated during a gliotic response. However, research has proven that in normal brains GFAP is mostly expressed in white matter astrocytes and not throughout the entire brain (Bignami, Eng et al. 1972; Eng, Ghirnikar et al. 2000; Zhang and Barres 2010).

Literature searches reveal that Aldh1L1 (aldehyde dehydrogenase) is currently considered to be the most pan-astrocytic marker in adult brains (Cahoy,

Emery et al. 2008). Yet this marker is not perfect; the molecular signatures seem to vary greatly making it difficult to have a completely pan-astrocytic marker. For example, in addition to GFAP, the A2B5 antibody only seems to stain fibrous astrocytes, and the GLAST (sodium-dependent glutamate/aspartate transporter)-CreER^{T2} mouse only labels about 30% of cortical astrocytes (Miller and Raff 1984; Mori, Tanaka et al. 2006). Different studies clearly show that a multitude of molecular markers are needed to identify mature astrocytes (Zhang, Wang et al. 2003; Bachoo, Kim et al. 2004). Current immunohistochemical markers for mature astrocytes include, but are not limited to, the following: A2B5, Aldh1L1, aldolase 3, apolipoprotein E, aquaporin 4, brain glycogen phosphorylase, connexin 30, cystatin C, fibroblast growth factor receptor 3, gap-junction channel protein 1-α, GFAP, GLAST, glutamine synthetase, Niemann Pick type C1, phospholipase A2 Group VII, S100β, and vascular cell adhesion molecule 1 (Holash, Harik et al. 1993; Patel, Suresh et al. 1999; Eng, Ghirnikar et al. 2000; Pringle, Yu et al. 2003; Bachoo, Kim et al. 2004; Mori, Tanaka et al. 2006; Slezak, Goritz et al. 2007).

The heterogeneity of astrocytes is very widespread, meaning that different populations can co-exist in the same regions of the brain, and each region has its own diverse population make up (Zhang and Barres 2010). For example, astrocytes which reside in the neurogenic niches of the adult brain have the capability to divide and proliferate, whereas astrocytes in the remainder of the

CNS rarely or never divide in normal physiological brains (Doetsch, Caille et al. 1999; Zhang and Barres 2010). Additionally, astrocytes in different regions of the adult brain express diverse surface molecules, which in turn influence their surrounding neurons (Prochiantz and Mallat 1988). There is also a marked heterogeneity in the receptors expressed by astrocytes (i.e. for glutamate and neurotransmitters) which also play a major role in the homeostasis of neuronal cell types (Hansson 1990; Hansson and Ronnback 1990; Matyash and Kettenmann 2010).

Due to the strong evidence of astrocyte heterogeneity, several different genetic mouse models have been developed to decipher the biological and functional roles of astrocytes expressing a specific transgene. While the number of genetic models has seen an increase in recent years, many more mice are going to be needed in order to fully grasp how astrocytes are the fulcrum for entire CNS biology.

Current Genetic Models Used to Study Astrocytes

While astrocytes play a crucial role in maintaining brain biology, there are relatively few genetic models available to fully investigate the impact of these cells. Of the current mouse models in the literature, very few truly study the normal behavior and phenotype of astrocytes throughout their entire lifespan;

most were mainly developed to study specific diseases and their related pathologies. The main diseases studied through the use of genetic models include the following: glioblastoma multiforme, neurofibromatosis, amyotrophic lateral sclerosis (ALS) (also known as Lou Gehrig's disease), Alzheimer's disease, Huntington's disease, Parkinson's disease, and Alexander disease (Bajenaru, Zhu et al. 2002; Lesche, Groszer et al. 2002; Maragakis and Rothstein 2006; Yamanaka, Chun et al. 2008; Marumoto, Tashiro et al. 2009). For review of transgenic technology and development, please refer to the following references: (Chan 1999; Yu and Bradley 2001; Kos 2004; Lee and Threadgill 2004; Crawley 2007; Gaveriaux-Ruff and Kieffer 2007).

Constitutive Cre mouse lines driven by the human GFAP promoter have proved to be very popular and were developed by several laboratories (Zhuo, Theis et al. 2001; Bajenaru, Zhu et al. 2002; Uhlmann, Wong et al. 2002; Garcia, Doan et al. 2004). In these mouse lines, Cre is coupled with a reporter strain (i.e. LacZ) and is driven by the expression of the transgene that has been incorporated into the genomic DNA (Zhuo, Theis et al. 2001). Transgene expression (i.e. human GFAP-Cre) permanently labels cells (through the reporter) and their progeny. In human GFAP-Cre mice, this normally occurs around embryonic day 12.5 (Zhuo, Theis et al. 2001). Due to the very strong expression of GFAP in radial glia during embryogenesis, these mice exhibit expression throughout the entire brain. This expression includes the neurons of the hippocampus when

analyzed at adult time points (Zhuo, Theis et al. 2001). This expression pattern hinders studying the normal function of adult astrocytes, as many embryonically fated astrocytes and neurons have been labeled along with the desired population (with the help of reporter lines such as Rosa26YFP or Rosa26LacZ) (Soriano 1999; Srinivas, Watanabe et al. 2001; Zhuo, Theis et al. 2001). Furthermore, using the constitutive Cre mice as mouse models for tumorigenesis (i.e. glioblastoma multiforme) is not advantageous as there are no reported cases of an embryonic glioblastoma. For an in-depth analysis and excellent reviews of Cre models, please refer to the cited journal articles (Brenner, Kisseberth et al. 1994; Brenner and Messing 1996; Messing, Head et al. 1998; Messing and Brenner 2003).

The current inducible Cre mouse models that label astrocytes are substantially limited, as they result in a very diverse expression pattern. In inducible Cre mouse models, such as CreER^{T2}, the Cre component is coupled with a mutated estrogen receptor that is activated after binding with tamoxifen (TAM). The receptor-TAM complex consequently translocates outside of the nucleus, resulting in the reporter strain labeling the cells with the transgene of interest (Indra, Warot et al. 1999). Transgenic CreER^{T2} mice that drive astrocyte specific genes include the following: GFAP, GLAST, connexin 30 (Cx30), apolipoprotein E (ApoE), aquaporin 4 (Aqp4), and fibroblast growth factor receptor 3 (Fgfr3) (Hirrlinger, Scheller et al. 2006; Mori, Tanaka et al. 2006;

Slezak, Goritz et al. 2007; Chow, Zhang et al. 2008; Young, Mitsumori et al. 2010). Both the ApoE-CreER^{T2} and Aqp4-CreER^{T2} transgenic lines had notably poor expression after recombination and were not extensively studied, but it was noted that these cells had astrocytic expression (Slezak, Goritz et al. 2007).

One of the first published astrocyte CreER^{T2} lines was the GLAST-CreER^{T2} lines created in Magdalena Gotz's lab. The original studies focused on both adult and embryonic recombination. Only trace numbers of cells, after adult recombination, colocalized with neuronal marker NeuN or oligodendrocyte markers, but several vastly colocalized with known astroglial markers (Mullen, Buck et al. 1992; Mori, Tanaka et al. 2006). The GLAST-CreER^{T2} labeled astrocytes were found throughout the white and gray matter, along with the neurogenic regions of the adult mouse brain. Within the cortex, it is estimated that only 30% of astrocytes are also GLAST-CreER^{T2} positive. Interestingly, this mouse model, GLAST-CreER^{T2}, is the only mouse model in literature that marks Müller glia of the retina in adults. After tamoxifen induction of GLAST-CreER^{T2} cells on embryonic day 12, expression was found in the lateral ganglionic eminence. Following an embryonic day 14 tamoxifen injection, the labeling was found in the dorsal and ventral telencephalon as well as the spinal cord (Mori, Tanaka et al. 2006). Additionally, Frank Pfrieger's lab also created and characterized their own GLAST-CreER^{T2} line. The characterizations of both of

the GLAST lines have been very complimentary to each other (Mori, Tanaka et al. 2006; Slezak, Goritz et al. 2007).

In reference to the GFAP-CreER^{T2} line, all cells labeled after tamoxifen injections appeared to have a glial morphology, and many colocalized with S100β; there was no detected colocalization with neuronal marker NeuN (Chow, Zhang et al. 2008). Another separate GFAP-CreER^{T2} line that also used the human GFAP gene construct had a broader expression throughout the entire brain, except the forebrain, which is not indicative of endogenous GFAP expression (Hirrlinger, Scheller et al. 2006).

Fgfr gene expression was first studied within the spinal cord both embryonically (ependymal zone) and postnatally (gray and white matter astrocytes) (Pringle, Yu et al. 2003; Hochstim, Deneen et al. 2008). Once a CreER^{T2} transgenic was generated for the Fgfr3 gene, it revealed that the induction of expression was found in both protoplasmic and fibrous astrocytes within the brain and spinal cord, as well as neural stem cells (Young, Mitsumori et al. 2010).

The Cx-30-CreER^{T2} transgenic line had similar morphologies and immunohistochemistry results previously discussed in relation to other CreER^{T2} lines. Its expression is found in the cerebellum, hypothalamus, thalamus, medulla, and cortex. Pfrieger's lab acknowledged that the GLAST-CreER^{T2} and

Cx-30-CreER^{T2} transgenic mouse models they created marked different populations of astrocytes, likely due to their inherit heterogeneous nature. They also stated that many CreER^{T2} astrocyte models are needed to fully cover the whole spectrum of astrocytes to fully understand and study astrocyte populations (Slezak, Goritz et al. 2007). Table 1.1 details the different inducible Cre mouse lines that are specific for astrocytes.

Gene Functions of Cystatin C and Phospholipase A2 Group VII

Due to an insufficiency of astrocyte specific markers, an extensive microarray screen of both *in vivo* and *in vitro* CNS samples was performed, which unearthed a wide variety of astrocyte specific markers. These genes were then assessed through a variety of bioinformatic tests to better understand their biological relevance (Bachoo, Kim et al. 2004). Cystatin C and phospholipase A2 group VII were two genes discovered in these studies.

Cystatin C

Cystatin C (CstC) is most commonly regarded as a cysteine-proteinase inhibitor with extensive expression in biological fluids, including seminal fluid, urine, serum, synovial fluids, and cerebrospinal fluid (Abrahamson, Olafsson et al. 1990). CstC is also expressed in several organs throughout the human body including the kidney, liver, pancreas, intestine, stomach, lung, placenta, and brain

(Abrahamson, Olafsson et al. 1990; Reed 2000; Newman 2002). In mouse studies, the expression of CstC exhibited in the brain parenchyma was at least five to six times higher than in other tissues examined (Huh, Hakansson et al. 1999). It is also produced in the choroid plexus, and thus displays extremely high concentrations of CstC in the cerebrospinal fluid – 5.5 times that of serum (Hasegawa, Naruse et al. 2007; Umegae, Nagai et al. 2008). The main clinical application for CstC is to act as a substitute for creatinine when measuring glomerular filtration rate. Creatinine levels can be skewed with abnormal levels of bilirubin and pyruvate, while CstC is not affected by these. CstC has a stable concentration in the blood, and in the kidney, it is filtered, but not secreted, and is fully reabsorbed and broken down in the renal tubules; this makes its serum level an accurate marker of glomerular filtration rate (Reed 2000).

On the p arm of chromosome 20, there is a gene called CS73 that encodes for CstC, a polypeptide chain of 120 amino acids with a low molecular weight (Abrahamson, Olafsson et al. 1990; Reed 2000; Newman 2002). CstC belongs to a super-family of cystatin cysteine protease inhibitors that includes 11 different members, which all act extracellularly (Abrahamson, Olafsson et al. 1990; Newman 2002). In mammals, cysteine proteases are found in lysosomes. The main cysteine protease that CstC has an effect on, as an inhibitor, is the entire family of cathepsins (i.e. cathepsins B, H, and L) (Reed 2000; Yetkin and Waltenberger 2009). Due to role of CstC in cathepsin regulation, CstC ends up

having an effect on protein catabolism, antigen presentation, and hormone processing, amongst other biological functions (Yetkin and Waltenberger 2009).

While Bachoo and colleagues found CstC expression in astrocytes through a careful screen, other previous, and much earlier, studies had already described CstC expression astrocytes (Bachoo, Kim et al. 2004). The first study that correlated CstC to astrocytes was performed in murine glial cells and found, through immunohistochemistry, that astrocytes contain and secrete CstC (Zucker-Franklin, Warfel et al. 1987). A later study performed by a separate laboratory found CstC expression mainly in the protoplasmic, gray matter astrocytes in rat, monkey, and human brain samples (Yasuhara, Hanai et al. 1993). These studies supported earlier suggestions that CstC is both created and secreted by CNS astrocytes (Yasuhara, Hanai et al. 1993).

CstC also plays a role in embryogenesis. It has been noted that on embryonic day 14 in mouse lines, there is low expression which greatly increases by E16. Additionally, when studying a cell culture APC line, AP16, treatment with transforming growth factor-β induced CstC (along with GFAP) expression and provided evidence that it is a differentiating factor for astrocytes (Kumada, Hasegawa et al. 2004). Analysis of embryonic gene expression reveals that CstC expression appears before traditional astrocyte marker GFAP and around the same time as GLAST. Another study revealed that, in the rat hippocampus, an N-

glycosylated form of CstC, induced in response to fibroblast growth factor-3 activity, resulted in neural stem cell proliferation (Taupin, Ray et al. 2000). Since an N-glycosylated form of CstC is only found in mice and rats but not in human, chick, or bovine endogenous CstC, these studies will not be discussed in depth (Hasegawa, Naruse et al. 2007). Although another study provided compelling evidence that CstC, as a neurosphere derived factor, encourages embryonic stem cells to differentiate into neural stem cells in *in vitro* studies with a conditioned medium for primary neurosphere cultures (Kato, Heike et al. 2006). All of the data taken together points towards CstC being excreted from APCs, and in an autocrine/paracrine fashion, supports astrocyte differentiation (Kumada, Hasegawa et al. 2004; Hasegawa, Naruse et al. 2007). From the studies in the literature (both those mentioned and not mentioned here), the expression of CstC makes it a prime candidate for the generation of a transgenic mouse to study astrocyte populations.

Cystatin C in Central Nervous System Pathophysiologies

CstC has been associated with numerous pathophysiologies within the CNS, most notably being hereditary cystatin C amyloid angiopathy (HCCAA) (Abrahamson, Olafsson et al. 1990). The disease causes amyloid deposits throughout multiple organ systems, in particular the brain, and results in recurring intracranial hemorrhages which usually present in afflicted individuals in their

third or fourth decade (Benson 2010). The majority of the amyloid deposits in the brain can be found in the following areas: arteries and arterioles, cerebral cortex, basal ganglia, brainstem, and cerebellum (Ghiso, Tomidokoro et al. 2010). Genetically, HCCAA is a result of an autosomal dominant point mutation in the CstC gene (Cystatin C Leu68Gln variant) and is typically found in people of Icelandic descent (Reed 2000; Nagai, Terashima et al. 2008; Benson 2010). Although the gene is mutated, it is still fully capable of acting as a cysteine protease inhibitor and inhibiting cathepsin B activity (Reed 2000). Due to the effect of the Cystatin C Leu68Gln variant mutation on amyloid deposits, an interest arose in the role of CstC in Alzheimer's disease, as Alzheimer's pathophysiology involves amyloid plaques and neurofibrillary tangles (Levy 2008). These amyloid plaques are primarily composed of amyloid-β, and it has been concluded that CstC colocalizes with amyloid-β in Alzheimer's disease (Levy, Jaskolski et al. 2006; Sundelof, Arnlov et al. 2008). Recent studies have shown that endogenous CstC will bind to amyloid-β within the brain, and overexpression of CstC in transgenic mice decreases the amount of amyloid-β found in the cerebrum (Kaeser, Herzig et al. 2007; Mi, Pawlik et al. 2007). Interpolating from this it can be hypothesized that the levels of endogenous CstC can either increase or decrease the risk of Alzheimer's disease individuals. The lower the CstC concentration, the more numerous the amyloid-β deposits and the higher risk for Alzheimer's development.

In addition to the effect that CstC has on amyloid deposits, CstC plays a role in other CNS pathophysiologies. These include stroke, epilepsy, multiple sclerosis (although evidence is not completely conclusive), and brain injury. Cysteine proteases have a role in multiple sclerosis, and several different studies have tried to correlate levels of CstC to the severity of multiple sclerosis. The results of these studies have been conflicting, finding both increased and decreased levels of CstC (Macpherson 1965; Lofberg, Grubb et al. 1980; Reed 2000). After a traumatic brain injury, there is a massive up regulation of proteases that can result in a secondary injury. A study conducted in Jonas Bergquist's lab (2009) sampled the cerebrospinal fluid in 3 patients after a traumatic brain injury, and it was revealed that there was an increased concentration of CstC to inhibit the proteases and protect against potential further injury (Hanrieder, Wetterhall et al. 2009). In a transgenic study using a CstC null mouse, there was a reduced amount of acute neuronal cell death after status epilepticus as well as global ischemia (Olsson, Nygren et al. 2004; Pirttila, Lukasiuk et al. 2005). Conversely, actual injection of CstC into the hippocampus was found to increase the amount of neuronal apoptosis within the granule cell layer of the dentate (Nagai, Ryu et al. 2002). Other CstC functions (in conjunction with cathepsins) that are not exclusive to the CNS, but are relevant, include cell migration and metastasis (Nagai, Terashima et al. 2008). Since CstC

plays such an important role in astrocyte biology, it is logical that CstC is intricately involved in several different CNS pathophysiologies.

Cystatin C Null Mice and Neurogenesis

CstC's ability to influence cancer cell migration and HCCAA made it a prime target for the development of a null transgenic mouse. The CstC null mouse when compared to control developed at the same rate and showed no histological difference when analyzed up to 6 months of age (Huh, Hakansson et al. 1999). The mice were able to procreate and produce a stable null mouse line. The one behavioral difference noted was that the null mice were hypoactive in comparison to wildtype mice with normal expression of CstC. The main focus of the initial transgenic mouse studies was not neurogenesis, but metastatic spread (Huh, Hakansson et al. 1999). A later study focused more on the role of CstC in neurogenesis and revealed that CstC null mice, at baseline, had fewer proliferating BrdU labeled cells in the subgranular zone of the dentate gyrus than their wildtype counterparts (Pirttila, Lukasiuk et al. 2005). The migration of newborn neurons in the granule cell layer of the dentate gyrus appeared to be hindered in CstC null mice. It is hypothesized that astrocytes may secrete factors that cause migration of immature neurons in the dentate gyrus. Additionally, CstC may participate in promoting neurogenesis, but it is neither the sole nor a highly dependent factor (Pirttila, Lukasiuk et al. 2005).

Phospholipase A2 Group VII

While there is considerable knowledge concerning CstC in the literature, phospholipase A2 Group VII (PLA2G7) has not been studied quite so extensively. A simple literature search will locate many scholarly journal articles detailing many different phospholipases, but the A2 Group VII isoform is not as popular. The enzymes that constitute the superfamily (comprised of 15 groups) of phospholipase A2 work by recognizing and catalyzing the sn-2 acyl bond of phospholipids and consequently releasing the fatty acids arachidonic acid and oleic acid (Six and Dennis 2000; Burke and Dennis 2009). Phospholipases were first described and recognized to occur in the brain in 1962 through the analysis of postmortem brain tissue (Gallai-Hatchard, Magee et al. 1962; Farooqui, Yang et al. 1997). The PLA2G7 isoform is also commonly referred to as platelet activating factor (PAF)-acetylhydrolase and is a 45 kDa calcium independent secreted protein (Six and Dennis 2000; McIntyre, Prescott et al. 2009). While most articles do not discuss PLA2G7 in reference to its function in the brain, it has been cited that PLA2G7 has robust expression in astrocytes of the central nervous system (Bachoo, Kim et al. 2004; Cahoy, Emery et al. 2008; McIntyre, Prescott et al. 2009). Unfortunately, the literature readily concedes that knowledge of PLA2G7 function in astrocytes is unknown (Cahoy, Emery et al. 2008).

PLA2G7 is catalytically associated with cholesterol metabolism through binding with low density lipoprotein and high density lipoprotein in the circulating blood (Burke and Dennis 2009). High levels of PLA2G7 is correlated to numerous diseases including coronary heart disease (atherosclerotic plaques contain PLA2G7), vascular inflammation, stroke, and dementia (Adibhatla and Hatcher 2008). PLA2G7 is used as marker for coronary heart disease in the clinical setting (Burke and Dennis 2009). It has been suggested that PLA2G7 plays a neuroprotective role through the removal of oxidatively damaged fatty acids (Adibhatla and Hatcher 2008).

Another known role of PLA2G7 in the CNS includes neuronal migration. As previously mentioned, PLA2G7 is also known as PAF-acetylhydrolase. In the disorder lissencephaly, the developed cortex only has 4 layers instead of the normal 6 layers of neurons. Two of the known mutations for lissencephaly are PAF and doublecortin (DCX), the latter of which is typically described as a marker for immature neurons in adult neurogenesis (Gleeson and Walsh 2000). This mutation in PAF does not allow for degradation of PAF-acetylhydrolase, which in turn leads to decreased neuronal migration (i.e. 4 neuronal layers instead of 6) (Gleeson and Walsh 2000). PAF acts as a regulator of neurodevelopment by mediating the development of the neuroblast cytoskeleton (Bix and Clark 1998; Lambert de Rouvroit and Goffinet 2001). The main known function of PLA2G7 in the cerebral cortex is through PLA2G7's regulation of PAF, which in turn

regulates neuronal migration. As PLA2G7 has strong expression in the brain tissue and recent screens have increased general interest in PLA2G7 as an astrocyte marker, it is only expected that the knowledge of PLA2G7 and its brain function will grow exponentially in the next several years.

Adult Neurogenesis in the Adult Murine Brain: Subventricular Zone and Subgranular Zone

Just as the idea that the world was flat was held as a known belief, the idea that the brain was a static organ with no new cell birth was an accepted concept and dogma. Why this idea was held on to so strongly is puzzling since many organs throughout the body knowingly and readily have new cell turnover. Nearly 50 years ago, Altman's lab, through studies with both rats and guinea pigs, provided convincing evidence that adult hippocampal neurogenesis exists (Altman and Das 1965; Altman and Das 1967). Within the past decade or two, the field of neurogenesis has exploded with many resources being dedicated to translate this research into a valuable therapeutic solution to many CNS diseases.

Mitotic Cell Labeling Techniques to Study Neural Stem Cells

The two areas of known and described neurogenesis in the adult murine brain are the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus. The field of neurogenesis has been

hindered due to few fully extensive ways to target and study the cells. Current methods used to study neural stem cells include labeling dividing cells with thymidine analog bromo-deoxyuridine (BrdU) coupled with immunohistochemistry (IHC), retroviral injection, and, most recently, genetic mouse models. Genetic mouse models used to study adult neurogenesis will be discussed more in depth later.

In 1988, a protocol for BrdU injection was created in order to label the DNA of dividing cells in S phase of mitosis in neural tissues (Miller and Nowakowski 1988). Cells labeled with BrdU and then analyzed shortly after injection highlight rapidly dividing and proliferating cells, and when studied at a later time point (such as a month), BrdU details a mature, more differentiated cell. However, BrdU does not fully label the population of cells that are largely quiescent and rarely divide. Also, after every cell cycle, the BrdU labeling sequentially dilutes and eventually, after enough cell divisions, is no longer detectable (Breunig, Arellano et al. 2007). With the use of IHC, different markers can be used to identify mitotically active cells in order to gain a better understanding of stem cell biology in both the SGZ and SVZ. Since BrdU cells can be stained with multiple other primary antibodies, it allows for identification of cell phenotype (Rakic 2002). For example, targeted cells that are analyzed shortly after injection (i.e. 2 hours) may colocalize with DCX as a neuroblast and cells analyzed at a later time point (i.e. 1 – 2 months) may colocalize with NeuN,

showing the mature differentiated cell type that the originally dividing cell became.

There are definitely drawbacks to BrdU labeling. Currently, there is not a cohesive and constant protocol for the dosage of BrdU to be given (Breunig, Arellano et al. 2007). This is important to note because different doses will label a different number of cells. Some protocols may label to saturation while others may only label some arbitrary percentage of cells in S-phase of mitosis. Additionally, too much BrdU can cause adverse cell side effects that may result in cell death or cause altered cell biology through abnormal proliferation or mutations, amongst other things (Breunig, Arellano et al. 2007).

Several different viral vectors may be injected into the brain to label a broad range of cells; these vectors include adenoviruses, lentiviruses, and retroviruses. All of the different viral vectors have the same drawback of forming a lesion where a needle injects the virus into the brain. This needle lesion causes altered biology, such as gliotic astrocytes, along the insertion tract (Cavanagh 1970). Of the three viral vectors, retroviruses prove to be the best method for targeting dividing cells (Breunig, Arellano et al. 2007). The resulting infection from an adenovirus is only transient, and both adenoviruses and lentiviruses infect a broad range of cells that are not specific to dividing cells, making them less than ideal for studying neural stem cells. Conversely, retroviruses are specific to

dividing cells (i.e. neurogenic cells) and label them permanently (Breunig, Arellano et al. 2007). The stereotaxic injection of retrovirus delivers the virus to a very specific brain location and has minimal spread throughout the tissue, thus making it even more cell type specific. Although retroviruses are the best suited for neurogenesis studies, there are still other drawbacks such as only one daughter cell being labeled. Also, there is no way to control how many cells are actually infected and labeled, making quantification incredibly difficult and lacking confidence (Noctor, Flint et al. 2001; van Praag, Schinder et al. 2002; Breunig, Arellano et al. 2007). These different cell labeling techniques in concert with IHC led the forefront of discovery into defining the cell maturation process of neural stem cells.

Subventricular Zone of the Lateral Ventricle

One area of neurogenesis that persists after sexual maturity in the rodent brain is the subventricular zone (SVZ) of the lateral ventricle. In rodent studies, the SVZ is first seen during embryogenesis, but as the animal ages and becomes an adult, only a slender remnant of the germinal layer becomes what is known as the adult SVZ (Lennington, Yang et al. 2003). The neural stem cells are flush with a single layer of ependymal cells that make actual contact with the ventricle (Doetsch, Garcia-Verdugo et al. 1999). The SVZ neurogenic system extends beyond this thin layer of neural stem cells that line the lateral ventricle. Immature

neurons, named neuroblasts, migrate out of the SVZ to the rostral migratory stream (RMS) (Lennington, Yang et al. 2003; Cleary, Uboha et al. 2006). The migrating neuroblasts in the RMS are tangentially guided by a physical network of glial tubes (Cleary, Uboha et al. 2006). A common immunohistological marker for RMS migration is polysialylated neural cell adhesion molecule (PSA-NCAM) (Gheusi, Cremer et al. 2000). Once the neuroblasts have migrated through the RMS/glial tube network, they arrive at the olfactory bulb where they continue to migrate radially and finally take residence and finish differentiating into various neuronal olfactory bulb cell types (Lennington, Yang et al. 2003; Cleary, Uboha et al. 2006). The olfactory bulb is comprised of different layers that include the glomerular layer, external plexiform layer, mitral cell layer, internal plexiform layer, and the granule cell layer (Pinching and Powell 1971; Petreanu and Alvarez-Buylla 2002).

Studies have shown that neural stem cells can be isolated throughout the entire SVZ pathway (including RMS and parts of the olfactory bulb) and are not just limited to the thin layer lining the lateral ventricle (Gritti, Bonfanti et al. 2002). The first experiments that revealed neurogenic activity within the SVZ system were cell dissection and *in vitro* studies. After treatment with growth factors (i.e. epidermal growth factor and fibroblast growth factor), these cultured cells showed immunoreactive and morphological properties (i.e. neurospheres) of neural stem cells and were able to differentiate into mature neuronal and glial

cells (Reynolds and Weiss 1992; Richards, Kilpatrick et al. 1992). Alvarez-Buylla and colleagues (using the techniques described in detail previously, or variations of said techniques) identified and named the four different cell types of the SVZ pathway: type A, type B, type C, and type E (no type D cell as of yet) (Lois and Alvarez-Buylla 1993; Doetsch, Garcia-Verdugo et al. 1997; Doetsch, Caille et al. 1999; Doetsch, Garcia-Verdugo et al. 1999; Doetsch 2003). Type A cells are considered to be the migrating neuroblasts of the SVZ are positive for PSA-NCAM (Doetsch, Garcia-Verdugo et al. 1997). Type B cells are astrocytes (which slowly proliferate) and are a source of type A cells. Additionally, the type B cells form part of the glial tubes that aid the migration of chains of type A cells through the RMS (Alvarez-Buylla and Garcia-Verdugo 2002). Rapidly dividing, neural precursor type C cells, which readily uptake thymidine analogs, are not found within the RMS (Doetsch, Garcia-Verdugo et al. 1997). Type B cells generate type C cells, which in turn become type A cells. The last cell type is the type E. Type E cells are ependymal cells that separate the actual lateral ventricle from the SVZ and have a close relationship with type B cells (Alvarez-Buylla and Garcia-Verdugo 2002). Figure 1.1 depicts the relationship of all the different cell types (A, B, C, and E) in the SVZ.

