THE ROLE OF GLUTAMATE TRANSPORTERS IN EARLY POSTNATAL HIPPOCAMPAL NEUROGENESIS.

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DEDICATION

This is dedicated to my parents, Max and Cindy Gilley. Thank you for always believing in me and inspiring me to go after my dreams. I could not have done this without your support. I love you.

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By

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The University of Texas Southwestern Medical Center at Dallas, 2010

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Adult neurogenesis has been well-characterized in the subgranular zone (SGZ) of the hippocampal dentate gyrus however, early postnatal development of the dentate gyrus and changes in the neurogenic niche during this time have not been well-studied. Using a well-characterized transgenic mouse that labels early neural progenitor cells with green fluorescent protein (GFP), we created a developmental profile of the dentate gyrus from postnatal day seven (P7) to six months of age. In addition, we determined that early progenitor populations within the developing dentate gyrus exhibit age-dependent changes in proliferation and differentiation which are controlled by cell-autonomous cues. To identify potential regulators of these phenotypes, we performed microarrays and identified several differentially expressed genes within the progenitor pools of

different aged mice. GltI, a glutamate transporter, was identified as a candidate which was upregulated 10 fold in progenitors from older animals. In astrocytes, GltI and Glast are required to maintain low levels of glutamate to prevent overstimulation of glutamate receptors. In neural precursors it has been suggested that glutamate stimulates proliferation by activating metabotropic glutamate receptors (mGluRs) which leads to increased intracellular calcium, however the function of glutamate transporters on these progenitors has not been identified. To elucidate the functional role of GltI and Glast, we performed in vitro experiments in glutamate-free media. By misexpressing GltI and Glast, we show that glutamate transporters negatively regulate calcium-dependent proliferation by controlling glutamate availability to mGluRs. To address the in vivo function of glutamate transporters in injury-induced neurogenesis, we characterized their expression after hypoxic-ischemic (HI) injury and noticed prolonged upregulation of both transporters on type I cells suggesting they may be involved in hypoxic preconditioning. To address this we induced expression of glutamate transporters with HI before exposing mice to traumatic brain injury (TBI). Compared to animals only injured with TBI, mice with both injuries displayed decreased progenitor proliferation suggesting an impaired capacity for repair. We have therefore identified a novel and clinically relevant role for GltI and Glast in progenitor proliferation during development and after injury.

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LIST OF ABBREVIATIONS

ALS – amyotrophic lateral sclerosis

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

ANOVA – analysis of variance

ApoE – apolipoprotein E

BDNF – brain-derived neurotrophic factor

BMP – bone morphogenetic protein

BrdU – bromodeoxyuridine

β-tubulin/Tuj1 – neuron-specific class III β-tubulin

cAMP – cyclic adenosine monophosphate

CCI – controlled cortical impact

CD47 – cluster of differentiation

Cldn10 – claudin 10

CNS – central nervous system

Cspg2 – chondroitin sulfate proteoglycan

DAB – 3,3'-diaminobenzidine

DAPI – 4',6-diamidino-2-phenylindole

Dcx – doublecortin

Eaat – excitatory amino acid transporter

bEGF – basic epidermal growth factor

ES cells – embryonic stem cells

FACS – fluorescent activated cell sorting

bFGF – basic fibroblast growth factor

GFAP – glial fibrillary acidic protein

GFP – green fluorescent protein

Gja4 – gap junction alpha 4

GL – granular layer

Glast – excitatory amino acid transporter 1

GltI – excitatory amino acid transporter 2

Gpc4 – glypican 4

GS – glutamine synthetase

HI – hypoxic-ischemia

IGF – insulin growth factor

iGluR – ionotropic glutamate receptor

IP – intraperitoneal

KD – knockdown

KR – kainate receptor

mGluR – metabotropic glutamate receptor

ML – molecular layer

NeuN – neuronal nuclei

NMDA – N-methyl-D-aspartate

OB – olfactory bulb

PBS – phosphate buffered saline

PDGFRα - platelet-derived growth factor receptor alpha

PFA – paraformaldehyde

PI – propidium iodide

PKC – protein kinase C

PSA-NCAM – polysialic neural cell adhesion molecule

RMS – rostral migratory stream

SGZ – subgranular zone

siRNA – small interfering ribonucleic acids

SVZ – subventricular zone

TBI – traumatic brain injury

Tbx5 – T-box 5

TGFβ – transforming growth factor beta

VEGF – vascular endothelial growth factor

VGLUTs – vesicular glutamate transporters

Vtc – vitronectin

Wnt - wingless

CHAPTER ONE

Introduction and Literature Review

Neurogenesis

Neurogenesis, or the creation of new neurons, gives rise to about 10^{11} neurons in the adult human brain (Pakkenberg and Gundersen, 1997). Unlike other organs of the body, cell divisions that create new neurons occur primarily during gestation (Rakic et al., 1974). Until recently it was a long held belief that it was impossible to generate new neurons in the adult brain. However, there are now two well-studied areas of the mammalian adult brain in which adult neurogenesis is known to occur. In addition to the subventricular zone (SVZ), the subgranular zone (SGZ) of the hippocampus is considered a neurogenic region of the adult brain (Altman and Das, 1965a, Altman and Bayer, 1990, Luskin, 1993, Eriksson et al., 1998, Gage et al., 1998, Doetsch et al., 1999, Alvarez-Buylla et al., 2002).

Embryonic neurogenesis

Embryonic neurogenesis is required to produce neurons that will eventually form the complex neuronal networks found in the adult brain (see (Gaspard and Vanderhaeghen, 2010) for review). A schematic of neurogenesis during mouse brain development can be found in Figure 1-1. During the first stages of brain development, embryonic stem (ES) cells undergo neurogenesis at which point neural induction occurs. During induction, bone morphogenic

proteins (BMPs), wingless (Wnt), fibroblast growth factor (FGF) and insulin growth factor (IGF) signaling pathways are coordinated to form the neural tube. Neural induction is complete around embryonic day 8.5 (E8.5) at which point regional patterning of the neural tube occurs and gives rise to the rostro-caudal and dorso-ventral axes of the developing brain. This process divides the neural tube into different areas which provide regional-specific niches for neural progenitors to produce neurons and glia. From E12.5-E17, ES-derived cortical progenitors undergo neurogenesis and generate different neuronal subtypes which form the layers of the cortex. This process is important because it promotes neuronal diversity in the developing brain. After this neurogenic wave occurs, progenitor cells begin to form astrocytes and oligodendrocytes (gliogenesis). Afterwards, myelination, axon guidance, synapse formation and programmed cell death all take place to complete brain maturation.

Postnatal Neurogenesis

Historical Significance

The concept of adult neurogenesis has taken decades to mature. Santiago Ramon y Cajal, a revered neuroscientist, first documented the lack of neuronal turnover in the adult brain in the nineteenth century (Ramon y Cajal, 1899). As a result, the notion of nonexistent adult neurogenesis became dogma for decades. It wasn't until 1962 that Joseph Altman initially reported the birth of new neurons in the adult brain (Altman, 1962). Afterwards a number of follow-up

studies began to examine the possibility of adult neurogenesis (Altman, 1963, Altman and Das, 1965a, b, 1966, Reznikov, 1975, Kaplan and Hinds, 1977). In the 1980's reports of neurogenesis in adult songbirds further fueled the debate and suggested a role for adult neurogenesis during memory formation (Paton and Nottebohm, 1984, Barnea and Nottebohm, 1994, Nottebohm, 2002). During the 1990's it was discovered that neural progenitor cells could form neurospheres in culture (Reynolds and Weiss, 1992). In addition, the formation of new neurons in the adult brain of humans was uncovered and characterized (Eriksson et al., 1998). These two studies along with others, further catapulted adult neurogenesis to the forefront of scientific investigation.

Neurogenesis in the Subventricular Zone

Along with the SGZ of the dentate gyrus, the SVZ near the lateral ventricles contain neural progenitor cells which give rise to adult neurons (Figure 1-2). Progenitors within the SVZ can be classified based on their capacity for cell division and their morphology. Type B cells divide slowly, have properties similar to astrocytes and are considered to be the resident stem cell population. They express glial fibrillary acidic protein (GFAP) and nestin, a marker commonly used to identify progenitor cells. Type C cells are highly proliferative transient-amplifying cells and are derived from type B cells. They lack expression of GFAP but maintain nestin expression. Type C cells give rise to type A cells which are considered immature neurons. These neuroblasts migrate along the rostral migratory stream (RMS) and localize to the olfactory bulb (OB) where they

become granular cells or interneurons and are thought to be important for the formation of olfactory memories (Doetsch et al., 1999, Alvarez-Buylla et al., 2002, Merkle et al., 2004, Sundholm-Peters et al., 2004). Although neurogenesis within the SVZ is well studied and beneficial in a number of scientific areas of investigation, the remainder of this thesis will focus on neurogenesis within the dentate gyrus.

Neurogenesis in the Dentate Gyrus

In order for a cell to be considered a "stem cell" it must demonstrate multipotentcy and the ability to self-renew and differentiate. During neurogenesis, early neural stem/progenitor cells persist and differentiate into mature neurons. The hippocampal dentate gyrus contains one of the most well studied neurogenic regions in the adult brain (Altman and Das, 1965a, Luskin, 1993, Gage et al., 1995, Suhonen et al., 1996) which continually produces new neurons that migrate into the granular layer (GL) of the dentate gyrus (Frederiksen and McKay, 1988, Suhonen et al., 1996, Gage et al., 1998, Fricker et al., 1999, Kempermann and Gage, 2000, Seaberg and van der Kooy, 2002, Abrous et al., 2005). During dentate gyrus neurogenesis, the stem cells or type I cells within the SGZ divide and produce progenitor cells that will eventually become mature neurons (Figure 1-3) (Cameron and McKay, 2001, Alvarez-Buylla et al., 2002). Type I cells are able to self-renew and differentiate into various lineages and express markers indicative of stem cells and astrocytes (Frederiksen and McKay, 1988, Cameron et al., 1998, Cameron and McKay,

2001, Alvarez-Buylla et al., 2002, Edgar et al., 2002, Joels et al., 2004, Encinas et al., 2006). Similar to type B cells, they possess a dendritic-like process which expresses GFAP while their cell body expresses nestin (Miles and Kernie, 2008, Yu et al., 2008).

The new progenitor cells derived from the stem cells are called type II cells which also express nestin but lack GFAP. These cells are mitotic and are characterized as transiently-amplifying cells. Unlike type I cells, they do not have a long visible process (Cameron and McKay, 2001, Seri et al., 2001).

Type II cells will divide to produce non-mitotic type III cells which express doublecortin (Dcx) and polysialic acid neural cell adhesion molecule (PSA-NCAM) (Seki and Arai, 1993, Francis et al., 1999, Gage, 2002, Seki, 2002, Joels et al., 2004, Christie and Cameron, 2006, Kronenberg et al., 2006, Duan et al., 2008, Peng et al., 2008). Type III cells are committed neuroblasts which will eventually differentiate into mature neurons that express neuronal nuclei (NeuN) (Mullen et al., 1992). For adult neurogenesis to be successful, these mature neurons must integrate and function within their microenvironment (Cameron and McKay, 2001, Goergen et al., 2002, Barkho et al., 2006).

The transition from embryonic to postnatal and adult neurogenesis

Generally speaking, there are fundamental differences that exist between embryonic and adult neurogenesis. In the embryonic brain, neurogenesis describes the production of new neurons on a population-wide scale. In other words all progenitor cells are undergoing the same simultaneous process during

embryogenesis. During prenatal development, neurogenesis is rampant and new neurons are produced from radial glia and neuroepithellial cells. Furthermore, the embryonic neurogenic niche surrounding these neural progenitors is specialized to promote proliferation, differentiation and migration. In the adult, neurogenesis is rare and occurs at a cellular level meaning all the different stages of neurogenesis can be found at any given time within the adult. Moreover, the cellular environment has to support ongoing neurogenesis in addition to the network of mature neurons that has already formed during development (Kempermann et al., 2004, Zhao et al., 2008). This transition from embryonic to adult neurogenesis has been documented in the SVZ, however few studies have characterized early postnatal neurogenesis in the SGZ.

Unlike the SVZ, the SGZ is absent during embryonic neurogenesis because the dentate gyrus is thought to form and mature rather late during the course of development (Altman and Bayer, 1990, Li and Pleasure, 2007). When it begins to develop, progenitor cells proliferate and expand to form granular cells within the primary dentate neuroepithelium which is near the ventricular zone (Fujita, 1962, 1963, Altman and Bayer, 1990, Frotscher et al., 2007, Li and Pleasure, 2007). At this point, the scaffold of the granular layer of the dentate gyrus has been formed and is termed the secondary dentate matrix (Fujita, 1964, Altman and Bayer, 1990, Frotscher et al., 2007, Li and Pleasure, 2007). Between embryonic day 13.5 (E13.5) and E17.5 in mice, progenitors within the secondary matrix proliferate and migrate to occupy the newly formed dentate gyrus. Also during this time, the tertiary matrix forms where the hilus will

eventually be and progenitors and granular cells migrate and begin to populate the two limbs of the nascent dentate gyrus (Altman and Bayer, 1990, Li and Pleasure, 2007). Once the neural progenitors are aligned within the SGZ, the granular cells layers are condensed (Altman and Bayer, 1990, Li and Pleasure, 2007) and their proliferation peaks one week after birth during the secondary and tertiary matrix (Schlessinger et al., 1975). Therefore at P7, dentate gyrus development is complete but has not fully matured.

Although the development of the dentate gyrus has been well-characterized, there are few studies that look at the functional role of radial glial cells (neural progenitors in the adult SGZ) during the transition between embryonic neurogenesis and adult hippocampal neurogenesis (Brunne et al., 2010). Some studies initially thought they serve as scaffolds for migrating neurons, similar to their function in the neocortex or hippocampus proper (Nadarajah and Parnavelas, 2002). However it is now known that during dentate gyrus development, the granular cell layer forms in such a way that new neurons do not have to migrate far from their site of origin (Stanfield and Cowan, 1979, Eckenhoff and Rakic, 1984). Other studies have identified potential functional roles for these early postnatal astroglial cells during neuronal differentiation, neurite outgrowth and the positioning of mature neurons in the granular layer (Hall et al., 2003, Zhou et al., 2004, Shapiro et al., 2005). However, there are few studies which definitively characterize roles for these cells early after birth.

Visualization of neural progenitor cells

The lack of functional data regarding early postnatal hippocampal neurogenesis has increased scientific interest in this population of cells. Early methods of detecting neural precursors include incorporation of a thymidine analogue, bromodeoxyuridine (BrdU), into actively dividing cells. As cells containing BrdU divide, they pass on BrdU expression to their progeny. Using an anti-BrdU antibody, proliferating cells can be reliably visualized. Used in combination with other cellular markers, BrdU allows for quantification and characterization of various cell populations (Kuhn et al., 1996, Kempermann et al., 1997, Eriksson et al., 1998, Kempermann and Gage, 2000, Cameron and McKay, 2001).

Although BrdU has widely been used to study adult neurogenesis, there are some disadvantages associated with its use. Because BrdU incorporation is passed onto progeny cells, the signal is constantly diluted with every cell division (Hayes and Nowakowski, 2002). In addition, live cell imaging is not possible because denaturation is required for its incorporation into DNA. Furthermore, because BrdU is a marker of DNA synthesis rather than mitosis, its incorporation can also indicate DNA repair (Selden et al., 1993).

To enhance the specificity of identifying neural progenitor cells in vivo, our lab has created and characterized a transgenic animal which specifically labels type I and II cells with green fluorescent protein (GFP) (Shi et al., 2007, Koch et al., 2008, Miles and Kernie, 2008, Yu et al., 2008). The GFP reporter is under control of the nestin promoter and enhancer elements found within the second intron of the nestin gene (Zimmerman et al., 1994). These nestin-eGFP

transgenic animals reliably express GFP exclusively in the SGZ of the dentate gyrus and the subventricular zone (SVZ) along the lateral ventricle and the neural tube (Yu et al., 2005). This transgenic mouse also has the ability to differentiate between type I progenitors and mature astrocytes. While both cell types express GFAP, only mature astrocytes express glutamine synthetase (GS) (Miles and Kernie, 2008). We have also shown that reactive astrocytes, which appear after hypoxic-ischemia (HI) and traumatic brain injury (TBI), lack expression of GFP but express GFAP and GS (Shi et al., 2007, Miles and Kernie, 2008, Yu et al., 2008). We have therefore created a transgenic mouse that specifically labels early mitotic progenitors (type I and II) which are distinguishable from mature astrocytes and reactive astrocytes induced by injury.

Glutamate

L-glutamate is a negatively charged amino acid which serves many purposes in the mammalian central nervous system (CNS). Not only does it play roles in basic brain function such as learning and memory and cognition (Fonnum, 1984), but it is also important in the development of the CNS (McDonald and Johnston, 1990, Johnston, 1995, Vallano, 1998). During this time, cells rely on glutamate for normal migration, death and differentiation (Komuro and Rakic, 1993). In addition, glutamate is involved in signaling to peripheral tissues and organs (Rossi and Slater, 1993).

Glutamate as a Neurotransmitter

Glutamate Synthesis and Metabolism

Glutamate serves as the most prominent excitatory neurotransmitter in the central nervous system (Danbolt, 2001, Fonnum and Lock, 2004). The Glutamate-Glutamine cycle is primarily responsible for producing glutamate in the adult brain. During synaptic transmission, glutamate is exocytosed from synaptic vesicles located within the presynaptic nerve terminal. It is then released into the extracellular space where it can activate fast-acting excitatory ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs) which are slower acting. Stimulation of glutamate receptors is required for propagation of the synaptic signal. Once in the synaptic cleft, glutamate must be removed by either postsynaptic or presynaptic neurons or by astrocytes. However, the latter are primarily responsible for glutamate uptake (Hediger, 1999, Daikhin and Yudkoff, 2000, Meldrum, 2000, Maragakis and Rothstein, 2004).

Once glutamate has been transported into the surrounding astrocytes, it is converted to glutamine which is then transported back out of the cell. Neurons are then able to transport glutamine into their cell body where it is converted back into glutamate by glutaminase. Vesicular glutamate transporters (VGLUTs) then transport glutamate into synaptic vesicles so that it may be used again during synaptic transmission (Hediger, 1999, Daikhin and Yudkoff, 2000, Meldrum, 2000, Maragakis and Rothstein, 2004). .

In order to maintain a low signal to noise ratio, extracellular concentrations of glutamate are tightly regulated. In the adult brain extracellular concentrations are in the range of 0.5-2 μM (Lerma et al., 1986). In nerve terminals however, this can increase to the mM range. Reports of high concentrations (100 mM) of glutamate within the synaptic vesicle have also been reported (Meldrum, 2000, Yudkoff et al., 2005). Compared to the embryonic extracellular fluid and neurogenic regions in the adult, glutamate concentrations are decreased in other parts of the brain including the cortex, hypothalamus and cerebellum (Miranda-Contreras et al., 1998, Benitez-Diaz et al., 2003). This suggests that glutamate concentrations are differentially and dynamically regulated during the course of brain development.

Extracellular Glutamate and Excitotoxicity

Stimulation of glutamate receptors and transmission of the signal is contingent on the concentration of glutamate within the synaptic cleft (Figure 1-4) (Fonnum, 1993, Westergaard et al., 1995). In order to prevent nerve damage caused by overstimulation of glutamate receptors, it is important to maintain low levels of extracellular glutamate via glutamate uptake (Logan and Snyder, 1971, Wofsey et al., 1971, Logan and Snyder, 1972). Astrocytes surrounding the nerve terminals are usually responsible for this function. Once glutamate is transported into astrocytes, it can be recycled and reused for synaptic transmission or may be used for metabolic purposes (Martinez-Hernandez et al., 1977, Hassel and Sonnewald, 1995).

Although glutamate is necessary for synaptic transmission, excessive amounts of glutamate can be toxic to the brain (Johnston, 1995). In addition, injuries such as HI and TBI result in increased concentrations of extracellular glutamate (Andine et al., 1991, Palmer et al., 1994, Globus et al., 1995, Bullock et al., 1998) which can ultimately lead to neuronal damage caused by excitotoxicity (Ikonomidou et al., 1989, Choi and Rothman, 1990, Jensen, 2002). Excessive release of glutamate, overstimulation of glutamate receptors, excitotoxicity and prolonged increase in extracellular glutamate all contribute to enhanced damage as a result of brain injury (Yi and Hazell, 2006).

Glutamate in Neurogenesis

In addition to its role as a neurotransmitter, glutamate has also been shown to have an effect on the proliferation of neural progenitors. Several studies have shown the effect glutamate has on the proliferation and differentiation of progenitor cells. In some of these studies glutamate has been shown to enhance the proliferation of neural progenitors from the SVZ, human neonatal cortex and striatum (Brazel et al., 2005, Luk et al., 2003, Suzuki et al., 2006). A proposed method for this positive regulation suggests that hippocampal progenitors respond to glutamate by increasing intracellular calcium levels which subsequently results in increased proliferation and neurogenesis (Choi, 1988, Choi et al., 1988). However the exact mechanisms underlying glutamate-induced proliferation remain unknown.

There is sufficient evidence in the literature to suggest a role for glutamate receptors in the regulation of hippocampal neurogenesis. For instance, several studies have used glutamate receptor antagonists and agonists to study the role of N-methyl-D-aspartate (NMDA)-receptors and metabotropic glutamate recptors (mGluRs) in neural progenitor proliferation (Di Giorgi-Gerevini et al., 2005, Castiglione et al., 2008, Petrus et al., 2009, Melchiorri et al., 2007) . In particular, activation of mGluR1, mGluR3 and mGluR5 receptors is thought to stimulate proliferation (Lu et al., 1997, Di Giorgi Gerevini et al., 2004, Yoshimizu and Chaki, 2004, Castiglione et al., 2008) . Blockade of NMDA receptors has also been shown to induce neural progenitor proliferation within the hippocampus (Cameron et al., 1995, Cameron et al., 1998, Nacher et al., 2001).

Glutamate Receptors

As extracellular glutamate is released into the synaptic cleft, it has the ability to bind to and stimulate different kinds of glutamate receptors: ionotropic and metabotropic (Table 1-1). iGluRs consist of two family members and act as glutamate gated ion channels. The first group of receptors is known as NMDA-receptors which bind to NMDA, a glutamate analog. In addition there are α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors which are stimulated by AMPA and kainate.

The mGluRs are G-coupled and can be further subdivided into three groups based on sequence homology and signal transduction pathways. Group I mGluRs consist of mGluR1 and mGluR5 isoforms while group II includes

mGluR2 and mGluR3. mGluR4, mGluR6, mGluR7 and mGluR8 make up group III mGluRs (see (Hollmann and Heinemann, 1994) for review).

Each subtype of mGluRs is coupled to G proteins which are either stimulatory or inhibitory. Group I mGluRs are coupled to stimulatory G_q proteins which activate phospholipase C and ultimately lead to activation of protein kinase C (PKC) and release of intracellular calcium stores (Schoepp, 2001). Group II and III mGluRs are coupled to the $G_{i/o}$ protein which inhibits synaptic transmission and ultimately results in decreased levels of intracellular cyclic adenosine mono phosphate (cAMP) (Zhang et al., 2003).

Glutamate Transporters

Sodium-dependent high affinity glutamate transporters are expressed within the plasma membrane of astrocytes and neurons (Storm-Mathisen, 1977, Storm-Mathisen and Wold, 1981) and actively transport glutamate into the cell in order to prevent excitotoxicity caused by excess glutamate (Danbolt et al., 1992)(Kanner and Sharon, 1978, Rothstein et al., 1996). During this process, one potassium ion is transported outside of the cell while three sodium ions, a hydrogen molecule and glutamate are passed into the cell (Figure 1-5) (Fonnum, 1984, Nicholls and Attwell, 1990). This process takes about 60-80 milliseconds (Wadiche et al., 1995, Otis and Jahr, 1998, Wadiche and Kavanaugh, 1998).

There are five excitatory amino acid transporters (EAAT) that have been identified thus far: glutamate-aspartate transporter (GLAST/EAAT1), glutamate transporter (GltI/EAAT2), excitatory amino acid carrier (EAAC/EAAT3), EAAT4

and EAAT5 (see (Danbolt, 2001) for review). GltI and Glast are primarily localized to astrocytes (Danbolt et al., 1992, Rothstein et al., 1994, Lehre et al., 1995) while the others are typically found on neurons (Arriza et al., 1997, Schmitt and Kugler, 1999). Although all five transporters share 50-60% amino acid identity with one another (Arriza et al., 1993, Shafqat et al., 1993, Arriza et al., 1994), GltI and Glast account for about 90% of synaptic glutamate clearance (Chaudhry et al., 1995, Peghini et al., 1997, Tanaka et al., 1997).

Only a handful of transgenic mice have been developed to study the function of GltI and Glast in vivo. GltI homozygous knockout mice have an increased susceptibility to brain injury and die embryonically from spontaneous epileptic seizures. In addition these mice display multiple developmental problems including several brain abnormalities (Tanaka, 2007). This suggests that GltI has a neuroprotective role under wild type conditions. GltI has also been shown to play a compensatory role in Glast null mice (Stoffel et al., 2004). In addition Glast knockouts also show motor dysfunction and increased propensity to brain injury (Watase et al., 1998). GltI and Glast double (homozygous) KOs also demonstrate various brain malformations and ultimately result in death at E17 (Matsugami et al., 2006). These results signify the necessity of GltI and Glast in normal brain development and function.

The Neurogenic Response to Injury

Although normal neurogenesis occurs throughout development, certain pathologies and brain injuries can also induce neurogenesis as a means of

repair. Several experimental injury models have been shown to stimulate neural progenitor proliferation within the dentate gyrus such as seizures, TBI and ischemia (Bengzon et al., 1997, Parent et al., 1997, Dash et al., 2001, Rola et al., 2006, Kernie et al., 2001, Magavi et al., 2000). Studies in our lab and others have systematically studied the effects of injury-induced neurogenesis on the neurogenic niche (Plane et al., 2004, Koch et al., 2008, Kernie et al., 2001, Shi et al., 2007, Yu et al., 2008) .

Injury Induced Neurogenesis

Hypoxic-Ischemia

Lack of oxygen (hypoxia) and decreased blood flow to the brain (ischemia), otherwise known as hypoxia-ischemia (HI), is a common form of acquired brain injury in humans. In newborns HI can lead to mental retardation, seizures, cerebral palsy and ultimately death (Raichle, 1983, Vannucci, 1990, Volpe, 1997).

As a result of HI, a number of cellular mechanisms can occur as well. Evidence of increased intracellular calcium mediated by NMDA receptors has been established (Deshpande et al., 1987, al-Mohanna et al., 1994). This is thought to play a role in injury-induced excitoxicity and neuronal death (Choi, 1995, Kristian and Siesjo, 1998). Other side effects of HI include the generation of free radicals, influxes in intranuclear calcium and cell membrane damage (Mishra and Delivoria-Papadopoulos, 1999).

HI has also been shown to stimulate neurogenesis in the adult brain (Plane et al., 2004, Miles and Kernie, 2008). More specifically, injury-induced neurogenesis caused by HI has been shown to peak three days after injury. During this time, type I and II cells are activated and proliferate to replace the loss of immature neurons. Furthermore, the subsequent neurogenesis caused by HI is required for long-term neuronal remodeling in the hippocampus (Miles and Kernie, 2008). Although the concept of injury-induced neurogenesis is well-studied, the exact mechanisms underlying this proliferative response are not understood.

Traumatic Brain Injury

TBI is the most common form of acquired brain injury and can be caused by blows to the head, falls and car accidents (Nortje and Menon, 2004, Heegaard and Biros, 2007). Following TBI a variety of brain damage can occur including intracranial lesions, neuronal depolarization and glutamate toxicity, which ultimately lead to neuronal death (Mattson and Scheff, 1994). Initial injuries are often exacerbated by secondary insults such as hypoxia, hypotension, edema and seizures (Enriquez and Bullock, 2004, Nortje and Menon, 2004, Heegaard and Biros, 2007).

As a result of TBI, patients will often incur a number of cognitive and emotional disabilities. Depending on the severity of the injury, amnesia and the inability to concentrate or communicate might arise (Bajo and Fleminger, 2002, Jellinger, 2004, Rickards, 2006). Furthermore, TBI can lead to a number of

behavioral modifications including irritability, anxiety and violence. In addition, patients suffering from TBI are more prone to developing phsychiatric issues (Bryant et al., 2001, Moore et al., 2006, Rees et al., 2007).

Neurogenesis is another well documented side effect of TBI. Studies in our lab and others have closely characterized TBI-induced neurogenesis within the dentate gyrus (Globus et al., 1995, Kernie et al., 2001, Rola et al., 2006, Richardson et al., 2007, Yu et al., 2008, Dash et al., 2010). Using a mouse model which inducibly ablates early progenitors, Yu and colleagues determined that type I cells are activated to proliferate after TBI in order to replace dying neuroblasts (type III cells). In addition, type I cells were shown to be required for brain remodeling and functional recovery in mice (Yu et al., 2008). These results and others suggest that TBI-induced neurogenesis is a mechanism of repair which is required to repopulate the injured hippocampus with new neurons.

Glutamate and Injury

Different forms of brain injury can induce activation of glutamate receptors as a result of increased extracellular glutamate levels (Andine et al., 1991, Globus et al., 1995, Bullock et al., 1998). Increased levels of glutamate stimulate AMPA and NMDA receptors which lead to elevated intracellular calcium levels which in turn initiate cell signaling pathways that can culminate in cell death (Berridge and Irvine, 1984, Choi, 1988, Choi et al., 1988, Siesjo, 1989, Siesjo et al., 1989, Choi and Rothman, 1990, Mattson, 1992, Pivovarova et al., 2004). However, several groups have identified ways to inhibit or reverse neuronal

toxicity ranging from pretreatment with steroids to altered gene expression (Hilton et al., 2006, Dash et al., 2001, Kitagawa, 2007, Dash et al., 2010, Choi et al., 2010, Liao et al., 2009, Menku et al., 2010) .

Several studies have begun looking at the effect of brain injuries on glutamate transporter expression and function, although the story remains unclear. In astrocytes different models of hypoxia and TBI have been shown to downregulate expression of GltI and Glast after injury. As a result their ability to transport glutamate is impaired (Torp et al., 1995, Martin et al., 1997, Fujita et al., 1999, Fukamachi et al., 2001, Rao et al., 1998, Pow et al., 2004, Rebel et al., 2005). However, others have shown an increase in glutamate transporter expression on astrocytes in models of cerebral ischemia and hypoxia (Gottlieb et al., 2000, Desilva et al., 2008). These contrasting results exemplify the need for additional studies examining the role of glutamate transporters in response to brain injury.

Specific Aims of this Study

(1) Create a developmental profile of early postnatal neurogenesis in the dentate gyrus. To achieve this I will utilize our transgenic mouse (Nestin-eGFP) and a number of in vitro and in vivo techniques to analyze the proliferation, differentiation, genetic makeup and distribution and quantification of the resident progenitor populations within the postnatal hippocampus.

(2) Characterize the expression and function of GltI and Glast in early neural progenitor cells. To identify various progenitor populations which express glutamate transporters, I will use well-characterized immunohistochemical markers. In addition, I will utilize small interfering ribonucleic acids (siRNAs) specifically targeted to GltI and Glast, I will determine the effect glutamate transporter loss of function has on neural progenitors in vitro. Furthermore, I will use plasmids that overexpress either GltI or Glast to further elucidate the function of glutamate transporters in hippocampal neurogenesis.

(3) Examine the in vivo relevance of glutamate transporters using models of acquired brain injury. To reach this goal I will use a well-characterized mouse model of HI to induce neurogenesis in our transgenic mice. Furthermore, I will characterize changes in glutamate transporter expression at several time points after injury. Finally, I will examine the role of glutamate transporters in hypoxic preconditioning by inducing HI before exposing the mice to TBI.

Figures

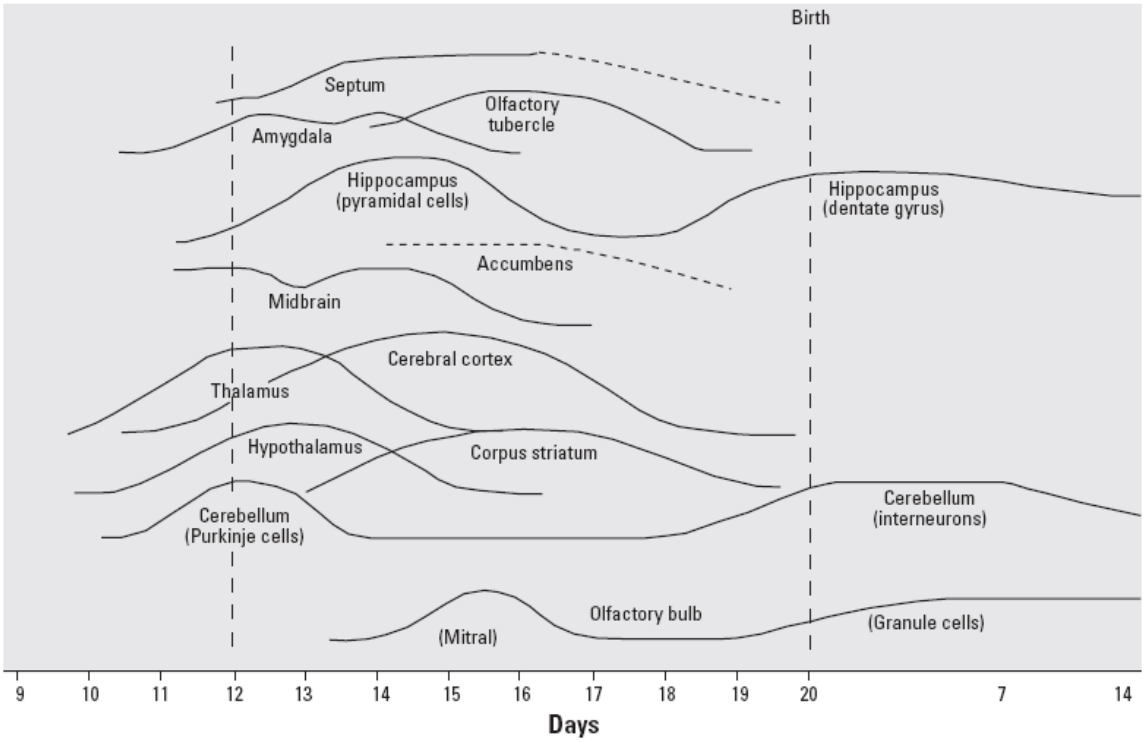

Figure 1-1: Embryonic and postnatal neurogenesis in various regions of the developing mouse brain. Most critical windows of peak neurogenesis occur during gestation, however the hippocampus and SVZ continually produce new neurons into adulthood. Taken from (Rodier, 1977).