Additionally, there are several reviews that also go into more detail about the SVZ system pathway and the neurotrophic factors that regulate it (Alvarez-Buylla and Garcia-Verdugo 2002; Goldberg and Hirschi 2009; Hagg 2009;

Whitman and Greer 2009; Bath and Lee 2010). Interestingly, migration similar to that of the SVZ pathway phenomenon has been described in primates, which could eventually tie laboratory animals studies to the clinical setting (Weickert, Webster et al. 2000; Kornack and Rakic 2001; Pencea, Bingaman et al. 2001).

Subgranular Zone of the Hippocampal Dentate Gyrus

The other area of adult neurogenesis in the rodent brain is the subgranular zone (SGZ) of the dentate gyrus. Like the neural stem cells that reside in the SVZ, the neural stem cells in the SGZ follow a maturation path that leads them to become neurons that are fully functional and integrate with existing neural circuits. The way these two neurogenic niches accomplish this though seems to be regulated and controlled by different processes, and it is necessary to study each niche separately to fully understand adult neurogenesis.

Neurogenesis in the SGZ of the hippocampus has been studied in many different mammalian species, most notably rodents. Across species, there is a great degree of inconsistency in the robustness of neurogenic activity. The amount of neurogenesis found in primates and bats is relatively low, but is very active in rodents (Eriksson, Perfilieva et al. 1998; Amrein and Lipp 2009). In human patients who suffer from either a stress-related disease or depression, their hippocampus is smaller than controls, postulating a lower basal neurogenic rate in the hippocampus (as observed in post-mortem histological samples) (Lee and Son

2009). It is important to note that even within rodents (i.e. laboratory rats and mice), there is a great degree of variability in neurogenesis (even from strain to strain), and because of that, the focus of this text is on mouse hippocampal neurogenesis, unless otherwise noted.

There is a great deal of interest in adult SGZ neurogenesis because it is believed that the SGZ and the hippocampus play an integral role in learning and memory. Some theorize that the role of SGZ neurogenesis is low in learning and memory since the amount of neurogenesis found in the hippocampus is relatively low (Amrein and Lipp 2009). But the overwhelming belief in the scientific community is that these cells do play an extremely active role, as shown in mouse behavioral studies (Schwegler, Crusio et al. 1988; Gerlai 2001; Bucan and Abel 2002; Zhang, Zou et al. 2008; Lee and Son 2009). Understanding the mechanisms that control and allow for neurogenesis at the basal level is important, as well as ways to alter this normal biology in order to study and understand CNS pathophysiology and potential therapeutic remedies. The following upregulate SGZ neurogenic activity: anti-depressants, exercise, learning, "enriched environment" (i.e. novel objects in the cage), hormones (such as estrogen), ischemic events, and seizure just to name a few (Kempermann, Kuhn et al. 1997; Tanapat, Hastings et al. 1999; van Praag, Christie et al. 1999; Malberg, Eisch et al. 2000; Jin, Minami et al. 2001; Shors, Miesegaes et al. 2001; Eisch and Nestler 2002; Pirttila, Lukasiuk et al. 2005). Conversely, there are

several factors that also suppress neurogenesis including: aging, stress (and related diseases such as depression), and drug abuse (Gould, Tanapat et al. 1998; Cameron and McKay 1999; Eisch and Nestler 2002).

Cell morphology and expression of certain immunohistological markers is currently how neurogenesis in the SGZ is defined. A brief introductory summary of hippocampal neurogenesis starts with mitotically active/proliferating cells with a triangular cell shape and an apical process that evolve into immature neurons. These either die or eventually differentiate into mature neurons and become incorpated into the granule layer of the dentate gyrus. These neurons interact with the CA3 region along the mossy fiber pathway and get input from the entorhinal cortex (Ming and Song 2005). The time from the initial proliferation of the neuroprogenitor to the mature neuronal cell is approximately a month, and mature neurons take anywhere from 4-7 weeks to become fully functional in the neuronal circuit (Jessberger and Kempermann 2003).

A more detailed view of the neurogenic activity that occurs in the region that seperates the hilus and the granule cell layer of the dentate gyrus is described using cell morphologies and specific markers . There is not a standardized classification of each cell type during the neuronal maturation process, so throughout the description, there will be alternative names included in parentheses (Seri, Garcia-Verdugo et al. 2001; Kempermann, Jessberger et al. 2004; Seri,

Garcia-Verdugo et al. 2004; Encinas, Vaahtokari et al. 2006; Encinas and Enikolopov 2008; Ihrie and Alvarez-Buylla 2008). For reference, figure 1.2 provides a visual representation of the maturation cascade.

The neurogeneic cascade starts with the type 1 cell (SGL type B cells, quiescent neural progenitors, radial astrocytes), which is a cell that morphologically is similar to radial glia and has a triangular soma that sits in the SGZ. The type I cell also projects an apical process through the granule cell layer and is topped with a tuft of extensive arborization (Ehninger and Kempermann 2008). The traditional molecular markers for these type 1 cells are GFAP and nestin. It has been reported that out of the total nestin expressing population of cells in the SGZ, type 1 cells compose about two-thirds of that population (Kempermann, Jessberger et al. 2004). Astrocytes that lack the strong apical process have been described in the SGZ and are called horizontal astrocytes, but as of yet, the neurogenic potential of this subset of astrocytes has not been unearthed (Ihrie and Alvarez-Buylla 2008).

Type 1 cells, as stem cells, give way to type II progenitors (type D1, amplyfying neural progenitors). Type II cells, which may be considered early progenitors, are divided into two subtypes (type IIa and type IIb), which are both proliferative and morphologically plump. The type II cells lie underneath the granule cell layer and have short, tangential proceses, but differ in expressed

antigenic markers (Kempermann, Jessberger et al. 2004; Encinas, Vaahtokari et al. 2006). Type IIa cells express nestin, as do type IIb, but type IIb are also DCX positive (Kempermann, Jessberger et al. 2004).

The last stage of mitotically active cells are the late progenitor, neuroblast type III cells (type D2, neuroblasts type-1). Type III cell nuclei are no longer plump and irregularly shaped, but are more rounded. The cell type itself is completely negative for nestin, but positive for DCX as well as PSA-NCAM. The maturation of type II to type III cells has been described as a "continuum" with overlapping characteristics, but they are distinct subsets of cells that offer differing reponses to stimuli (Kempermann, Jessberger et al. 2004).

Post-mitotically, the type III cells become an immature granule cell/neuron (type D3, neuroblasts type-2, immature neurons). The rounded nuclei of the type III cell begins to send out apical dendrites and axons in preparation of becoming fully functional (Encinas and Enikolopov 2008). The immature granule cell has strong expression of DCX, PSA-NCAM, and NeuN, with transient expression of calretinin (Kempermann, Jessberger et al. 2004; Encinas, Vaahtokari et al. 2006). These cells stay as immature granule cells anywhere from 10-15 days or 2-3 weeks (depending on the source) before becoming fully mature granule cell neurons that no longer express any calretinin, but do stably

express both calbindin and NeuN (Kempermann, Jessberger et al. 2004; Encinas, Vaahtokari et al. 2006).

The mature granule neurons have extensive dendritic branching that reaches through the granule cell layer, and the actual granule cell neurons have taken residence inside the granule cell layer (no longer in the subgranular zone) (Encinas, Vaahtokari et al. 2006). Postmitotic mature granule cell neurons are the last stop in the maturation process of the SGZ; these cells make functional contacts within in the hippocampal circuitry and play an active proposed role in learning and memory.

Unfortunately, the current understanding and model of SGZ neurogenesis treats all neural progenitor cells as a very homogenous population of cells that march through a very regimented maturation cascade. In actuality, the population of neural stem cells are most likely an extremely heterogenous population that requires more investigation to fully understand and grasp the biology of this compartment.

Current Models of Mammalian Adult Neurogenesis

While the use of thymidine analogs, retroviral labeling, and IHC analysis has allowed for the discovery and description of the neurogenic niches in the adult

mammalian brain, they definitely have their drawbacks. Utilizing mouse models to label and track specific cells (especially in inducible systems) gives a more controlled study for neurogenesis. IHC maturation studies identified the different cell makers (i.e. nestin, GFAP, DCX) that could be potential genes for mouse models. Two popular genes that inspired the creation of mouse models (especially the inducible CreER^{T2} system) are GFAP and nestin. For this text, models discussed will focus on SGZ neurogenesis.

Knock-Out Mouse Models

Several labs have tried to create knock-out (KO) mouse models (loss of a specific gene expression) to study adult neurogenesis, but most of these mice did not survive to adulthood (Filipkowski, Kiryk et al. 2005). "Genetic models to study adult neurogenesis," is an excellent review that outlines many relevant knock-out mouse models (Filipkowski, Kiryk et al. 2005). Two genetic knock-out mouse models were successful in completely reducing neurogenesis in adult mice: cyclin D2 KO mice and orphan nuclear receptor/*tailless* (Tlx) KO mice (Kowalczyk, Filipkowski et al. 2004; Shi, Chichung Lie et al. 2004). Cyclin D2 KO mice showed that cyclin D2 is a crucial component of adult neurogenesis in the SGZ, and KO mice have few newborn neurons in the SGZ. The entire brain morphology also changes, especially in areas of active adult neurogenesis, such as a smaller hippocampus, olfactory bulb, cerebellum, and sensory cortex

(Kowalczyk, Filipkowski et al. 2004; Filipkowski, Kiryk et al. 2005). Tlx KO mice also exhibit smaller CNS structures (olfactory bulb, dentate gyrus), but a larger lateral ventricle (Shi, Chichung Lie et al. 2004). Interestingly, GFAP KO mice showed that GFAP is not necessary for adult neurogenesis, and functional neural stem cells still reside in the neurogenic niches (Messing and Brenner 2003; Morshead and van der Kooy 2004). While KO mice can provide important and novel information, they can completely alter the CNS biology thus limiting their capabilities in studying the functions and biology of adult neural stem cells.

Cre Mouse Models

There are two main types of Cre mice: the constitutive Cre and the inducible Cre mouse. In constitutive Cre mouse models, Cre expression begins as soon as the promoter for the gene of interest is turned on, whereas in inducible Cre mice (i.e. $CreER^{T2}$), the expression can be turned on at any time point after induction (i.e. injection with tamoxifen). Cells with activated Cre expression permanently express Cre, as do any of their progeny (Indra, Warot et al. 1999; Hirrlinger, Scheller et al. 2006). As type-1 cells in the SGZ are defined as GFAP positive, GFAP-Cre mice were used to try to study adult neurogenesis. The caveat with using GFAP-Cre mice for adult studies is that GFAP expressing radial glia experience Cre activation during development; observation of Cre expression in the adult SGZ is muddled because of the expression remnants from

development. Several labs have generated their own Nestin-Cre mice, but the focus of many of these studies is on embryonic development (Zimmerman, Parr et al. 1994; Isaka, Ishibashi et al. 1999; Tronche, Kellendonk et al. 1999; Dubois, Hofmann et al. 2006). (Another mouse model, nestin-GFP, exhibited expression in the neurogenic niches in the adult, specifically in the dentate gyrus as type 1 cells (Mignone, Kukekov et al. 2004)). While some studies using either the GFAP-Cre or Nestin-Cre were performed in adult mice, the majority of discernable information is found in embryonic development and not the adult due to early activation of the Cre that persists through adulthood, making it difficult to describe what is embryonically fated versus an adult stem cell.

To get around this problem, inducible Cre systems (such as CreER^{T2} or CreER^{TM}) were created. Current genes that have been used for inducible Cre systems include nestin, GFAP, GLAST, and Fgfr3 (Mori, Tanaka et al. 2006; Lagace, Whitman et al. 2007; Chow, Zhang et al. 2008; Chen, Kwon et al. 2009; Young, Mitsumori et al. 2010). Interestingly, three of the four genes just listed (GFAP, GLAST, Fgfr3) were also discussed in the section concerning astrocyte mouse models, suggesting that astrocytes play a very essential role in neurogenic niches.

There have been several nestin driven inducible Cre mouse models developed (even at the same academic institution) (Balordi and Fishell 2007;

Battiste, Helms et al. 2007; Burns, Ayoub et al. 2007; Lagace, Whitman et al. 2007; Chen, Kwon et al. 2009). One difference noticed in different Nestin-CreER^{T2} mouse models is the amount of expression in different brain locations. For example, Parada's group showed that their Nestin-CreER^{T2} mice demonstrated expression in the cerebellum whereas the mouse generated in the Eisch lab did not (Lagace, Whitman et al. 2007; Chen, Kwon et al. 2009). The initial paper describing the mouse from the Parada lab focused on expression in the SVZ. Conversely, Eisch and colleagues mainly described expression and dynamics in the SGZ neurogenic niche. The neural stem cells marked by the Eisch nestin-CreER^{T2} mouse model mature into NeuN positive adult neurons that integrate into the granule cell layer of the dentate gyrus, and it's important to note that it is uncommon for any nestin-CreER^{T2} cells to become astrocytes (Lagace, Whitman et al. 2007). Nestin-CreER^{T2} animals coupled with the inducible knockout for Notch experimentally showed that Notch was responsible for maintaining neurogenesis in nestin positive SGZ cells at both the basal level and an increased exercise-induced level (Ables, Decarolis et al. 2010). While that is a very brief summary of the findings from that study, it is of importance to note that these inducible Cre lines can be used for more than just cell fate tracking.

In the GLAST-CreER^{T2} mouse line, expression was studied in the SVZ along with the RMS and olfactory bulb, but the main concentration of the original article focuses on the role of GLAST-CreER^{T2} cells as radial glia during

embryogenesis. A later article provided evidence for GLAST cells to also contribute mature neurons to the granule cell layer of the dentate gyrus (Ninkovic, Mori et al. 2007). The most recent inducible Cre mouse discussed in literature is driven by the Fgfr3 transgene, and again, the neural stem cells studied were in the SVZ pathway (Young, Mitsumori et al. 2010).

While there are several different inducible Cre transgenic lines that can be induced at adult time points to study the dynamics of neurogenesis, more transgenes need to be studied to fully understand the heterogeneous nature of the adult stem cell compartment and how different neural stem cells interact with each other. Although the dynamics of the SVZ and SGZ are very different (resulting in a laboratory to typically focus on one niche), it would be interesting to study role of Fgfr3 and other specific markers in both the SVZ and SGZ to gain a better understanding of their functions.

Evidence for Astrocytes as the Origins of Neural Stem Cells

Growing volumes of evidence have emerged establishing that astrocytes may be the true origins of neural stem cells in the adult brain (SVZ and SGZ). As was just previously discussed, many of the current inducible Cre mouse models used to study adult neurogenesis are also present in populations of mature astrocytes throughout the entire brain (i.e. GLAST and Fgfr3 transgenes) (Mori,

Tanaka et al. 2006; Young, Mitsumori et al. 2010). While it has been hypothesized that a different subset or class of astrocytes are the neural stem cells, it should be noted that astrocytes that act as both neural stem cells and mature astrocytes in other parts of the brain have similar electrophysiological signatures (Zhang and Barres 2010). The more likely explanation is that astrocytes respond to local environmental cues that are found in these neurogenic niches such as secreted factors and vasculature (Lim and Alvarez-Buylla 1999; Palmer, Willhoite et al. 2000; Song, Stevens et al. 2002).

Arturo Alvarez-Buylla's lab has performed anti-mitotic studies utilizing cytosine-β-D-arabinofuranoside (AraC) in order to research the *in vivo* dynamics of both the SVZ and the SGZ (Doetsch, Caille et al. 1999; Doetsch, Garcia-Verdugo et al. 1999; Seri, Garcia-Verdugo et al. 2001). From these studies it was inferred that astrocytes were the primary precursor for all of adult neurogenesis.

In the SGZ, AraC was used to decimate the neurogenic activity, and BrdU was utilized to determine the neurogenic precursor. As early as 2 days after AraC treatment ceased, there were BrdU labeled cells in the SGZ, and these cells were concluded to be astrocytes (by both morphology and GFAP labeling) that gave rise to migrating neuroblasts and eventually differentiated neurons (Seri, Garcia-Verdugo et al. 2001). Similar studies have been performed in the SVZ system, and again, astrocytes were found to be the neuronal precursors (Doetsch, Caille et

al. 1999). In the context of the SGZ, another experiment utilized a retrovirus driven by the GFAP promoter to label neural stem cells. The GFAP retrovirus was stereotactically injected into the dentate gyrus, and after 8 days, some infected astrocytes had the characteristics of immature neurons which later were tracked to mature granule cell neurons (Seri, Garcia-Verdugo et al. 2001). *In vivo* studies have provided convincing evidence that astrocytes are the true neural stem cells within the neurogenic niches of the adult brain.

Other *in vitro* studies compliment the *in vivo* findings that astrocytes either are adult neural stem cells or play a very important and crucial role to the maintenance of the neural stem cell pool. First, it seems that astrocytes expressing GFAP (in neurogenic locations) have differing neurogenic potential when dissected and cultured. GFAP positive astrocytes removed from the spinal cord do not act as neural stem cells in culture, but ones from the hippocampus do (Lim and Alvarez-Buylla 1999; Song, Stevens et al. 2002). While there are many hypotheses for how astrocytes control the neurogenic niches, such as secreted factors, actual cell contact with astrocytes may be an accurate one (Horner and Palmer 2003). Astrocytes may possess specific cell surface makers that propagate neurogenesis, postulated from the fact that even dead astrocytes in culture, when making cellular contact, promote neurogenic activity (Song, Stevens et al. 2002).

Since astrocytes are extremely heterogeneous, astrocytes in different regions may express different cell markers based off the factors in that particular niche, or astrocytes that are born/originate from varying parts of the brain may have different expression patterns. Astrocytes differ in their neurogenic activity depending on both brain locale and the vasculature of the region. It is well documented that astrocytes play a very important role in maintaining and interacting with the vasculature in the CNS (reviewed earlier in this text; i.e. astrocyte endfeet wrapping around vessels), and other studies have found neural stem cells to closely interact with the capillaries of the vasculature (Palmer, Willhoite et al. 2000; Jin, Zhu et al. 2002; van Praag, Schinder et al. 2002; Kempermann, Gast et al. 2003; Shen, Wang et al. 2008; Tavazoie, Van der Veken et al. 2008). Clusters of cells with neurogenic potential reside in tandem with the vasculature in both the SVZ and SGZ and, in rodents, vascular endothelial growth factor (VEGF) influences neural stem cells (increased concentration relates to increased neurogenesis) (Horner and Palmer 2003).

One current problem for analyzing and understanding the dynamics of astrocytes in the stem cell compartment is a lack of markers to distinguish astrocytes that can be classified as neural stem cells and fully mature astrocytes in other parts of the brain such as the cortex. Currently, many GFAP positive astrocytes can be considered neural stem cells, but there are also GFAP positive astrocytes that are have absolutely no neurogenic potential (i.e. spinal cord

astrocytes as far as current science understanding can describe neural stem cells). Once novel markers (or other distinguishing factors) are discovered that are specific for astrocytes (functioning as neural stem cells), the field of adult neurogenesis will flourish.

In conclusion, astrocytes and adult neural stem cells are two areas of neuroscience research that have emerged to the forefront in recent times. As more evidence emerges, it is becoming abundantly clear that astrocytes do play a very vital role in not only embryonic development, but also adult neurogenesis in both the subventricular zone of the lateral ventricle and the subgranular zone of the hippocampal dentate gyrus. My doctorial research, discussed in this thesis, focused on utilizing Cystatin C-CreER^{T2} and phospholipase A2 Group VII-CreER^{T2} inducible mouse models to not only investigate normal astrocyte biology and function, but also the intricate roles these two genes play in adult neurogenesis.

TABLE 1.1

Inducible Cre Transgene	Apolipoprotein E	Aquaporin 4	Connexin 30	Fibroblast Growth Factor Receptor 3	Glial Fibrillary Acidic Protein	Sodium-Dependent Glutamate/Aspartate Transporter
Abbreviation	ApoE	Aqp4	Cx30	Fgfr3	GFAP	GLAST
Astrocyte Expression	Yes Overall poor transgenic expression	Yes Overall poor transgenic expression	Yes	Yes Gray and white matter astrocytes	Yes	Yes Gray and white matter astrocytes and Müller glia in the retina.
GFAP and S100β Colocalization	Not Analyzed	Not Analyzed	Yes	GFAP – Not extensive S100β – Yes	Yes	Yes
NeuN Colocalization	Not Analyzed	Not Analyzed	None	None	None	Rare Cells
Neurogenic Activity	Not Analyzed	Not Analyzed	Not Analyzed	SGZ – Not Analyzed SVZ – Yes	SGZ – Yes SVZ – Yes	SGZ – Yes SVZ – Yes
Reference	(Slezak, Goritz et al. 2007)	(Slezak, Goritz et al. 2007)	(Slezak, Goritz et al. 2007)	(Young, Mitsumori et al. 2010)	(Chow, Zhang et al. 2008)	(Mori, Tanaka et al. 2006) (Ninkovic, Mori et al. 2007) (Slezak, Goritz et al. 2007)

Table 1.1: Inducible Cre Mice for Studying Astrocyte Biology

Table 1.1 outlines the different inducible Cre mice (i.e. CreER^{T2}) that are currently available to study and analyze astrocyte biology in mouse brains. These different mouse models have varying degrees of expression in different regions in the brain and can be used in combination to get a better overall view of normal astrocyte function and location. Additionally, some of these mice exhibit expression also found in the neurogenic compartments of the adult mouse brain, providing further insight into the dynamics of that compartment.

FIGURE 1.1

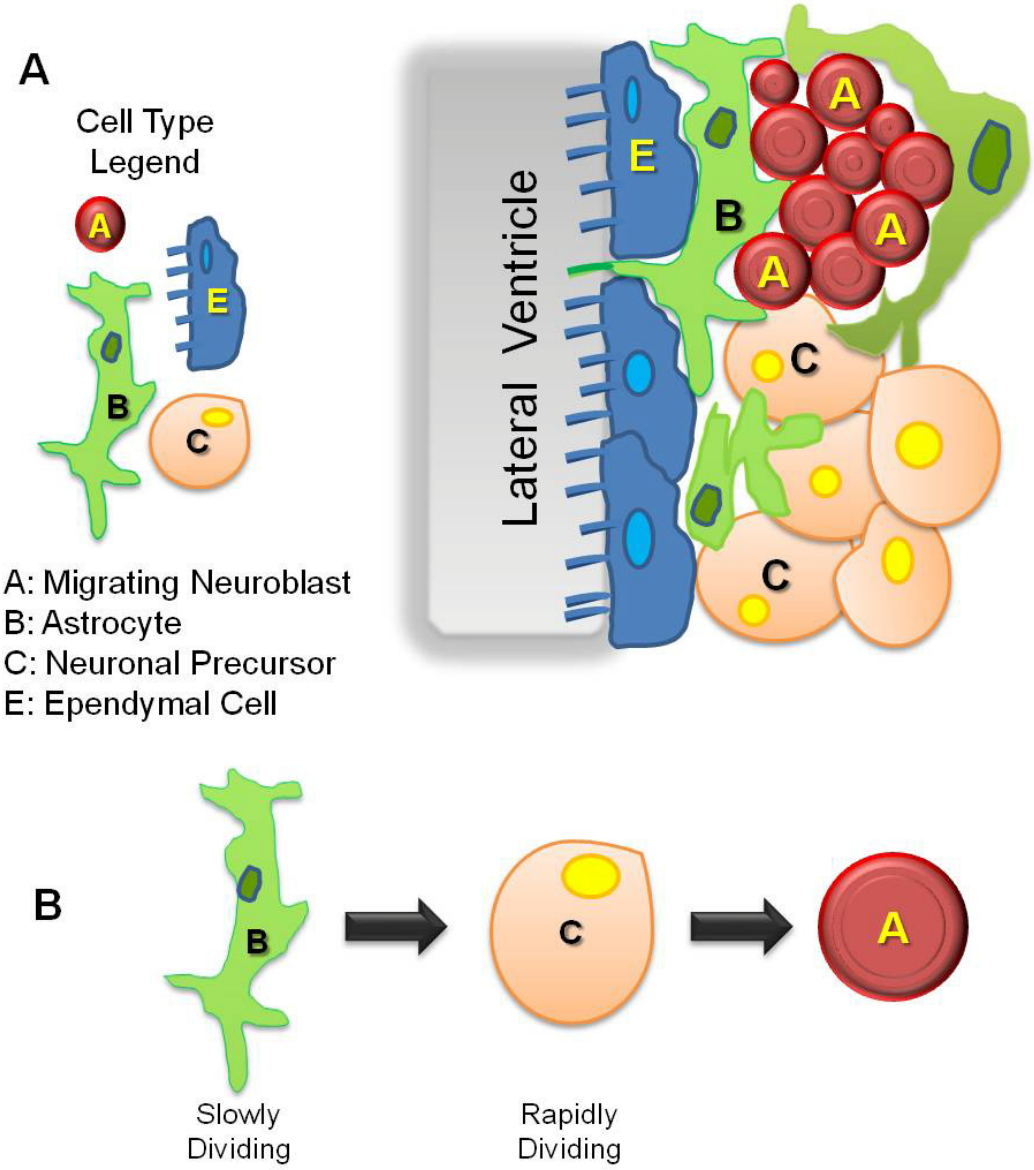
A
Cell Type
Legend
A
E
B
C
A: Migrating Neuroblast
B: Astrocyte
C: Neuronal Precursor
E: Ependymal Cell
Lateral Ventricle
E
B
A
A
A
C
C
B
B
C
A
Slowly
Dividing
Rapidly
Dividing

Figure 1.1: Four different cell types reside in the subventricular zone.

There are at least four different types of cells that reside in the SVZ. The type E cell is an ependymal cell that lines the cavity of the lateral ventricle (A). Type B cells are slowly dividing astrocytes that generate type C cells and make contact with type E cells and the wall of the lateral ventricles (A, B). Type B cells can further be distinguished as type B1 (touching the type E cells) and type B2 (not in contact with type E cells). Type C cells, which are very mitotically active, give rise to type A cells (B) and are arranged in clusters that interact with chains of type A cells (A). These type A cells are immature neurons that migrate in chains (with the help of type B cells) through the RMS to the olfactory bulb, where they will differentiate into interneurons (Doetsch, Caille et al. 1999; Lim and Alvarez-Buylla 1999).

FIGURE 1.2

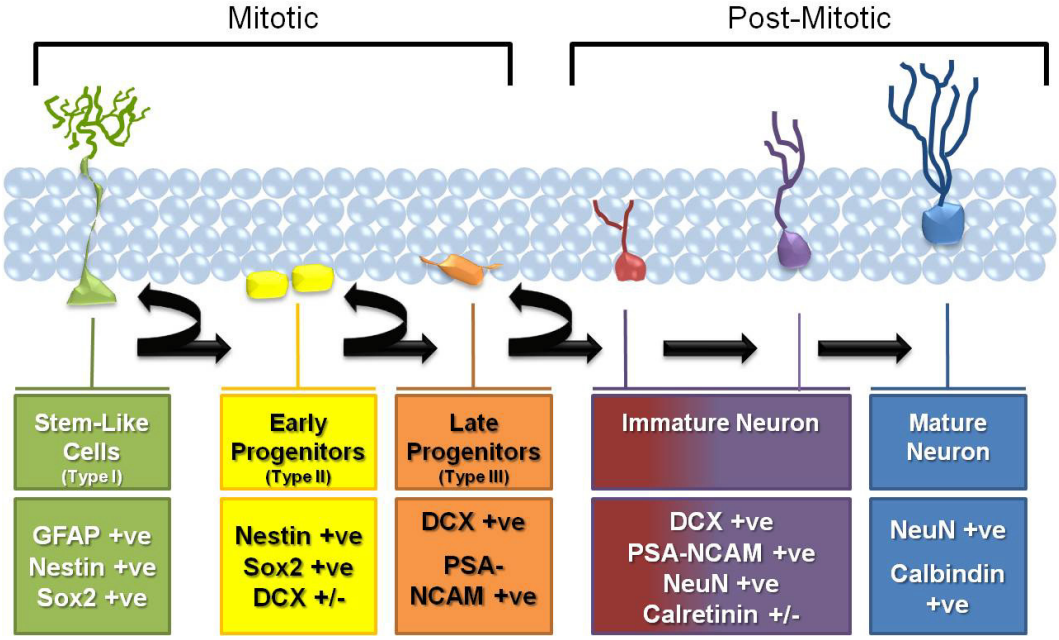
Mitotic
Post-Mitotic
Stem-Like Cells (Type I)
GFAP +ve Nestin +ve Sox2 +ve
Early Progenitors (Type II)
Nestin +ve Sox2 +ve DCX +/-
Late Progenitors (Type III)
DCX +ve PSA-NCAM +ve
Immature Neuron
DCX +ve PSA-NCAM +ve NeuN +ve Calretinin +/-
Mature Neuron
NeuN +ve Calbindin +ve

Figure 1.2: Neural stem cells in the subgranular zone of the dentate gyrus undergo a neurogenesis maturation cascade.

The current model for adult hippocampal neurogenic niche is that neural stem cells progress through stages of maturation before becoming a fully integrated and mature granule cell neuron. The type I stem-like cells share many features with astrocytes such as GFAP expression, but never express astrocytic marker S100β. The putative neural stem cells which express nestin and Sox2, in addition to GFAP, become early progenitors, losing their GFAP expression and gaining DCX expression in some cells. Early progenitors lose all early stem cell markers (nestin and Sox2) as they become late progenitors, picking up PSA-NCAM expression. DCX and PSA-NCAM are classic markers for neuroblasts. The type III late progenitors are the last mitotically active cell in the SGZ neurogenesis maturation cascade. Immature neuronal cells gain mature neuronal markers such as NeuN and transiently express calretinin. Fully mature granule cells that integrate into the granule cell layer finally shed all their neuroblast characteristics and transient calretinin expression. These cells are marked by calbindin and NeuN (Kempermann, Jessberger et al. 2004; Encinas and Enikolopov 2008).