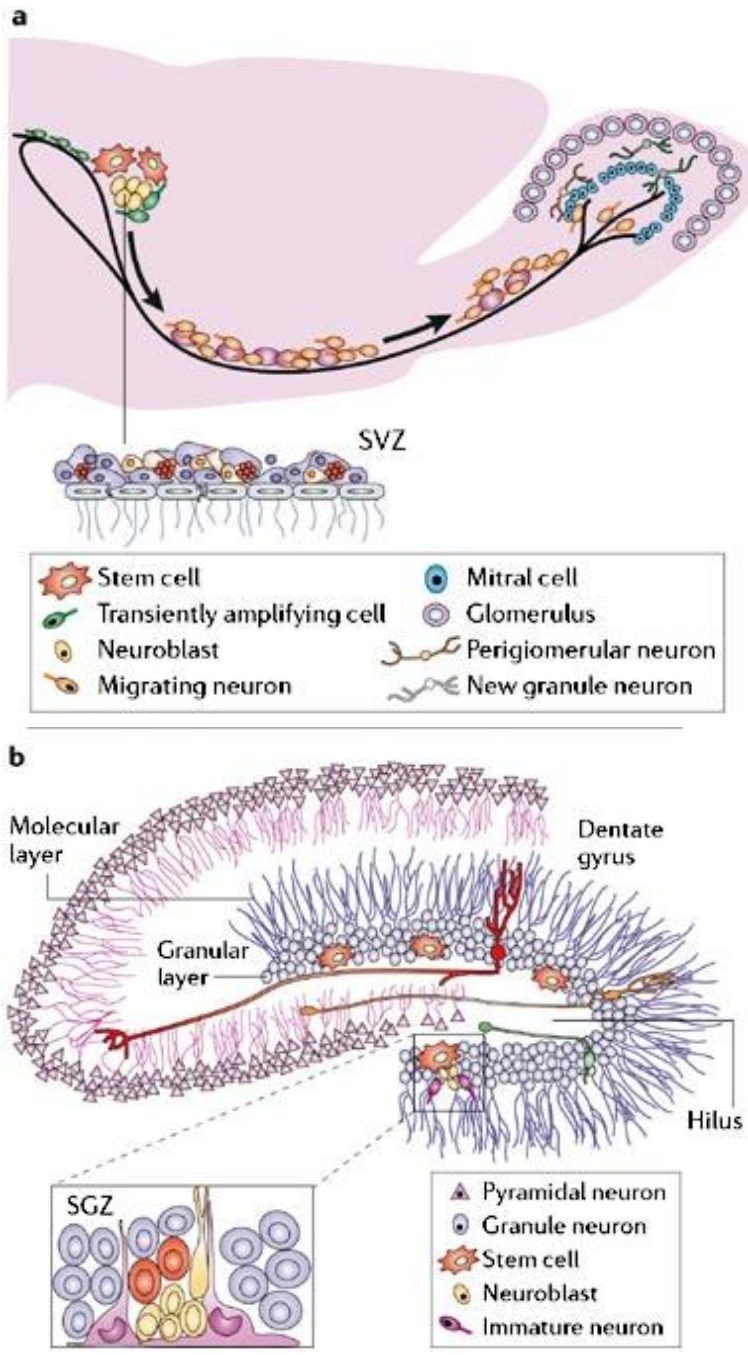

Figure 1-2: Adult neurogenesis in humans and mice. (A) In the SVZ stem cells along the wall of the lateral ventricle divide and produce transient-amplifying cells, which eventually become neuroblasts. They then migrate along the RMS to the OB where they become periglomular or granule neurons. (B) Progenitors within the hippocampal dentate gyrus also divide to form neuroblasts which will ultimately become granular cell neurons that contribute to the overall circuitry of the hippocampus. OB, olfactory bulb; RMS, rostral migratory stream; SVZ, subventricular zone; SGZ, subgranular zone. Adapted from (Vescovi et al., 2006).

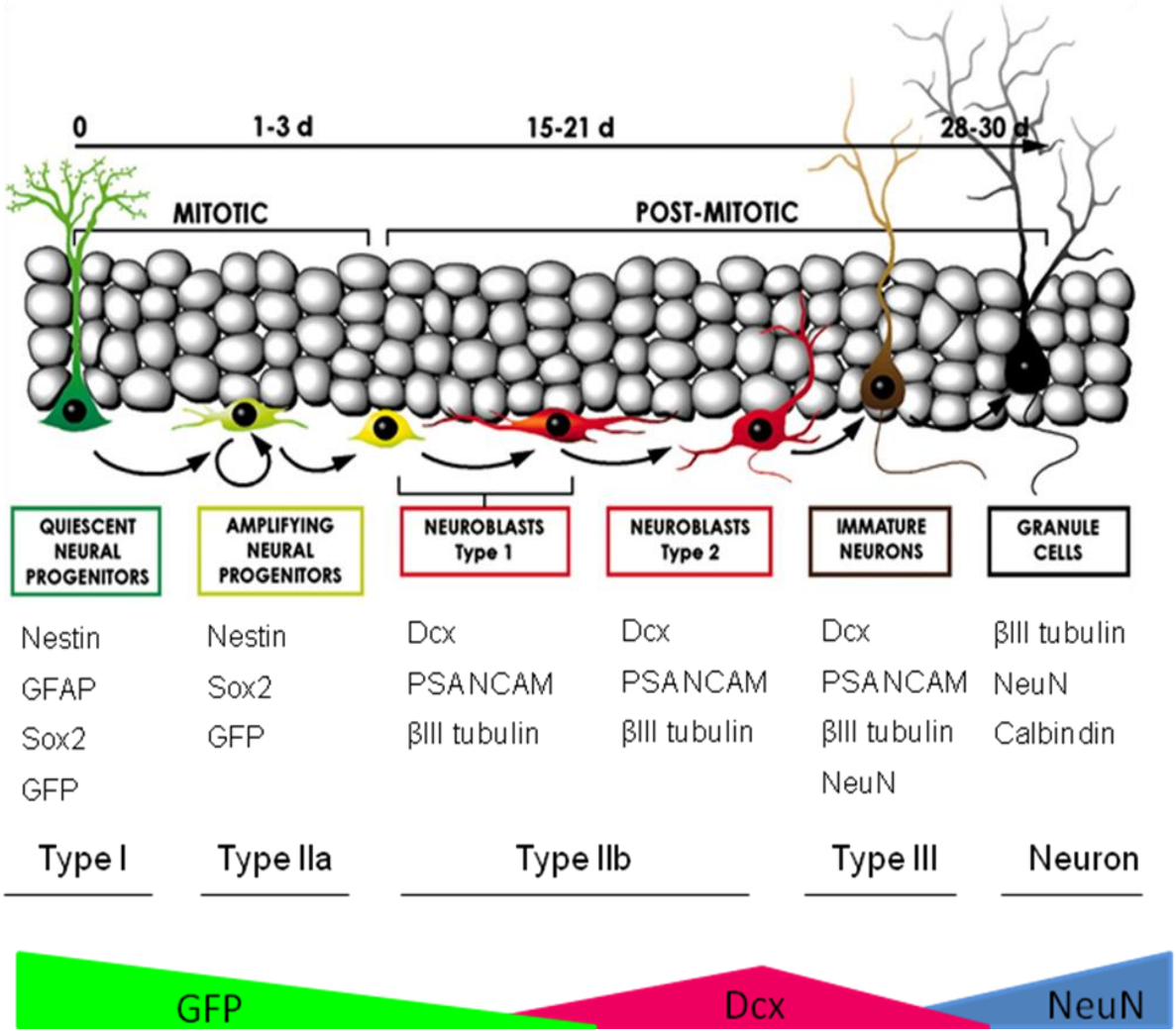

Figure 1-3: Characteristics of adult neurogenesis in the dentate gyrus. During adult neurogenesis GFAP-expressing type I putative stem cells within the SGZ self-renew and divide to form mitotic neural progenitor cells (type IIa) which express nestin. Type IIa cells transform into nestin- and Dcx-expressing type IIb cells before becoming Dcx-positive type III late progenitors at which point cells are committed to a neuronal lineage. Eventually these type III progenitors differentiate into mature granular cell neurons which express calbindin and NeuN. Abbreviations: GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; Dcx, Doublecortin; NeuN, neuronal nuclei. Image adapted from (Encinas et al., 2006).

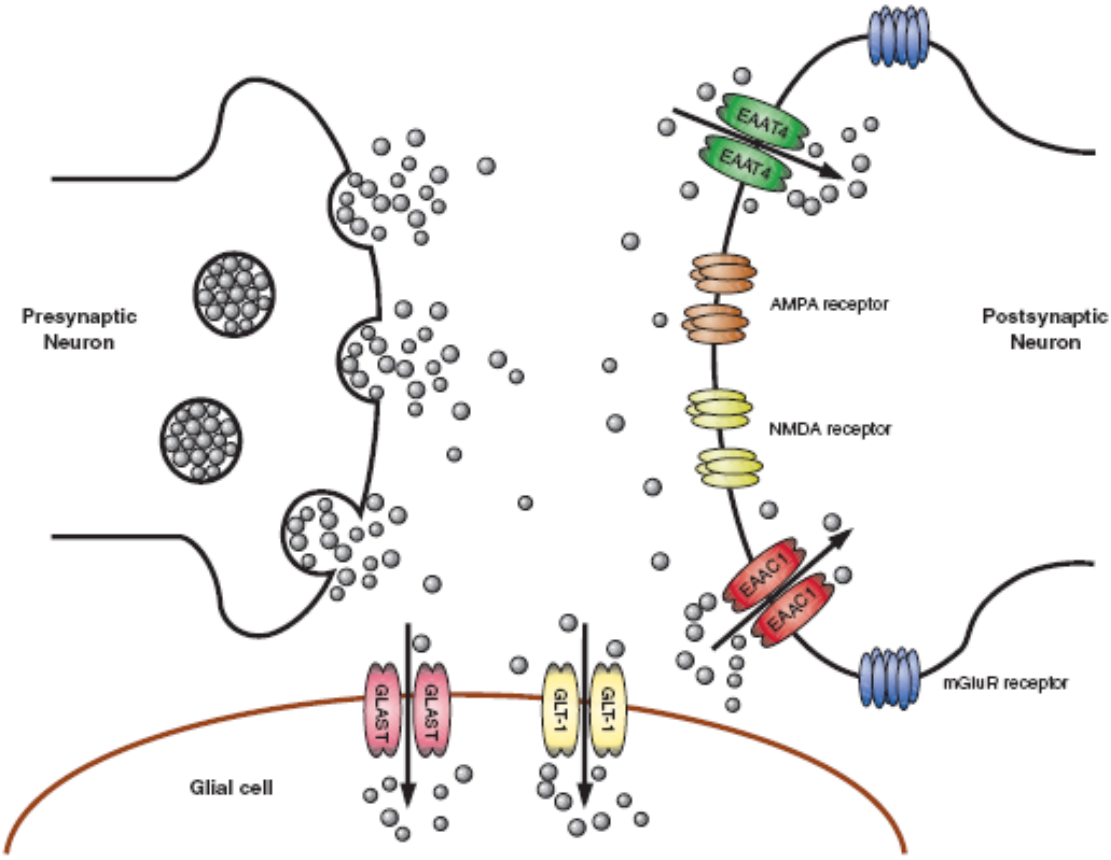

Figure 1-4: Glutamate in the synaptic cleft. During synaptic transmission glutamate is released from the presynaptic neuron and enters the synaptic cleft. In order to perpetuate the signal, glutamate must bind to and activate one of several receptors on the postsynaptic neuron. However, overstimulation of glutamate receptors is harmful to neurons and so the extracellular glutamate concentrations must be maintained at appropriate levels. Glutamate transporters GltI and Glast are expressed on astrocytes and are required to uptake excess glutamate from the synaptic space. Once glutamate has been transported into the astrocyte, it is converted to glutamine before being released from the cell. At this point presynaptic neurons can transport glutamine into their cell body and convert it back into glutamate so that it can be reused as a neurotransmitter. EAAT4, excitatory amino acid transporter 4; EAAC1, excitatory amino acid transporter 3; mGluR, metabotropic glutamate receptor; NMDA, N-methyl-D-aspartate; AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate. Image taken from (Gonzalez and Robinson, 2004).

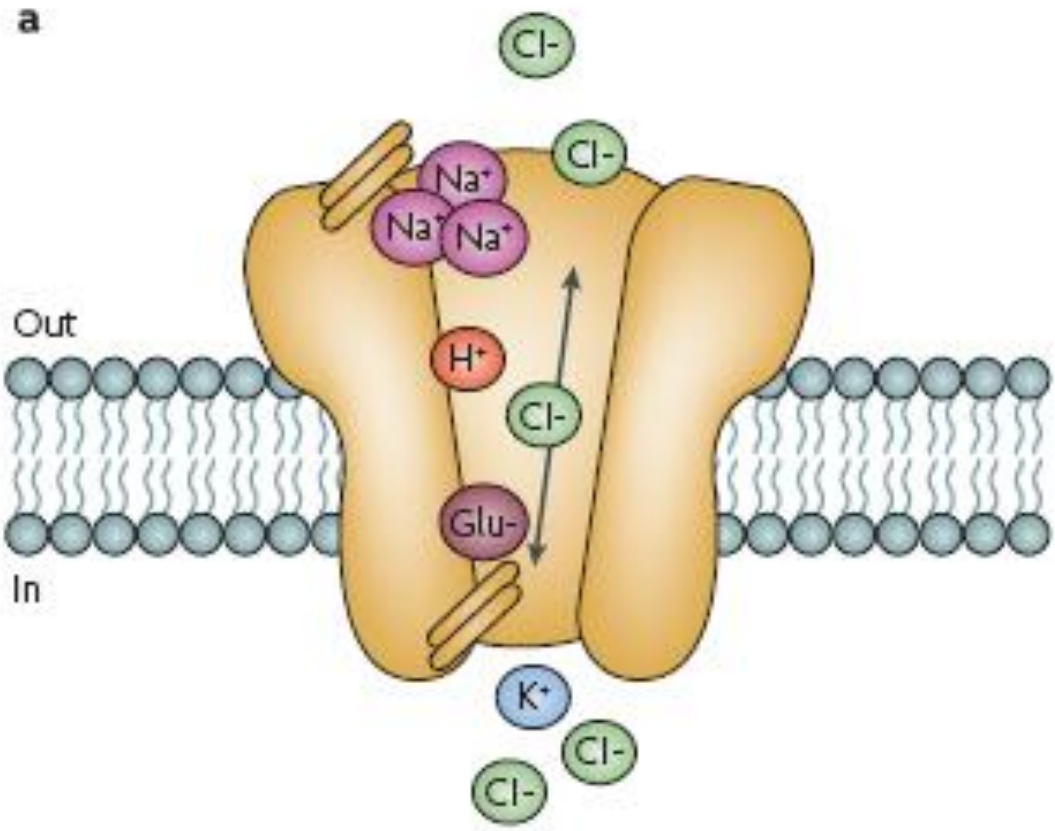

Figure 1-5: Dynamics of glutamate transporter function. Glutamate transporter function is necessary to prevent neuronal excitotoxicity in the synaptic cleft. During this process, one glutamate, one hydrogen and three sodium molecules are all actively transported into the cell while one potassium ion is passed out of the cell. In addition to their role as sodium-dependent transporter, GltI and Glast also function as passive chloride channels, which allow the constant flow of chloride in and out of the cell. Glu, glutamate; H^+, hydrogen; Na^+, sodium; Cl^-, chloride; K^+, potassium. Taken from (Tzingounis and Wadiche, 2007).

Glutamate Transporters

vesicular	VGLUT1, VGLUT2, VGLUT3
Na^+ ion-dependent	EAAT1 (Glast), EAAT2 (GltI), EAAT3 (EAAC1), EAAT4, EAAT5

Glutamate Receptors

	Subtype	Subunit	Signal
ionotropic	NMDA	NR1, NR2A-D, NR3A-B	Ca^{2+} and Na^+
	AMPA	GluR1-4	Na^+, (Ca^{2+})
	Kainate	GluR5-6, KA-1, KA-2	Na^+, (Ca^{2+})
metabotropic	Group I	mGluR1, mGluR5	G_q, PLC; IP_3/Ca^{2+}
	Group II	mGluR2-3	G_i; cAMP decrease
	Group III	mGluR4, mGluR6-8	G_i/G_o; cAMP decrease

Table 1-1: Glutamate transporters and receptors in the CNS. Image adapted from (Schlett, 2006).

CHAPTER TWO

Developmental Profile of Hippocampal Neural Progenitors

Introduction

Adult neurogenesis has been well-characterized in the dentate gyrus, however, studies that specifically profile its postnatal development are lacking. Several studies have described “adult” neurogenesis in the hippocampus but the time point at which the adult phenotype is acquired has not been well-studied. The range of studies in adult neurogenesis varies from four to twelve weeks of age in mice (Kuhn et al., 1996, Tropepe et al., 1997, Wagner et al., 1999, Lemaire et al., 2000, Bailey et al., 2004, Enwere et al., 2004, Molofsky, 2006, Bastos et al., 2008, Bulloch et al., 2008, Goncalves et al., 2008, Yang et al., 2009). Therefore, one of the aims of the current study is to developmentally profile the postnatal neurogenic niche because it primarily matures after birth and not much is known about its development after the first week.

Previous studies have described age-dependent decreases in neurogenesis, but few have identified intrinsic factors responsible for this observed phenotype. Although some genes have been shown to regulate proliferation and differentiation, many unidentified candidates remain to be discovered (Abrous et al., 2005). We therefore wanted to search for relevant genes that may contribute to the regulation of the neurogenic niche because the role of extrinsic factors such as hypoxia, neurotransmitters, hormones and others have already been well-characterized (Cameron and Gould, 1994, Calof, 1995, Cameron et al., 1995, Ghosh and Greenberg, 1995, Muller et al., 1995, Heller et

al., 1996, Kempermann et al., 1997, Cameron et al., 1998, Kernie et al., 2001, Goergen et al., 2002, Barkho et al., 2006, Kronenberg et al., 2006, Kaneko and Sawamoto, 2008).