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CHAPTER TWO

CYSTATIN C AND PHOSPHOLIPASE A2 GROUP VII IDENTIFY NOVEL MATURE ASTROCYTE MARKERS IN THE SPINAL CORD AND BRAIN IN BOTH EMBRYONIC AND ADULT TIME POINTS

ABSTRACT

Due to the speculated heterogeneous and abundant nature of astrocytes in the central nervous system, several markers are consequently needed to understand their proposed diversity. Current astrocyte markers and models do not reveal the full extent of the role that astrocytes play in both embryonic development and neurogenesis. This study describes two novel astrocyte tamoxifen inducible CreER^{T2} transgenic mouse lines, CstC-CreER^{T2} and PLA2G7-CreER^{T2}, that label different populations of morphologically spongiform cells without colabeling with oligodendrocyte or neuronal markers in embryonic and adult animals. These two genes were discovered from previous work that studied the astrocyte transcriptome and identified several candidates. In both lines, adult recombination shows mature astrocyte populations throughout the entire brain, including, but not limited to, the cortex, brainstem, and cerebellum. Embryonic recombination, as early as e12.5, labels both radial glia marked by BLBP and mature astrocytes in both the spinal cord and the brain.

Additionally, PLA2G7-CreER^{T2} cells marked at embryonic day 12.5 and studied at P28 show neurogenic expression in the subventricular zone. The expression of each line in both embryonic and adult animals will allow for studies not only in normal astrocyte biology, but also the pathogenesis of disease.

INTRODUCTION

There are three main cell types in the adult mammalian brain: astrocytes, oligodendrocytes, and neurons. Of these three cell types, astrocytes are the most abundant, but until recent times, the most ignored, cell type. Many in the field felt that astrocytes merely played a supportive role for neuron homeostasis and had minimal dynamic capabilities of their own. As interest in astrocytes peaked, so did the need for practical protocols to study and analyze them. Currently, inducible Cre mice (CreER^{T2}) prove to be an exceptionally valuable tool to analyze astrocytes at all ages and stages during the life of the mouse. While there are currently a few viable astrocyte inducible Cre lines (Cx30, Fgfr3, GFAP, GLAST), considerably more are needed to fully understand the inherent heterogeneous biology of astrocytes (Hirrlinger, Scheller et al. 2006; Mori, Tanaka et al. 2006; Slezak, Goritz et al. 2007; Chow, Zhang et al. 2008). A study comparing the Cx30-CreER^{T2} to the GLAST-CreER^{T2} mouse line indicated that these two populations were similar but distinct (Slezak, Goritz et al. 2007). To

complement the already published astrocyte-specific CreER^{T2} mouse models, new mouse lines were generated from the transgenes for cystatin C (CstC) and phospholipase A2 group VII (PLA2G7).

Astrocytes in the developing and mature mammalian brain play a variety of roles that prove critical for the normal, basal function of the central nervous system (CNS). These roles and functions include regulation of the CNS vasculature, neurotransmitter reuptake and release, glutamate concentration homeostasis, and synaptogenesis, to name a few. Currently, adult neurogenesis is a research topic in neuroscience that is extremely popular. One of the most interesting proposed roles of mature astrocytes is that they are the true neural stem cell in both the subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricle (Doetsch, Caille et al. 1999; Seri, Garcia-Verdugo et al. 2001). Radial glia, a form of astrocyte precursors, are responsible for the guidance of neurons in the developing brain and the birth of mature glia. As such, they have a critical role in neurodevelopment and maintenance of the adult neurogenic niches. Due to these functions, it was pivotal for this study to identify new astrocyte-specific inducible Cre lines and describe their expression and roles throughout the entire CNS.

Here, I describe the expression of both the PLA2G7-CreER^{T2} and CstC-CreER^{T2} mouse lines in combination with the Rosa26YFP reporter line (Srinivas,

Watanabe et al. 2001). These mice were generated by utilizing transgenes derived from BACs (bacterial artificial chromosomes) in Ron DePinho's lab at the Dana Farber Institute in Boston after a careful screen of potential astrocyte-specific genes (Bachoo, Kim et al. 2004). The expression of both of these lines were analyzed throughout development (embryogenesis and early postnatal) as well as in fully mature adult animals. Recombination in both CreER^{T2} mouse lines targeted a wide variety of astrocytes (i.e. fibrous, protoplasmic, Bergmann, neural stem cells). Results from these studies show that both CstC-CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP prove to be viable astrocyte-specific inducible Cre mouse models.

MATERIALS AND METHODS

Animal Studies

All transgenic animals were housed in a pathogen-free, climate controlled facility at The University of Texas Southwestern Medical Center at Dallas. Animal studies and procedures were approved by the Institutional Animal Care and Use Committee (APN: 2009-0373) at UT Southwestern. The reporter strain, Rosa26YFP, was generously provided by Dr. Amelia Eisch's lab and originally described by Dr. Frank Costantini's Lab (Srinivas, Watanabe et al. 2001). The CreER^{T2} lines were generated and provided by Dr. Ron DePinho's laboratory at

the Dana Farber Institute in Boston based off the initial findings by Bachoo and colleagues (Bachoo, Kim et al. 2004). The CreER^{T2} construct contains a mutated estrogen receptor that is activated by tamoxifen, but not endogenous estrogens (Indra, Warot et al. 1999).

Genotyping

Animals had their tails clipped at 10 days of age for genotyping. Tails were digested in 100 µl DirectPCR lysis buffer (Viagen, Catalog Number: 102-T) with 1 µl proteinase K (AMRESCO, catalog number: E195) at 55ºC overnight and then denatured at 85ºC for 1 hour. Tail DNA was used for genotyping both CreER^{T2} and Rosa26YFP genes. For CreER^{T2} genotyping, the following forward and reverse primers were used: Forward: 5' – ATT TGC CTG CAT TAC CGG TC - 3'; Reverse: 5' – ATC AAC GTT TTC TTT TCG G - 3'. The following are primers for Rosa26YFP genotyping: Primer 1: AAA GTC GCT CTG AGT TGT TAT; Primer 2: GCG AAG AGT TTG TCC TCA ACC; Primer 3: GGA GCG GGA GAA ATG GAT ATG. Using Invitrogen Taq Polymerase (Catalog Number: 18038-042), the following conditions were applied for both the CreER^{T2} and Rosa26YFP PCR reactions with an initialization step of 94ºC for 1 minute, followed by a denaturing step at 94ºC for 30 seconds, an annealing step of 53ºC for 30 seconds, and an elongation step at 72ºC for 1 minute. Each of these steps was repeated 36 times. Last, there was a final elongation step performed at 72ºC

for 10 minutes and a final holding step at 4ºC. Positive CreER^{T2} animals produced a band at 350 bp, and the CreER^{T2} animals were kept as heterozygotes. Rosa26YFP genotyping had product sizes of 310 bp, with the wildtype being 560 bp. Rosa26YFP animals could either be heterogeneous (having both bands) or homogenous (310 bp only) and still observe expression after tamoxifen induced recombination.

Tamoxifen Inducible CreER^{T2} Recombination

For adult studies, transgenic CreER^{T2}/Rosa26YFP animals were treated with 5 consecutive doses of 180 mg/kg of tamoxifen (TAM) through intraperitoneal injections (IP). Tamoxifen (Sigma; catalog number T5648-5G) for the injection was made by weighing out 30 mg of TAM into an 1.65 ml eppendorf tube and adding 100 µl of 100% high grade ethyl alcohol (PHARMCO-AAPER, catalog number: 111ACS200) that was followed by vortexing. Next, 900 µl sunflower seed oil (Sigma; catalog number S5007) was added, vortexed, and then sonicated for 10 minutes. After sonication, the tamoxifen was vortexed again to confirm that all the tamoxifen had dissolved. Tamoxifen is exceptionally light sensitive, and therefore was covered with aluminum foil, and was only viable for 1 week at 4ºC.

For embryonic time points, breeding mothers were monitored daily for sperm plugs. Once plugged, mothers were placed in their own individual cages,

and that day was counted as embryonic day 0.5. Pregnant females were injected with a single dose of 3.0 mg. Early postnatal pups (P0 and P4) were injected with a single injection of 1.5 mg subcutaneously on their dorsum. Older animals, P14, were injected with 3 consecutive doses of 180 mg/kg of tamoxifen.

Tissue Processing

Animals were subjected to an intraperitoneal injection with 5-Bromo-2'-deoxyuridine (BrdU) (Sigma, Catalog Number: B9285) at a dose of 50 mg/kg both 4 hours and 2 hours before sacrifice by transcardial perfusion. Before transcardial perfusion, mice were deeply anesthetized with avertin (through IP injection) with a weight-based dose. The perfusion procedure did not begin until a lack of reflexes was observed when pressing on the foot pad of the mouse. Mice were transcardially perfused with 10 mL of ice cold phosphate buffered saline (PBS) pH 7.4 followed by 10 mL of 4% of paraformaldehyde (PFA) (Sigma, catalog number: 158127) in PBS pH 7.4. Brains and spinal cords were dissected and post-fixed in 4% PFA overnight at 4ºC. Spinal cords were dissected out of the vertebral column under a Leica MZ6 dissecting microscope. After post-fixation, tissue was washed in PBS three times and then placed in 30% sucrose (Fisher, catalog number: S2-500) in PBS with 0.1% sodium azide (Sigma, catalog number: S8032) for a period of either 48 hours or until the brain or spinal cords sank. Sections were cut on a Leica CM3050S Cryostat at 30 μm sagittal

sections, and sections were kept in PBS with 0.1% sodium azide at 4°C. Brains that were used for quantification were cut in 30 µm coronal sections on a Leica SM2000R microtome in series of 12.

Immunofluorescence for CreER^{T2} Characterization

Thirty micron sagittal sections were mounted on plus-charged slides (Fisher, catalog number: 12-550-15) and allowed to dry overnight at room temperature. Using a pap pen (Fisher, catalog number: 23-769-300), a barrier was drawn around the borders of the tissue on the slide. Endogenous peroxidases were quenched using 0.3% hydrogen peroxide (Sigma, catalog number: H1009) diluted in tris buffered saline (TBS) for 30 minutes at room temperature. Tissue was then blocked using 3% normal donkey serum (Jackson Immuno Research, catalog number: 017-000-121) with 0.3% Triton X100 (Sigma, catalog number: X100) for 1 hour and then using the primary antibody diluted in 3% normal donkey serum with 0.3% Tween 20 (BioRad, catalog number: 170-6531) overnight at room temperature. The following antibodies and dilutions were used: chicken anti-green fluorescent protein (GFP) at 1:10000 (Aves, catalog number: GFP1020), rabbit anti-glial fibrillary acidic protein (GFAP) at 1:500 (DAKO, catalog number: Z0334), mouse anti-NeuN at 1:50 (Millipore, catalog number: LV1457494), rabbit anti-Olig2 at 1:200 (Chemicon, catalog number: LV1359426), rabbit anti-S100β at 1:300 (DAKO, A5110), rat anti-mouse CD31

at 1:50 (BD Pharmingen, catalog number: 550274), and rabbit anti-K67 at 1:200 (Abcam, catalog number: ab15580). Slides stained for chicken anti-GFP needed amplification and, therefore, a biotinylated donkey anti-chicken secondary antibody (Jackson Immuno Research, catalog number: 703-065-155) was diluted 1:200 in 1.5% normal donkey serum diluted in TBS and incubated at room temperature for one hour. Avidin-biotin complex (Vector Laboratories, catalog number: PK-6100) was made 30 minutes before application by diluting 20 µl of avidin in 960 µl of TBS and vortexing, then adding 20µl of biotin and vortexing again. The avidin-biotin complex was applied to the tissue for 60-90 minutes at room temperature after washing the biotinylated-secondary antibodies off the slides 3 times with TBS. After the avidin-biotin complex incubation was complete, tyramide (which provides color) was applied for 10 minutes. Either Fluorescein (Perkin Elmer, catalog number: STA701) or Cyanine 3 (Perkin Elmer, catalog number: STA704A) can be used for tyramide signal amplification. For slides that had double labeling, primary antibodies, other than the chicken anti-GFP, had a four hour incubation at room temperature with the appropriate species and fluorophore Alexa Fluor secondary antibody (Invitrogen).

Characterization of CreER^{T2} Lines

After the staining of sections on slides, imaging was performed using a DM5500B Leica Upright microscope and Leica's Advanced Fluorescence imaging software (Leica Microsystems Inc., Wetzlar, Germany).

Immunofluorescence for Colocalization Quantification

Colocalization quantification for both CstC-CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP (stained for with GFP) was calculated with the following markers: CstC, and GFAP. One series of every 12th section that were cut at 30 μm on a Leica SM2000R microtome were placed in a well of a 12-well plate and washed in 1X PBS with 0.02% sodium azide for 10 minutes. Sections were then placed in PBS with 0.3% Triton X and 0.02% sodium azide and either goat anti-Doublecortin antibody from Santa Cruz (catalog number: sc-8066) diluted 1:1000, rabbit anti-CstC antibody from Abcam (catalog number: ab33487) diluted 1:5000, or rabbit anti-GFAP antibody from DAKO (catalog number: Z0334) diluted 1:500, and all sections were stained with chicken anti-GFP from Aves (catalog number: GFP1020) diluted 1:5000. The primary antibody was incubated for 48 hours at room temperature (free floating sections). Sections were then washed with PBS with 0.02% sodium azide 3 times at 10 minutes each. Sections were placed in PBS with 0.02% sodium azide and with the appropriate secondary antibody: donkey anti-goat (catalog number: 705-165-147) or donkey

anti-rabbit (catalog number: 711-165-152) Cy3 secondary antibody from Jackson Labs diluted 1:300 for donkey anti-goat and 1:500 for donkey anti-chicken (catalog number: 703-225-155) Cy2 secondary antibody from Jackson Labs. Sections were incubated in the secondary antibody for 48 hours. After the secondary antibody incubation, sections were incubated with 4'-6-diamidino-2-phenylindole, dihydrocholoride (DAPI) (Invitrogen, catalog number: D1306) for 10 minutes free floating at 1:5000 dilution. Sections were washed 3 times for 10 minutes each in PBS with 0.02% sodium azide. After the washes, sections were finally mounted on 1% gelatin coated slides and then coverslipped with lab-made, glycerol based, fluorescence mounting media.

Colocalization Quantification

After immunofluorescence staining for quantification, Z-stacks for every 12^{th} section were compiled using a 10x objective lens on a Leica DM5500B upright microscope in conjunction with Leica Advanced Fluorescence imaging software. Z-stacks were saved as Leica files (.lif) and then opened using Imaris software (Bitplane Scientific Software AG, Zurich Switzerland) for quantification. Via volume reconstruction (from the Z-stacks) and thresholding (parameter defined as: "exclusion of intensity pairs that exhibit no correlation"), colocalization of CstC and GFAP with either CstC-CreER^{T2}/Rosa26YFP or PLA2G7-CreER^{T2}/Rosa26YFP, positive cells (stained for with chicken anti-GFP

antibody) were calculated. This quantification procedure using the Imaris software was developed by Costes & Lockett at the NIH, NCI/SAIC.

Statistics

GraphPad Prism software (version 5.00 for Windows, GraphPad Software, San Diego California USA) was used to calculate means and SEM (standard error of the mean). All values are shown as means $\pm$ SEM.

RESULTS

Cystatin C-CreER^{T2} and Phospholipase A2 Group VII-CreER^{T2} Transgenic Animals Label Mature Astrocytes in the Adult Murine Brain

Data collected previously through an unbiased study that utilized microarray data coupled with biological filters and bioinformatics unearthed both cystatin C (CstC) and phospholipase A2 Group VII (PLA2G7) as novel markers for mature astrocytes (Bachoo, Kim et al. 2004). Based on this information, two inducible transgenic mouse models were developed using BAC clones which contained the control modulatory region (CRM) for both CstC and PLA2G7 (Hoggatt, Simon et al. 2002). A section of the CRM (about 10 kilobase pairs) was subcloned into a plasmid vector that contained a promoterless Cre recombinase. These mice were generated through microinjection of the BAC clone (including

the CRM) by Dr. Ron DePinho's laboratory. These inducible Cre lines utilize the CreER^{T2} construct that has a mutated estrogen receptor that is activated by tamoxifen.

Both cystatin C-CreER^{T2}/Rosa26YFP (CstC-CreER^{T2}/Rosa26YFP) and phospholipase A2 Group VII-CreER^{T2}/Rosa26YFP (PLA2G7-CreER^{T2}/Rosa26YFP) lines demonstrated no significant difference in expression between male and female animals.

Genetic analysis of these two lines through the Rosa26YFP reporter shows that both lines have abundant expression throughout the entire adult murine brain (Figure 1). The CNS represented the majority of CreER^{T2} expression in both astrocyte specific lines, with minimal expression outside of the CNS. When compared to the traditional astrocyte marker, GFAP (glial fibrillary acidic protein), the expression is clearly more abundant. GFAP expression appears to be restricted to the pia, white matter tracts, and neurogenic regions (Figure 1C), which clearly does not represent the 80% of the brain that astrocytes are theorized to comprise (Nedergaard, Ransom et al. 2003). When compared to markers for the other two cell types in the brain, neurons (NeuN) and oligodendrocytes (Olig2), both the CstC-CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP astrocyte populations are clearly distinct (Figure 2). S100β, which marks not only astrocytes, but also oligodendrocytes, has some overlap with both CstC-

CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP (Figure 2 C and G). The results depicted in Figure 2 do not contradict the astrocyte-Cre-ER^{T2} lines already described and published: Cx30-CreER^{T2}, Fgfr3-CreER^{T2}, GFAP-CreER^{T2} and GLAST-CreER^{T2} (Mori, Tanaka et al. 2006; Slezak, Goritz et al. 2007; Chow, Zhang et al. 2008; Young, Mitsumori et al. 2010). The fidelity of CstC-CreER^{T2}/Rosa26YFP was measured through colocalization with a CstC polyclonal antibody. Quantification results for CstC-CreER^{T2}/Rosa26YFP cells and CstC polyclonal antibody are 51.94$\pm$8.70% in the internal granular layer of the cortex and 57.73$\pm$21.08% in the dentate gyrus (Figure 2.1 D). For PLA2G7-CreER^{T2}/Rosa26YFP, the colocaliztion in the cortex is 43.81$\pm$8.86% and 42.01$\pm$8.08% within the dentate gyrus. Expression of the CstC-CreER^{T2}/Rosa26YFP cells was more abundant than endogenous CstC labeled by the polyclonal antibody. The difference in the colocalization numbers could be due to a variety of factors such as the polyclonal antibody for CstC not being highly sensitive or expression of the CstC transgene not having 100% fidelity.

The fluffy, spongiform morphology is a common feature of both CstC-CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP astrocytes throughout the entire brain (Figure 2.2). The common conception that astrocytes are simply a stellate structure is based upon imaging of GFAP-positive astrocytes. Unfortunately, GFAP only allows for the visualization of the cytoskeleton but not the entire astrocyte including the cytoplasm (like both of the CreER^{T2}/Rosa26YFP

lines) (Nedergaard, Ransom et al. 2003). The visualization of the spongiform morphology shows that astrocytes occupy their own individual, non-overlapping microdomains (Figure 2.3 A and B). While astrocyte populations are extremely heterogeneous (i.e. a single astrocyte can be labeled by a variety of different markers), only one astrocyte can occupy a particular space and volume within the brain (Bushong, Martone et al. 2002). Within these microdomains, astrocytes play a very supportive role to the NeuN positive neurons in the adult cortex, and an individual astrocyte volume will encompass 4 to 5 NeuN positive neurons. In Figure 2.3 C, PLA2G7-CreER^{T2}/Rosa26YFP astrocytes are given as an example. Literature has cited that astrocytes can encompass up to 100 neurons with their fine processes (Hamilton and Attwell 2010). While visualization of both CstC-CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP astrocytes did not support the anatomical construct of a single astrocyte coming in contact with up to a 100 neurons, the Rosa26YFP reporter may not be able to fully fill all of the processes in a fashion that allows for the visualization of the full extent of the arborization of astrocytes. In addition to the supportive role astrocytes provide to NeuN positive neurons, they also play a very intimate role in the regulation of blood vessels (Gordon, Mulligan et al. 2007). The foot processes of astrocytes have been shown to wrap around the blood vessels that were stained with CD31 (Figure 2.3 D). This interaction with the blood vessels is of note since there is documented evidence that, in the neurogenic niches of the adult brain, the

interaction of astrocytes with the vasculature creates an environment permissive for dividing neuronal precursors (Horner and Palmer 2003).

Astrocytes in the Adult Neurogenic Regions: Subventricular Zone (SVZ) and Subgranular Zone (SGZ)

Since the expression of GFAP is very limited outside of the areas of neurogenesis and white matter tracts, colocalization expression of both CstC-CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP was studied in the SGZ and SVZ, as well as the quantification of the amount of colocalization in the SGZ (Figure 2.4). Within the SGZ for both transgenic lines, GFAP only represents a small fraction of stem-like cells. The total amount of GFAP that colocalizes with the CstC-CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP is 32.83 $\pm$ 8.55% and 32.83 $\pm$ 8.83% respectively (Figure 2.4 C and F respectively). Additionally, GFAP positive cells were found in both horizontal and vertical CstC-CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP cells in the SGZ of the dentate gyrus. According to the literature, neurogenic GFAP positive cells have only a vertical morphology, and a role for astrocytes with the horizontal morphology has yet to be discovered (Ihrie and Alvarez-Buylla 2008). The GFAP positive astrocytes with the radial/vertical morphology in the SGZ are termed “type I” cells (Ihrie and Alvarez-Buylla 2008).

The astrocyte populations within the neurogenic niches are not only mitotically active, but also going through the neuronal maturation process. Ki67, a marker of actively dividing cells, is present in both the SGZ and SVZ and experiences colocalization with both transgenic astrocyte markers. Not all of the Ki67 positive cells are colabeled with either CstC-CreER^{T2}/Rosa26YFP or PLA2G7-CreER^{T2}/Rosa26YFP, meaning that some transgenic astrocytes are no longer mitotically active (Figure 2.5 E, F, G, H), and/or the neural stem cell population is very heterogeneous and not just labeled by one or two markers that mark a homogenous cell population. As these cells mature from a Ki67 positive cell, they begin to express doublecortin (DCX), which marks immature neurons (Figure 2.5 A, B, C, D).

Examining the SVZ neurogenic pathway in more detail, neural stem cells leave the subventricular zone of the lateral ventricle where they travel through the rostral migratory stream (RMS) as immature neurons (neuroblasts) and then finally arrive in the olfactory bulb where they differentiate into neurons (Cleary, Uboha et al. 2006). Figure 2.6 depicts PLA2G7-CreER^{T2}/Rosa26YFP labeled cells in all parts of the SVZ pathway. The neural precursor cells in the SVZ have been described as GFAP positive astrocytes and, in Figure 2.6 A, there are PLA2G7-CreER^{T2}/Rosa26YFP cells lining the SVZ that have GFAP processes radiating out from their cell bodies (Doetsch, Caille et al. 1999). These PLA2G7-CreER^{T2}/Rosa26YFP neural stem cells also express Ki67 in the SVZ (Figure 2.5

H) and travel throughout the RMS as neuroblasts (Figure 2.6 B). Once these immature neurons reach the olfactory bulb, they migrate out radially to the different layers of the olfactory bulb where they differentiate into either mature astrocytes or interneurons (Figure 2.6 C and D). Interestingly, some of the PLA2G7-CreER^{T2} labeled cells colocalized with GFAP while others did not, again demonstrating the heterogeneity of astrocyte populations (Figure 2.6 D). The PLA2G7-CreER^{T2}/Rosa26YFP interneurons in the olfactory bulb were stained with NeuN, calretinin, calbindin, PSA-NCAM (polysialylated neural cell adhesion molecule), DCX, and tyrosine hydroxylase, but the PLA2G7-CreER^{T2} labeled cells did not colocalize with any of them. Either these cells are part of an interneuron class that were not stained for, or they represent their own unique cell type in the olfactory bulb that has not been previously described.

Embryonic and Early Postnatal Expression

Studies of induced recombination were not limited to adult time points only; both inducible astrocyte transgenic lines also underwent tamoxifen induced recombination at both embryonic and early postnatal time points. In PLA2G7-CreER^{T2}/Rosa26YFP animals, embryonic expression induced on day e12.5 and sacrificed on e14.5 demonstrated expression of immature glia (i.e. radial glia) in the spinal cord (Figure 2.8 A). Surprisingly, at this early stage, there is already limited expression of morphologically mature astrocytes in the spinal cord.

Interestingly, by P28, these PLA2G7-CreER^{T2}/Rosa26YFP cells have spread throughout the entire spinal cord in both white and gray matter. Traveling up through the neuroaxis towards the brain, PLA2G7-CreER^{T2} cells with radial glia morphology completely line the floor of the fourth ventricle (Figure 2.8 D). Expression in the spinal cord increases significantly when animals were injected at e15.5 and sacrificed 48 hours after tamoxifen injection (on e17.5) (Figure 2.8 C).

CstC-CreER^{T2}/Rosa26YFP has very robust expression when induced at post natal day 4 and 14 and sacrificed for analysis on P28 (Figure 2.7). Within the neurogenic regions, CstC-CreER^{T2}/Rosa26YFP labeled cells at both P4 and P14 are still mitotically active at P28. The cortex, for both time points, is chockfull of large spongiform astrocytes. The PLA2G7-CreER^{T2}/Rosa26YFP expression is similar to the CstC-CreER^{T2}/Rosa26YFP expression, but not as robust, especially in the cortex (Figure 2.7). The lesser expression is not completely unexpected as the PLA2G7-CreER^{T2} expression is less than CstC-CreER^{T2} expression in animals treated at P28 and analyzed at various time points after adult recombination (Figure 2.1). In the areas of neurogenesis in PLA2G7-CreER^{T2}/Rosa26YFP animals treated at e12.5 and observed at P28, there are no cells in the SGZ, but there are remnants of cells in the SVZ, along with cells in the rostral migratory stream (RMS) and mixed neurons and astrocytes in the olfactory bulb (Figure 2.9). Cells recombined at birth (P0) and studied at adult

time point P28, show an abundance of cells in both the SGZ and SVZ, and the same applies for P4 and P14 time points (Figure 2.9). All of the cells in the SGZ and SVZ show colocalization with DCX. Animals induced with tamoxifen at later time points have progressively more astrocytes in the areas surrounding the neurogenic regions and throughout the entire brain.

Early Post-Natal Neurogenesis in the SGZ of PLA2G7-CreER^{T2}/Rosa26YFP Animals

Development of the granule cell layer of the hippocampal dentate gyrus continues until about 2 weeks after the birth of the animal (Tanapat, Galea et al. 1998). The PLA2G7-CreER^{T2}/Rosa26YFP cells labeled at both P0 and P4 contribute to the development of the dentate gyrus of the hippocampus (Figure 2.10). These cells develop into NeuN positive neurons that are sprinkled throughout the entire dentate gyrus and are not restricted to just the inner third granule cell layer. Additionally, these cells labeled at P4 are still NeuN positive cells when the animal is 3 months of age (Figure 2.10 C).

DISCUSSION

Considering there are a few existing inducible astrocyte-specific CreER^{T2} models already in literature, more models are needed for a better insight into true

astrocyte biology. Astrocytes have become a "recent star" in neuroscience research, warranting more resources and time to be devoted to them. As such, more models are needed to truly understand their heterogeneous nature (Matyash and Kettenmann 2010). A few roles of astrocytes that were researched and discussed within this text include astrocyte microdomains, relationships with cortical neurons, interactions with the vasculature, and their expression in development and adult neurogenesis.

Both CstC-CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP tamoxifen inducible CreER^{T2} mouse models represent a diverse population of cells that are astrocyte and neural stem cell specific. There was no colocalization with neuronal or oligodendrocyte markers anywhere in the brain with a special emphasis on the cortex, hippocampus, and cerebellum. In the cortex, for example, the CstC-CreER^{T2}/Rosa26YFP positive astrocytes fill the entire cortex in distinct, non-overlapping microdomains (Figure 2.3 C) (Bushong, Martone et al. 2002). These astrocyte domains encompass neurons and provide homeostatic support. Being able to selectively ablate these astrocytes could provide extremely powerful data concerning the nature of these astrocyte microdomains and how astrocytes respond to the death of a neighbor. One such way to accomplish this would be through a deleter strain such as the Rosa26LacZ Diphtheria Toxin Fragment A mouse line (Brockschnieder, Lappe-Siefke et al. 2004). In the cerebellum for the PLA2G7-CreER^{T2} mouse, there was detection of an extremely

strong concentration of PLA2G7-CreER^{T2}/Rosa26YFP astrocytes serving as Bergmann glia (Figure 2.1 B). CstC-CreER^{T2}/Rosa26YFP astrocytes also comprise part of the Bergmann glia population of the cerebellum, although not as extensively (Figure 2.1 A). Such strong Bergmann glia expression makes both of these tamoxifen inducible Cre mouse models potentially intriguing models for pediatric pilocytic astrocytomas. PLA2G7-CreER^{T2}/Rosa26YFP mice could be used in combination with a floxed Braf gene (most common oncogene in pilocytic astrocytomas) and induced at postnatal time points in hopes of creating a mouse model (Dubuc, Northcott et al. 2010).