By using our nestin-eGFP mouse, we are able to reliably label type I and II progenitors within the SGZ of the hippocampal dentate gyrus. This transgenic allows us to distinguish early progenitor cells from mature astrocytes due to the absence of GS expression and although mitotic neural progenitors and reactive astrocytes both express GFAP, the latter lacks GFP expression (Miles and Kernie, 2008). Moreover, our GFP-expressing progenitors do not colocalize with ionized calcium binding adaptor molecule 1 (Iba1), a microglial marker (unpublished results). We are therefore able to consistently label nestin-expressing neural precursors with GFP and distinguish these cells from other cell lineages with similar expressional markers.

Here we create a developmental profile of postnatal neurogenesis in hopes of identifying changes in gene expression which may influence the hippocampal neurogenic region. In addition, we provide functional data which suggests a proliferative advantage for progenitors from younger mice compared to older ones. We also show in our differentiation assay that progenitors from P7 animals are more neurogenic than those from P28 transgenics. Characterization of the dentate gyrus progenitor population demonstrates a steady decline in the percentage of GFP-expressing cells until two months of age. This study also quantifies absolute numbers of various cell types using stereological methods. Finally, using a stem cell-specific array, we have identified candidate genes that

are differentially expressed between early neural progenitors from different aged transgenic animals. These results indicate that cell-autonomous factors change over time and contribute to overall regulation of declining neurogenesis observed in vivo.

Results

Developmental profile

Using our nestin-eGFP transgenic animal that has previously been characterized (Shi et al., 2007, Koch et al., 2008, Miles and Kernie, 2008, Yu et al., 2008), we immunohistochemically labeled different cell types within the dentate gyrus during early postnatal development. Because eGFP is expressed under control of the nestin promoter and enhancer element found within the second intron (Zimmerman et al., 1994), we were able to identify early nestin-expressing progenitors (type I and II) by labeling with a GFP antibody. To label type III transiently amplifying progenitors we stained with Dcx and to look at mature neurons, we used NeuN. Confocal images of triple-stained sections from P7, P14, P21, P28, two-month, four-month and six-month old transgenics suggest that the development of the dentate gyrus occurs slowly over time (Figure 2-1). Populations of both GFP- and Dcx-expressing cells look to be migrating from the GL of the dentate gyrus to the SGZ over the course of development. In addition, the morphology of GFP-expressing progenitors appears to change with time. Furthermore, the number of early and late

progenitors seems to decrease as the animals age. In particular, the GFP-positive population appears to stabilize at two months of age.

Lack of platelet-derived growth factor receptor alpha (PDGFRα) expression suggests that dentate gyrus dissections are void of SVZ progenitors

In addition to the SGZ of the hippocampus, the SVZ along the wall of the lateral ventricle is another neurogenic region in the postnatal brain (Altman and Das, 1965a, Luskin, 1993, Gage et al., 1995, Suhonen et al., 1996). Both contain nestin-expressing type I and II early progenitors which are adjacent to one another. In fact, dissection of the hippocampus can lead to mixed progenitor populations from these two regions (Seaberg and van der Kooy, 2002, Becq et al., 2005, Tonchev and Yamashima, 2006). To distinguish between SVZ and SGZ progenitors, we used the differential expression pattern of PDGFRα in combination with GFP immunostaining. Because SVZ progenitors coexpress GFP and PDGFRα and those from the SGZ do not (Jackson et al., 2006), dentate gyrus dissections lacking PDGFRα expression should be void of SVZ progenitors.

To distinguish between the two populations, GFP and PDGFRα expression patterns were analyzed in vivo. SVZ and SGZ immunostaining of P7, P14, P21 and P28 animals show that GFP-expressing progenitors within the dentate gyrus do not colocalize with PDGFRα (Figure 2-2, A-D) while those from the SVZ do (Figure 2-2, E-H). In addition, coronal whole mounts of the hippocampus (H), dentate gyrus (DG) and hippocampus without the dentate

gyrus (H-DG) demonstrate dissections free of SVZ progenitors (Figure 2-2, I-K). To further show that our dentate gyrus dissections are independent of PDGFRα-positive progenitors from the SVZ, we performed Western blots on lysates isolated from P14 transgenic animals. Using protein from the hippocampus, dentate gyrus and hippocampus without dentate gyrus, we illustrate the presence of GFP in all lysates and the absence of PDGFRα expression in dentate gyrus dissections (Figure 2-2, L). These results, taken together, suggest that GFP-expressing progenitors isolated from the dentate gyrus are free of SVZ progenitors.

Progenitor proliferation is cell-autonomous in vitro

As mentioned earlier, we observed morphological age-dependent differences between dentate gyrus progenitors. This lead us to hypothesize that type I and II progenitors might show different growth phenotypes in vitro. To address this we first isolated dentate gyrus from P7 and P28 animals, dissociated the tissue into single cells and then plated them at a uniform density in semisolid media. Neurospheres were allowed to form and after 14 days in vitro they were analyzed. Only neurospheres of at least 50 μm in diameter were quantified. The average number of neurospheres derived from P7 animals was 260 while those from older animals only averaged 35 neurospheres (Figure 2-3, C). In addition to forming more neurospheres, progenitors from P7 animals demonstrated increased diameters when compared to P28 neurospheres (Figure 2-3, A-B).

These results indicate that neurospheres derived from P7 animals are more proliferative than P28 neurospheres.

Since neurospheres derived from the dentate gyrus of different aged animals are heterogeneous, we next wanted to determine if this observed phenotype was cell-autonomous. Taking advantage of our transgenic mouse, we used fluorescent activated cell sorting (FACS) to isolate GFP-positive neural progenitors from P7 and P28 dentate gyrus and repeated the neurosphere assay. Results again showed that P7 progenitors formed more neurospheres than P28 progenitors (62.1 versus 3.6, respectively) (Figure 2-3, F). Because we were able to isolate our particular progenitor population of interest using flow cytometry methods, these results suggest that progenitor proliferation in vitro is cell-autonomous.

To confirm this we performed a single-cell proliferation assay. GFP-expressing cells from P7 and P28 dentate gyrus were sorted in 96-well plates at a density of one cell/well and were allowed to grow for 14 days before quantification. Only P7 progenitors were able to form any neurospheres at all. P28 progenitors could not form any neurospheres while those from P7 animals formed an average of four neurospheres per plate (data not shown). These results further indicate that the differential growth phenotypes observed between P7 and P28 progenitors are in fact cell-autonomous.

In vitro differentiation suggests P7 progenitors are more neurogenic than P28 progenitors

To determine if P7 and P28 progenitors had the same neurogenic potential, we performed an in vitro differentiation assay on primary neurospheres in the absence of growth factors and in the presence of serum. After cells were allowed to differentiate for five days, they were immunohistochemically stained for mature neurons with neuron-specific class III β-tubulin (Tuj1) and astrocytes and undifferentiated progenitors with GFAP. 4'6-diamidino-2-phenylindole (DAPI) was also used to stain the nucleus of cells. 61.7% of cells derived from P7 progenitors expressed Tuj1 while only 33.7% of P28 progenitors were Tuj1-positive (Figure 2-4, C). When the percentages of astrocytes and neurons were compared between P7 and P28, they were significantly different (***P<0.0001). Almost all of the DAPI-expressing cells expressed markers of either astrocytes or neurons (~99%). These results suggest that P7 progenitors are more neurogenic than P28 progenitors in vitro.

Quantification of early progenitors with FACS suggest that the neurogenic niche stabilizes during early development

To further quantify the percentage of GFP-expressing progenitors over the course of postnatal development, we collected all GFP-positive and GFP-negative cells from several dentate gyri via FACS. As expected the percentage of GFP-expressing cells diminished over time and P7 and P14 animals had the highest percentages and were statistically significantly different to all other time points measured (***P<0.0001) (Figure 2-4, D). Quantification of two-, four -, and six-month old animals revealed similar percentages of GFP-expressing cells.

These data are comparable to what we observed in the developmental profile (Figure 2-1). In addition, the percentage of GFP-positive cells derived from P7 animals was 27-fold higher when compared to P28 animals. These results demonstrate a declining progenitor population until about two months of age at which point the GFP-expressing cells become stabilized.

Age-dependent changes in cell number occur between GFP-, Dcx- and BrdU-expressing populations

In addition to characterizing the percentage of early progenitor present at different time points, we chose also to more closely analyze exact cell counts using design-based stereology. Using 3,3'-diaminobenzidine (DAB) Immunohistochemistry, we quantified the number of early progenitors (GFP-positive), late progenitors (Dcx-positive) and dividing cells (BrdU-positive) in P7, P28 and two month old transgenic animals. The amount of early progenitors present in P7, P28 and two month animals were 27,000, 47,000 and 16,000, respectively (Figure 2-5, D). All three time points were significantly different from one another as indicated by One Way Analysis Of Variance (ANOVA) and Bonferonni *post hoc* analysis. Overall, P28 animals displayed the highest number of GFP-positive cells.

Unlike the early progenitors, the highest number of Dcx-expressing cells was observed at P7 and this population declined with increasing age. The number of late progenitors was 76,000 at P7, 40,000 at P28 and 36,000 at two months of age (Figure 2-5, H). The population of dividing cells was similar to

later progenitors in that the total number of BrdU-positive cells was inversely related to age and the highest cell count was observed at P7 (Figure 2-5, L).

Using dual-labeled immunofluorescence and confocal microscopy, we quantified the percentage of GFP- and BrdU-expressing double-positive cells within the dentate gyrus at P7, P28 and 2 months. The percentage of GFP and BrdU colocalization was 50% for P7, 64% at P28 and 38% for 2 month-old animals. We therefore extrapolated these data and compared them to the stereological quantification of progenitor cells. Although the highest percentage of colocalization was observed at P28 (Figure 2-5, P), quantification of BrdU-expressing cells was highest in P7 animals. Furthermore, when comparing P28 and P7 animals, the ratio of GFP-positive progenitors to Dcx-expressing progenitor was highest at P28. This suggests that increased numbers of type III progenitors at P7 (Figure 2-5, H) might account for the lower percentage of GFP and BrdU colabeled cells observed at that time point (Figure 2-5, P). Therefore the increased percentage of colocalization observed at P28 may be a consequence of lower numbers of Dcx-expressing cells at P28 and 2 months (Figure 2-5, H).

Nine differentially expressed stem cell-specific genes exist among progenitors from different aged animals

The vast changes that occur in the hippocampal niche over the course of postnatal development including progenitor number and their proliferative capacity has lead us to hypothesize that genetic factors may be responsible for

these observed phenotypes. To identify differentially expressed genes in the GFP-expressing progenitor population, we sorted cells from P7 and P28 animals and performed a microarray screen in triplicate. We used a stem cell-specific platform spotted (in quadruplicate) with relevant mouse genes found within a number of different stem/progenitor populations including but not limited to those from the CNS. Distinct markers of multiple stem cells, genes expressed during their differentiation and various positive and negative controls were included. This platform also assayed for genes specific to neural progenitor cells such as nestin, Of 916 genes assayed, many demonstrated significant changes in expression from P7 to P28 progenitors. Only genes that were significantly upregulated or downregulated in all three trials were considered candidates. The results of the microarray analysis can be seen in Table 2-1. Of the nine genes that met this stringent analysis, three were downregulated compared to P7 while six were upregulated compared to the P7 control. Although our stringent analysis only produced nine candidates, the complete list of tested genes were submitted to NCBI's Gene Expression Omnibus (Edgar et al., 2002) and may be viewed at the following website; http://www.ncbi.nlm.nih.gov/geo/querry/acc.cgi?acc=GSE15085 (GEO Series accession number is GSE15085).

Discussion

The results from this study indicate that differentially expressed genes distinguish early progenitors from young and old animals and may ultimately

contribute to functional differences. Several groups have previously analyzed dentate gyrus progenitor proliferation using BrdU incorporation (Kuhn et al., 1996, Heine et al., 2004, McDonald and Wojtowicz, 2005, Rao et al., 2006), therefore age-related declines in neurogenesis have been well-documented. Others have also linked age-dependent changes in progenitor proliferation to the expression patterns of several genes including transforming growth factor beta (TGFβ), brain-derived neurotrophic factor (BDNF), insulin growth factor 1 (IGF1), vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2) (Hattiangady et al., 2005, Shetty et al., 2005, Buckwalter et al., 2006). It has also been shown that caspase activity is an indicator of postnatal neurogenesis in the SGZ and SVZ (Gemma et al., 2007, Tang et al., 2009). However, unlike the current study, these previous works have only looked at changes in the entire brain or within the progenitor niche over time and not cell-autonomous differences within the stem/progenitor population.

Results from our immunostaining begin to characterize the different progenitor populations present within the dentate gyrus during the course of development (Figure 2-1) while our quantification data focus more closely on early mitotic progenitors (type I and II) (Figure 2-5). The developmental profile in Figure 2-1 and quantification by FACS analysis (Figure 2-4) both demonstrate age-dependent decreases in GFP-expressing cells. We also demonstrate that these age-related changes occur within the progenitor cells themselves. Furthermore, evidence from our developmental profile suggests that the progenitor niche is continuously undergoing dynamic changes until two months of

age. Therefore, other studies which claim to investigate “adult” neurogenesis prior to this time point are vulnerable to studying the developmentally immature dentate gyrus whose progenitor population has yet to stabilize.

Certain microenvironmental factors have been shown to regulate the proliferative potential of progenitors within various tissues and organs (Fliedner et al., 1985, Ivasenko et al., 1990, Yanai et al., 1991, Jenkinson et al., 2003, Zhu et al., 2004, Boyle et al., 2007). For example, the proliferation of thymic epithelial progenitor cells is dependent on various growth factors provided by the surrounding mesenchyme (Jenkinson et al., 2003). However, results from our neurosphere cultures indicate that proliferative potential varies amongst progenitors from different aged animals in which the microenvironment is constant. This suggests that declining proliferation of P28 progenitors (compared to P7) is due at least in part to intrinsic factors.

Unlike other studies, our neurosphere assay studies were performed using cells strictly derived from the dentate gyrus (Figure 2-2). This prevents other cells with different proliferative potentials, like those within the SVZ, from contaminating our cultures. This is important because SVZ progenitors are more heterogeneous and proliferative than dentate gyrus progenitors (Golmohammadi et al., 2008) and they respond differently to FGF2 (Becq et al., 2005). In addition, in vitro phenotypes have been shown to be region-specific within the brain (Armando et al., 2007). It was therefore essential that our in vitro proliferation studies were based solely on neural progenitors from the dentate gyrus

We have shown that neurospheres from P7 progenitors are more proliferative than those from P28 and these differences are due in part to cell-autonomous factors. However we do not distinguish between two different possibilities explaining this phenotype. One possibility is that P7 progenitors are in themselves more proliferative than P28 progenitors. It is also possible that the dentate gyrus from P7 animals contain a higher number of proliferative cells than P28. Although either of these explanations is plausible, we can still conclude that P7 and P28 neurospheres are differentially regulated by genetic factors.

Results from our differentiation assay also suggest that neural progenitors derived from different aged animals are differentially regulated. Figure 2-4 suggests that P7 progenitors are more neurogenic than P28 progenitors because of their increased capacity to differentiate into neurons. This finding might be beneficial to certain stem cell-based therapies in which certain progenitor populations often fail to adequately stimulate neurogenesis.

To quantify the GFP-positive progenitors within the dentate gyrus over the course of postnatal development, we utilized unbiased stereological quantification and FACS analysis. Although results from Figure 2-4 indicate that the percentage of GFP-expressing cells is highest at P7, stereological quantification suggests that the highest actual number of early progenitors are present at P28. Morphological differences among dentate gyrus from P7, P28 and 2 month old transgenics may contribute to these discrepancies. The size of the brain and hippocampus in P7 animals is much smaller compared to older animals. Furthermore, the dentate gyrus is still maturing at this time point and

GFP-positive cells are not yet confined to the SGZ (Figure 2-5, A). As a result, GFP-expressing cells outside of the SGZ and the granular layer would not have been quantified using stereological techniques. In contrast, the dentate gyrus of P28 and two month old animals is more developed and the progenitors are restricted to the SGZ.

In addition to our nine candidate genes, we also identified several other differentially expressed genes that may also contribute to age-specific phenotypes we observed. For instance, Sox4, which is thought to be involved in the positive regulation of cell proliferation (Sinner et al., 2007), is downregulated in P28 progenitors by a ratio of 0.41. Genes involved in stem cell maintenance such as wingless (Wnt1) (Sato et al., 2004, Kleber et al., 2005, Lowry et al., 2005) also indicate decreased expression in P28 progenitors compared to P7 (ratio of 0.46). In addition, VEGF which has been shown to be associated with age-dependent decreases in neurogenesis (Hattiangady et al., 2005, Shetty et al., 2005, Buckwalter et al., 2006), is also differentially expressed in our microarray. Although these genes and others could not be included as candidates due to variability between samples, they provide validation for our techniques and may be compelling targets for future study.

There are, however some limitations associated with this microarray platform mostly due its specificity. Because only stem cell-specific genes were screened, we were not able to assay for genes inclusive to the entire genome. Moreover, this screen was unable to reflect the presence of epigenetic changes or non coding RNAs such as microRNAs which could alter gene expression

patterns cell-autonomously. Additional more inclusive experiments are required to account for the transcriptional regulation and its effect on gene expression.

Although the link between neurogenesis and its effect on the aging or diseased brain are complex and not well-understood, age-dependent progenitor effects on proliferation, differentiation and function have implications in stem cell-based therapies such as ex vivo transplantation and activation of endogenous progenitor proliferation (Limke and Rao, 2002, 2003). In order to capitalize on the benefits of cell-based therapies, we must first understand the mechanisms and implications of age-dependent changes within the dentate gyrus and its neural progenitor population.

Figures

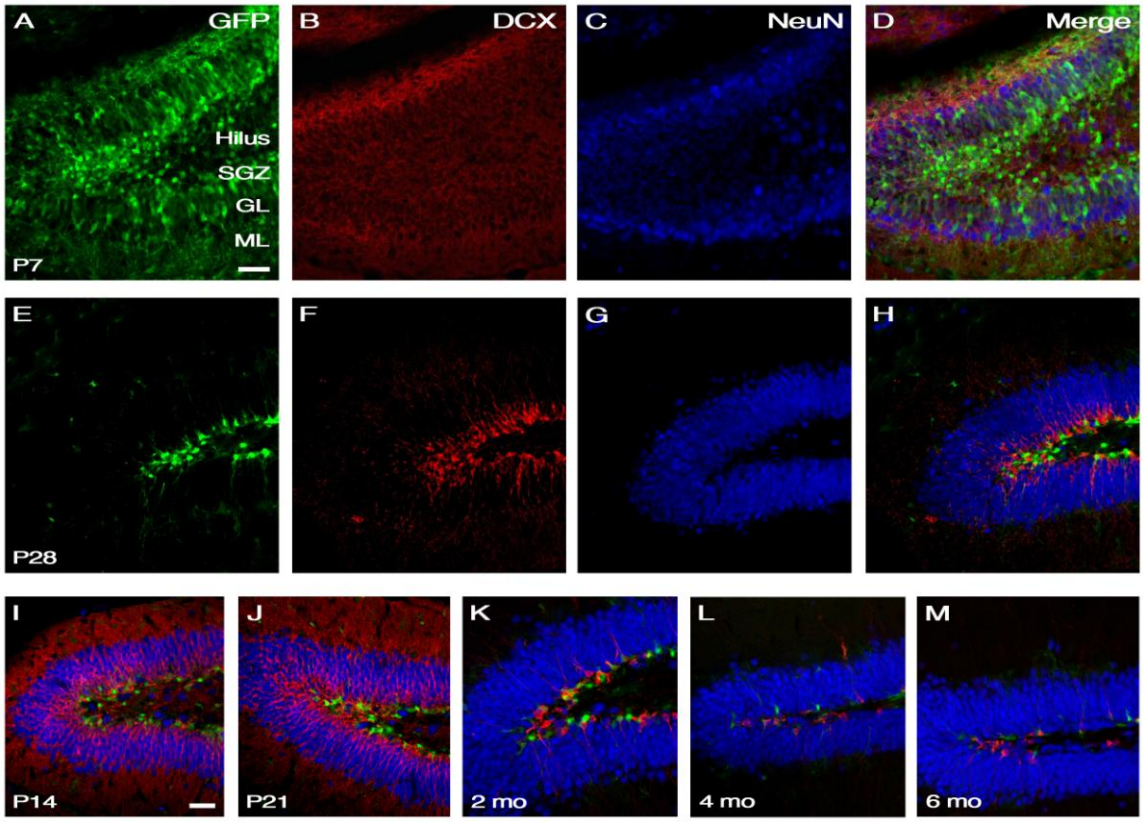

Figure 2-1: Developmental profile of the postnatal dentate gyrus. GFP labels nestin-expressing type I and II cells, Dcx identifies type III cells and NeuN marks mature neurons. (A-D) shows immunofluorescence from P7 transgenic mice and (E-H) depict sections from P28 mice. Merged images from P14, P21, two month, four month and six month animals are shown in (I-M). The population of early progenitors (GFP-positive) seems to decline as postnatal development progresses, however it begins to stabilize at two months of age. Scale bars in (A) and (I) are 50 μm. SGZ, subgranular zone; GL, granular layer; ML, molecular layer. Taken from (Gilley et al., 2009).

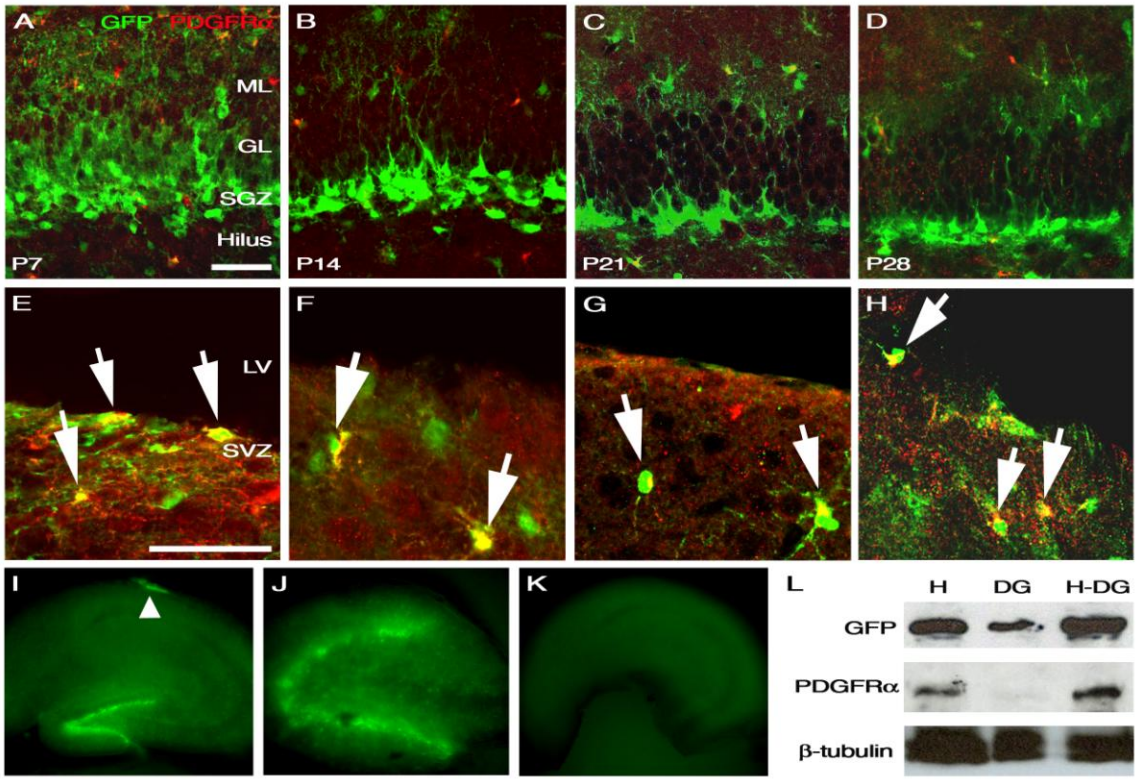

Figure 2-2: Isolation of PDGFRα-negative progenitors from the dentate gyrus. Differential expression patterns of PDGFRα were used to distinguish GFP-expressing progenitors from the SGZ and SVZ. In P7, P14, P21 and P28 transgenic animals, early type I and II progenitors in the dentate gyrus lack expression of PDGFRα (A-D) while oligodendrocyte precursors are labeled with it (see arrows, E-H). Caronal whole mounts of the hippocampus (I), dentate gyrus (J) and hippocampus without the dentate gyrus (K) also emphasize proper dissection techniques. Of importance to note is the population of GFP-positive cells from the SVZ (arrowhead) located along the CA1 region of the hippocampus (I). Western blot analysis was used to confirm that no PDGFRα-expressing cells were found in dentate gyrus isolated from the rest of the hippocampus (L). Scale bars in (A) and (E) represent 50 μm. SGZ, subgranular zone; GL, granular layer; ML, molecular layer; LV, lateral ventricle; SVZ, subventricular zone; H, hippocampus; DG, dentate gyrus; H-DG, hippocampus without dentate gyrus. Taken from (Gilley et al., 2009).

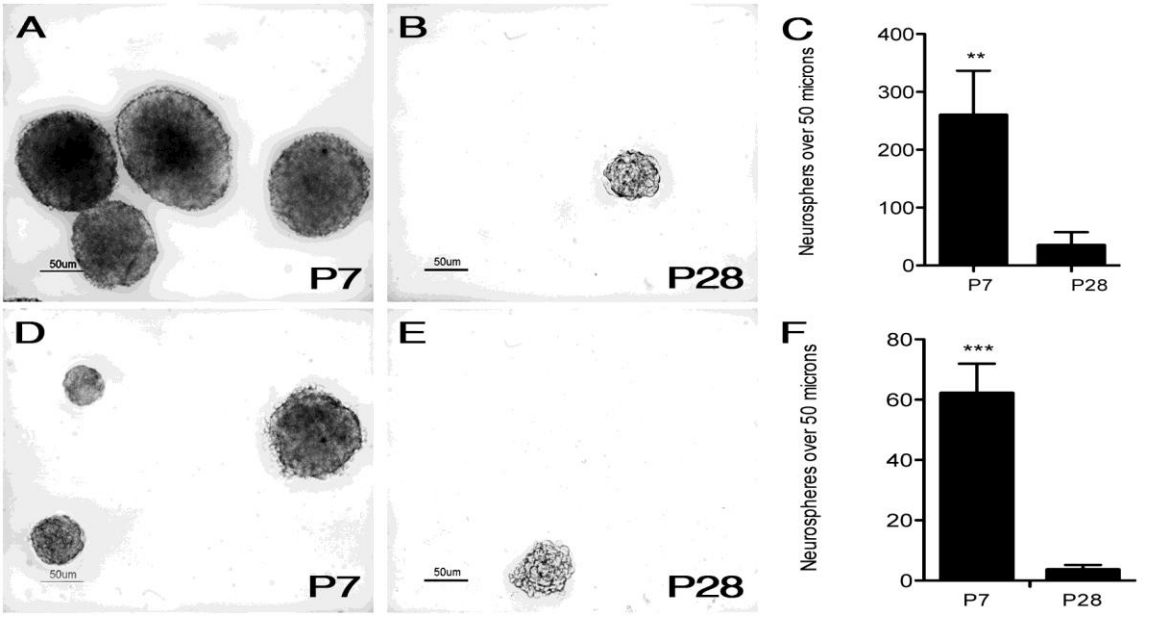

Figure 2-3: Age-dependent effects on neurosphere proliferation are cell-autonomous. Neurosphere assays were utilized to determine different proliferative potential of progenitors isolated from P7 and P28 dentate gyrus. Neurospheres derived from the dentate gyrus (A-B) and FAC-sorted cells (D-E) are shown alongside quantification of neurospheres over 50 μm (C and F). Progenitors obtained from younger animals appear to have a growth advantage compared to those derived from P28 mice. This phenotype is consistent in cell-autonomous (D-F) and non cell-autonomous (A-C) experiments. The number of neurospheres quantified in both experiments is significantly different. Error bars represent standard deviation and *P*-values are ***$P<0.0001$ and **$P<0.001$. Unpaired *t*-tests were used to obtain significance. Scale bars are 50 μm. Taken from (Gilley et al., 2009).

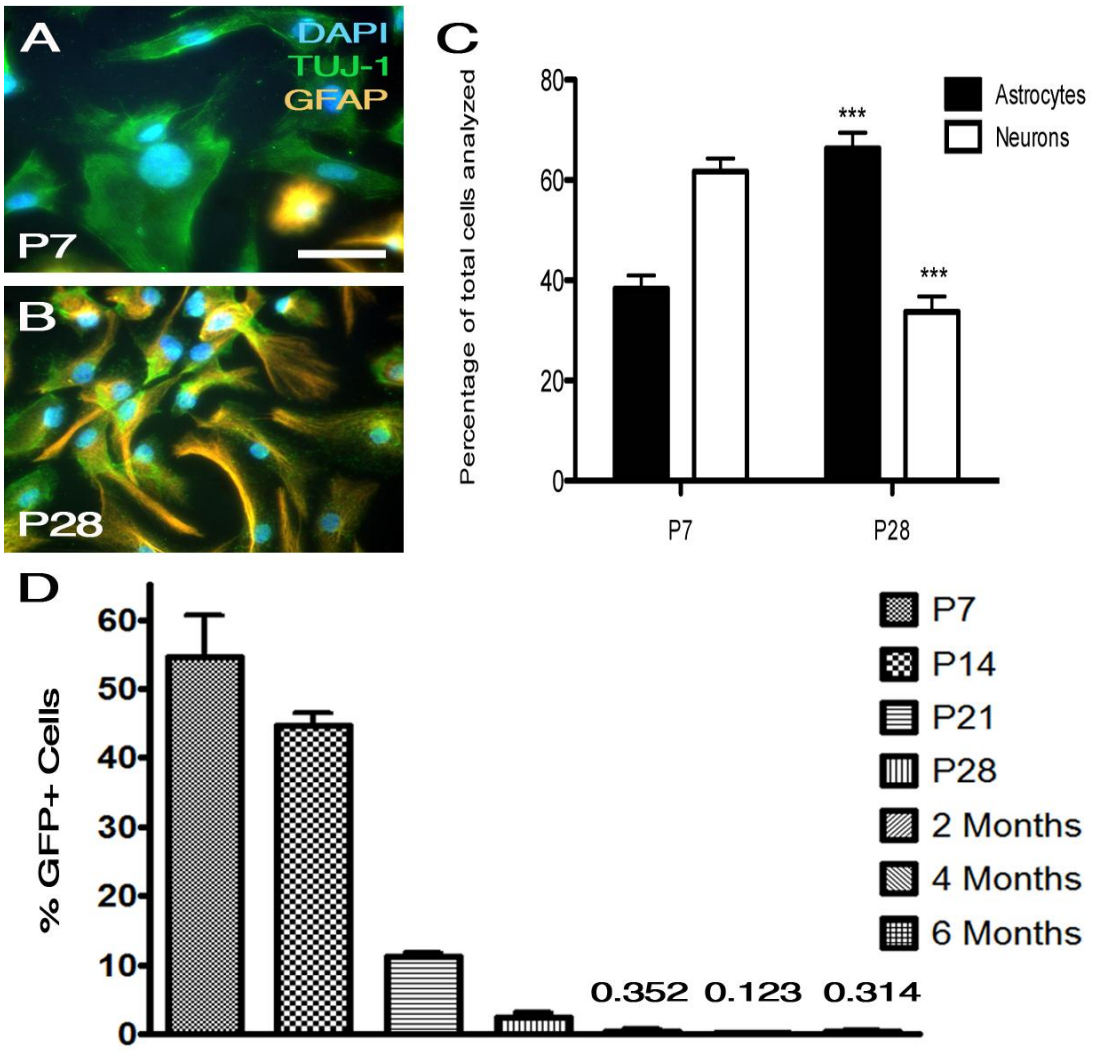

Figure 2-4: Differentiation and quantification of progenitors suggests early adulthood senescence. Early progenitors from P7 and P28 dentate gyrus were differentiated in vitro and stained for the presence of neurons (Tuj1), astrocytes and undifferentiated cells (GFAP) and nuclei (DAPI) (A and B). P7 progenitors primarily become neurons while those from P28 preferentially become astrocytes suggesting that progenitors from younger animals are more neurogenic (C). The percentage of astrocytes and neurons are significantly different (****P*<0.0001). Quantification of early progenitors via FACS demonstrates a steady decline in the percentage of GFP-expressing cells. However, the population appears to stabilize at two months of age suggesting early adulthood senescence. ANOVA and Bonferonni *post hoc* analysis were used to determine significance between groups. P7 and P14 are not significantly different from one another but are significant to all other time points (****P*<0.0001). Error bars represent standard deviation and the scale bar in (A) is 50 μm. DAPI, 4',6-diamidino-2-phenylindole; Tuj-1, neuron-specific class III beta-tubulin; GFAP, glial fibrillary acidic protein. Taken from (Gilley et al., 2009).