In addition to being great models for adult astrocytes, both CstC-CreER^{T2} and PLA2G7-CreER^{T2} represent populations of neural stem cells in both the neurogenic niches of the subgranular zone and subventricular zone. Utilization of both transgenic mouse lines could be used in tandem to uncover the proposed heterogeneity of the adult neural stem populations (Kempermann, Jessberger et al. 2004). Both transgenic lines show mitotic potential through Ki67 colocalization and then progress through the neuronal maturation cascade (i.e. from GFAP positive to DCX positive) that has been proposed through several studies (Figures 2.4 and 2.5) (Kempermann, Jessberger et al. 2004; Bordey 2006; Encinas and Enikolopov 2008). The PLA2G7-CreER^{T2} mouse model would be excellent for studying the dynamics of the SVZ. Plentiful numbers of PLA2G7-CreER^{T2}/Rosa26YFP cells line the lateral ventricle (Figure 2.5 D and H) and

migrate out through the RMS and fill the olfactory bulb with interneurons (Figure 2.6). As Alvarez-Buylla and colleagues described astrocytes as the true neural stem cell in the SVZ, studying PLA2G7-CreER^{T2} cells would allow for a deeper understanding into the full role of astrocytes in that context (Doetsch, Caille et al. 1999). For example, are some of these astrocytes situated for niche support? How do some PLA2G7-CreER^{T2} neural stem cells ultimately result in interneurons and others astrocytes? Do some PLA2G7-CreER^{T2} cells that are originally found lining the lateral ventricle end up comprising part of the glial tube network of the RMS? A popular way to study the SVZ compartment is to ablate the neural stem cell population through anti-mitotic drugs (such as AraC). Additionally, many studies have illustrated neural stem cells residing along the vasculature found in the neurogenic niches (Goldberg and Hirschi 2009). The cells marked by the astrocyte-specific models introduced in this text have a tight relationship with blood vessels (as shown through CD31 staining in Figure 2.3 D) in the neurogenic regions as well as throughout the brain.

While the PLA2G7-CreER^{T2}/Rosa26YFP cells have expression in the developing embryo, in the spinal cord and brain, the early postnatal (P0 and P4) expression and contribution of PLA2G7-CreER^{T2}/Rosa26YFP cells is extremely interesting. Animals injected with TAM at P0 showed weak to moderate astrocyte expression throughout the entire brain when sacrificed and analyzed at P3 (data not shown). This expression, by P28, becomes widespread and abundant

in all areas especially the cortex, brainstem, and neurogenic areas. Most strikingly, the PLA2G7-CreER^{T2}/Rosa26YFP positive cells contribute to the development of the granule cell layer of the hippocampal dentate gyrus (Figure 2.10). One interesting possibility to further study the contribution of PLA2G7-CreER^{T2} labeled cells in dentate gyrus would be to give injections to mouse pups over several days (i.e. P0 thru P7). This would open a window into the role of PLA2G7-CreER^{T2} labeled cells throughout development of the granule layer during the early postnatal time, instead of just a snapshot of one day (i.e. P0 or P4). Additionally, a study demonstrates that pups of mothers who voluntarily exercise during pregnancy and lactation have greater postnatal neurogenesis and larger granule cell layer (Bick-Sander, Steiner et al. 2006). When analyzed at P36, the granule cell layer was reported to be up to 40% larger than those of controls (Bick-Sander, Steiner et al. 2006). It would be intriguing to allow dams pregnant with PLA2G7-CreER^{T2}/Rosa26YFP progeny to have voluntary access to a running wheel throughout pregnancy and lactation and analyze the difference in proliferative capabilities in running and control groups.

In summary, these two novel astrocyte-specific tamoxifen inducible Cre lines, CstC-CreER^{T2} and PLA2G7-CreER^{T2}, are complementary to the astrocyte-specific lines already in literature, but also provide insight into new, diverse populations of astrocytes. More evidence continues to emerge indicating traditional astrocyte markers are not sufficient for study, and that the population

of astrocytes are very diverse morphologically, molecularly, functionally, etc. Several markers and transgenic lines are necessary for a full understanding of the role astrocytes play in the CNS and its associated pathophysiologies and functions.

FIGURE 2.1

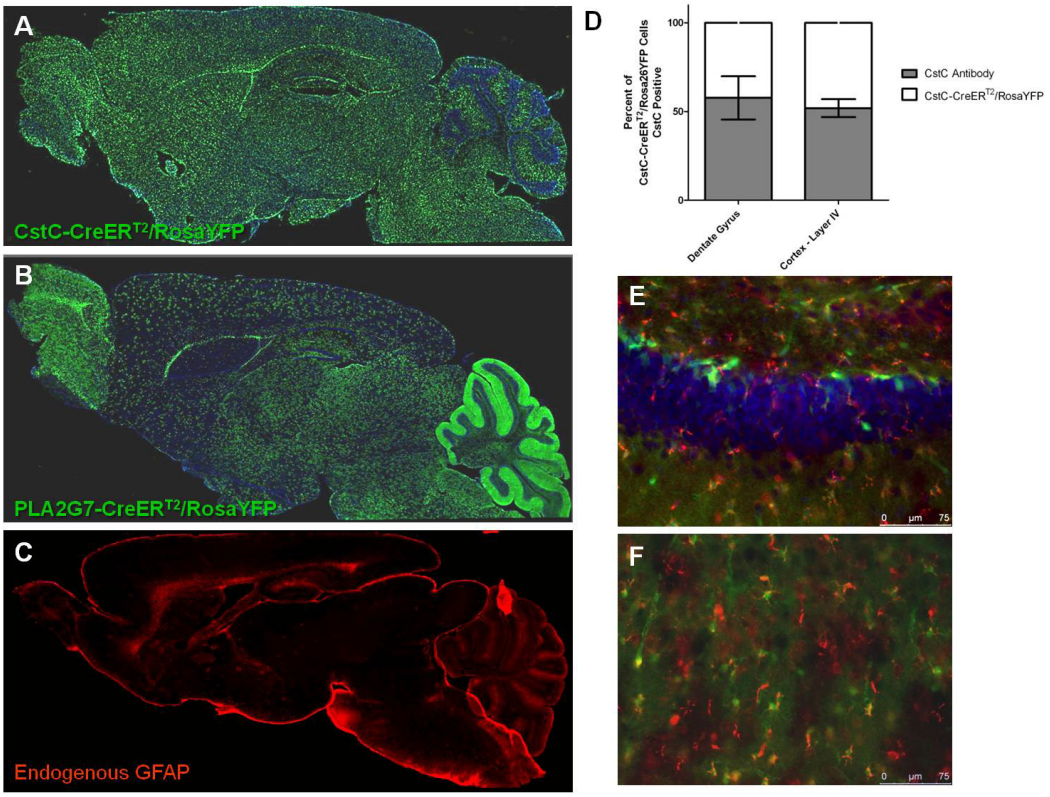

Figure 2.1: CstC-CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP label mature astrocytes in the adult murine brain.

Whole brain expression of transgenic CstC-CreER^{T2}/Rosa26YFP (A) and PLA2G7-CreER^{T2}/Rosa26YFP (B) cells in mice treated with tamoxifen at P28 and then sacrificed 1 month later show mature astrocyte expression throughout the entire brain. When compared to endogenous expression of traditional astrocyte marker, GFAP, (C) both transgenic lines show significantly more expression. Quantification of endogenous cystatin C expression (through polyclonal antibody) shows 51.94$\pm$8.70% in the internal granular layer of the cortex (layer IV) and 57.73$\pm$21.08% in the dentate gyrus for CstC-CreER^{T2}/Rosa26YFP transgenic cells (D). E and F show CstC-CreER^{T2}/Rosa26YFP expression in green and CstC polyclonal antibody in red in the subgranular zone of the dentate gyrus (with DAPI, blue) and layer IV of the cortex (outlines of these areas are shown in A).

FIGURE 2.2

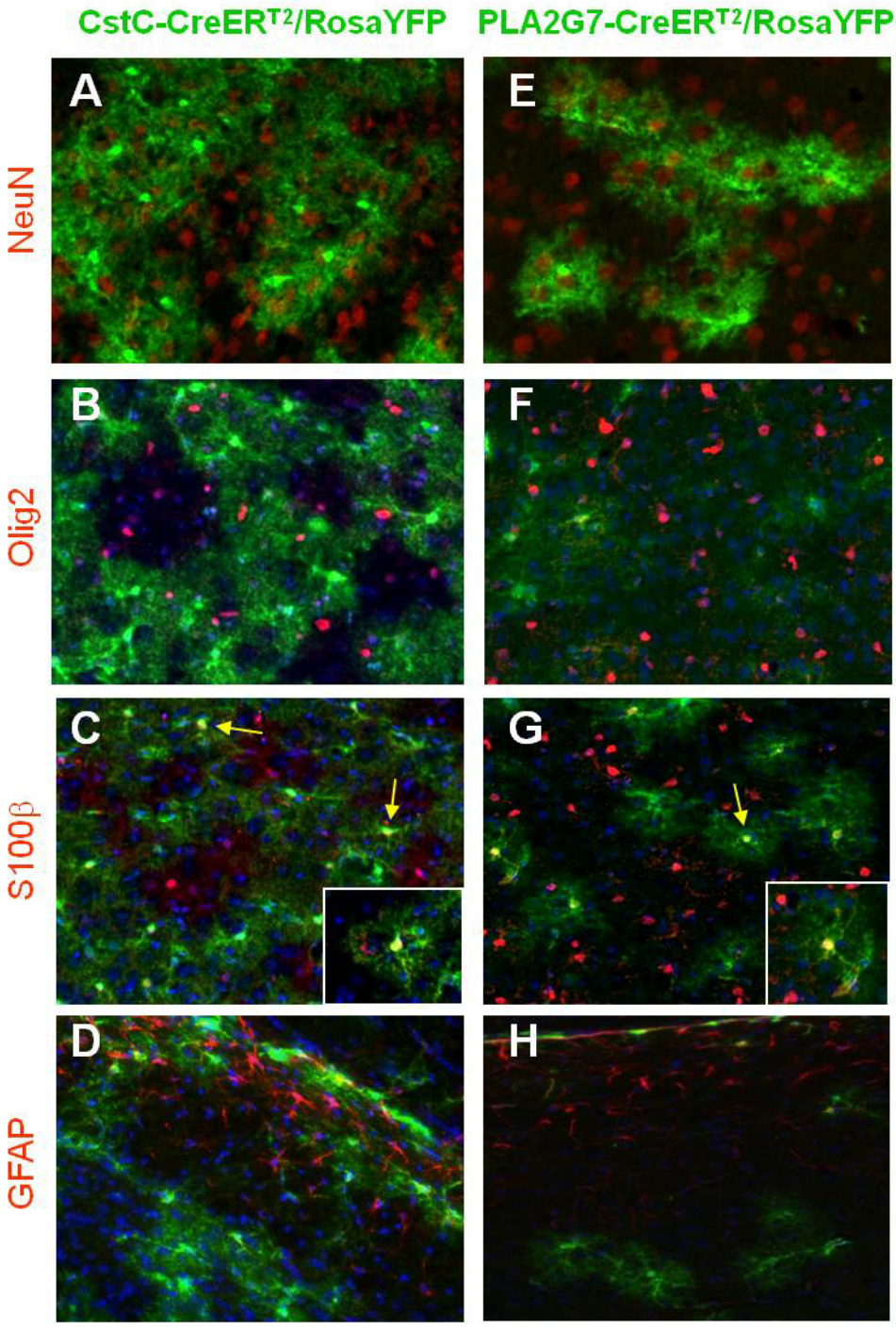
CstC-CreER^{T2}/RosaYFP
PLA2G7-CreER^{T2}/RosaYFP
NeuN
Olig2
S100β
GFAP
A
B
C
D
E
F
G
H

Figure 2.2: Inducible recombination outside of the neurogenic areas is astrocyte specific for both CstC-CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP.

For both transgenic lines, CstC-CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP, expression is astrocyte specific, but more extensive than traditional markers GFAP (shown in white matter tract of the corpus callosum) and S100β (C, D, G, H). Transgenic astrocytes do not colocalize with neurons (NeuN, A, E) or oligodendrocytes (Olig2 B, F).

FIGURE 2.3

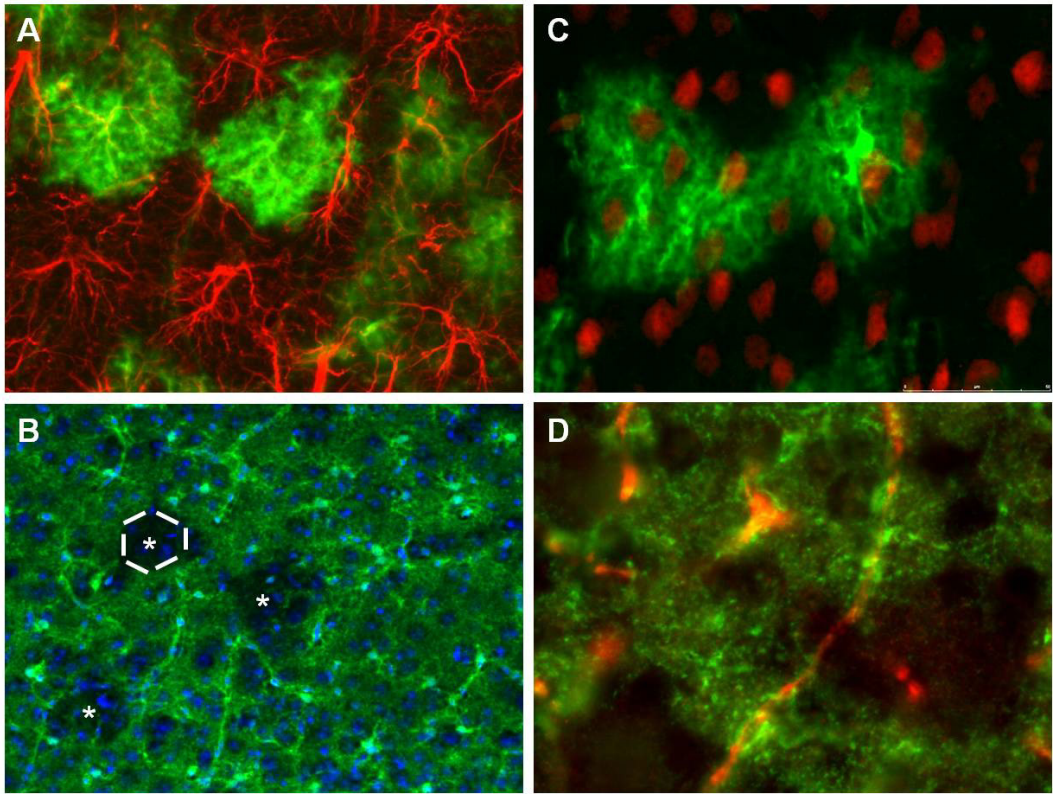

Figure 2.3: Astrocytes occupy non-overlapping domains.

Astrocytes occupy distinct, non-overlapping micro domains. The transgenic CstC-CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP labeled astrocytes follow this principle; GFAP astrocytes (red) and CstC-CreER^{T2}-Rosa26YFP astrocytes (green) in (A) show two distinct astrocyte populations in the subiculum. The difference in morphology between the CstC-CreER^{T2} and GFAP astrocytes is due to the fact that CreER^{T2} astrocytes have Rosa26YFP expression that fills the cytoplasm, but GFAP is restricted to the cytoskeleton. Each individual astrocyte occupies its own domain that does not encroach into its neighbor's domain (the same can be in seen in B in the cortex, without GFAP). PLA2G7-CreER^{T2}/Rosa26YFP astrocytes in the cortex of the adult murine brain encompass 4 to 5 NeuN (red) positive neurons (C). Both transgenic astrocyte lines also have a close relationship with blood vessels as outlined by CD31 (red) (D).

FIGURE 2.4

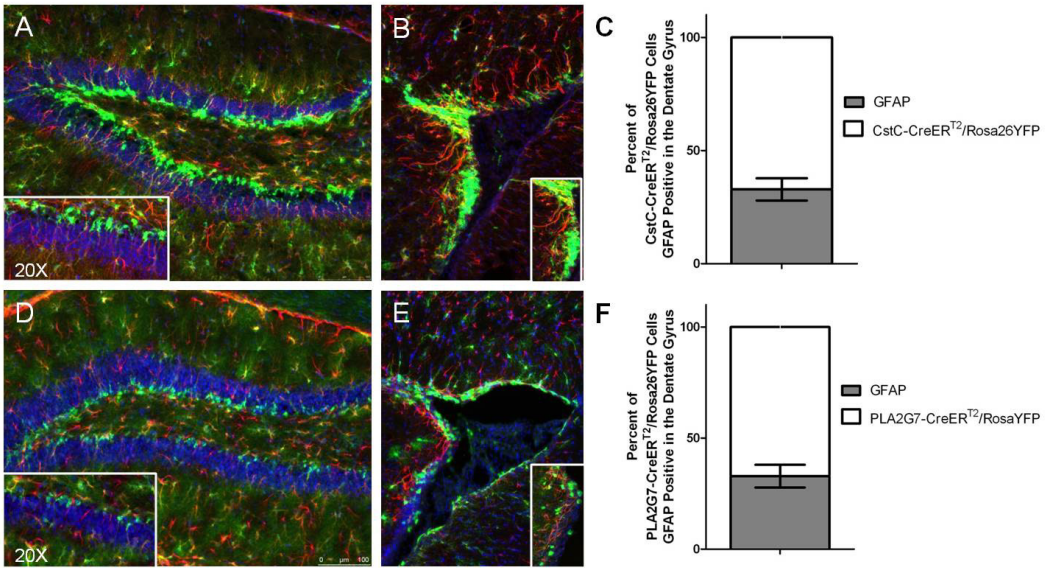

Figure 2.4: Comparison of CstC-CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP astrocytes to GFAP positive astrocytes.

In the neurogenic areas of the subgranular zone and subventricular zone, GFAP marks both astrocytes and stem cells. Both inducible cre lines, CstC-CreER^{T2} (A and B) and PLA2G7-CreER^{T2} (D and E), have much more extensive expression in the neurogenic areas and express the cell morphology of both a GFAP positive stem cell and a mature astrocytes. In panels C and F (respectively), quantification of the colocalization of both CstC-CreER^{T2} and PLA2G7-CreER^{T2} in the subgranular zone with GFAP shows 32.83 $\pm$ 8.55% and 32.83 $\pm$ 8.83%, respectively.

FIGURE 2.5

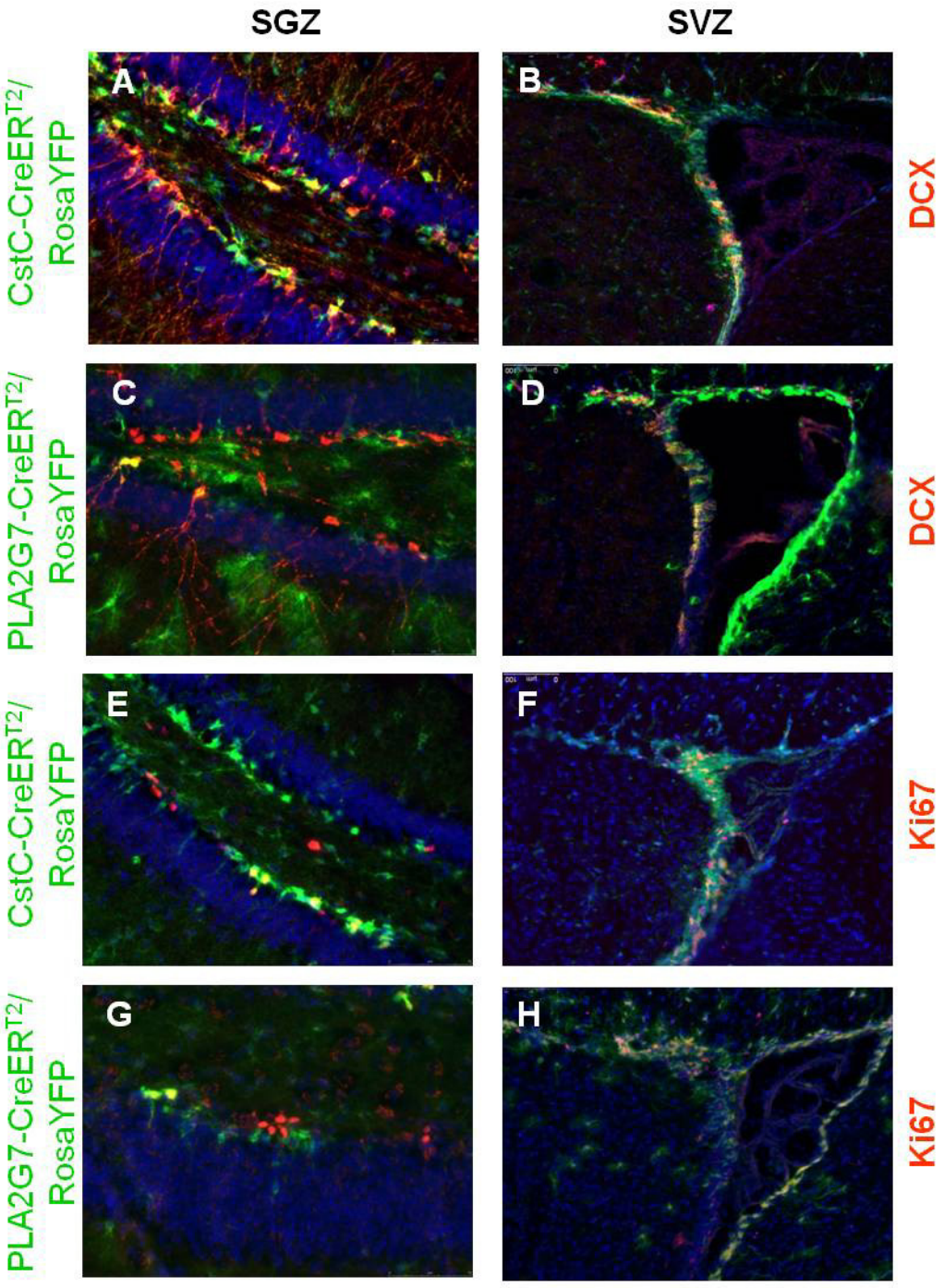
SGZ
SVZ
CstC-CreER^{T2}/ RosaYFP
PLA2G7-CreER^{T2}/ RosaYFP
CstC-CreER^{T2}/ RosaYFP
PLA2G7-CreER^{T2}/ RosaYFP
A
B
C
D
E
F
G
H
DCX
DCX
Ki67
Ki67

Figure 2.5: Both inducible CreER^{T2} lines have neural stem cell populations in the SVZ and SGZ.

Both CstC-CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP cells in the SVZ and SGZ are actively undergoing cell proliferation in the both the SGZ (E, G) and SVZ (F, H) as marked by colocalization with Ki67. The cells mature from a more immature state (Ki67 positive) to a more mature neuroblast that is DCX positive (A, B, C, and D).

FIGURE 2.6

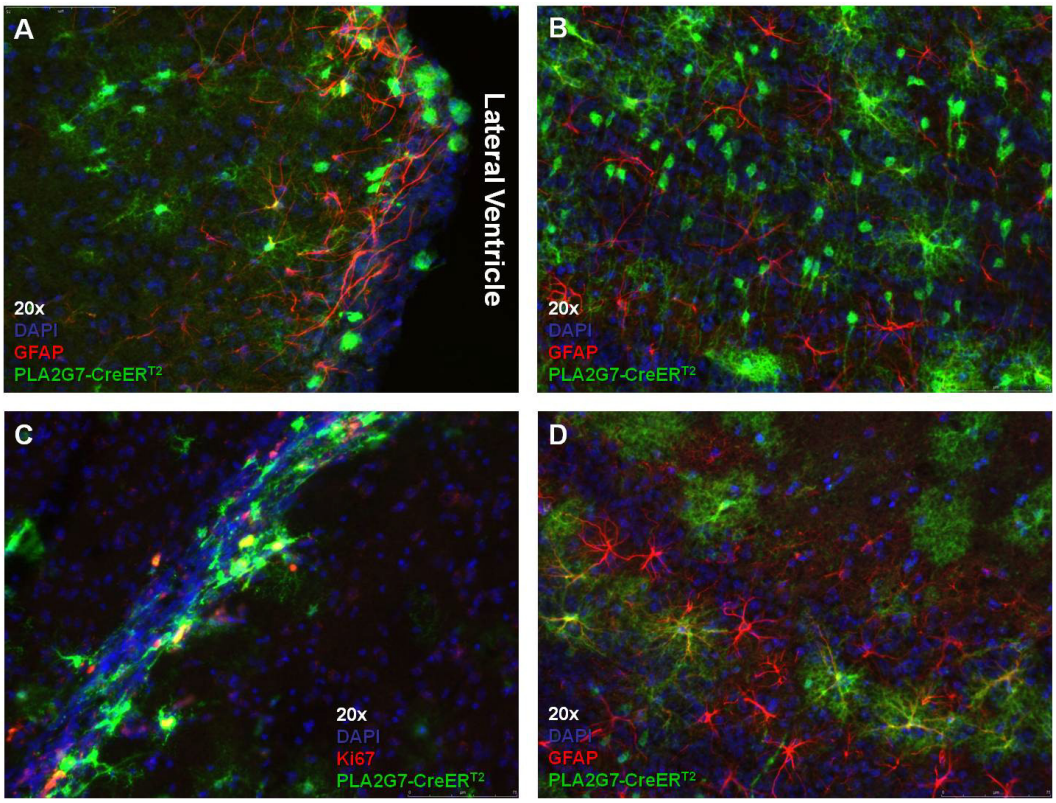

Figure 2.6: PLA2G7-CreER^{T2}/Rosa26YFP has strong expression in the subventricular zone, rostral migratory stream, and olfactory bulb neurogenic pathway.

PLA2G7-CreER^{T2}/Rosa26YFP cells have strong neurogenic expression throughout the entire neurogenic SVZ pathway. The cells that are in the SVZ (A) and RMS are mitotically active. In the RMS (B), PLA2G7-CreER^{T2}/Rosa26YFP cells colabel with proliferation marker Ki67. Within in the olfactory bulb, they differentiate into both astrocytes (C and D) and interneurons (C).

FIGURE 2.7

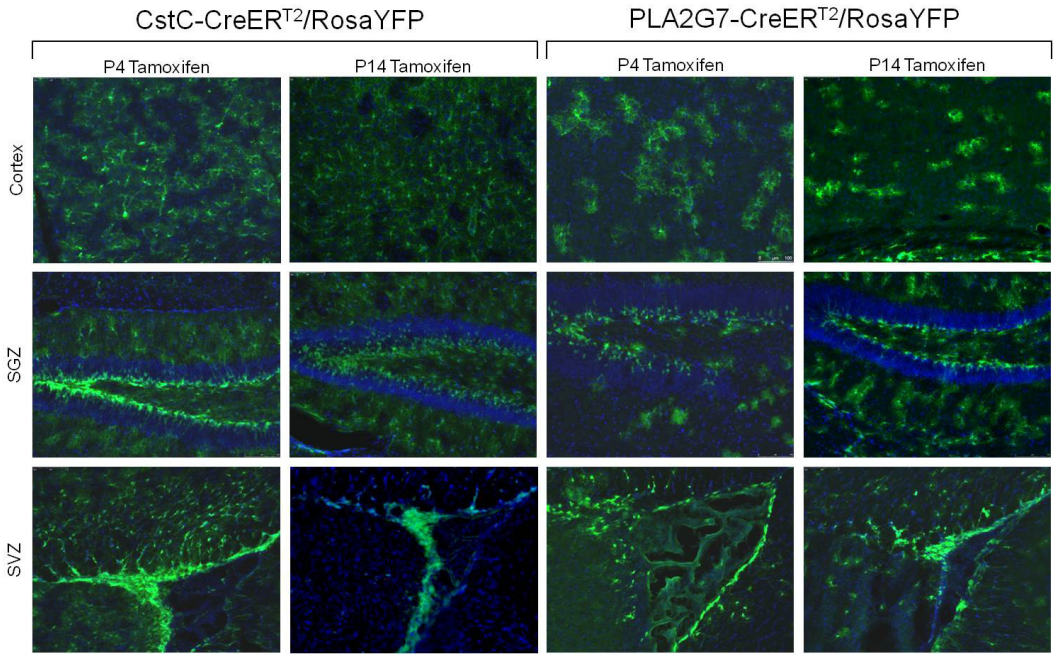

Figure 2.7: Early postnatal temporal analysis of CstC-CreER^{T2} and PLA2G7-CreER^{T2}.

Tamoxifen induced recombination in CstC-CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP animals performed at either P4 or P14 and sacrificed at P28 show mature astrocyte expression throughout the entire brain similar to what is seen in adult recombination.