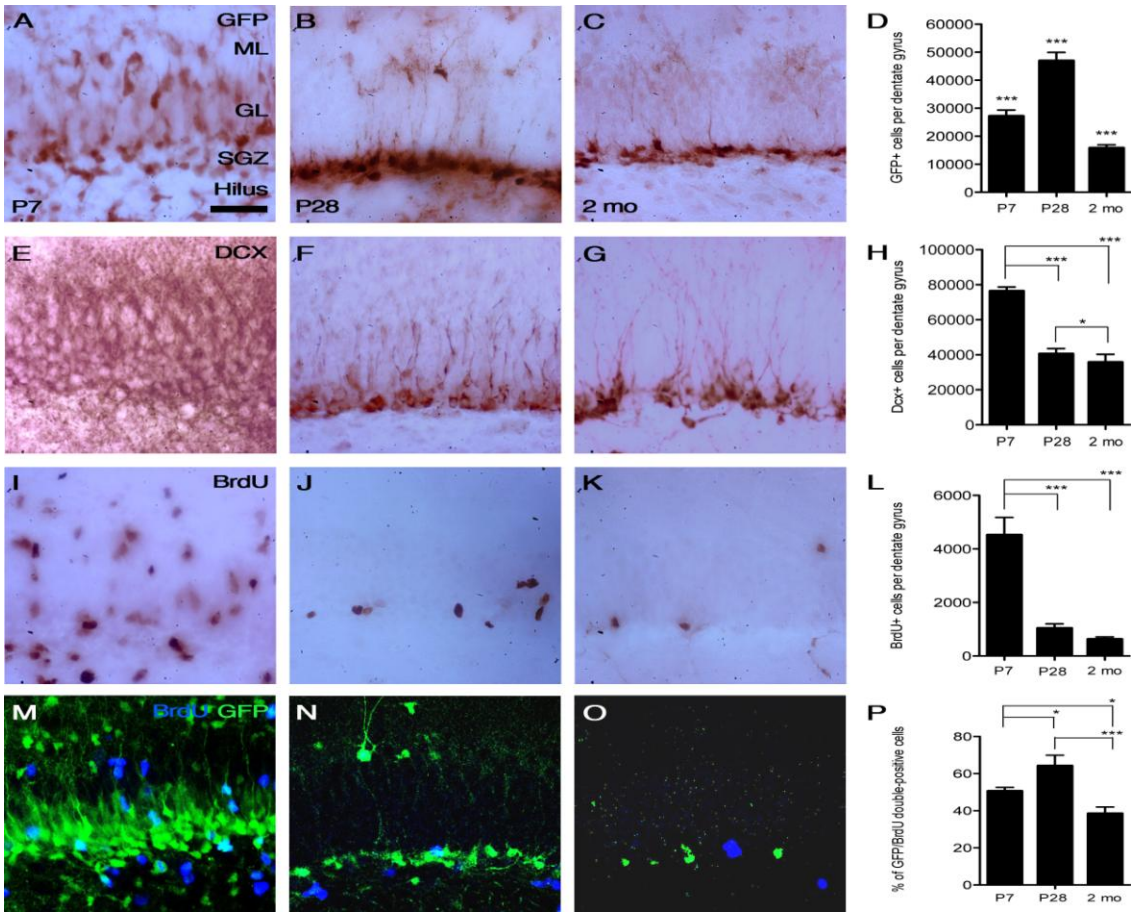

Figure 2-5: Age-dependent changes in early and late progenitor populations. Unbiased stereological and immunohistochemical methods were used to quantify absolute cell number within the SGZ and GL of the dentate gyrus. Representative DAB histological pictures and quantifications of GFP- (A-D), Dcx- (E-H) and BrdU-expressing (I-L) cells are shown. In addition, immunofluorescent images of cells colabeled with GFP and BrdU and their corresponding quantifications are shown (M-P). The highest number of GFP-expressing cells is in P28 animals (D) while P7 animals contain the most Dcx-positive cells compared to the other time points (H). Similar to type III progenitors, BrdU expression (I-K) and quantification (L) is highest at P7 and lowest at two months of age. The percent of GFP and BrdU dual-labeled cells is highest at P28 (P) despite the fact that GFP and BrdU immunofluorescence declines with respect to age (M-O). ANOVA and Bonferonni *post hoc* analysis was used to determine significance. Quantification of GFP, Dcx, BrdU and dual-labeled cells were all significant and error bars represent standard deviation. The *P*-values follow: ****P*<0.0001, ***P*<0.001 and **P*<0.05. The scale bar in (A) represents 50 μm. ML, molecular layer; GL, granular layer; SGZ, subgranular layer. Taken from (Gilley et al., 2009).

GENE	AVG RATIO	CELLULAR FUNCTIONS
Downregulated compared to p7		
CD47	0.217	cell adhesion
CSPG2	0.24	cell adhesion and development
Tbx5	0.49	transcriptional regulator
Upregulated compared to p7		
ApoE	4.35	regulation of lipid metabolism
Cldn10	5.75	cellular adhesion
Eaat2	9.77	glutamate transport
Gja4	6.62	gap junction protein
Gpc4	4.31	cell proliferation and morphogenesis
Vtn	7.32	cell adhession and immune response

Table 2-1: Microarray candidate genes. CD47, cluster of differentiation 47; CSPG2, chondroitin sulfate proteoglycan 2; Tbx5, T-box 5; ApoE, apolipoprotein E; Cldn10, claudin 10; Eaat2, excitatory amino acid transporter 2; Gja4, gap junction alpha 4; Gpc4, glypican 4; Vtn, vitronectin. Taken from (Gilley et al., 2009).

Materials and Methods

Mice

All animals were housed and cared for in a humane manner within the Animal Resource Center at UT Southwestern Medical Center. In addition, The Institutional Animal Use and Care Committee at UT Southwestern approved all animal experiments.

The nestin-eGFP transgenic mice used in all experiments have previously been described and well-characterized (Shi et al., 2007, Koch et al., 2008, Miles and Kernie, 2008, Yu et al., 2008). In order to express eGFP specifically within the neural tube and neural progenitor populations, a nestin-rtTa-eGFP construct was used to generate transgenic mice. In addition, the nestin promoter was driven by a neural progenitor-specific enhancer element within the second intron of the nestin gene (Zimmerman et al., 1994).

Tissue Preparation

Mice were perfused and fixed overnight in 4% paraformaldehyde (PFA) before they were imbedded in 3% agarose and sectioned on a Leica vibratome at 50 μm. Every sixth section was used for all immunostaining experiments. Sections were washed three times with 0.3% TritonX phosphate buffered saline (PBS), blocked in 10% normal donkey serum and then stained with primary antibodies (with 5% normal donkey serum) for at least two hours. Sections were then washed again and stained with fluorescent secondary antibodies for at least two

hours before three final wash steps and mounting on glass slides with Immu-Mount.

Cell Culture Media

The activated papain used to digest tissue consisted of the following: 42 μl papain (Worthington), 27 μl 100 mM Cystein-HCl, 6 μl of 100 mM EDTA and 425 μl DMEM/F12 (Gibco) or Neurobasal A (Gibco).

Neural stem cell media consisted of 1% N2 supplement (Invitrogen), B27 1:50 (Gibco), 1% Penicillin/Streptavidin, 10 μg/mL Heparin, 1% glutamine, 20 ng/mL bFGF (Sigma) and 20 ng/ml bEGF (Invitrogen) in DMEM/F12 or Neurobasal A.

Serum containing media consisted of 1% calf serum and 1% antibiotics in either DMEM/F12 or Neurobasal A media.

Semisolid media was made up of 60% neural stem cell media (in DMEM/F12 or Neurobasal A) and 40% 1.6% methylcellulose (Sigma)

Developmental Profile

P7, P14, P21, P28, two month, four month and six month old transgenic animals were perfused, vibratomed and stained with various antibodies to determine cell type. Antibodies used include rabbit anti-GFP 1:500 (Molecular Probes), goat anti-Dcx 1:200 (Santa Cruz), and mouse anti-NeuN 1:500 (Chemicon).

PDGFRα Characterization

P14 transgenic animals were analyzed for their GFP and PDGFRα expression. Sections were stained with chicken anti-GFP 1:500 (Aves Labs) and rat anti-PDGFRα 1:250 (BD Pharmingen) primary antibodies followed by incubation with the appropriate fluorescent secondary antibodies 1:200 (Santa Cruz).

Neurosphere Assays

Dentate gyrus was microdissected from nestin-eGFP transgenic mice and was further cut into smaller pieces with scissors. After incubation in activated papain for 20 minutes at 37 degrees, the tissue was triturated with a fire-polished Pasteur pipette in serum containing media and filtered into a single-cell solution using a 30 μm filter (Partec). Cells were then washed, counted and plated at the same density (20 cells/μl) in semisolid media or used for cell sorting via FACS. Using a MoFlo cell sorter (DAKO), GFP-expressing neural progenitors were collected and then plated in semisolid media at a density of 20 cells/μl. 14 days after initial plating, neurospheres over 50 μm were quantified to measure proliferation. These experiments were performed in neural stem cell media prepared with DMEM/F12 which contains glutamate and aspartate.

A similar experiment was performed which tried to determine if progenitor proliferation was cell-autonomous. GFP-expressing cells from P7 and P28 animals were sorted into 96-well plates containing neural stem cell media at a density of one cell/well and were allowed to form neurospheres. 14 days after

plating the total number of neurospheres were quantified. Culture media used in this experiment contained glutamate and aspartate.

Cell Differentiation

Neurospheres derived from P7 and P28 dentate gyrus were dissociated into single cells and then plated on coated chamber slides in the presence of serum-containing media (with glutamate) and were allowed to differentiate in culture for five days. Cells were then washed and immunohistochemically stained with mouse anti-Tuj1 1:2000 (Covance), rabbit anti-GFAP 1:2000 (Invitrogen) and DAPI 1:1000 (FLUKA) to visualize neurons, astrocytes and nuclei, respectively. Differentiated cells were quantified using an Olympus BX50 microscope and Photometrics CoolSNAP camera. At least 200 DAPI-expressing cells were counted per sample and the experiment was repeated four times. We used an unpaired *t*-test to analyze statistical significance (***P<0.0001).

Cell Sorting

All cells were sorted using a MoFlo from DAKO. For quantification of GFP-expressing cells, dentate gyrus was microdissected at several time points (P7, P14, P21, P28, 2 months, 4 months and 6 months) and digested with 20 μl DNase I, 2 ml 10x Dispase II and 10 ml 2x Papain (Worthington) in 20 ml DMEM/F12 for 20 minutes at 37 degrees. The tissue was then triturated with a p1000 pipette tip in serum-containing media before being passed through a 70 μm filter (BD Biosciences). Propidium iodide (PI) was added to the single cell solution before GFP-positive and GFP-negative cells were collected. In

determining the percentage of GFP-expressing cells at multiple ages (P7-6 months), the number of GFP-positive cells was divided by the total number of cells collected. At least six animals were used per time point and each time point was repeated at least three times. To check the population purity of sorted cells, GFP-positive and –negative cells were analyzed under a fluorescent scope. Statistical analysis was performed using a One Way ANOVA followed by Bonferonni post hoc analysis (***P<0.0001).

For all other sorting experiments, tissue was digested using activated papain and a flame-polished pipette (see Neurosphere Assay). After being sorted, GFP-expressing cells were used for neurosphere assays, Q-PCR analysis or microarray analysis.

Stereological Staining and Quantification

All sections were prepared using the same methods (see Tissue Preparation). Sections from P7, P28 and 2 month old transgenic animals were stained overnight with rabbit anti-GFP 1:500 (Invitrogen), goat anti-Dcx 1:100 or rat anti-BrdU 1:400 primary antibodies and then washed with PBS and incubated with biotinylated secondary antibodies (1:350). Afterwards, sections were treated with horseradish peroxidase-based Vectastain ABC Kit (Vector Laboratories) followed by incubation with 3,3'-diaminobenzidine (DAB) substrates (Vector Laboratories) to enhance the staining.

Cell quantification was performed using the Optical Fractionator Probe within the Stereo Investigator software (MBF Bioscience, MicroBrightField, Inc.) on a Olympus BX51 System Microscope and a MicroFIRE A/R camera (Optronics). Stained cells were counted with an unbiased counting frame (Gundersen, 1980, West, 1993) while utilizing a 40x oil immersion lens. Cells were counted within the SGZ and GL of the dentate gyrus on one side of the brain and at least 200 cells were counted per animal. The average mounted thickness after tissue processing was approximately 35 μm and the average number of sections counted per animal was ten.

Several measures were undertaken in order to reduce bias between samples. We used a height sampling fraction of 30 μm to account for observed tissue shrinkage after processing and only sections with homogenous staining were counted. Furthermore, only samples with all sections present were analyzed. The area sampling fraction for BrdU stained sections was one while the area sampling fraction for GFP- and Dcx-positive cells was 1/8. The section sampling fraction used for quantification was six (every sixth section).

BrdU Incorporation

Two hours prior to perfusion, P7 and P28 transgenic animals were pulsed with BrdU (100 μg/kg Sigma). After sectioning, they were stained with rabbit anti-GFP 1:500 (Invitrogen) and rat anti-BrdU 1:400 (Abcam) primary antibodies followed by incubation with fluorescent secondary antibodies 1:200 (Santa Cruz).

Using methods adapted within the lab, transiently transfected neurospheres were pulsed with 10 μM BrdU for 15 minutes, dissociated with activated papain and triturated into a single-cell solution before fixation in 100% ethanol. The DNA was then denatured with 2N HCl/0.5% TritonX in PBS for 30 minutes at room temperature and afterwards the reaction was neutralized with 0.1 M NaB_4O_7. Samples were then incubated in staining solution (1.3 μl BrdU-APC, 5 μl RNase and 50 μl 0.5% Tween/1% BSA in PBS) overnight at 4 degrees. Finally, cells were washed and resuspended in PI-containing PBS and analyzed for BrdU incorporation. Only BrdU-positive and PI-negative cells were quantified.

Confocal Microscopy

A Zeiss LSM 510 confocal microscope was used for all confocal microscopy experiments and imaging. For cell quantification, Argon 488 and He 633 lasers were used to quantify GFP and BrdU double-labeled cells within the SGZ and GL of P7, P28 and two month transgenic animals. A Zeiss Neofluar 40X/1.3 oil lens was used to focus through the *z*-axis of cells in order to determine complete colocalization. All BrdU-positive cells within the SGZ and GL of the dentate gyrus were analyzed for their coexpression with GFP. Percentages are presented as the ratio of double-labeled cells to BrdU-expressing cells. One Way ANOVA was used to calculate overall significance followed by a Bonferonni *post hoc* analysis to determine significance between groups (**P*<0.05, ***P*<0.001, ****P*<0.0001).

Microarray Analysis

GFP-expressing cells were sorted from the dentate gyrus of P7 and P28 transgenic animals for microarray analysis as described in Gilley et al, 2010 (Gilley et al., 2009). Briefly, cells were lysed according to manufacturer's protocol and shipped on dry ice to Miltenyi Biotec where RNA was extracted, cDNA was synthesized and amplified before being hybridized to a stem cell-specific array. Validated protocols developed by Miltenyi Biotec were used to perform the microarray and analyze the data (Landgraf et al., 2007). Microarrays were performed in triplicate and only statistically relevant genes were included in our final list of candidate genes.

CHAPTER THREE

Characterization of GltI and Glast

Introduction

Previous results from our lab demonstrate the presence of various cell-autonomous cues among early type I and II progenitors isolated from P7 and P28 transgenic mice (Table 2-1). We have also shown differences in the proliferative and differentiation potentials for these cells in culture (Figure 2-3, 4). These observed phenotypes in vitro might therefore contribute to the development of the postnatal dentate gyrus and help explain the in vivo landscape of different progenitor populations within the hippocampus at various time points (Figure 2-5). From this, we can hypothesize that one of the genes identified in our microarray screen (Table 2-1) might be responsible for the cell-autonomous phenotypes observed in vitro. It is the goal of this study to examine the functional role of one of those candidate genes, GltI, on hippocampal progenitors.

GltI and its family member Glast are normally expressed on astrocytes within the synaptic cleft and actively transport extracellular glutamate to prevent excitotoxic damage to neurons (Rothstein et al., 1994, Rothstein et al., 1996, Tanaka et al., 1997, Anderson and Swanson, 2000, Liang et al., 2008). Although glutamate transporters have a well-characterized role in astrocytes (Tanaka, 2007), not much is known about their function in neurogenesis. However, the differential expression pattern of GltI on early neural progenitors (Table 2-1) suggests that it may play a role in regulating the neurogenic niche within the

dentate gyrus. While the functional role of glutamate transporters in neural progenitors remains unknown, we know they play a key role in protecting the brain from excessive glutamate, the most abundant neurotransmitter in the brain.

Glutamate transporters have been well-characterized in a number of traditional knockout mice (Tanaka et al., 1997, Watase et al., 1998, Matsugami et al., 2006), however few have studied their function in a temporally or spatially regulated fashion. GltI and Glast knockout mice are prove useful to the current study of adult neurogenesis because homozygous mutant mice die before birth (Tanaka et al., 1997, Watase et al., 1998, Matsugami et al., 2006) and conditional mutants have not been generated. Therefore, for these studies, we have chosen to investigate the relevance of GltI and Glast in hippocampal progenitors using a number of in vitro techniques.

Here we utilize immunohistochemistry to characterize the expression of glutamate transporters on various progenitor populations within the developing dentate gyrus. In addition, we have taken advantage of the growth properties of P7 neurospheres to study the role of GltI and Glast in neural progenitors. We have genetically manipulated expression of GltI and Glast in vitro and identified a novel functional role for glutamate transporters in progenitor proliferation.

Results

Q-PCR and Western blot analysis confirm microarray data

Excitatory amino acid transporter 2 (Eaat2 or GltI) was the candidate gene whose expression changed the most between P7 and P28. Table 2-1 shows that

GltI was upregulated 9.77-fold in P28 animals when compared to P7. To confirm this we performed quantitative PCR analysis on GFP-positive cells sorted from P7 and P28 transgenic animals. Our results are in agreement with our microarray data as relative GltI expression was 9.65 times higher in P28 compared to P7.

We also undertook Western blot analysis to confirm GltI protein expression at these time points. Protein lysates were collected from the dentate gyrus of P7 and P28 animals and blotted for the presence of GltI. GltI protein levels at P28 are also higher than those observed at P7 (Figure 3-1, B). We also looked at GltI protein in the cortex, hippocampus, dentate gyrus and hippocampus without dentate gyrus (Figure 3-1, C) of P14 transgenic to further analyze its widespread distribution throughout the forebrain.

GltI and Glast localize to early type I and II progenitors but not to later type III progenitors

Results from our microarray data indicate that GltI is expressed on progenitor cells. However, since the nestin-GFP transgenic animal labels both type I and type II cells, we wanted to specifically examine GltI and Glast on these cell types. Using immunofluorescent techniques, we used GFP and GFAP to label type I cells, GFP expression to identify type II cells and Dcx to indicate later type III progenitors. In accordance with our microarray data, GltI is expressed on GFP-positive cells at higher levels in P28 animals (Figure 3-2). Glast however, has higher expression in P7 animals compared to P28 (Figure 3-3).

Furthermore, a majority of GltI- and Glast-expressing cells also express GFAP indicating that these glutamate transporters are present on type I progenitors (Figures 3-2,3). These GltI and Glast-expressing cells did not colocalize with doublecortin-expressing cells, suggesting that GltI and Glast are not found on type III cells.