FIGURE 2.8

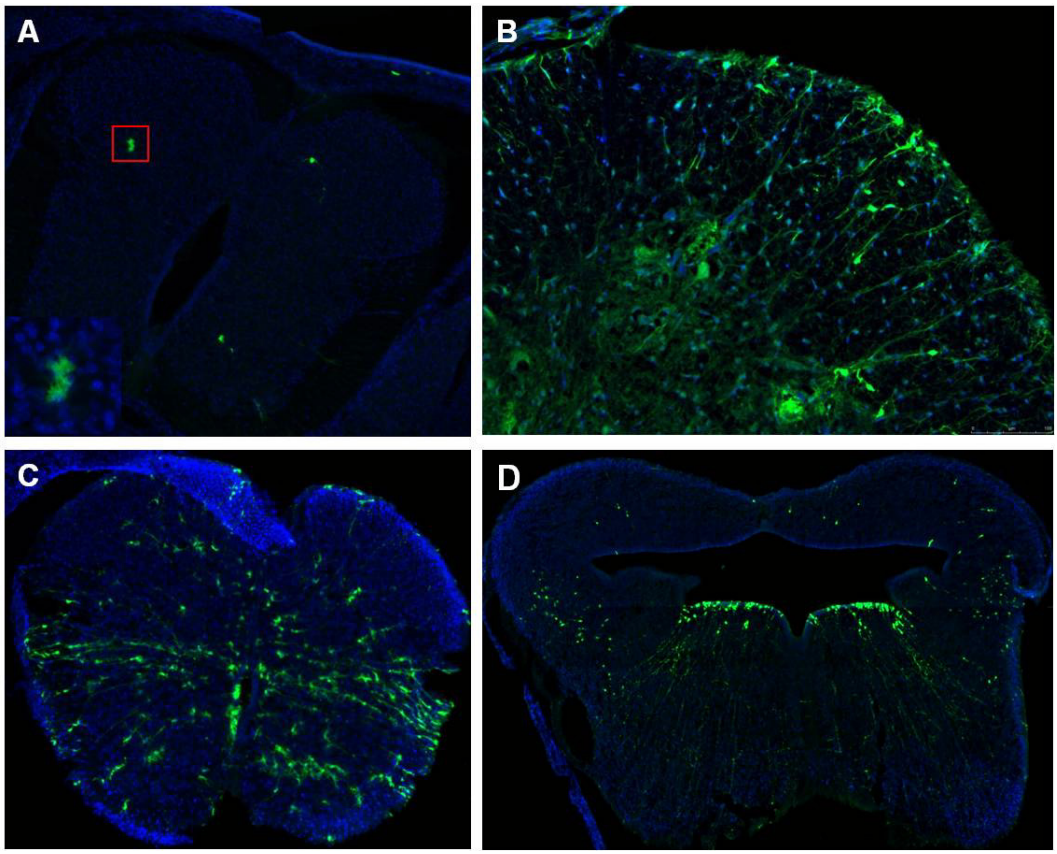

Figure 2.8: Embryonic PLA2G7-CreER^{T2}/Rosa26YFP expression along the neuroaxis.

PLA2G7-CreER^{T2}/Rosa26YFP embryos injected with tamoxifen on embryonic day 12.5 and analyzed 48 hours later show low expression of immature glia in the thoracic region of the spinal cord, with some mature astrocytes being visualized (A). By day P28 (B), in the thoracic region of the spinal cord, mature astrocytic expression has spread throughout the entire region. PLA2G7-CreER^{T2}/RosaYFP radial glia also cover the floor of the fourth ventricle (D). Embryos were also treated on e17.5 (and sacrificed 48 hours later), and much more extensive expression throughout the entire spinal cord was noted (C).

FIGURE 2.9

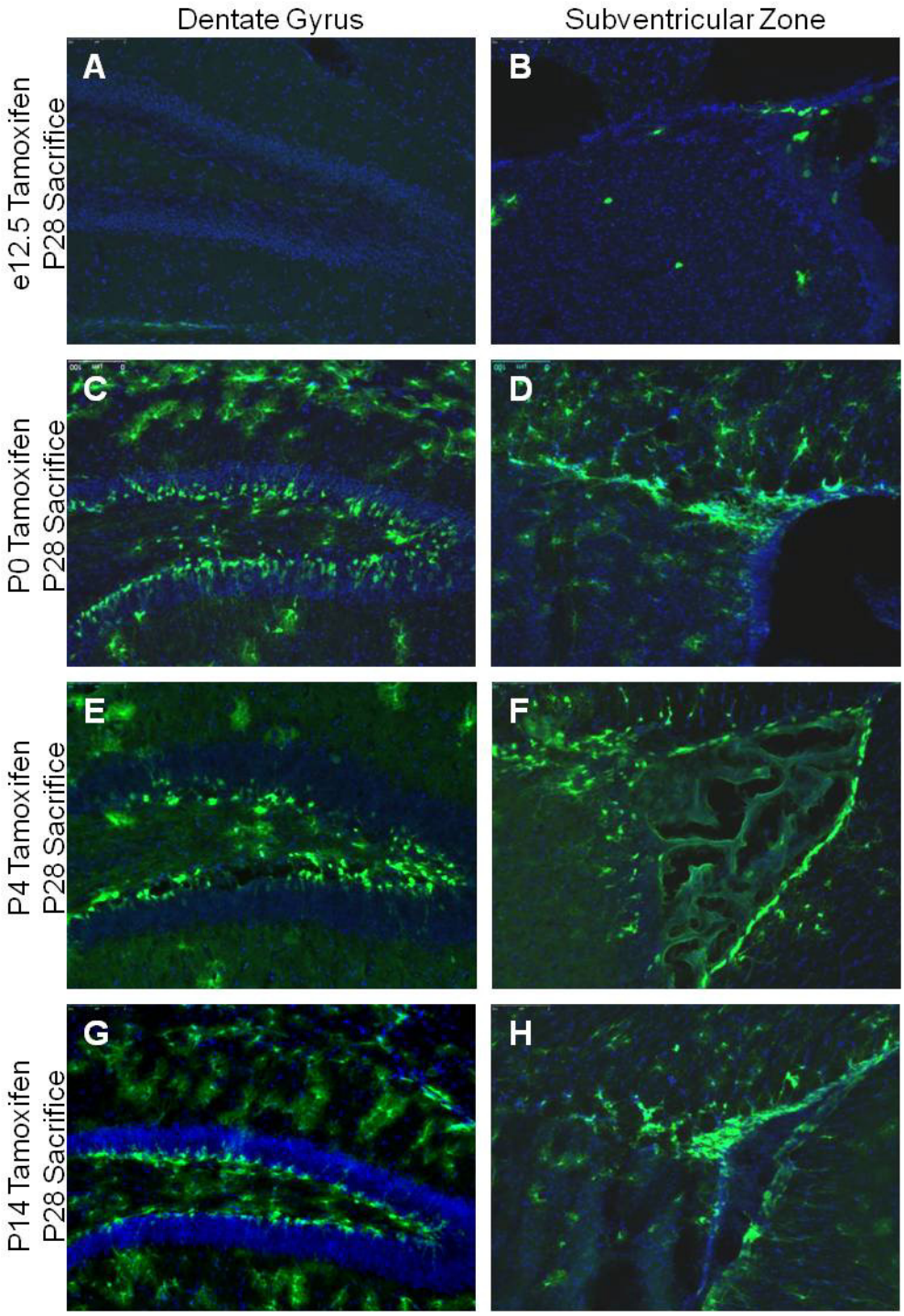
Dentate Gyrus
Subventricular Zone
e12.5 Tamoxifen
P28 Sacrifice
P0 Tamoxifen
P28 Sacrifice
P4 Tamoxifen
P28 Sacrifice
P14 Tamoxifen
P28 Sacrifice
A
B
C
D
E
F
G
H

Figure 2.9: Early post-natal recombination shows PLA2G7-CreER^{T2}/Rosa26YFP populations in the neurogenic regions.

Animals were tamoxifen injected at various time points, and all were sacrificed at P28. When PLA2G7-CreER^{T2}/Rosa26YFP embryos were tamoxifen injected on embryonic day 12.5 and studied at P28, there were no positive cells in the subgranular zone of the dentate gyrus (A) and only a few cells in the subventricular zone (B). In contrast to this, all animals treated at early postnatal time points P0, P4, P14 showed large population of mitotically active cells in the SGZ (C, E, G) and SVZ (D, F, H). This indicates that PLA2G7-CreER^{T2}/Rosa26YFP populations from the early postnatal period play an important and active role in adult murine neurogenesis.

FIGURE 2.10

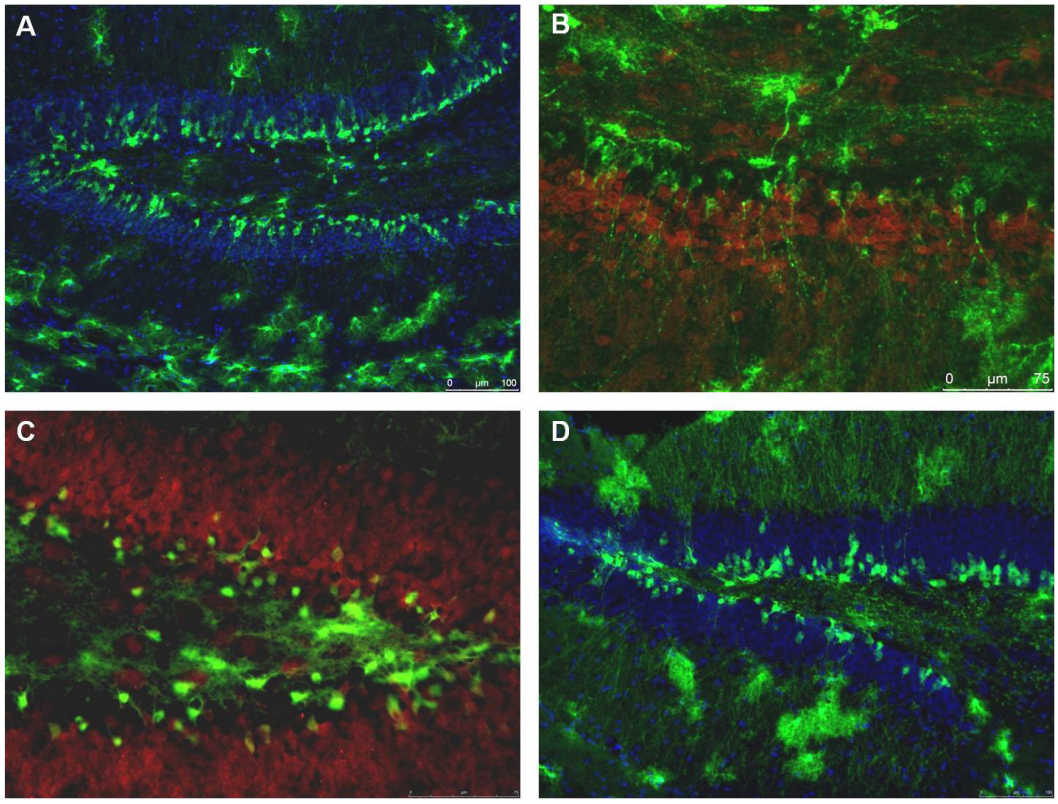

Figure 2.10: Early postnatal PLA2G7-CreER^{T2}/Rosa26YFP cells in the SGZ produce adult hippocampal neurons.

PLA2G7-CreER^{T2}/Rosa26YFP positive pups treated at birth (P0) and then analyzed at P28 show a large proportion of cells in the SGZ (A). These cells either possess neurogenic potential or are mature neurons (NeuN, red) that have integrated into the inner boundary of the granule cell layer of the dentate gyrus (B). Animals treated later on P4 again show mature NeuN (red) positive neurons in the granule cell layer at P28 (C), and when analyzed at 3 months of age, show many mature neurons with long processes that are integrated throughout the granule cell layer (D).

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CHAPTER THREE

GENETIC IDENTIFICATION OF LONG-LIVED NEURAL STEM CELLS AND TRANSIENT AMPLIFYING PROGENITORS IN THE SUBGRANULAR ZONE OF THE ADULT MOUSE BRAIN

ABSTRACT

The study of adult neurogenesis in the murine brain has superficially focused on the role of mature astrocytes in both identifying and describing neural stem cells. In the context of this study, the roles of two tamoxifen-inducible transgenic Cre lines that label mature astrocyte populations, cystatin C-CreER^{T2} and phospholipase A2 group VII-CreER^{T2}, were investigated. Both transgenic astrocyte lines mark stem cell populations in both neurogenic niches of the adult brain - the subventricular zone (SVZ) and the subgranular zone (SGZ). This study focused on the dynamics of these Cre lines in the SGZ. Through temporal analysis and cell quantification, it was determined that the CstC-CreER^{T2} cells identify a transient amplifying population of stem cells, while PLA2G7-CreER^{T2} cells identify a long-lived stem cell that survives throughout the life of the animal. Cell deletion studies, using an inducible diphtheria toxin line, were able to specifically target the CstC-CreER^{T2} and PLA2G7-CreER^{T2} cells and cause cell-specific apoptosis. Deletion of both cell types shows evidence of a marked

decrease in immature neuron marker doublecortin and cell proliferation marker Ki67. The loss of these neurogenic markers steadily increases over time and is not reversed 9 months after cell deletion. In addition to loss of neurogenic cells in the SGZ, both the CstC-CreER^{T2} and PLA2G7-CreER^{T2} transgenic lines express marked degeneration of the granule cell layer of the dentate gyrus, and PLA2G7-CreER^{T2} demonstrates cerebellar degeneration and loss of the peduncles. The utilization of these lines allows for not only the study of adult neurogenesis dynamics, but also can be utilized as a potential mouse model to study neural degeneration.

INTRODUCTION

One of the most interesting phenomena in the adult mammalian brain is the persistence of active neurogenesis. There are two established areas of neurogenesis in the adult rodent brain: the subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricle. While the two neurogenic niches are very different, there are some similarities that both compartments share. For example, studies have shown that in both the SVZ and SGZ, astrocytes (or astrocyte-like cells) are the true neural stem cell (Doetsch, Caille et al. 1999; Seri, Garcia-Verdugo et al. 2001). This proposes a particularly interesting role for astrocytes in the adult brain that needs to be studied further.

Within in the SGZ, the neural precursor cells go through a maturation process in which their morphology and antigen expression evolves (Kempermann, Jessberger et al. 2004; Encinas and Enikolopov 2008). The maturation cascade starts with the putative stem-like cell (type I) which has been previously described as a glial fibrillary acidic protein (GFAP) positive and nestin positive astrocyte-like cell. These type I cells have a morphology similar to radial glia, in particular a triangular soma and a strong apical process that breaks through the granule cell layer of the dentate gyrus and is topped with a tuft of branching processes (Ehninger and Kempermann 2008). Type I cells mature into early progenitors (type II) and late progenitors (type III) that no longer express GFAP but still express Nestin (type II). At this point, they also begin expressing doublecortin (DCX) (type II and III) and polysialylated neural cell adhesion molecule PSA-NCAM (type III). Type III cells are the last mitotically active cell in this maturation process. As type III cells become immature neurons, they gain expression for neuronal markers such as NeuN (neuronal nuclei) and calretinin. Cells remain as immature neurons for two to three weeks before maturing into NeuN and calbindin expressing granule cell neurons (Kempermann, Jessberger et al. 2004; Ehninger and Kempermann 2008). The age of these mature granule cell neurons ranges from one to two months before they become fully integrated in the dentate gyrus and identical to older neurons (Kempermann, Jessberger et al. 2004). The foregoing describes the current accepted theory for SGZ

neurogenesis, but this theory does not elucidate that neural progenitors are probably a diverse and heterogeneous population.

In this study, I focused on the mitotic progression and cell dynamics of the subgranular zone of the dentate gyrus through careful stereologic quantification. Considering that there is strong evidence for astrocytes being neuronal precursors, I hypothesized that two astrocyte-specific inducible CreER^{T2} lines participated in SGZ neurogenesis. These studies revealed the very diverse roles of both cystatin C-CreER^{T2}/Rosa26YFP and phospholipase A2 Group VII-CreER^{T2}/Rosa26YFP labeled astrocytes and their neurogenic potential in the SGZ of the hippocampus. The neurogenic potential of these two transgenic lines was analyzed through cell fate tracking and the use of a diphtheria toxin fragment A mice deleter strain. A better understanding of the dynamics of SGZ neurogenesis can provide therapeutic remedies to a wide array of CNS pathophysiologies.

MATERIALS AND METHODS

Animal Studies

Transgenic animals were housed in The University of Texas Southwestern Medical Center at Dallas' pathogen-free, climate controlled facility. All animal

studies and procedures were approved by the Institutional Animal Care and Use Committee (APN: 2009-0373) at UT Southwestern.

Both the CstC-CreER^{T2} and PLA2G7-CreER^{T2} lines were generated through Dr. Ron DePinho's lab after determining both the CstC and PLA2G7 genes through an astrocyte transcriptome study (Bachoo, Kim et al. 2004). The diphtheria toxin fragment A mice (Rosa26LacZ/diphtheria toxin fragment A, referred to as DTA BpA) was generously provided by Dr. Amelia Eisch's lab and originally described by Dr. Dieter Riethmacher (Brockschnieder, Lappe-Siefke et al. 2004). The Rosa26YFP reporter mouse line, described by Dr. Frank Costantini's Lab, was also provided by Dr. Amelia Eisch's lab (Srinivas, Watanabe et al. 2001).

Genotyping

When the mice were 10 days old, their tails were clipped for genotyping DNA and digested in 100 µl DirectPCR lysis buffer (Viagen, Catalog Number: 102-T) with 1 µl proteinase K (AMRESCO, catalog number: E195) at 55ºC overnight and then denatured at 85ºC for 1 hour. The DNA was centrifuged for 10 minutes and then used for genotyping. For CreER^{T2} genotyping, the following forward and reverse primers were used: Forward: 5' – ATT TGC CTG CAT TAC CGG TC - 3'; Reverse: 5' – ATC AAC GTT TTC TTT TCG G - 3', and DTA BpA genes: Forward: 5' – CGA CAA TAA ATA CGA CGC TGC GGG – 3';

Reverse: 5' – CAT CGC ATC TTG GCC ACG TTT TCC – 3'. The following are primers for Rosa26YFP genotyping: Primer 1: AAA GTC GCT CTG AGT TGT TAT; Primer 2: GCG AAG AGT TTG TCC TCA ACC; Primer 3: GGA GCG GGA GAA ATG GAT ATG. Using Invitrogen Taq Polymerase (Catalog Number: 18038-042), the following conditions were used for both the CreER^{T2}, DTA BpA, and Rosa26YFP PCR reactions, commencing with an initialization step of 94ºC for 1 minute, followed by a denaturing step at 94ºC for 30 seconds, an annealing step of 53ºC for 30 seconds, and an elongation step at 72ºC for 1 minute. All these steps were repeated 36 times. Lastly, there was the final elongation step at 72ºC for 10 minutes and a final holding step of 4ºC. Positive CreER^{T2} animals produced a band at 350 bp (CreER^{T2} animals were kept as heterozygotes) and DTA BpA genotyping has a positive product size of about 300 bp. Rosa26YFP expression can be observed in both homozygotes (product size of 310 bp) or heterozygotes (product sizes of both 310 bp and 560 bp).

Tamoxifen injections for Inducible CreER^{T2} Recombination

Both Rosa26YFP and Rosa26LacZ/DTA BpA expression can be induced after tamoxifen treatment of animals that carry the CreER^{T2} gene. The following animals exhibited induced expression at P28 after 5 consecutive days of 180 mg/kg tamoxifen treatment: CstC-CreER^{T2}/Rosa26YFP, CstC-CreER^{T2}/DTA BpA, PLA2G7-CreER^{T2}/Rosa26YFP, and PLA2G7-CreER^{T2}/DTA BpA.

Preparation of tamoxifen (TAM) (Sigma; catalog number T5648-5G) was prepared by weighing out 30 mg of TAM and combining with 100 µl of 100% high grade ethyl alcohol (PHARMCO-AAPER, catalog number: 111ACS200) in a 1.65 ml eppendorf tube and vortexing. Next, 900 µl sunflower seed oil (Sigma; catalog number S5007) was added and vortexed. The mixture was sonicated for 10 minutes. After sonication, the mixture was vortexed a final time to confirm that all the TAM dissolved. TAM is very light sensitive and consequently was covered with aluminum foil, remaining viable for only 1 week at 4ºC

Memantine Injection

Both CstC-CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP mice were injected with TAM for 5 consecutive days at a dose of 180 mg/kg. One week after the last TAM injection, the mice were then injected with a single 50 mg/kg dose (prepared in 0.9% saline) of NMDA receptor antagonist drug memantine (Sigma, catalog number: M9292). Ten days following the memantine injection, animals were treated with 50 mg/kg 5-Bromo-2'-deoxyuridine (BrdU) (Sigma, Catalog Number: B9285) both 4 hours and 2 hours before sacrifice by transcardial perfusion.

Temozolomide Injection

Animals were treated with the typical tamoxifen regimen at P28, and then one week following the last tamoxifen injection, the animals began a temozolomide (TMZ) (LKT Labs, Catalog Number: T1849) regimen. Mice were administered a dose of 80mg/kg TMZ three times a week for four weeks for the purpose of suppressing neural stem cell activity. Animals were then perfused according to protocol.

Tissue Processing

When animals were ready for sacrifice, they received two intraperitoneal (IP) injections of 50 mg/kg BrdU at 4 hours and 2 hours before transcardial perfusion. Animals were deeply anesthetized through an IP injection of Avertin using a weight-based dose. Reflexes were tested by pressing down on the footpad on the mouse, and the perfusion did not start until an absence of reflexes was observed. Transcaridal perfusion was performed using 10 mL of ice cold phosphate buffered saline (PBS) immediately followed by ice cold 4% paraformaldehyde (PFA) (Sigma, catalog number: 158127) prepared in PBS with a pH of 7.4. After perfusion, brains were dissected (and their weight recorded), then post-fixed in 4% PFA overnight at 4°C, and finally (after post-fixation) washed in PBS 3 times. If brains were to be retained for long term storage, they were placed in a PBS with 0.1% sodium azide (Sigma, catalog number S8032)

solution at 4°C, and if the brains were to be cut for analysis, they were placed in a 30% sucrose (Fisher, catalog number S2-500) solution in PBS with 0.1% sodium azide. Once the brain tissue sank in the 30% sucrose solution, it then was ready to be cut for analysis. Brains were sliced coronally at 30 μm on a Leica SM2000R microtome. Sections were cut into serial sections (every 12th section) and kept at 4°C in PBS with 0.1% sodium azide.

Immunofluorescence for Quantification

One series of every 12th section was placed in a well of a 12-well plate and washed in 1X PBS with 0.02% sodium azide for 10 minutes. Sections were then placed in PBS with 0.3% Triton X and 0.02% sodium azide and either goat anti-Doublecortin antibody from Santa Cruz (catalog number: sc-8066) diluted 1:1000 or mouse anti-Ki67 antibody from BD Pharmingen (catalog number: 550609) diluted 1:400. Sections were incubated, free floating, for 48 hours at room temperature. Sections were next washed in PBS with 0.02% sodium azide 3 times at 10 minutes each. Sections were subsequently placed in PBS with 0.02% sodium azide and with either donkey anti-goat (catalog number: 705-165-147) or donkey anti-mouse (715-165-150) Cy3 secondary antibody from Jackson Labs diluted 1:300. Sections were incubated in the secondary antibody for 48 hours. After the secondary antibody incubation, sections were incubated with 4'-6-diamidino-2-phenylindole, dihydrocholoride (DAPI) (Invitrogen, catalog number:

D1306) for 10 minutes free floating at a 1:5000 dilution. Then, sections were washed 3 times for 10 minutes each in PBS with 0.02% sodium azide. After the washes, sections were mounted on 1% gelatin coated slides and then coverslipped with lab-made, glycerol-based, fluorescence mounting media. For any sections where GFP was needed to stain for Rosa26YFP, chicken-anti GFP (Aves, catalog number: GFP1020) was used at a dilution of 1:5000. The incubation was performed with the other primary antibody for 48 hours. The secondary antibody used (again with the other secondary) was donkey anti-chicken Cy2 (Jackson Labs, catalog number: 703-225-155) diluted 1:500.

Colocalization Quantification

Once immunofluorescence staining for every 12th section was completed, Z-stacks of the dentate gyrus for every section were taken using a 10x objective lens on a Leica DM5500B upright microscope using the Leica's proprietary Leica Advanced Imagining software (Leica Microsystems Inc., Wetzlar Germany). After the Z-stacks were compiled (and saved as Leica .lif files), they later were opened using Bitplane's Imaris software (Bitplane Scientific Software AG, Zurich, Switzerland). Imaris performs quantification by volume reconstruction of the Z-stacks and stringent thresholding parameters. The quantification procedure used was developed by Costes and Lockett at the NIH, NCI/SAIC. Quantification

for the colocalization of both DCX and CstC-CreER^{T2}/Rosa26YFP or PLA2G7-CreER^{T2}/Rosa26YFP was analyzed.

Doublecortin Quantification

To study both the number of DCX cells as a mouse ages, and the loss of DCX cells in both CstC-CreER^{T2}/DTA BpA and PLA2G7-CreER^{T2}/DTA BpA mice, an unbiased stereological approach was used. An Olympus BX51 microscope was used in tandem with StereoInvestigator analysis software (MicroBrightField Inc, Williston, VT), along with the optical fractionator method to count total DCX cell estimates. Using the StereoInvestigator, the subgranular zone of the dentate gyrus (region of interest) was traced using a 20x objective lens. Once the area of interest was designated, the counting parameters were set. The counting frame was designated as 12 μm x 12 μm; the counting frame was set so that 1 to 5 cells can be counted per counting frame. The grid layout was set at 50 μm by 50 μm over the region of interest. At every intersection of the grid layout, a counting frame was placed, providing systematic random sampling. The average mounted thickness of the section was between 20 and 22 μm. For the optical dissector, the thickness of the section was measured at every counting frame site, and cells in the inner 12 μm (height sampling fraction) were counted, giving an outer guard zone of 4-5 μm. Counting of cells was done live using a 100x oil-immersion objective. Cells were only counted at the largest part of their

diameter. This procedure was performed for every 12th section (section sampling fraction). Once every section had been counted, the total number of cells was estimated by the following equation: total number of cells = (total number of cells counted) *x* (1/section sampling fraction) *x* (height sampling fraction). The section sampling fraction is defined as (1/section interval), and height sampling fraction is defined as (dissector height/average mounted section thickness).

Ki67 Quantification

Ki67 total cell estimates were also counted to study both the dynamics of the Ki67 compartment in the SGZ over time and to quantitate the amount of Ki67 loss in both CstC-CreER^{T2}/DTA BpA and PLA2G7-CreER^{T2}/DTA BpA mice. Since the Ki67 cells are sparse in the SGZ (region of interest), every individual cell is counted. Once the region of interest was traced using a 20x lens on an Olympus BX51 microscope, the meander scan option was used on the StereoInvestigator software. The meander scan places counting frames throughout the entire region of interest so the entire region of interest is counted. Again, the mounted section thickness (between 20 to 22 μm) was measured at every counting site, and a dissector height of 12 μm was used. Ki67 cells were only counted when their diameter was the largest. This procedure was repeated for every 12th section throughout the brain that contained SGZ. The total number of cells was calculated using the following equation: total number of cells =

(number of cells counted) *x* (1/section sampling fraction) *x* (height sampling fraction).

Cavalieri Volume Estimates of the Granule Cell Layer of the Dentate Gyrus

Using the Cavalieri volume estimation, the regional volume of a structure (such as the granule cell layer) can be determined by calculating the area of the structure in parallel sections (i.e. every 12^{th} section) throughout the structure. The region of interest was traced using a 20x objective lens, and counting points were placed 80 μm apart, Every DAPI cell in the granule cell layer at 20x that touched the counting point (in the upper right hand corner) was included in the structure area estimation. Areas for every 12^{th} section throughout the granule cell layer of the dentate gyrus were calculated and used for the volume estimation.

Adult Hippocampal Neurospheres

Adult hippocampal neurospheres were derived from CstC-CreER^{T2}/Rosa26YFP mice treated with 180 mg/kg of tamoxifen for 5 days at 3 weeks of age and sacrificed at 4 weeks of age. At sacrifice, the dentate gyrus of the hippocampus of these mice were dissected out and dissociated into individual cells. These cells were cultured in neural stem cell media with epidermal growth factor and fibroblast growth factor (EGF/FGF) (Sigma, catalog number: E4122 and Sigma, catalog number: F0291) and 2% fetal bovine serum (FBS) (HyClone,

catalog number: SH30396). Cultured neurospheres were then differentiated by the removal of both EGF/FGF and FBS for 4 days. The differentiated mature cell types were then stained for mouse anti-Tuj-1 (Sigma, catalog number T8660) at a 1:300 dilution, rabbit anti-GFAP (DAKO, catalog number: Z0344) at a 1:500 dilution, and mouse anti-CNPase (Chemicon, catalog number MAB326) at a 1:200 dilution. All cells were additionally stained with chicken anti-GFP (Aves, catalog number GFP1020) at a dilution of 1:5000 for the CstC-CreER^{T2}/Rosa26YFP expression. All primary antibodies were incubated overnight at room temperature followed by washes and a four hour secondary incubation at room temperature. The following secondary antibodies were used: donkey anti-chicken Cy2 (Jackson Labs, catalog number: 703-225-155) diluted 1:500, donkey anti-mouse Alexa Fluor 594 (Invitrogen, catalog number: A21203), and donkey anti-rabbit Alexa Fluor 594 (Invitrogen, catalog number: A21207) diluted 1:1000.