Glutamate stimulates dentate gyrus neurosphere growth

To determine if glutamate positively regulates neurosphere proliferation under our mitogen-free culture conditions, we plated dissociated P7 neurospheres in semisolid media in the presence and absence of 5 μM glutamate and allowed spheres to form for seven days. We determined that the addition of glutamate increased neurosphere size but did not affect the overall number of neurospheres present (Figure 3-4) or overall cell death (observation). The average number of neurospheres per well was 27.43 and 28.25 with and without exogenous glutamate, respectively. The average size of neurospheres without glutamate was 65.63 μm while those stimulated with glutamate had an average size of 270 μm. These latter results were statistically significant (***P<0.0001) and analyzed by ANOVA and Bonferonni *post hoc* analysis. This result confirms other studies which have demonstrated glutamate's role in regulating proliferation and cell survival (Luk et al., 2003, Brazel et al., 2005, Suzuki et al., 2006).

Overexpression of GltI or Glast results in decreased BrdU incorporation

In order to elucidate the function of glutamate transporters in neural progenitor cells without stimulating glutamate-dependent proliferation (observed in Figure 3-4), we knocked down and overexpressed GltI and Glast in P7 neurospheres (in quadruplicate) using simple reverse transfection protocols in media lacking glutamate. Seven days after neurospheres were transfected, small samples were assayed to confirm misexpression. Relative mRNA levels were normalized to GltI and Glast expression in cells transfected with DsRed2, a negative control plasmid. Therefore, GltI and Glast relative expression in DsRed2 control is one. To assure that our GltI and Glast siRNAs are specifically targeting their genes, we also transfected a scrambled siRNA negative control which should have similar expression levels of GltI and Glast to cells transfected with DsRed2.

In this experiment all mRNA levels are compared to expression of GltI and Glast in cells transfected with DsRed2 (Figure 3-5, A-B). In cells transfected with scrambled siRNA (Scramble), the average relative expression of GltI was 1.138 while the average expression of Glast was 1.078. Gene silencing with either GltI or Glast siRNA (KD) resulted in RNA levels of 0.558 and 0.324, respectively indicating that both genes are knocked down by at least 50%. Cells transfected with plasmids overexpressing (OE) either GltI or Glast had average expression levels of 11.014 and 3.47, respectively. It should be noted that this level of GltI expression (11.014) is similar to in vivo expression levels observed in P28 transgenic mice (see microarray data in Table 2-1). From these data we can conclude that GltI and Glast are consistently knocked down or overexpressed

when compared to controls. Furthermore, overexpression of GltI in P7 neurospheres resembles similar levels in P28 mice.

Once misexpression was confirmed, the remaining neurospheres were pulsed with BrdU and stained with an anti-BrdU-APC fluorescent antibody overnight. To asses proliferation rates of cells transfected under various conditions we used flow cytometry analysis. The results are reported as a percentage of BrdU-positive, PI-negative cells and are shown in Figure 3-5, C. The negative controls suggest a baseline BrdU incorporation of 1.792% (DsRed2) and 1.95% (Scramble). Knockdown of GltI resulted in an increase of BrdU-positive, PI-negative cells (2.88%) while its overexpression resulted in decreased BrdU incorporation (0.505%). Similarly, silencing of Glast increased the percentage of BrdU-expressing cells (2.60%) while overexpression decreased it (0.149%) when compared to controls. These results suggest that GltI and Glast regulate the proliferation of neural progenitor cells in vitro.

Knockdown of glutamate transporters results in increased neurosphere proliferation

To confirm this proliferative phenotype, we performed quadruplicate neurosphere assays on cells transfected with controls, siRNA or overexpression plasmids. After seven days in culture, neurospheres were assayed with Q-PCR to confirm misexpression (Figure 3-6, A-B). GltI relative mRNA was 1 in DsRed2, 0.862 in Scramble, 0.344 in KD and 10.27 in OE. Glast expression was 1 in DsRed2, 1.2 in Scramble, 0.318 in KD and 3.68 in OE. These results are

similar to those observed in Figure 3-5, A-B and suggest that we are able to consistently knockdown and overexpress GltI and Glast in P7 neurospheres.

Once relative mRNA levels were acquired, the remaining transfected neurospheres were dissociated and plated at a density of 20,000 cells/ml in semisolid media containing neural stem cell media and 1.6% methylcellulose. Neurospheres were allowed to form for seven days in vitro before they were quantified. Results are presented as the number of neurospheres per well that are over 50 μm in size (Figure 3-6, C). Cells previously transfected with the control DsRed2 plasmid formed an average of 64 neurospheres while those with scrambled siRNA formed an average of 57.25 neurospheres. GltI knockdown produced 98.8 spheres per well and its overexpression resulted in an average of 67.75 neurospheres. Glast knockdown saw an increase in sphere formation (108.1) while its overexpression only showed modest increases the number of spheres (69.18) when compared to controls. Results from this experiment confirm that knockdown of glutamate transporters causes an increase in proliferation as shown by neurosphere assays.

Intracellular glutamate levels do not change in the presence of GltI or Glast siRNA

Once again misexpression was confirmed seven days after transfection (Figure 3-7, A-B). GltI relative expression levels are as follows: 1 in DsRed2, 0.920 in Scramble, 0.102 in KD and 11.535 in OE. Glast mRNA was 1 in DsRed2, 0.983 in Scramble, 0.542 in KD and 3.493 in OE. These relative mRNA

levels are consistent with previous experiments and confirm knockdown and overexpression of GltI and Glast.

After mRNA levels had been determined, neurospheres were dissociated and subject to an intracellular glutamate assay kit (Glutamate Assay Kit, BioVision). Baseline levels of intracellular glutamate were around 7 nmol as shown in the negative controls (Figure 3-7, C). Knockdown of GltI and Glast resulted in cellular glutamate concentrations of 5.413 nmol and 5.942 nmol, respectively while overexpression exhibited non-significant differences in glutamate (7.608 for GltI OE and 7.114 for Glast OE). The lack of exogenous glutamate in the media likely explains this phenotype.

Glutamate transporter misexpression alters intracellular calcium levels

The mechanism underlying glutamate-induced proliferation is unclear, however changes in intracellular calcium caused by activation of glutamate receptors may be involved (Choi, 1988, Choi et al., 1988, Deisseroth et al., 2004, Nacher and McEwen, 2006, Nacher et al., 2007). To determine how glutamate transporter expression effects glutamate-mediated calcium flux, we incubated transfected neurospheres with a Fluo-4 AM ester calcium indicator and assayed for changes in intracellular calcium using flow cytometry methods. Upon stimulation of cells with 5 μM glutamate, we measured increased fluorescence indicating a proportional increase in intracellular calcium. Prior to stimulation with glutamate, baseline levels of intracellular calcium were also recorded. The results are presented as a percentage of cells that are Fluo-4 AM-positive. In

addition the results are normalized to the percentage of positive cells at baseline levels before stimulation with glutamate (Induction/Baseline) (Figure 3-8, C).

Of cells transfected with negative controls (scrambled siRNA or DsRed2 plasmid) we determined that in the absence of exogenous glutamate, about 4% of progenitor cells were Fluo-4-positive. Upon stimulation with glutamate, the ratio of induced cells to baseline cells increased by 1.5275 for DsRed2 and 1.4775 for Scramble. Knockdown of either GltI or Glast resulted in a 2.01 or 1.95 fold induction of cells expressing the Fluo-4 calcium indicator (respectively). Compared to controls, these percentages are significantly different. In contrast, overexpression of glutamate transporters resulted in an overall decrease in the number of cells expressing the calcium marker. The percentage of positive cells was 1.15 for GltI overexpression and 1.20 for Glast overexpression.

These results taken together suggest that knockdown of GltI and Glast results in increased intracellular levels of calcium while their overexpression leads to decreased intracellular calcium. In other words, lack of GltI and Glast lead to decreased glutamate transport and increased signaling through glutamate receptors. Activation of these receptors ultimately results in the release of intracellular calcium stores. We can therefore conclude that the role of glutamate transporters on neural progenitors is to regulate the availability of glutamate available to glutamate receptors in order to control proliferation.

Discussion

Using Q-PCR we were able to validate our in vivo microarray results (Table 2-1) and manipulate expression levels of GltI and Glast in vitro (Figures 3-5,6,7). Similar to the array results, our mRNA quantification suggests that GltI is upregulated about 10-fold in P28 progenitors compared to P7 progenitors (Figure 3-1, A). In addition we were able to consistently manipulate GltI and Glast expression levels in vitro in order to mimic certain in vivo phenotypes. For instance, all of our experiments using neurospheres transfected with the GltI overexpression plasmid demonstrated relative mRNA levels in the range of 10.27-11.54 (Figure 3-5,6,7). This intensity of expression is consistent with GltI levels observed in P28 progenitors from our microarray (Table 2-1). This suggests that we are genetically upregulating GltI in vitro to physiologically relevant levels observed in vivo. The same is true for Glast overexpression as well. The 3.47-3.68 fold increase in Glast expression observed in P7 neurospheres (Figures 3-5,6,7) is consistent with in vivo levels of Glast observed in P7 progenitors isolated from transgenic mice (data not shown). It is therefore important to point out that we were able to manipulate GltI and Glast in our in vitro experiments while maintaining physiological relevant expression levels. Moreover, the ability to mimic GltI expression patterns in P28 animals using P7 neurospheres allows us to take advantage of the increased proliferative potential of P7 neurospheres compared to P28 neurospheres (Figure 2-3, C and F).

Results from our transfected neurosphere assays (Figure 3-6) can be used to explain the differential proliferative capacity of P7 and P28 progenitors in

culture (Figure 2-3). As mentioned previously, overexpression of GltI in P7 neurospheres resembles the expression profile of P28 progenitors in vivo (Table 2-1 and Figures 3-5,6,7). In previous experiments we have shown that P28 progenitors are less proliferative in culture compared to P7 progenitors (Figure 2-3). We have also shown that GltI is upregulated in P28 mice compared to P7 (Figure 3-1,2 and Table 2-1). Furthermore, knockdown of glutamate transporters results in increased proliferation (Figures 3-5, C and 3-6, C). Unlike the BrdU incorporation results (Figure 3-5, C), overexpression of GltI and Glast did not produce a significant decrease in the number of spheres formed in the neurosphere assay (Figure 3-6, C). However, we believe the variable sensitivities of these assays may explain this discrepancy. These results taken together strongly suggest that GltI and Glast negatively regulate neural progenitor proliferation in vitro and in vivo. However these experiments should be repeated in the presence of glutamate to confirm this novel functional role for glutamate transporters.

The fact that we used glutamate-free media in all of our in vitro experiments might explain why we did not see an increase of intracellular glutamate when the cells were overexpressing GltI or Glast. The only glutamate available to the cell is derived from the glutamine included in the neural stem cell media. Therefore, the baseline concentration of extracellular glutamate is probably already low. Although GltI and Glast were successfully overexpressed, there might not have been enough extracellular glutamate to transport into the cell that would warrant a change in intracellular glutamate levels. In other words,

there was not enough extracellular glutamate to saturate the increased number of glutamate transporters. As a result, there was no change in glutamate levels when they were overexpressed. Knockdown of glutamate transporters, on the other hand, would result in decreased intracellular glutamate because there are baseline levels of glutamate within these progenitors which may be decreased due to genetic manipulation. This is what we present in Figure 3-7. Attempts were made to examine intracellular glutamate in the context of double knockdown of GltI and Glast, however consistent results were never obtained.

In addition we show that knockdown of GltI and Glast results in increased concentrations of intracellular calcium (Figure 3-8, C). This suggests that glutamate transporters actively regulate glutamate concentrations in order to control the amount of available glutamate to stimulate glutamate receptors. A proposed mechanism of the role of glutamate transporters and their regulation of neural progenitor proliferation is shown in Figure 3-9.

Results from the current study suggest that GltI and Glast regulate neural progenitor proliferation by controlling access to extracellular glutamate. Their function in neural progenitor cells is therefore similar to their transporter roles in astrocytes. This is not surprising considering the astrocytic characteristics of neural stem and progenitor cells (Doetsch et al., 1999, Gotz et al., 2002, Goldman, 2003, Merkle et al., 2004, Sundholm-Peters et al., 2004, Ihrie and Alvarez-Buylla, 2008, Malatesta et al., 2008, Kriegstein and Alvarez-Buylla, 2009). Like astrocytes, neural progenitors respond to glutamate through NMDA receptors and mGluRs (Cameron et al., 1995, Nacher et al., 2001, Luk et al.,

2003, Deisseroth et al., 2004, Di Giorgi Gerevini et al., 2004, Di Giorgi-Gerevini et al., 2005, Nacher and McEwen, 2006, Suzuki et al., 2006, Melchiorri et al., 2007, Castiglione et al., 2008). Our results show how the trafficking of glutamate by GltI and Glast affects proliferation of neural progenitor cells. When glutamate transporter expression is low, there is no way to regulate extracellular glutamate concentrations. As a result there is more available glutamate that can stimulate NMDA receptors and mGluRs on the surface of the progenitors. Conversely, in the presence of GltI and Glast, extracellular glutamate is transported into the cell thereby reducing the stimulation of glutamate receptors which results in decreased proliferation. We have therefore identified a novel functional role for GltI and Glast on neural precursors derived from the hippocampus. A model of the proposed mechanism is shown in Figure 3-9.

The regulation of progenitor proliferation by glutamate transporters has several therapeutic implications. Numerous studies have underscored the importance of glutamate transport in diseases such as Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS) and Parkinson's disease as well as many others (Gray et al., 1980, Rothstein et al., 1992, Dodd et al., 1994, Rothstein, 1995, Rothstein et al., 1995, Beal, 1998, Canton et al., 1998, Masliah et al., 2000, Plaitakis and Shashidharan, 2000, Lievens et al., 2001, Maragakis and Rothstein, 2001, Behrens et al., 2002, Scott et al., 2002, Maragakis et al., 2004). Manipulation of GltI and Glast might therefore be advantageous in elucidating mechanisms of disease. In addition, these diseases are all associated with neurodegeneration. Modulating glutamate transporter

function in progenitors might be a useful way of stimulating neurogenesis as a means of repair and regeneration. Our results are therefore relevant and will prove useful in the ongoing search for disease therapies and cures.

Using a variety of in vitro techniques, we are able to demonstrate that GltI and Glast are expressed in early neural progenitors and function to regulate proliferation by sequestering extracellular glutamate. Although their role as glutamate transporters on progenitor cells is similar to their function in astrocytes, this is the first report on the influence of GltI and Glast on hippocampal neurogenesis. These findings contribute to and help elucidate the molecular mechanism of hippocampal adult neurogenesis and provide insight and therapeutic potential for many human diseases.

Figures

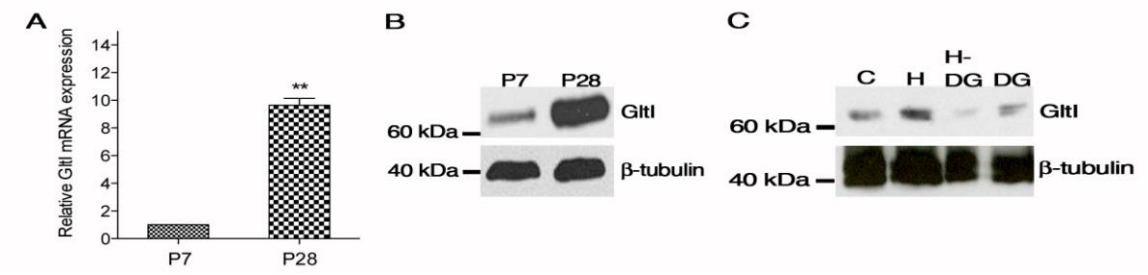

Figure 3-1: Analysis of GltI mRNA and protein expression confirms microarray data. (A) RNA was isolated from P7 and P28 FAC-sorted cells, reverse transcribed into cDNA and analyzed for expression of GltI using Q-PCR. (B) Whole cell lysates from P7 and P28 dentate gyrus demonstrate an upregulation of GltI protein in P28 animals. (C) GltI expression is widespread throughout the P14 brain. C, cortex; H, hippocampus; DG, dentate gyrus; H-DG, hippocampus with the dentate gyrus dissected. An unpaired *t*-test was used in (A) to determine significance (**P<0.001). Taken from (Gilley et al., 2009).