Paraffin Sections

CreER^{T2}/DTA BpA mouse brains processed for paraffin sections were handled by the Histology Core at UT Southwestern. Midbrain sagittal sections were cut at 5 μm and stained with Nissl and hematoxylin and eosin (H&E). Paraffin slides with histological stains were imaged on a Leica DMI6000B

inverted microscope using Leica's LAS software (Leica Microsystems Inc., Wetzlar, Germany).

Statistics

GraphPad Prism software (version 5.00 for Windows, GraphPad Software, San Diego California USA) was utilized for statistical analysis using either a one or two-way ANOVA with Bonferroni's post-hoc test. Statistical significance was represented by a p value less than 0.05. All values are shown as means $\pm$ SEM (standard error of the mean).

RESULTS

Dynamics of the Subgranular Zone in Aging Mice

While it is an accepted fact that neurogenesis decreases as an animal ages, a comprehensive study to fully understand the dynamics of neurogenesis in the subgranular zone has neither been completed or reported in the literature. My research comprehensively analyzed the total number of actively dividing cells (Ki67 positive) in the subgranular zone of the dentate gyrus beginning with a 1 month animal and completing with a 10 month animal. The number of actively dividing cells experienced a net decrease between 1 month and 2 months of age. The decrease between 2 and 4 months of age was only moderate, and by 10

months of age, the total number of Ki67 cells in the SGZ was only 10.49 ± 8.45% of the number in a 1 month old mouse (Figure 3.1 A). An additional study was performed to determine the number of immature neurons labeled by doublecortin (DCX) in the SGZ. Overall, the general trend of decrease was the same for the number of DCX cells, and the total number of cells at 10 months was 4.32 ± 10.51% of a 1 month old animal (Figure 3.1 B).

Cystatin C-CreER^{T2}/Rosa26YFP Labeled Cells in the Subgranular Zone Mark Transient Amplifying Stem Cell Populations

In order to study the dynamics of the CstC-CreER^{T2} cells in the SGZ, CstC-CreER^{T2}/Rosa26YFP animals were injected with 5 consecutive doses of tamoxifen on P28. Animals were sacrificed and analyzed 1 week, 1 month, 2 months, 3 months, and 9 months later. CstC-CreER^{T2}/Rosa26YFP cells colocalized with Ki67 positive cells in the subgranular zone, and these cells also advanced to become DCX positive. One month after tamoxifen induced recombination, the number of CstC-CreER^{T2}/Rosa26YFP positive cells that colocalized with DCX was found to be 70.23 ± 11.17%, but 3 months after tamoxifen, only 16.15 ± 4.84% of cells colocalized (Figure 3.2 D). Such a drastic decrease in the number of colocalized cells begged the question if the CstC-CreER^{T2}/Rosa26YFP labeled cells were "dying out." Mice at 5 months of age were injected with the typical tamoxifen regime and then sacrificed 2 weeks after

injection (Figure 3.2 F). Analysis of these animals showed approximately a 30% colocalization of CstC-CreER^{T2}/Rosa26YFP and DCX when injected with tamoxifen at 5 months of age, proving that the CstC-CreER^{T2} compartment in the SGZ does not die off, but has some role throughout the life of the animal. From these observations, CstC-CreER^{T2}/Rosa26YFP positive cells in the SGZ were termed, "transient amplifying cells." Additionally, these cells are not always DCX positive from the beginning. In Figure 3.3 A, animals were injected with tamoxifen and then sacrificed the day after the last injection. It is apparent, that at as early as one day after tamoxifen, the CstC-CreER^{T2} positive cells and the DCX positive cells mostly form two separate compartments. However, if they are analyzed a week after the last injection, the two compartments are mostly colocalized (Figure 3.3 B). To confirm that CstC-CreER^{T2} cells are neural stem cells, tamoxifen induced recombination was performed at 3 weeks. Animals were then sacrificed one week later, the hippocampus was dissected out, and adult neurospheres were made (as described in the methods). Upon differentiation of these neurospheres, the following mature cell types were identified: neurons (Tuj-1 positive), astrocytes (GFAP positive) and oligodendrocytes (Olig-2 positive) (Figure 3.4). CstC-CreER^{T2}/Rosa26YFP positive cells in the SGZ identify a multi-potent, transient amplifying stem cell compartment. Identification and characterization of this transient amplifying compartment is important to gain a

better understanding into the heterogeneous nature of the stem cell compartment in the SGZ.

Phospholipase A2 Group VII-CreER^{T2}/Rosa26YFP Labeled Cells in the Subgranular Zone Mark Long Term Stem Cell Populations

While the number of PLA2G7-CreER^{T2}/Rosa26YFP cells that colocalize with DCX are never as numerous as CstC-CreER^{T2}/Rosa26YFP (Figure 3.2), they do colocalize throughout the life of an animal when labeled through tamoxifen recombination at P28. PLA2G7-CreER^{T2}/Rosa26YFP animals were injected with tamoxifen to induce recombination on P28 and analyzed 1 week, 1 month, 2 months, 3 months, 4 months, 6 months, 9 months, and 1 year later. Colocalization of PLA2G7-CreER^{T2}/Rosa26YFP and DCX cells was quantified for both 1 month and 3 months post-tamoxifen injection. One month after recombination, there was 52.14 $\pm$ 4.91% colocalization between the two cell populations, and at 3 months 37.67 $\pm$ 11.94% of PLA2G7-CreER^{T2}/Rosa26YFP cells still colocalized with DCX in the SGZ (Figure 3.5 D). Animals studied 1 year after P28 recombination still exhibited PLA2G7-CreER^{T2}/Rosa26YFP cells committed to the neuronal maturation fate and some that colocalized with DCX (Figure 3.5 C). PLA2G7-CreER^{T2}/Rosa26YFP cells also became neurons in the granule cell layer of the dentate gyrus by 4 months after tamoxifen, and at 9 months after recombination, these neurons were no longer restricted to just the inner one third

of the granule cell layer (Figure 3.5 E and F respectively). The PLA2G7-CreER^{T2} compartment in the SGZ represents a particularly important key to understanding the entire neural stem cell network. Within this study, two different compartments within the neural stem cell network were identified: the transient amplifying cells and long-lived stem cells. These two different cell types identified play in tandem throughout the life of the animal to create a functional neural stem cell compartment.

Effects of Memantine on both CstC-CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP Cells in the SGZ

Previous literature has established that the Alzheimer's disease drug memantine increases the number of neural stem cells that reside in the SGZ of the dentate gyrus (Namba, Maekawa et al. 2009). Both astrocyte transgenic lines were injected with tamoxifen at P28. Then, one week after the last injection, they were treated with a single dose of memantine and perfused 10 days after that. Memantine roughly doubled the total number of DCX cells lining the SGZ, and both CstC-CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP cells also increased in number (Figure 3.6). Overall, the total percentage of cells from both stem cell compartments that colocalized with DCX did not change. Since memantine is a known neural stem cell manipulator, this study provided evidence that both stem cell compartments, the transient amplifying stem cells and long-

lived neural stem cells, are true stem cells and are capable of being influenced by known manipulators.

Cell Deletion Studies Using an Inducible Diphtheria Toxin Line

Through the memantine studies, the manipulation of the CstC-CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP cells was observed. Next, these two astrocytic transgenic lines were crossed to a Rosa26LacZ Diphtheria Toxin line and deletion of these cell populations was studied. After deletion of both astrocyte cell populations, there was a massive gliosis response with large, thick GFAP astrocytes compensating for the loss of their CreER^{T2} neighbors. This upregulated GFAP expression was found throughout the entire brain, but the response is greater in the CstC-CreER^{T2}/DTA BpA animals than the PLA2G7-CreER^{T2}/DTA BpA animals (Figure 3.7 D, E, F, G, H, I). The gliosis was very strong 1 week after P28 induced recombination, but 1 month after recombination, most of the gliosis had subsided with only weakly upregulated GFAP expression left over in the CstC-CreER^{T2}/DTA BpA mice (Figure 3.7 E, H). Astrogliosis is a common reaction to both brain injury and disease, so it is not surprising that a major cell death of astrocytes throughout the entire brain resulted in a gliotic response (Chvatal, Anderova et al. 2008). Also investigated was the response of cystatin C observed through polyclonal antibody staining; control animals showed abundant CstC polyclonal antibody expression, PLA2G7-

CreER^{T2}/DTA BpA had decreased expression, and CstC-CreER^{T2}/DTA BpA animals had further decreased expression that was barely detectable (Figure 3.7 A, B, C). This result was expected as PLA2G7-CreER^{T2}/Rosa26YFP cells are a different astrocyte population with only minimal overlap with cells labeled with a polyclonal CstC antibody.

Aside from the effect that neighboring astrocytes experienced from the loss of CstC-CreER^{T2} and PLA2G7-CreER^{T2} astrocytes, the effect on the neural stem cell compartment of the SGZ was also studied. As little as one week after cell deletion of both compartments, there was a significant decrease in the number of Ki67 cells that resided in the SGZ. Deletion of CstC-CreER^{T2} expressing cells caused a 58.16 $\pm$ 2.23% decrease in the total number of Ki67 cells, and PLA2G7-CreER^{T2} deletion resulted in a 35.71 $\pm$ 4.46% decrease (Figure 3.8 E, F). The decrease in the number of Ki67 cells was not reversible at 1 month, 3 months, or 9 months after deletion. By 9 months after deletion, 73.11 $\pm$ 7.31% of Ki67 cells were lost in the CstC-CreER^{T2}/DTA BpA animals and 63.55 $\pm$ 10.29% in the PLA2G7-CreER^{T2}/DTA BpA animals (Figure 3.8 E, F). There was a similar trend in total number of DCX cells lost, but not as dramatic (Figure 3.8 A, B, C). The death of both CstC-CreER^{T2} and PLA2G7-CreER^{T2} astrocytes within the SGZ caused a significant disruption in both the Ki67 and DCX populations. These impressive effects conclusively prove that both the transient amplifying and long-lived neural stem cells of the two CreER^{T2} compartments play a serious role

in maintaining the neural stem cell compartment of the SGZ and the environmental niche that accompanies it.

Loss of these cells also resulted in degeneration of the granule cell layer of the dentate gyrus. This degeneration became progressively worse with time and did not show any signs of reversal by 9 months after cell specific deletion of either CstC-CreER^{T2} or PLA2G7-CreER^{T2} cells (Figure 3.9 C, D, E). In CstC-CreER^{T2}/DTA BpA animals, volume loss of the dentate gyrus occurs as early as 1 week after tamoxifen induced cell deletion and by 3 months in PLA2G7-CreER^{T2}/DTA BpA animals. To eliminate the possibility that the dentate gyrus volume loss was due to a loss of neurogenesis, animals were treated with temozolomide three times a week for 4 weeks. Temozolomide kills any cells that are actively dividing. There was no noticeable difference in the volume of the granule cell layer of the dentate gyrus in the temozolomide treated animals and age matched controls. Additionally, there was no significant difference between PLA2G7-CreER^{T2}/DTA BpA animals, but a significant difference existed between temozolomide treated and CstC-CreER^{T2}/DTA BpA animals (p=0.006) (Figure 3.9 B). The decrease in the volume of the granule cell layer is not due to the decrease in neurogenesis, but overall is due to a cell niche effect.

Another brain region where degeneration was present was within the cerebellum in PLA2G7-CreER^{T2}/DTA BpA mice injected with tamoxifen on P28.

This recombination induced cell deletion and was analyzed 9 months later. The cerebellum of the PLA2G7-CreER^{T2}/DTA BpA was smaller in size, and also appeared to have a loss of peduncles (Figure 3.10 I). This loss was not observed in either control animals or CstC-CreER^{T2}/DTA BpA animals at any time point (Figure 3.10). This loss was only seen in PLA2G7-CreER^{T2}/DTA BpA animals, which was probably due to the extremely high expression of PLA2G7-CreER^{T2} cells in the cerebellum (Bergmann Glia), which are also GFAP positive (data not shown).

Interestingly, behavioral studies for both fear-conditioning, learning, and memory showed no statistical difference between control animals (DTA BpA) and PLA2G7-CreER^{T2}/DTA BpA animals. This reveals the plasticity of the brain. Such a loss and degeneration does not necessarily relate to a noticeable change in behavioral studies.

DISCUSSION

Astrocytes have previously been described as adult neural stem cells in both the SVZ and SGZ, but few studies focus exclusively on the role of mature astrocytes in these neurogenic niches. Through the utilization of two astrocyte-specific inducible CreER^{T2} mouse lines, CstC-CreER^{T2} and PLA2G7-CreER^{T2},

this study focused on the roles of these two astrocyte populations in the dynamics of subgranular zone neurogenesis.

To date, there has not been a comprehensive quantitative study of the Ki67 and DCX populations in the SGZ as an animal ages; even though it is an accepted belief that neurogenesis decreases with age (Kuhn, Dickinson-Anson et al. 1996; Molofsky, Slutsky et al. 2006). One month old mice had very robust expressions of both mitotic marker Ki67 and neuroblast marker DCX. This study followed both the Ki67 and DCX populations as the animal aged to 10 months of age. By the time the animal was 2 months of age, there was already an impressive decrease in these populations, and when analyzed again at 4 months, the amount was only slightly less. The most impressive decrease was at 10 months when the Ki67 population was about 10% of that at 1 month, and the DCX population was approximately only 4% of that at 1 month. While not studied, it could be hypothesized that these low base-line levels of neurogenesis (represented through both mitotic activity and immature neurons) would persist throughout the life of the animal. Since there is not a great need for ongoing neurogenesis in an aged mouse, it is reasonable to conclude that mitotic activity in the SGZ experienced a 90% loss. However, several studies have shown that this base line of SGZ neurogenesis can be stimulated through a variety of factors: voluntary exercise, anti-depressants, learning, enriched environments, hormones, ischemic events, seizures, etc (Kempermann, Kuhn et al. 1997; Tanapat, Hastings et al. 1999; van

Praag, Christie et al. 1999; Malberg, Eisch et al. 2000; Jin, Minami et al. 2001; Shors, Miesegaes et al. 2001; Eisch and Nestler 2002; Pirttila, Lukasiuk et al. 2005).

Astrocytes have previously been named the neural stem (type I) cell in the SGZ maturation cascade (Ehninger and Kempermann 2008). Using two distinct astrocyte CreER^{T2} lines, the astrocyte dynamics in the neural stem cell compartment was studied. Interestingly, each astrocyte population represented a different population of neural stem cells. CstC-CreER^{T2}/Rosa26YFP positive cells represented a transient amplifying stem cell compartment while PLA2G7-CreER^{T2}/Rosa26YFP positive cells identified a long-lived neural stem cell compartment. There is a definite and important distinction between these populations, as one persists throughout the life of the animal (long-lived neural stem cells), and the other is a transient stem cell that has a limited life expectancy of about 1 – 2 months. In regards to the classical maturation cascade (please refer to Figure 1.2) that is currently used to describe the dynamics of SGZ neurogenesis, the long-lived neural stem cell population is most analogous to the quiescent stem-like type I cells and the transient amplifying cells representative of the early progenitor type II cells.

Currently, the type I cells are most widely accepted to express GFAP, Nestin, and Sox2 molecular markers (Encinas and Enikolopov 2008). Identifying

the PLA2G7-CreER^{T2}/Rosa26YFP positive cells as a source for long-lived neural stem cells is important because not only has a new molecular marker been identified, but also a whole cell population. At one year of age, PLA2G7-CreER^{T2}/Rosa26YFP mice that were tamoxifen induced at P28 still harbor mitotically active cells as well as cells marching through the maturation cascade by expressing immature neuronal marker DCX. These cells also went on to produce cells that morphologically resemble mature neurons throughout the entire granule cell layer of the hippocampal dentate gyrus.

A diphtheria toxin fragment A mouse was used in concert with the PLA2G7-CreER^{T2} mouse strain to study the dynamics of this whole cell population. The PLA2G7-CreER^{T2}/DTA BpA mice displayed significant ablation of the neural stem cell compartment measured through the loss of mitotic cells and later DCX positive neuroblasts. Since the PLA2G7-CreER^{T2} population is more representative of a type I cell, it is probably largely quiescent and has a very slow rate of turnover. This proposed slow turnover can be hypothesized to correlate to the pattern of decreased Ki67 and DCX expression observed after the cell-specific ablation of PLA2G7-CreER^{T2} positive astrocytes in the subgranular zone. Initially (1 week after ablation), there was approximately a 40% decrease in Ki67 positive cells in the SGZ, and by 1 month, it progressed to 60%. This approximate 60% decrease remained steady up to 9 months after the cell ablation. Since the PLA2G7-CreER^{T2} cell population has a slow turn-over, the full net

approximate decrease of 60% was not observed at the early 1 week post-ablation time point. The same remains relevant for the DCX neuroblast population; the initial drop was not as dramatic as the later time points (3 and 9 months post tamoxifen-induced ablation) (Figure 3.8). This partial ablation of Ki67 and DCX activity also collaborates the hypothesis that the neural stem cell compartment is heterogeneous and that the ablation of just one population is not enough to completely silence the activity. Another reason why there was residual activity could be due to the ablation not having total efficacy. However, efficiency of the recombination in the DTA BpA mouse was described in the original paper and was found to be highly effective (Brockschnieder, Lappe-Siefke et al. 2004).

Conversely, the CstC-CreER^{T2}/DTA BpA cells depleted in the SGZ experienced a much more dramatic loss of Ki67 cells in comparison to the PLA2G7-CreER^{T2}/DTA BpA population. This transient amplifying compartment experiences a high rate of turnover, and thus represents a large proportion of the current actively dividing cells. Of the DCX population, about 30% was lost 1 week to 1 month after CstC-CreER^{T2} cell specific ablation induced at P28 (Figure 3.8). This loss continued to increase as the animal aged to 10 months. Since these transient amplifying cells only have a life of about 1 – 2 months, it is a little counterintuitive that the loss of Ki67 and DCX populations would continue to increase at 3 and 9 months post-tamoxifen (Figures 3.2 and 3.8). Of note, a glycosylated form of CstC has been shown to regulate neural stem cells in an

autocrine/paracrine fashion and could account for part of the disruption of the neural stem cell compartment (Taupin, Ray et al. 2000).

Loss of CstC-CreER^{T2} cells also relates to a significant decrease in the volume of the granule cell layer of the dentate gyrus that was apparent as early as 1 week post-tamoxifen induced ablation. To test whether this loss was due to a loss of neurogenic activity or to a loss of a cell niche factor, neurogenesis was suppressed through temozolomide (anti-mitotic drug) injections (Newlands, Stevens et al. 1997). After a month of TMZ injections in normal animals, careful stereological quantification of the granule cell volume was performed, and no significant difference was observed when compared to controls. The difference between the granule cell volume in CstC-CreER^{T2}/DTA BpA mice and controls was significantly different (Figure 3.9). From this observation, I postulate that the loss of the CstC-CreER^{T2} population of cells in the SGZ results in a loss of essential neurogenic niche factors that not only encourage normal neural stem cell biology but also the neuronal homeostasis of granule cell neurons.

In both mouse lines PLA2G7-CreER^{T2}/DTA BpA and CstC-CreER^{T2}/DTA BpA, tamoxifen-induced cell ablation resulted in a transient gliosis throughout all brain regions. In particular, the cortex, which is typically filled with both CstC-CreER^{T2} astrocytes (very abundant) and PLA2G7-CreER^{T2} astrocytes (abundant), there was a strong expression of reactive, GFAP positive

astrocytes (Figure 3.7 E and F). Typically, GFAP expression in the cortex is very minimal (Figure 3.7 D). This gliotic response was probably a "neighbor-effect" of other astrocytes responding to the shock of the death of their astrocyte neighbor. A gliotic response also occurs after an injury such as a traumatic brain injury (Norton, Aquino et al. 1992; McKeating and Andrews 1998). Normal, healthy astrocytes have extensive arborization and extend thousands of processes out to create a network (Hamilton and Attwell 2010). The loss of an astrocyte results in the neighboring astrocytes in the network compensating for that loss (i.e. gliosis).

Both astrocytes and neural stem cells represent very heterogeneous and complex populations of cells. Through cell-specific ablation studies, the dynamics of both the CstC-CreER^{T2} and PLA2G7-CreER^{T2} populations were studied as neural stem cells and mature cortical astrocytes. Loss of mature cortical astrocytes results in a massive transient reactive gliosis one week after cell death, which by one month, had mostly subsided. Gliosis was also observed in the hippocampus, but within this anatomical region, the main interest was in the significant decrease of neurogenic activity. From the cell dynamics observed, PLA2G7-CreER^{T2} positive cells are long-term neural stem cells that rarely divide over the lifetime of the animal. The CstC-CreER^{T2} population of cells are short-lived (1 – 2 months), transient amplifying cells that also play a critical role in maintaining the health of the SGZ and its associated structures (i.e. granule cell

layer of the dentate gyrus). These findings are important as they add another molecular signature for identifying adult neural stem cells in the SGZ.

FIGURE 3.1

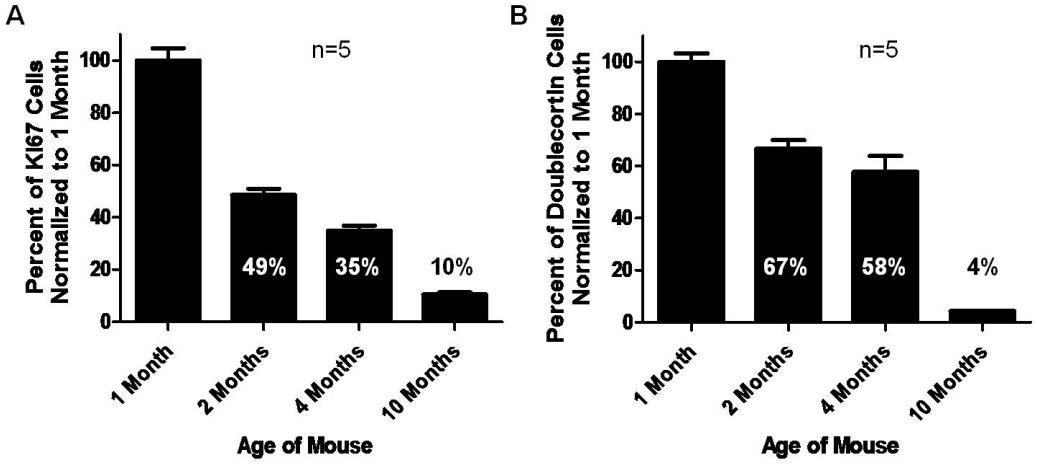

Figure 3.1: Dynamics of neurogenesis in the adult subgranular zone in the murine brain.

As mice age, neurogenesis steadily decreases. Animals were analyzed at 1, 2, 4, and 10 months of age for the amount of both Ki67 (cells of rapid turnover) and DCX (cells of more latent turnover). All numbers are normalized to animals 1 month of age.

FIGURE 3.2

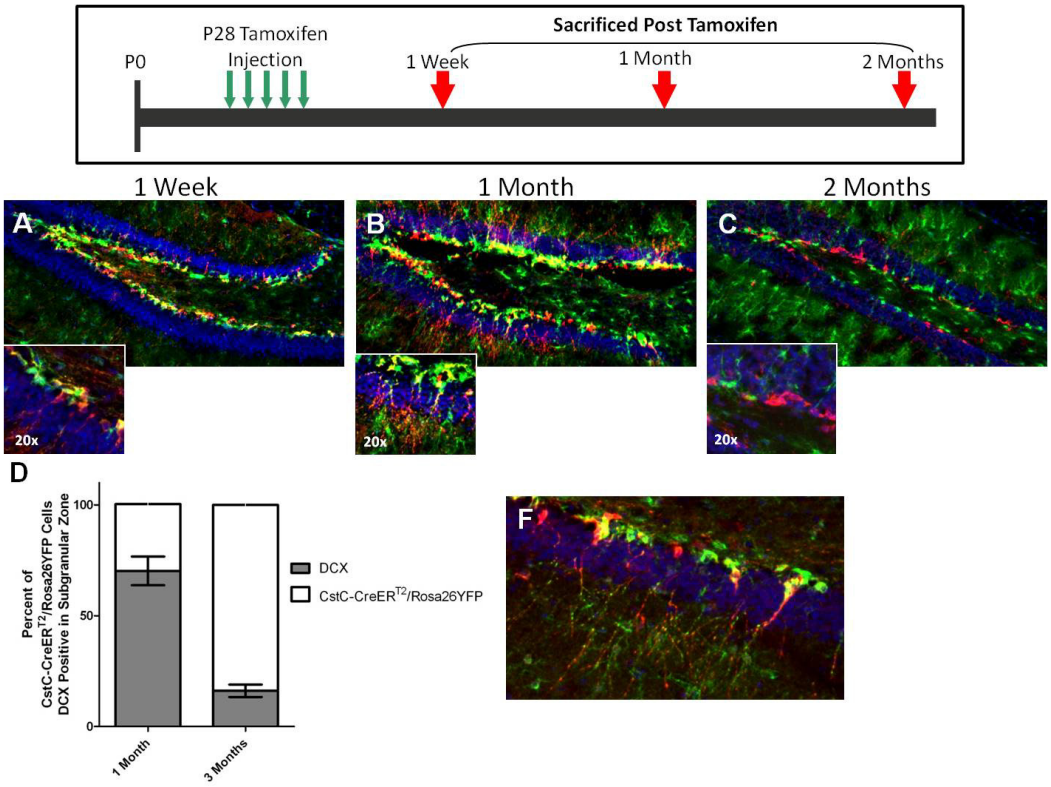

Figure 3.2: CstC-CreER^{T2}/Rosa26YFP cells identify a transient amplifying stem cell population.

CstC-CreER^{T2}/Rosa26YFP animals were treated on P28 with 5 injections of tamoxifen, and then analyzed 1 week (A), 1 month (B), and 2 months (C) later. Strong colocalization with DCX was shown at 1 month later (70.23% $\pm$ 11.17), and by 3 months later, the amount of colocalization has decreased to 16.15% $\pm$4.84 (D). When animals were injected with 5 injections of tamoxifen at the age of 5 months and then analyzed 2 weeks later, colocalization with DCX was 32.97% (F). This shows that the DCX/CstC-CreER^{T2}/Rosa26YFP population did not run out or die off, but can be induced at later time points in the adult life of mice.

FIGURE 3.3

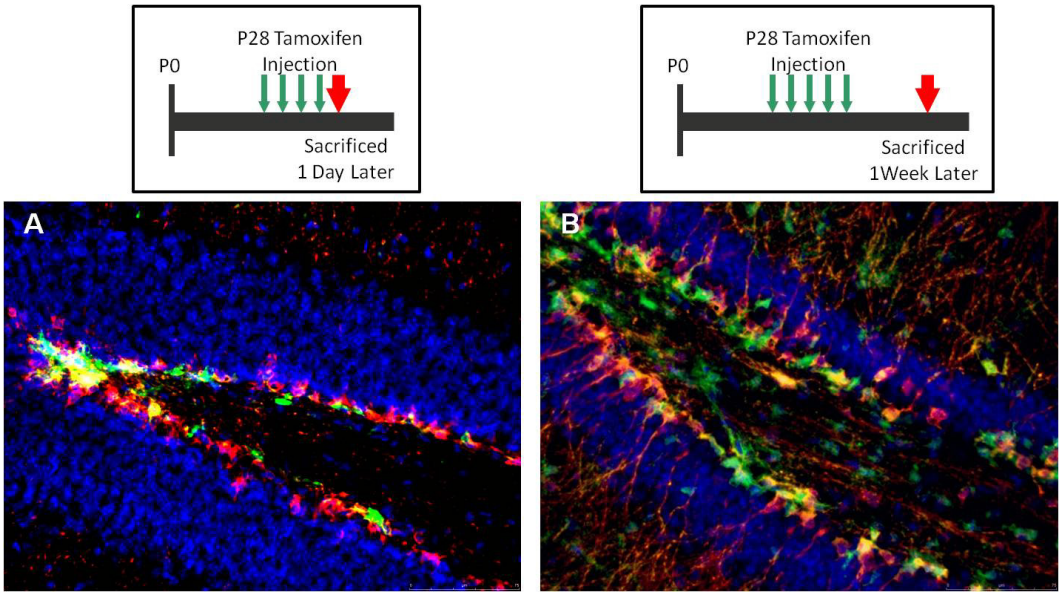

Figure 3.3: Temporal analysis of CstC-CreER^{T2}/Rosa26YFP labeled cells in the SGZ identifies neural stem cells.

Adult P28 CstC-CreER^{T2}/Rosa26YFP animals were injected with four doses of tamoxifen and then sacrificed one day after the last injection, after which the animal was perfused. Analysis showed that the CstC-CreER^{T2}/Rosa26YFP (green) cells mature into DCX (red) positive cells since there are several DCX cells that are not colocalized (A). In panel B, the animal was treated with 5 doses of tamoxifen at P28 and then sacrificed 1 week later. Many of these cells mark DCX positive populations in yellow, postulating that CstC-CreER^{T2}/Rosa26YFP cells become DCX positive over a course of a week.

FIGURE 3.4

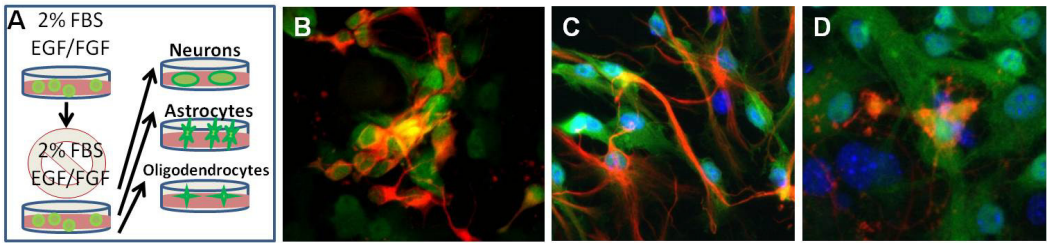

Figure 3.4: Cystatin C-CreER^{T2} expression in adult hippocampal neurospheres.

Cystatin C-CreER^{T2}/Rosa26YFP animals were treated with tamoxifen at 3 weeks of age and sacrificed one week later. At sacrifice, hippocampal neurospheres were made and kept in culture using neural stem cell media with EGF/FGF and 2% FBS (A). Upon removal of EGF/FGF and 2% FBS for 4 days, neurospheres differentiated into mature cell types *in vitro*. Differentiated neurospheres in culture produced Tuj-1 positive neurons (B), GFAP positive astrocytes (C), and CNPase positive oligodendrocytes (D).

FIGURE 3.5

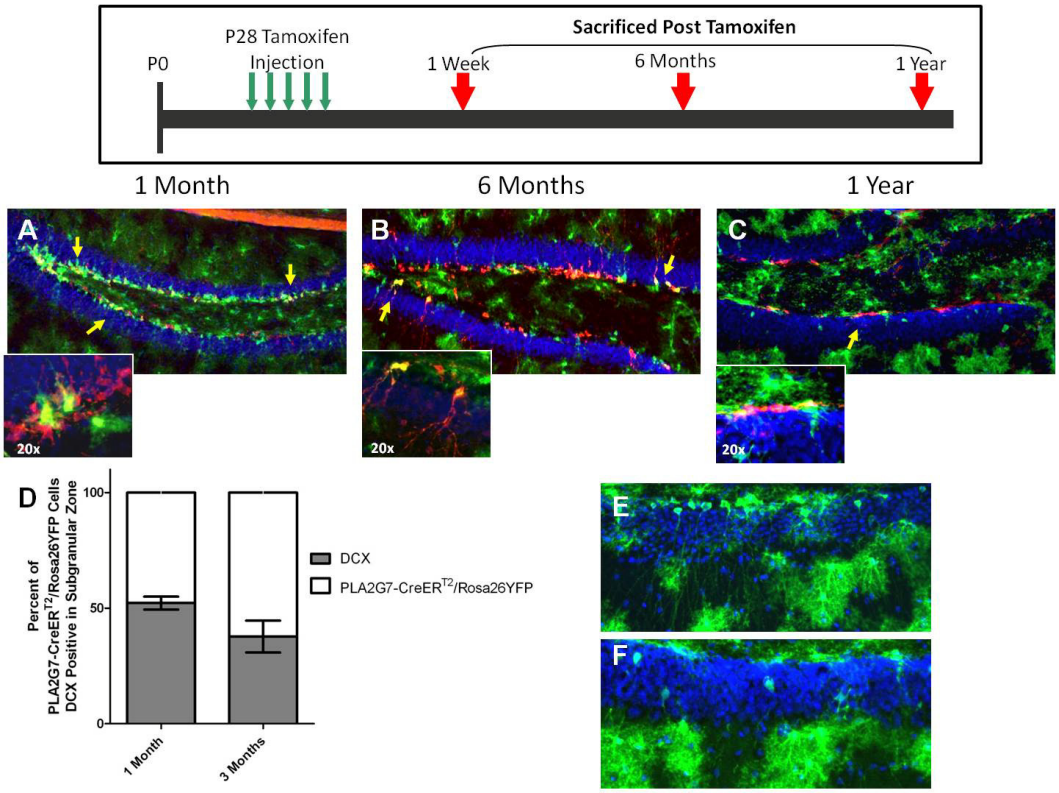

Figure 3.5: PLA2G7-CreER^{T2}/Rosa26YFP cells mark a long term stem cell population.

PLA2G7-CreER^{T2}/Rosa26YFP cells in the subgranular zone of the dentate gyrus continuously make DCX positive cells throughout the life of the animal. Animals were injected with tamoxifen on P28 and sacrificed and analyzed for DCX colocalization 1 month (A), 6 months (B), and a year later (C). Quantification at 1 month and 3 months (post P28 tamoxifen injections) showed a colocalization of 52.14$\pm$4.91% and 37.67$\pm$11.94% respectively (D). Additionally, these cells eventually become a small percentage of neurons in the granule cell layer of the dentate gyrus as shown at 4 months (E) and 9 months (F) after tamoxifen injection at P28.

FIGURE 3.6

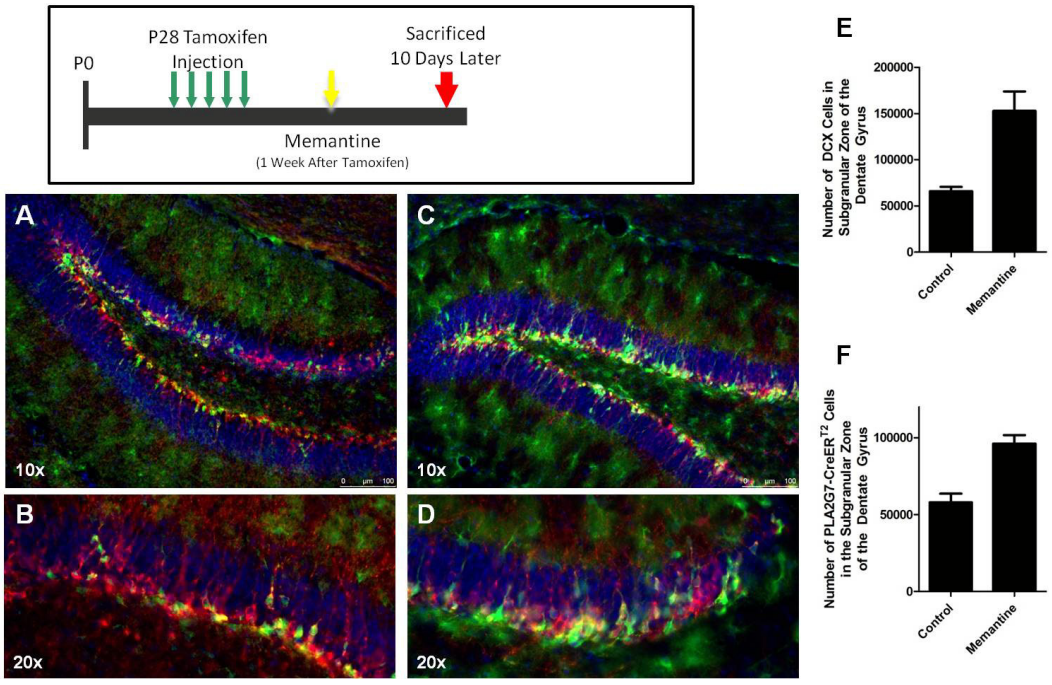

Figure 3.6: Alzheimer's drug memantine stimulates both the CstC-CreER^{T2} and PLA2G7-CreER^{T2} stem cell compartments.

Animals were first injected for five consecutive days with 180 mg/kg of tamoxifen. One week later, they were treated with a single dose of 50mg/kg of memantine and sacrificed 10 days later. Analysis of the subgranular zone of these animals show both an increase in the DCX (red) population as well as the CstC-CreER^{T2}/Rosa26YFP (green) (A and B) and PLA2G7-CreER^{T2}/Rosa26YFP (green) (C and D). The overall percent colocalization of these stem cell populations with DCX does not change, but populations increase by the same percent when analyzed in mice at 2 months of age (E and F).

FIGURE 3.7

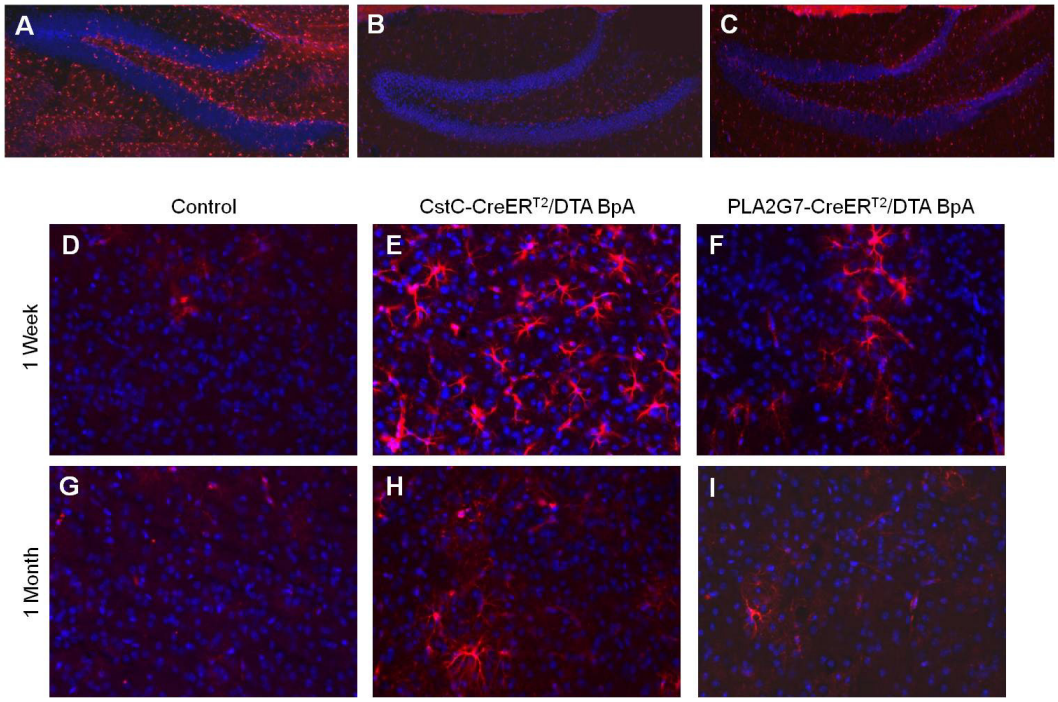

Figure 3.7: One week after diphtheria toxin induced deletion of CreER^{T2} cells, loss of endogenous CstC is observed along with gliosis throughout the entire brain.

Endogenous cystatin C expression was analyzed by a polyclonal antibody at one week after diphtheria toxin induced deletion of both CstC-CreER^{T2} cells (B) and PLA2G7-CreER^{T2} cells (C). When compared to a control animal (A), the endogenous cystatin C expression was depleted in the CstC-CreER^{T2} animal (B), and there was a marked decrease in expression in the PLA2G7-CreER^{T2} animal (C). Gliosis, shown by GFAP (red), is observed throughout the entire brain in the inducible Cre animals, with the majority of gliosis happening at 1 week and mostly subsiding by 1 month. CstC-CreER^{T2}/DTA BpA animals had the most noticeable gliosis, especially in the internal granular layer of the cortex (E, H) when compared to control (D, G). PLA2G7-CreER^{T2}/DTA BpA brains also experienced gliosis (F, I).

FIGURE 3.8

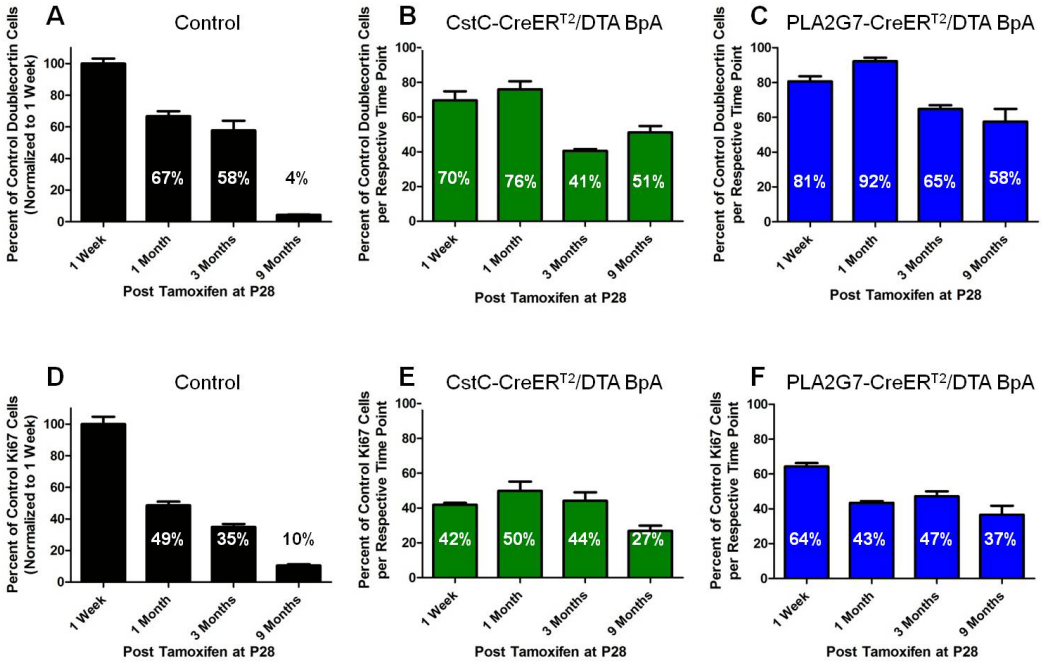
A
Control
Percent of Control Doublecortin Cells (Normalized to 1 Week)
67%
58%
4%
1 Week
1 Month
3 Months
9 Months
Post Tamoxifen at P28
B
CstC-CreER^{T2}/DTA BpA
Percent of Control Doublecortin Cells per Respective Time Point
70%
76%
41%
51%
Post Tamoxifen at P28
C
PLA2G7-CreER^{T2}/DTA BpA
Percent of Control Doublecortin Cells per Respective Time Point
81%
92%
65%
58%
Post Tamoxifen at P28
D
Control
Percent of Control Ki67 Cells (Normalized to 1 Week)
49%
35%
10%
Post Tamoxifen at P28
E
CstC-CreER^{T2}/DTA BpA
Percent of Control Ki67 Cells per Respective Time Point
42%
50%
44%
27%
Post Tamoxifen at P28
F
PLA2G7-CreER^{T2}/DTA BpA
Percent of Control Ki67 Cells per Respective Time Point
64%
43%
47%
37%
Post Tamoxifen at P28

Figure 3.8: Deletion of CstC-CreER^{T2} and PLA2G7-CreER^{T2} cells results in a loss of both Ki67 and DCX in the subgranular zone of the dentate gyrus.

The Ki67 and DCX populations naturally decline as an adult mouse ages (please refer to Figure 3.1). Deletion of these two Cre populations show a significant drop in both DCX and Ki67 populations, with CstC-CreER^{T2} having a much more dramatic loss than PLA2G7-CreER^{T2}. The initial 1 week to 1 month decrease in DCX for both cre lines show a moderate initial drop, followed by a more severe drop 3 and 9 months after tamoxifen injections (C, D). All time points for both cre lines when compared to control were statistically significant with a p value of <0.0001. For the Ki67 population, the drop was exaggerated for all groups and was most significant at 9 months (when compared to control). For the CstC-CreER^{T2}/DTA BpA line (A), the most significant change in loss of Ki67 cells happened at 9 months ($p<0.005$). In contrast, for the PLA2G7-CreER^{T2}/DTA BpA animals (B), the most significant change in loss of Ki67 happened between 1 week and 1 month ($p<0.0001$).

FIGURE 3.9

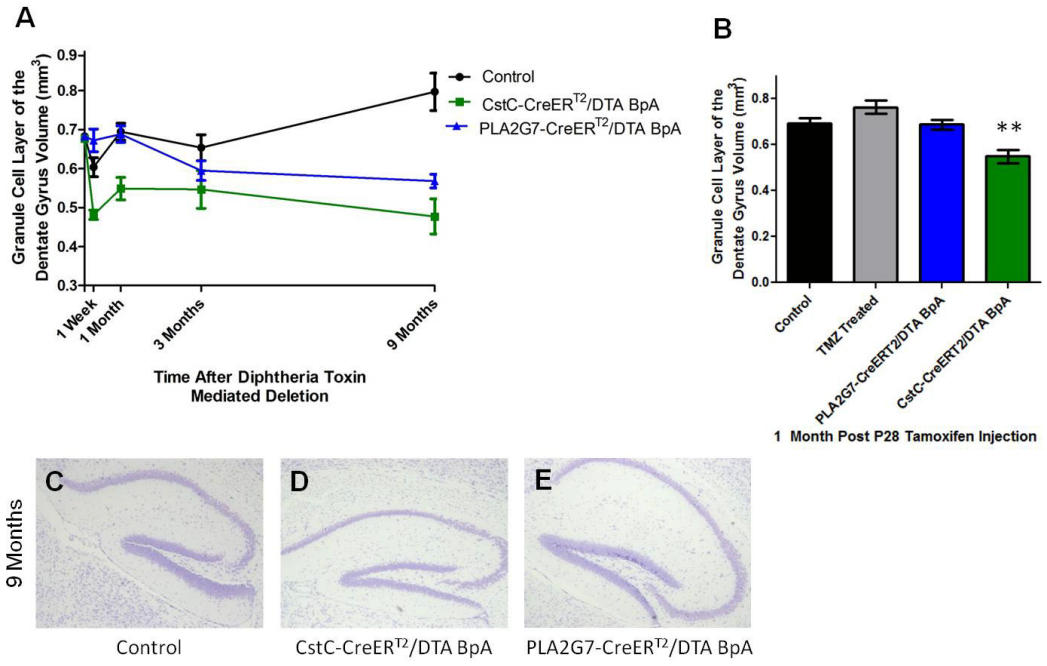

Figure 3.9: Degeneration of the dentate gyrus after cell specific ablation.

Loss of PLA2G7-CreER^{T2}/DTA BpA causes irreversible volume depletion of the granule cell layer of the dentate gyrus over time (A, E); most loss is noticed at 3 months and worsens at 9 months. Loss of dentate gyrus volume for CstC-CreER^{T2}/DTA BpA cells happens as early as 1 week to 1 month after cell deletion and persists as long as 9 months after cell deletion (A,D). Figures C, D, and E show Nissl staining of the hippocampus. B shows that the degeneration of the dentate gyrus is not an effect from the loss of the neural stem cells, but due to a stem cell niche effect. Animals injected with TMZ (which suppresses the neural stem cell compartment) show no loss of volume of the dentate gyrus, but there is a significant difference between the TMZ animals and the CstC-CreER^{T2}/DTA BpA animals (** $p = 0.006$) (Figure 3.9 B).

FIGURE 3.10

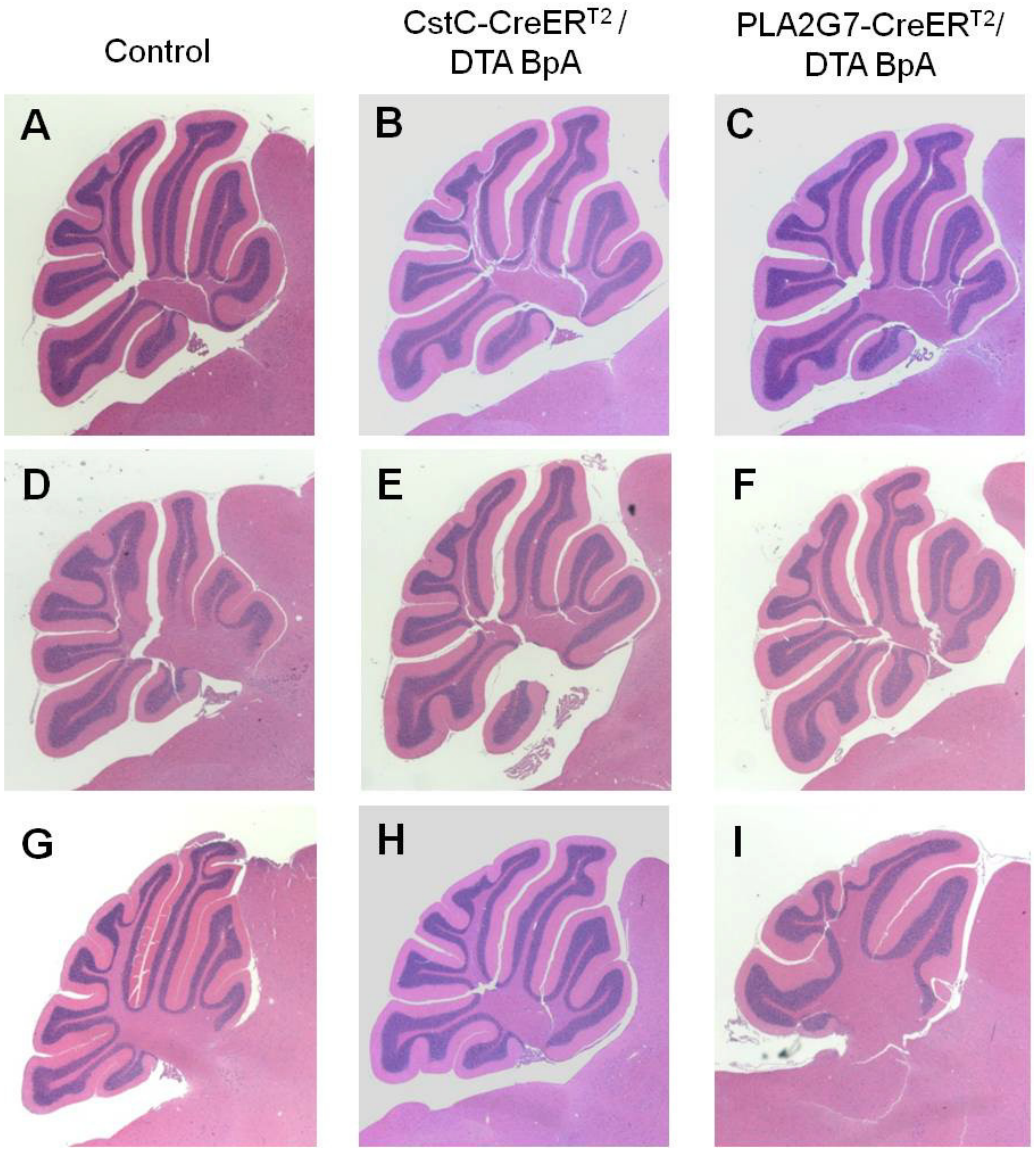
Control
CstC-CreER^{T2} / DTA BpA
PLA2G7-CreER^{T2}/ DTA BpA
A
B
C
D
E
F
G
H
I

Figure 3.10: Loss of PLA2G7-CreER^{T2} astrocytes causes cerebellum degeneration.

PLA2G7-CreER^{T2} astrocytes have very strong expression in the cerebellum. After losing these astrocytes through diphtheria toxin mediated cell death, animals experience both volume and cerebellum peduncle loss by 9 months when compared to control (G and I). No noticeable change in cerebellum morphology is noticed at 1 month and 3 months between the control (A, D) and the PLA2G7-CreER^{T2}/DTA BpA mouse (C, F). CstC-CreER^{T2}/DTA BpA animals never seem to experience any differences in cerebellum morphology or volume (B, E, H).

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CHAPTER FOUR

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Though astrocytes are the most abundant cell type in the brain, their great importance in brain physiology has been largely understated throughout the history of neuroscience research. As the entire field of neuroscience progresses, so does the need for more comprehensive and practical ways to study the biology of mature astrocytes in the central nervous system (CNS). Traditionally, astrocytes have been viewed as merely providing support for neurons and being immunohistologically positive for glial fibrillary acidic protein (GFAP) and S100β (a calcium binding protein) (Sofroniew and Vinters 2010). Advances made in recent times have propelled astrocyte biology into the spotlight in relation to their critical responsibilities in maintaining brain homeostasis and molecular heterogeneity. A study focused on analyzing the "molecular fingerprint" of astrocytes described many new astrocyte-specific markers including cystatin C (CstC) and phospholipase A2 Group VII (PLA2G7) (Bachoo, Kim et al. 2004). Consequently, from these studies, tamoxifen inducible $CreER^{T2}$ mice were generated to more closely assess astrocyte biology.

Utilizing the two novel astrocyte specific transgenic mouse models, CstC-CreER^{T2} and PLA2G7-CreER^{T2}, I addressed two critical questions: what population does each transgenic model label, and what is their role in both development and adult neurogenesis? Focused on the concept of astrocyte heterogeneity, I hypothesized that CstC-CreER^{T2} and PLA2G7-CreER^{T2} positive populations represent separate, but minimally overlapping, mature astrocyte populations. In both the subgranular zone (SGZ) and subventricular zone (SVZ), astrocytes have been identified as a neural stem cell (Doetsch, Caille et al. 1999; Laywell, Rakic et al. 2000; Seri, Garcia-Verdugo et al. 2001; Ihrie and Alvarez-Buylla 2008). Moreover, I hypothesized that both CstC-CreER^{T2} and PLA2G7-CreER^{T2} populations have an active role in adult neurogenesis.

A comprehensive embryonic and adult time course analysis of both astrocyte specific transgenic animal lines revealed the heterogeneity between the CstC-CreER^{T2} and PLA2G7-CreER^{T2} astrocyte populations. Both lines were analyzed at early and late embryonic time points, early post-natal periods (i.e. P4 and P14), and a wide range of adult time points. Extensive studies were performed on the long term survival of astrocytes that underwent recombination at P28. The most intriguing embryonic recombination was in the PLA2G7-CreER^{T2}/Rosa26YFP population of cells. Recombination on embryonic day 12.5 resulted in morphologically mature astrocyte expression in both the spinal cord and brain when analyzed on embryonic day 14.5. This is of importance since it is

widely accepted that most mature astrocyte populations arise late embryonically and postnatally. If embryos could survive multiple tamoxifen injections over consecutive days, one would postulate that a reasonable sized population of morphologically mature astrocytes would be observed throughout the CNS. Additionally, embryonic PLA2G7-CreER^{T2}/Rosa26YFP expression also colocalized with radial glia (represented with by binding with brain lipid binding protein (BLBP)). Further, postnatal expression (P0 and P4) of PLA2G7-CreER^{T2}/Rosa26YFP, when analyzed at 1 and 3 months, shows a contribution to the mature neurons in the granule cell layer of the dentate gyrus. Conversely, early postnatal expression of the CstC-CreER^{T2} compartment shows minimal mature neuronal contribution to the granule cell layer of the dentate gyrus. As such, of the two lines, the PLA2G7-CreER^{T2} population of cells plays a much more active role in the embryonic and postnatal development in the murine brain.

In adult animals, the PLA2G7-CreER^{T2} positive cells continue to produce mature neurons in the neurogenic regions. In the subventricular zone (SVZ), PLA2G7-CreER^{T2} astrocytes show mitotic activity, supporting the idea that astrocytes are true neural stem cells. These cells migrate through the rostral migratory stream until they arrive in the olfactory bulb and differentiate into interneurons. The dynamics of the SVZ was not the main focus of my studies, but rather the other neurogenic niche of the adult brain, the subgranular zone (SGZ). In the SGZ, the PLA2G7-CreER^{T2} astrocytes appear to slowly divide and have

neurogenic activity throughout the majority of the life of a mouse. In contrast, the CstC-CreER^{T2} neurogenic astrocytes have a limited lifespan of 1 – 2 months. From this observation, these cells were termed transient amplifying progenitors. Even when the CstC-CreER^{T2}/Rosa26YFP animals were 6 months or 1 year of age, tamoxifen-induced recombination showed positive cells in the SGZ colocalizing with both Ki67 and DCX. Cells induced later in life (past P28) still have a life span of 1 – 2 months. Presently, the SGZ neurogenesis is described through changes in cell morphology and immunohistological markers as the cell continues through a maturation cascade (Kempermann, Jessberger et al. 2004; Encinas and Enikolopov 2008). Identification of these two astrocyte populations as possessing stem cell properties adds another marker to identify stem cells, but more importantly, provides a practical way to trace these stem-like cells throughout their lifetime. Table 4.1 outlines the similarities and differences between the CstC-CreER^{T2} and PLA2G7-CreER^{T2} transgenic mouse lines.

To attain an in depth understanding of SGZ dynamics, both CstC-CreER^{T2} and PLA2G7-CreER^{T2} mouse lines were coupled with a floxed diphtheria toxin fragment A (DTA BpA) deleter strain of mice. After a cell-specific ablation of either the CstC-CreER^{T2} or PLA2G7-CreER^{T2} populations in the SGZ, there is not a complete loss of either the Ki67 or DCX population. This argues in favor of the concept that the putative stem cell population is heterogeneous and cannot be represented entirely by just one cell type (i.e. GFAP astrocytes) or by a few

molecular markers (i.e. GFAP, Nestin, Sox2). To fully understand the normal biology of the neural stem compartment researchers will need to cast a wider net.

From the CstC-CreER^{T2}/DTA BpA results, it could be hypothesized that CstC-CreER^{T2} positive cells not only play the role of a transient amplifying progenitor but also have a function in maintaining the neurogenic niche. Since CstC-CreER^{T2} cells have a lifespan of 1 – 2 months, one would expect the loss of both Ki67 and DCX cells to return by 3 months after ablation. In actuality, neither cell population returns, but continues to decrease, even 9 months later. This is not the first time that CstC has been implicated in neurogenic niche maintenance. A previous study recognized that a glycosylated form of CstC functioned in an autocrine/paracrine fashion to sustain the neurogenic niche (Taupin, Ray et al. 2000). This data underlines the importance of astrocytes in not only the actual neural stem cell maturation cascade, but also in creating an environment conducive for neurogenic activity. Figure 4.1 illustrates a proposed model for the roles of both PLA2G7-CreER^{T2} and CstC-CreER^{T2} in the SGZ stem cell compartment.

Future Directions

The studies presented here could be of great use for future neuroscience research focusing on the diverse roles astrocytes play in CNS pathophysiologies.

Although only briefly analyzed in the context of these studies, both the CstC-CreER^{T2} and PLA2G7-CreER^{T2} populations presented a strong presence in both the embryonic and adult spinal cords. Embryonically, it has been theorized that astrocyte progenitors are distinct and give rise to only astrocytes. These astrocyte progenitor populations may express mature astrocyte molecular markers, for example, fibroblast growth factor 3 (Fgfr3) (Pringle, Yu et al. 2003). PLA2G7-CreER^{T2} positive cells may also represent an astrocyte progenitor population which may differentiate new mature astrocyte populations, not previously described herein.

In spinal cord injuries, astrocytes can function to protect the tissue from further damage (Faulkner, Herrmann et al. 2004). Reactive gliosis is a widespread astrocyte reaction to injury induced not only in the spinal cord, but throughout the entire brain (Pekny and Nilsson 2005). Gliosis has been observed in a range of neurodegenerative diseases (i.e. Alzheimer's disease, AIDS dementia complex, prion disease, etc) and can eventually lead to a glial scar. Depending on the context, the glial scar can have either beneficial or detrimental effects on brain repair (Prusiner and DeArmond 1994; Vajda 2002; Pekny and

Nilsson 2005). As both CstC-CreER^{T2} and PLA2G7-CreER^{T2} transgenic animals label large populations of mature cortical astrocytes, they will possibly provide discerning models for traumatic brain injury gliosis (i.e. cortical weight-crushing and stab models).

In reference to postnatal development of the hippocampal dentate gyrus, numerous and varied studies focusing on the role of the PLA2G7-CreER^{T2} compartment are required. Since the hippocampus is recognized to be crucial in learning and memory in adult animals, the postnatal development has great implications for future cognitive abilities. Maternal care for rodent pups greatly affects the hippocampal development and cognitive abilities of offspring (Liu, Diorio et al. 2000; Bredy, Grant et al. 2003; Bick-Sander, Steiner et al. 2006). It has been shown that allowing pregnant and lactating mothers to voluntarily exercise appears to increase neuronal volume of the pup's dentate gyrus (Bick-Sander, Steiner et al. 2006). This would be an easy way to assess if A) PLA2G7-CreER^{T2} expression can be upregulated through voluntary exercise, and if so, B) what is the impact of increased PLA2G7-CreER^{T2} expression? Conversely, pups with negligent mothers (i.e. lack of grooming) or reduced nutrient intake experience impaired cognitive hippocampal development (Bredy, Grant et al. 2003). Changes in neuronal contribution to dentate gyrus development in the early postnatal periods could translate into altered adult neurogenesis in the SGZ.

The PLA2G7-CreER^{T2} compartment is an attractive target for the study of neurogenic potential from early postnatal to adult.

One eminently appealing translational neuroscience research topic is neural stem cell replacement therapy in cases of neurodegenerative disease. Cell specific ablation through the diphtheria toxin mice in combination with either CstC-CreER^{T2} or PLA2G7-CreER^{T2} transgenics reflected a significant loss of mitotic activity in the neurogenic regions, granule cell volume loss in the dentate gyrus, and death of cortical neurons. Conversely, both astrocyte transgenic lines experience stimulation in the neural stem cell compartment after exposure to memantine. One future study could focus on stimulating neurogenesis through memantine injection in animals that have already undergone diphtheria toxin cell ablation in order to try to rescue the effects of ablation. Memantine is a NMDA (N-methyl-D-aspartate) receptor antagonist and has been reported to increase neurogenesis (Fuchs and Gould 2000; Nacher and McEwen 2006; Namba, Maekawa et al. 2009). More naturally, there are also a variety of ways to increase adult hippocampal neurogenesis, such as voluntary exercise (van Praag, Kempermann et al. 1999). Therefore, in addition to treatment with NMDA receptor antagonists, animals with depleted SGZs could also be exposed to voluntary exercise situations to increase neurogenic activity.

Many current replacement studies require implantation of neural stem cells, but after engraftment, these neural stem cells are not guaranteed to differentiate into the desired cell types (Rossi and Cattaneo 2002). While the studies previously proposed may be elementary, they will provide some insight into how to recruit endogenous neural stem cells from a damaged stem cell compartment. By recruiting existing stem cells, the intracellular machinery is potentially already primed and ready to allow for maturation and differentiation into the mature cell types desired.

Aside from the gliotic response to injury and neurodegeneration, astrocytes also contribute to other pathophysiologies such as brain tumors. Glioblastoma multiforme (GBM) is an extremely aggressive and malignant intracranial tumor. These highly aggressive and treatment resistant tumors are comprised of great cell type heterogeneity, but are predominantly of glial origin (Furnari, Fenton et al. 2007). Since CstC-CreER^{T2} and PLA2G7-CreER^{T2} transgenic lines represent both adult neural stem cells and mature astrocytes, they make attractive candidates for GBM mouse models. Initial research has already been performed for GBM mouse model research (see Appendix A). These studies focused on cell-specific deletion of tumor suppressors PTEN and p53. PTEN and p53 are some of the most common mutations found in GBMs, but since the molecular signature of GBMs are highly diverse, it is crucial to target more mutations to both the astrocytes and neural stem cells in the generation of a

faithful GBM mouse model (Furnari, Fenton et al. 2007; Parsons, Jones et al. 2008; Zheng, Ying et al. 2008; Alcantara Llaguno, Chen et al. 2009). Since p53 and PTEN are both tumor suppressors, it would be logical to also target an oncogene mutation to these cells, making them more representative of the cancerous cells found in GBMs. Some such oncogenes for targeting include B-raf, KRAS, and EGFR (epidermal growth factor receptor) (Parsons, Jones et al. 2008). Targeting specific tumor suppressor and oncogene mutations to either the CstC-CreER^{T2} or PLA2G7-CreER^{T2} population of cells is a very attractive option for the modeling of adult GBMs. These populations of cells target, in adult animals, both the neural stem compartments and mature astrocytes – two cell populations hypothesized to be responsible for malignant GBMs.

As the field of neuroscience grows, so does the realization of the importance of astrocyte biology. Astrocytes have been implicated in a wide range of CNS pathophysiologies from neurodegeneration to cancer. While there have been previous discoveries of a few inducible astrocyte specific transgenic models, more are needed to fully understand the underlining heterogeneity of astrocytes and the neural stem cells they can represent. In this study, I successfully characterized two novel astrocyte specific transgenic lines, CstC-CreER^{T2} and PLA2G7-CreER^{T2}, and their roles in both normal astrocyte biology (i.e. expression patterns, relationship to vasculature, microdomains, and role in embryonic development) and functions in the neural stem cell compartment.

Future studies can build on the results presented in this thesis for further expansion of the scientific community's understanding of astrocyte biology.

TABLE 4.1

	Cystatin C-CreER^{T2}	Phospholipase A2 Group VII-CreER^{T2}
Astrocyte Expression	Yes	Yes
Cortical Expression	Yes, Extremely Strong	Yes, Abundant
Bergmann Glia	Yes	Yes, Extremely Strong
Neuronal Expression	No	No
Oligodendrocyte Expression	No	No
Neurogenic Activity	Yes, Transient Amplifying Stem Cell in the SGZ	Yes, Long-Lived Neural Stem Cell in the SGZ
Subgranular Zone Expression?	Yes	Yes
Subventricular Zone Expression?	Yes	Yes
Embryonic Expression	Low Expression	Moderate Expression
Postnatal Expression	Yes, Strong	Yes, Strong
Spinal Cord Expression	Yes	Yes

Table 4.1: Expression patterns of CstC-CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP transgenic mice.

Table 4.1 compares the expression similarities and differences of the two inducible transgenic mouse lines, CstC-CreER^{T2}/Rosa26YFP and PLA2G7-CreER^{T2}/Rosa26YFP.

FIGURE 4.1

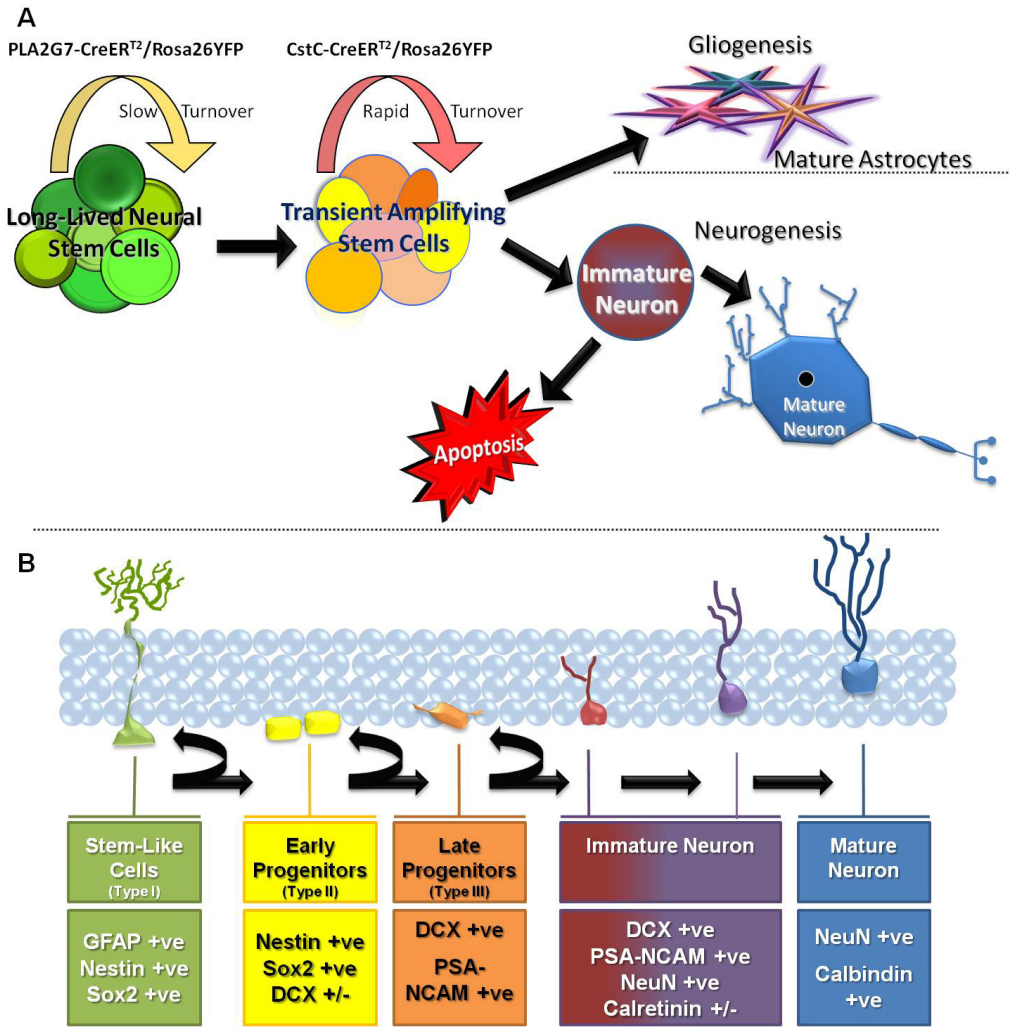
A
PLA2G7-CreER^{T2}/Rosa26YFP
CstC-CreER^{T2}/Rosa26YFP
Slow Turnover
Rapid Turnover
Gliogenesis
Mature Astrocytes
Long-Lived Neural Stem Cells
Transient Amplifying Stem Cells
Immature Neuron
Neurogenesis
Mature Neuron
Apoptosis
B
Stem-Like Cells (Type I)
Early Progenitors (Type II)
Late Progenitors (Type III)
Immature Neuron
Mature Neuron
GFAP +ve Nestin +ve Sox2 +ve
Nestin +ve Sox2 +ve DCX +/-
DCX +ve PSA-NCAM +ve
DCX +ve PSA-NCAM +ve NeuN +ve Calretinin +/-
NeuN +ve Calbindin +ve

Figure 4.1: Roles of PLA2G7-CreER^{T2} and CstC-CreER^{T2} in the subgranular zone neural stem cell compartment.

Both PLA2G7-CreER^{T2} and CstC-CreER^{T2} have distinct roles in the SGZ. PLA2G7-CreER^{T2} positive cells are most representative of the already described stem-like, type I cells, while CstC-CreER^{T2} positive cells are similar to early/late progenitors. This illustration combines the currently accepted SGZ maturation cascade (B) along with the findings from the CreER^{T2} positive cells (A). The illustration in A takes into account that stem cells may go through gliogenesis and that immature neurons that do not fully mature will eventually die.

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APPENDIX A

GENERATION OF AN ADULT GLIOBLASTOMA MULTIFORME MOUSE MODEL THROUGH THE UTILIZATION OF NOVEL ASTROCYTE SPECIFIC TRANSGENIC MOUSE LINES

BACKGROUND

Of all primary intracranial tumors, glioblastoma multiforme (GBM) is the most frequent and aggressive malignancy. These tumors (also referred to as grade IV astrocytomas) have a particularly high mortality rate, as they prove exceedingly resistant to current available treatments (Furnari, Fenton et al. 2007). The present standard in therapeutic care involves surgical resection followed by radiotherapy coupled with temozolomide chemotherapy (Robins, Chang et al. 2007; Clarke, Butowski et al. 2010). Combination therapy with temozolomide, an alkylating agent, subtly increased median survival time from 12.1 months to 14.6 months (Robins, Chang et al. 2007). There are several known genetic lesions found in these grade IV astrocytomas (Parsons, Jones et al. 2008). Tumor suppressors include PTEN and p53, and oncogenes EGFRvIII (epidermal growth factor receptor variant III), B-raf and KRAS, just to name a few (Furnari, Fenton et al. 2007; Parsons, Jones et al. 2008; Clarke, Butowski et al. 2010). Considering that there have been relatively few advances in the treatment therapy of GBMs in

the past several decades, it is of importance to create transgenic mouse models that are representative of GBM presentation and progression.

OBJECTIVE

Presently, researchers do not have great options for animal modeling of GBMs with a high degree of fidelity. For example, many studies have targeted GFAP-Cre mice with known genetic lesions (Furnari, Fenton et al. 2007). Endogenous GFAP (glial fibrillary acidic protein) expression can be found early in embryonic development representing radial glia. Radial glia act as precursor cells for brain development; thus when observing GFAP-Cre expression in mature adult animals, expression is not limited to only astrocytes, but all cell types including neurons (Zhuo, Theis et al. 2001). Since GBMs are hypothesized to be mainly of glial origin, these GFAP-Cre mouse models clearly are not accurate representatives for the cell of origin. Additionally, as GFAP expression occurs during early embryogenesis, these tumors were not fated during adulthood, but instead embryonically. No embryonic GBM exist.

Recently, the neural stem cell compartment was targeted through Nestin-CreER^{T2} animals in concert with heterozygous loss of Nf1 (neurofibromatosis type 1) and PTEN coupled with complete loss of p53. This resulted in a

histopathological grade IV astrocytomas in adult animals (Alcantara Llaguno, Chen et al. 2009). While these studies hold the advantage of inducing mutations in adult animals, they did not adequately address the roles of astrocytes in GBMs. The 2007 review written by Furnari and colleagues clearly details the current status of GBM knowledge, and Table 2 within that text highlights key genetic mouse models previously described in the literature (Furnari, Fenton et al. 2007).

Presently, no genetic models exist which address the roles of mature adult astrocytes combined with known GBM genetic lesions. Due to this fact, I generated transgenic mice with floxed p53 and PTEN alleles targeted to both the CstC-CreER^{T2} and PLA2G7-CreER^{T2} astrocyte specific inducible Cre mouse lines. I hypothesized that targeted loss of tumor suppressors p53 and PTEN to either CstC-CreER^{T2} or PLA2G7-CreER^{T2} mature astrocytes will provide insight into the initiation and progression of GBM malignancies.

RESULTS

Loss of p53 and PTEN in PLA2G7-CreER^{T2} Mice Results in Expression of Immature Glial Markers

PLA2G7-CreER^{T2}/p53$^{f/+}$/PTEN$^{f/f}$ were generated and underwent tamoxifen-induced recombination at P28. Animals were sacrificed 2 months after recombination (resulting in floxing of p53 and PTEN alleles) and analyzed through immunohistochemistry on 30μm sagittal sections.

Analysis of the astrocytes and stem cells in both the cortex and subgranular zone (SGZ) of the hippocampus showed upregulated expression of immature glial marker BLBP (brain lipid binding protein) as well as phospho-Akt (p-Akt) (Figure A.1) and pS6-ribosomal protein (data not shown). Figure A.1 A and B clearly display p-Akt activation in the SGZ neural stem cells accompanied by BLBP expression that is not apparent in age-matched, vehicle-injected control animals. While these changes in the neural stem cell compartment are striking, the immunoreactivity of these markers in mature cortical astrocytes is even more surprising. Mature astrocytes coexpressing both BLBP and p-AKT are observed throughout the entire cortex, and it could be hypothesized that these astrocytes, given enough time, would initiate malignant progression towards an astrocytoma.

Loss of p53 and PTEN in CstC-CreERT2/Rosa^{26}YFP Mice Extends the Life of Transient Amplifying Progenitors

Previous studies (Chapter 3) have shown CstC-CreER^{T2}/Rosa26YFP mice to represent a transient amplifying progenitor compartment in the SGZ. Transient amplifying progenitors have a life span of 1 – 2 months, and by 3 months have either matured into a neuronal phenotype or died. To study the effects of p53 and PTEN loss in this compartment, I generated a CstC-CreER^{T2}/Rosa26YFP/p53$^{f/+}$/PTEN$^{f/f}$ transgenic animal model.

Heterozygous loss of the p53 allele and complete loss of the PTEN allele resulted in an extended life span of CstC-CreER^{T2}/Rosa26YFP cells in the SGZ. As transient amplifying progenitors, CstC-CreER^{T2}/Rosa26YFP cells strongly express neuroblast marker doublecortin (DCX) 1 – 2 months after recombination at P28. In the CstC-CreER^{T2}/Rosa26YFP/p53$^{f/+}$/PTEN$^{f/f}$, colocalization with DCX was observed at 3 months and beyond in the SGZ after P28 recombination (Figure A.2). This result indicates that the loss of p53 and PTEN alleles in the transient amplifying compartment increases the neurogenic ability of these cells. Since transient amplifying cells represent a cell that is not fully differentiated, it has great potential for aberrant cellular programming that could result in tumor initiation.

SUMMARY/CONCLUSIONS

Glioblastoma multiforme represents a challenge for those in the neuro-oncology field as it is a highly malignant, infiltrative, and therapy resistant cancer that has seen slow research and clinical progress. One hurdle in the GBM research is the creation of a transgenic mouse model that not only mimics tumor initiation with fidelity, but also creates tumors which are histopathologically similar to those found in human patients. This brief study shows the effect of allelic loss of p53 and PTEN in not only mature cortical astrocytes, but also in long-lived neural stem cells and transient amplifying progenitors. By 3 months after tamoxifen-induced recombination, there are signs of cellular change, but not full tumor initiation. Figure A.3 describes the consequences of genetic lesions to the different cell compartments (i.e. mature cortical astrocytes and neural stem cells). These studies only targeted tumor suppressor genetic lesions to CstC-CreER^{T2} and PLA2G7-CreER^{T2} cell types. In conclusion, I hypothesize that inclusion of oncogenes, such as B-raf, and a longer latency time could result in a histopathological malignancy that shares characteristics of a grade IV astrocytoma.

FIGURE A.1

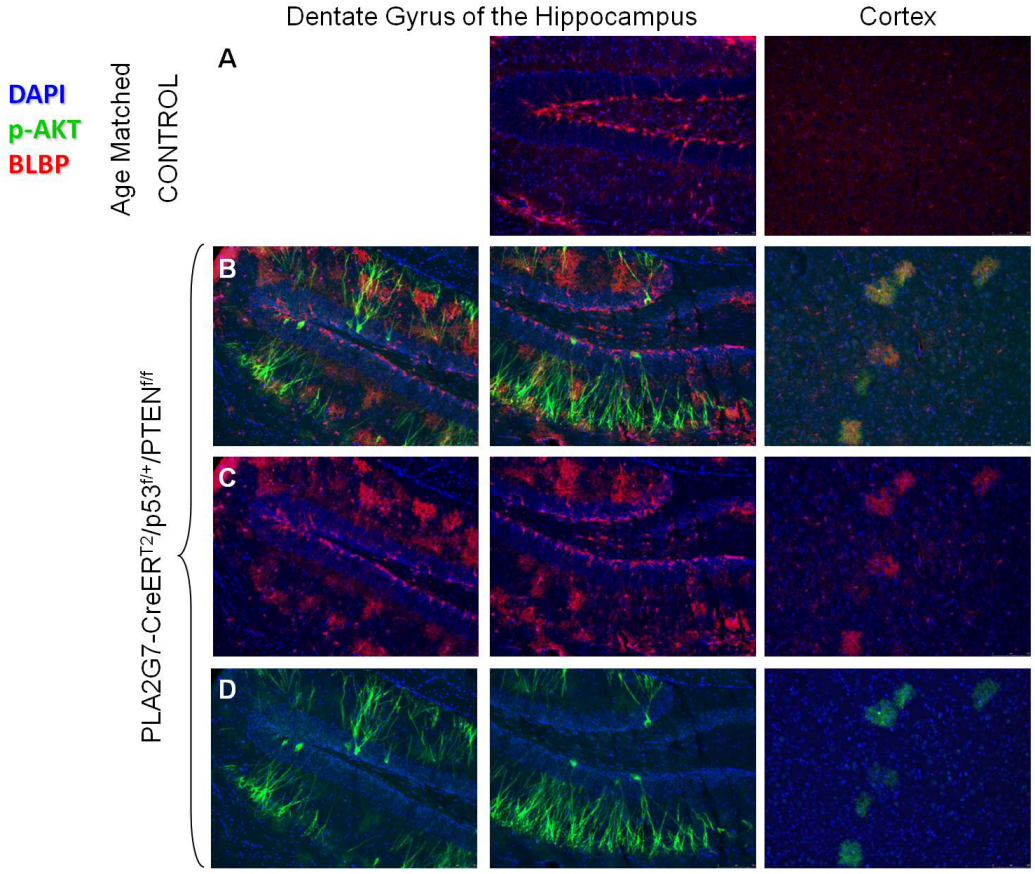

Figure A.1: Allelic deletion of p53 and PTEN in PLA2G7-CreER^{T2}/Rosa26YFP cells.

The effects of p53 and PTEN deletion in PLA2G7-CreER^{T2} cells results in disturbances in both the SGZ stem cell compartment and cortical mature astrocytes. There is extensive expression of p-AKT in the neural stem cells of the SGZ as shown in B and D. The increased of expression of BLBP in the hippocampus is not the most impressive, but the expression in morphologically mature astrocytes in the cortex is (B and C). Interestingly in the cortex, both p-AKT and BLBP expression colocalized in cortical astrocytes.

FIGURE A.2

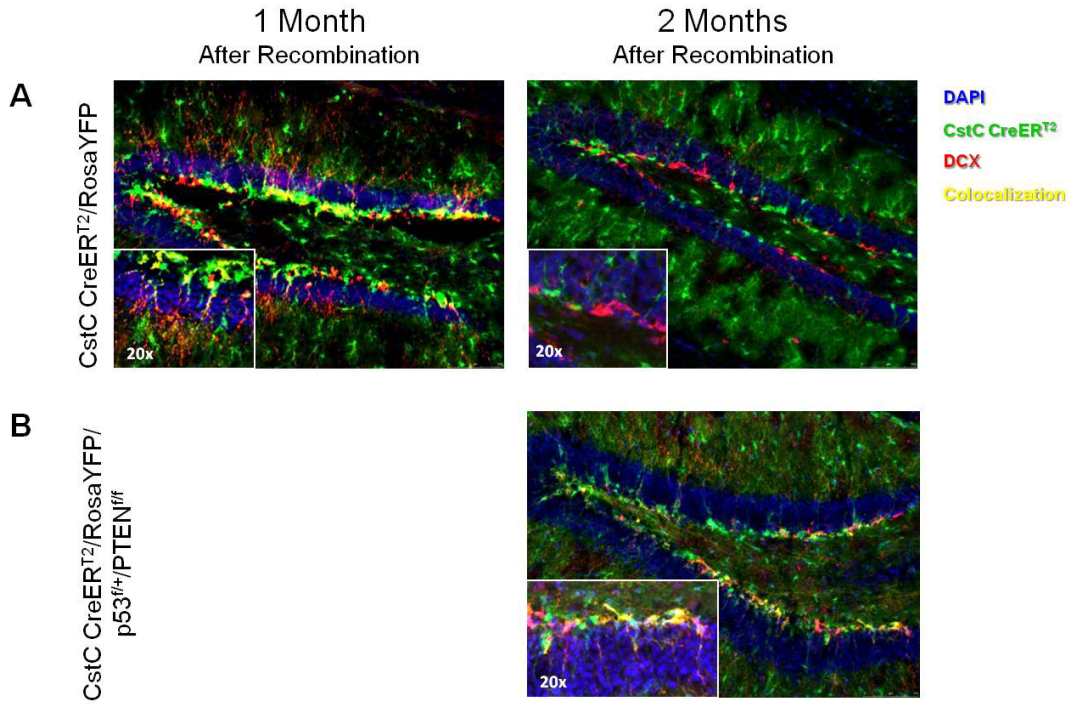

Figure A.2: Allelic deletion of p53 and PTEN extends the life of CstC-CreER^{T2}/Rosa26YFP cells.

CstC-CreER^{T2}/Rosa26YFP cells in the SGZ represent a transient amplifying compartment. These transient amplifying cells no longer express DCX by 2 months of age (A). By contrast, allelic heterozygous deletion of p53 and homozygous deletion of PTEN results in CstC-CreER^{T2}/Rosa26YFP cells colocalizing with neuroblast marker DCX at 2, 3 months, and beyond (B). This change in cellular programming may lead to hyperproliferation.

FIGURE A.3

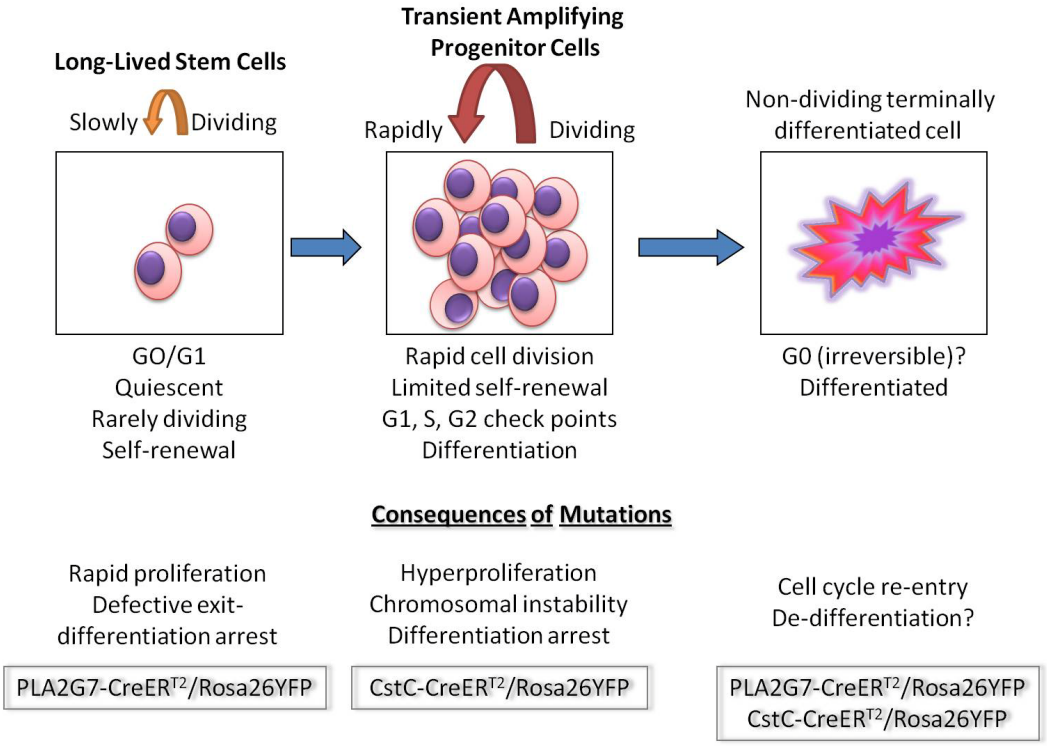

Figure A.3: Targets of genetic transformation.

In the study of glioblastomas, there are several main cell types in which genetic lesions can be targeted. One, the neural stem cells, which can be subdivided into the long-lived, slowly dividing stem cells and the transient amplifying stem cells. Second, the mature, differentiated cell types such as the astrocytes. Each targeted cell compartment represents different cell cycle capabilities, and thus they differ in the resulting consequences of targeted specific mutations.

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