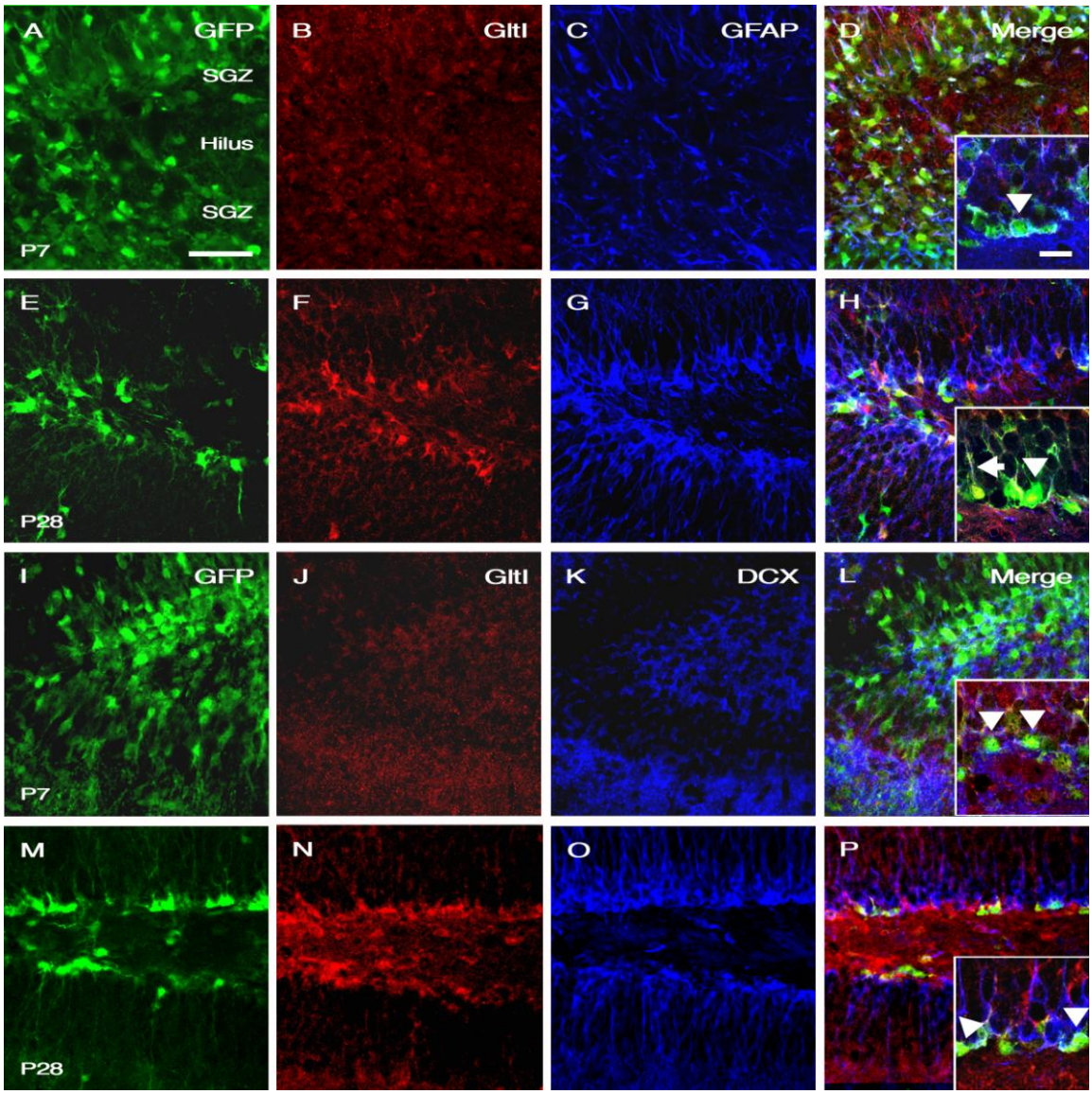

Figure 3-2: GltI is expressed on early type I but progenitors. Colocalization of GltI with GFAP and GFP in P7 (A-D) and P28 (E-H) SGZ suggests that it is expressed on early type I progenitors. Although the magnified image in (D) shows background levels of GltI, GFP- and GFAP-double-labeled cells are present (arrowhead). A type I cell expressing all three markers is illustrated in (H) (arrow) along with a type II progenitor that only expresses GFP and GltI (arrowhead). (I-L) and (M-P) demonstrate that Dcx does not colocalize with GltI-expressing cells in P7 and P28 animals, respectively. In (L) and (P) there are GFP-expressing cells that lack Dcx expression altogether (arrowheads) suggesting that GltI is not expressed on Dcx-expression type III progenitors. Scale bar in (A) is 50 μm and the one in (D) is 35 μm. SGZ, subgranular zone. Taken from (Gilley et al., 2009).

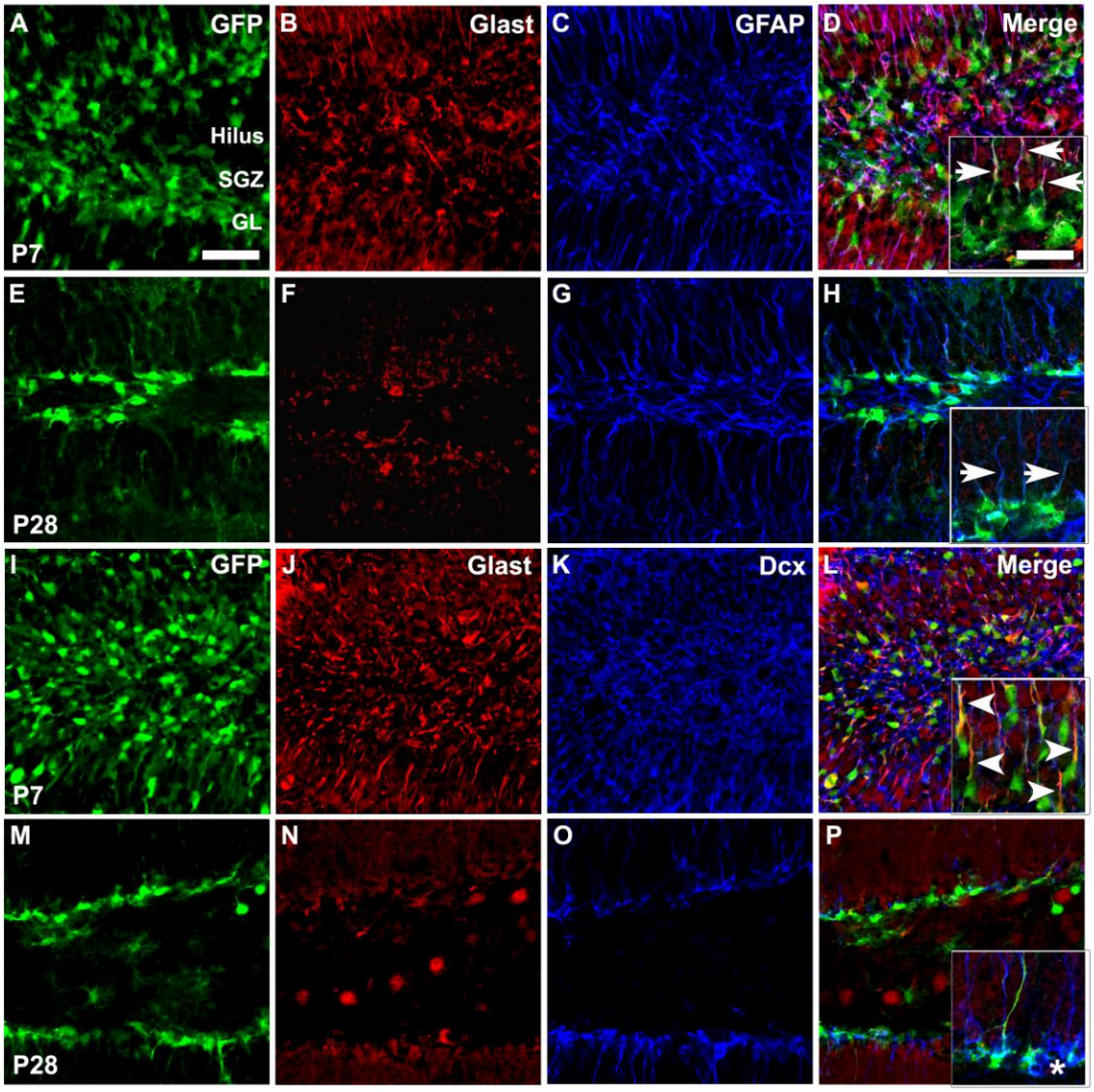

Figure 3-3: Glast is expressed on early type I progenitors. Colocalization of Glast with GFAP and GFP in P7 (A-D) and P28 (E-H) SGZ suggests that it is expressed on early type I progenitors. Although the magnified image in (H) shows background levels of Glast, GFP- and GFAP-double-labeled cells are present (arrows). A type I cell expressing all three markers is illustrated in (D) (arrow). (I-L) and (M-P) demonstrate that Dcx does not colocalize with Glast-expressing cells in P7 and P28 animals, respectively. In (L) there are GFP-expressing cells that lack Dcx expression altogether (arrowheads). The asterisk in (P) shows a cluster of type III cells that lack GFP and Glast expression. This suggests that Glast is only expressed on type I progenitors. Scale bar in (A) is 50 μm and the one in (D) is 35 μm. SGZ, subgranular zone; GL, granular layer.

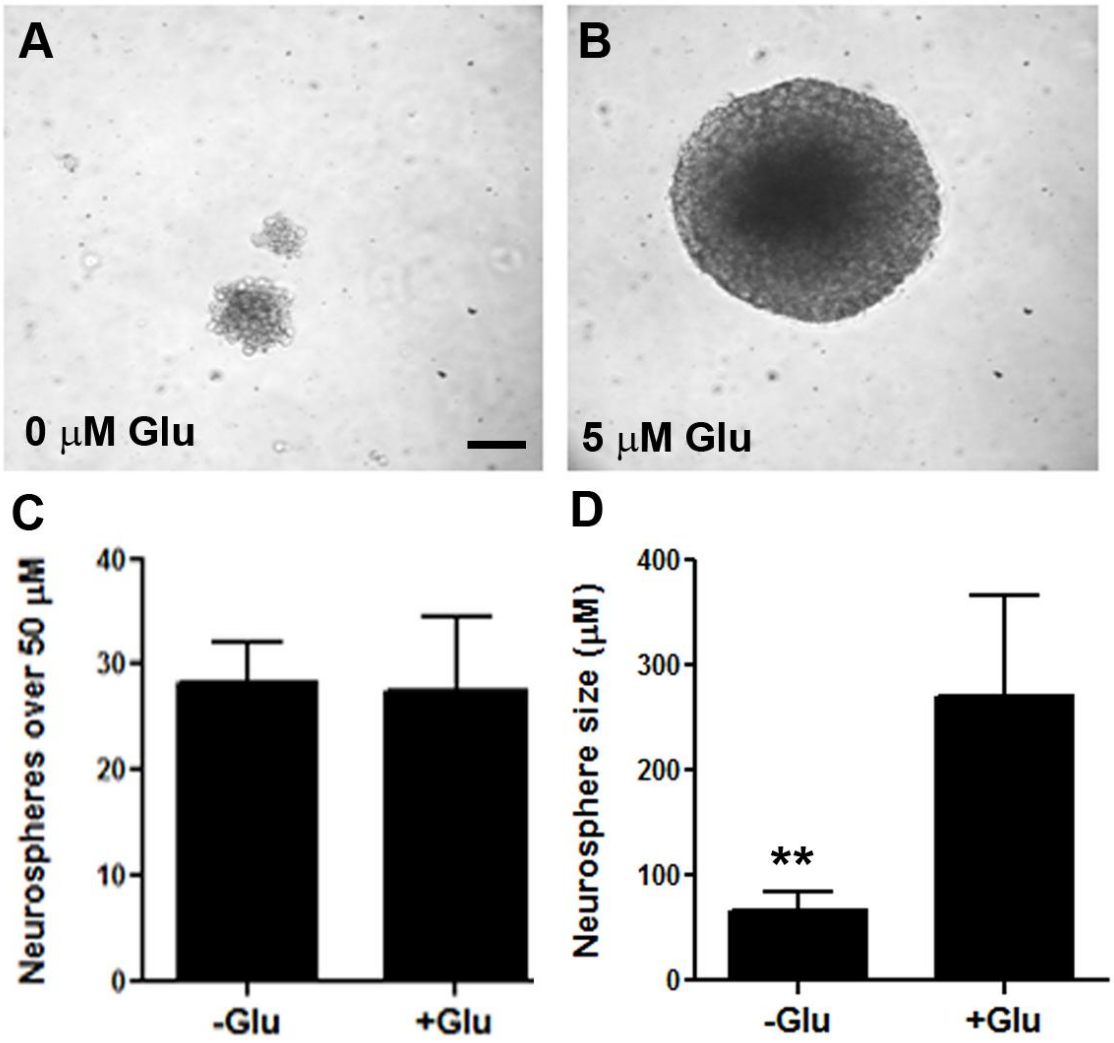

Figure 3-4: Glutamate enhances dentate gyrus-derived neurosphere proliferation. Wild type P7 neurospheres were dissociated and plated in either the presence of absence of exogenous glutamate and were quantified after seven days. Neurospheres grown in the absence and presence of glutamate are shown in (A) and (B). Cells stimulated with glutamate formed larger neurospheres (C), however the overall number of neurospheres was unchanged (D). The scale bar in (A) is 50 μm and error bars in (C) represent standard deviation. Statistical significance was calculated using One Way ANOVA and Bonferonni post hoc analysis (P<0.0001).

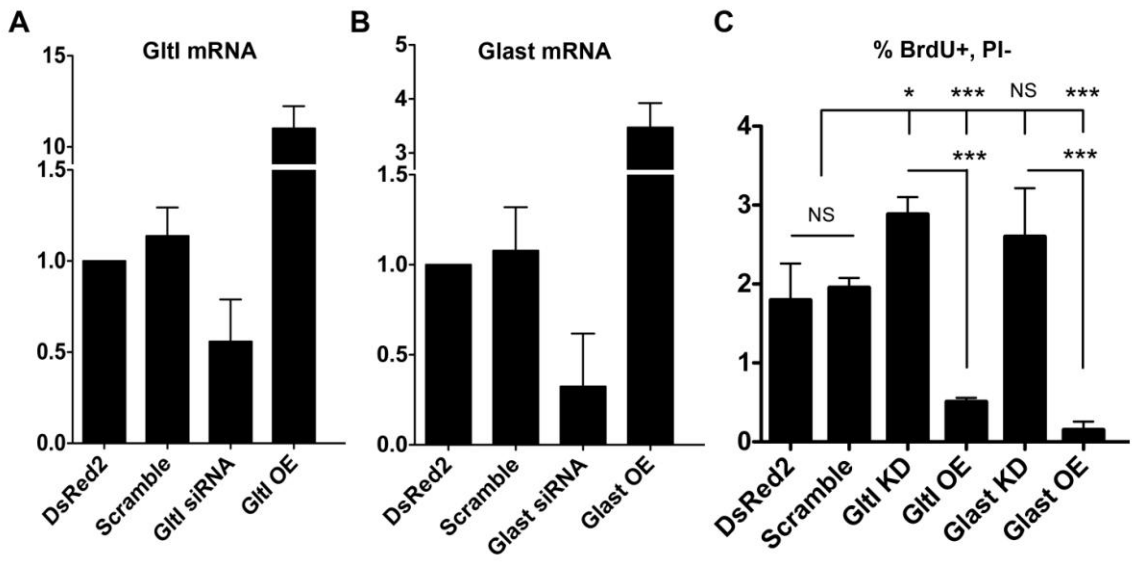

Figure 3-5: Knockdown of glutamate transporters results in increased BrdU incorporation. Neurospheres transfected with DNA constructs or siRNA were assayed for GltI and Glast expression levels after seven days in culture (A-B). Relative mRNA levels of GltI (A) and Glast (B) mRNA are normalized to their respective expression levels in samples transfected with the DsRed2 plasmid. Neurospheres exhibit consistent knockdown and overexpression of GltI (A) and Glast (B). Seven days after transfection neurospheres were pulsed with BrdU and assayed for proliferation using flow cytometry. (C) In the presence of GltI or Glast siRNA the percentage of BrdU-positive cells increases while their overexpression results in decreased percentages of proliferative cells. This suggests that glutamate transporters function to regulate progenitor proliferation in vitro. Statistical analysis in (C) utilized a One Way ANOVA followed by a Bonferonni *post hoc* analysis (*P<0.05; ***P<0.0001). OE, overexpression; KD, knockdown; PI, propidium iodide; NS, not significant.

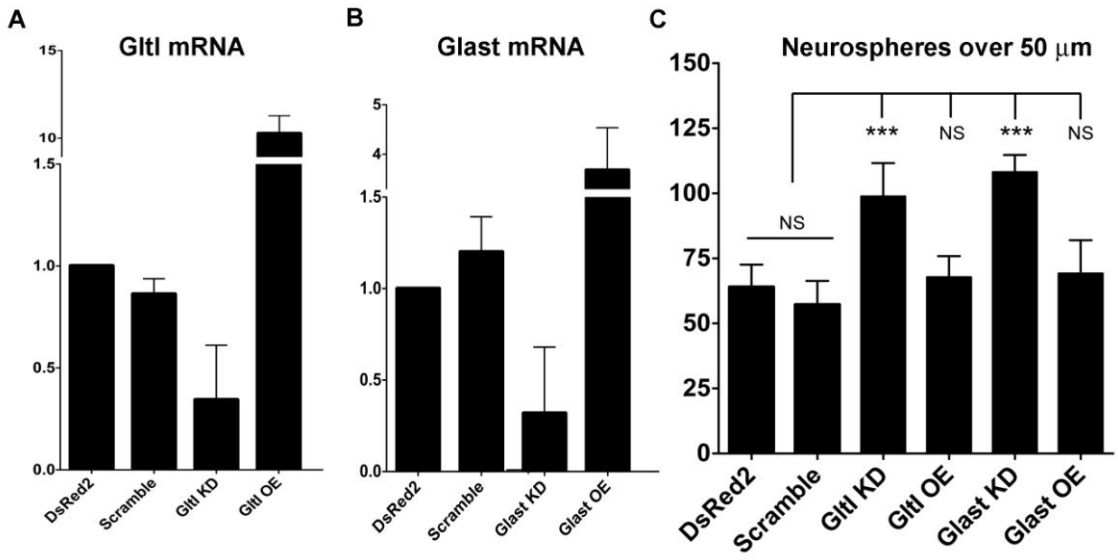

Figure 3-6: Knockdown of GltI or Glast increases neurosphere proliferation. Seven days after P7 neurospheres were transfected, they were assayed for misexpression using Q-PCR methods. In (A) and (B) GltI and Glast mRNA is knocked down and overexpressed under the appropriate transfection conditions. After misexpression was confirmed, neurospheres were dissociated and plated in semisolid media and allowed to form neurospheres for seven days in culture. Quantification of neurospheres over 50 μm is shown in (C). Cells transfected with either GltI or Glast siRNA formed more neurospheres than cells transfected with control plasmid (DsRed2) or scrambled siRNA. Overexpression of GltI and Glast had little effect on neurosphere proliferation. This suggests that glutamate transporters negatively regulate neurosphere proliferation. Statistical analysis in (C) utilized a One Way ANOVA followed by a Bonferonni *post hoc* analysis (***P<0.0001). OE, overexpression; KD, knockdown; NS, not significant.

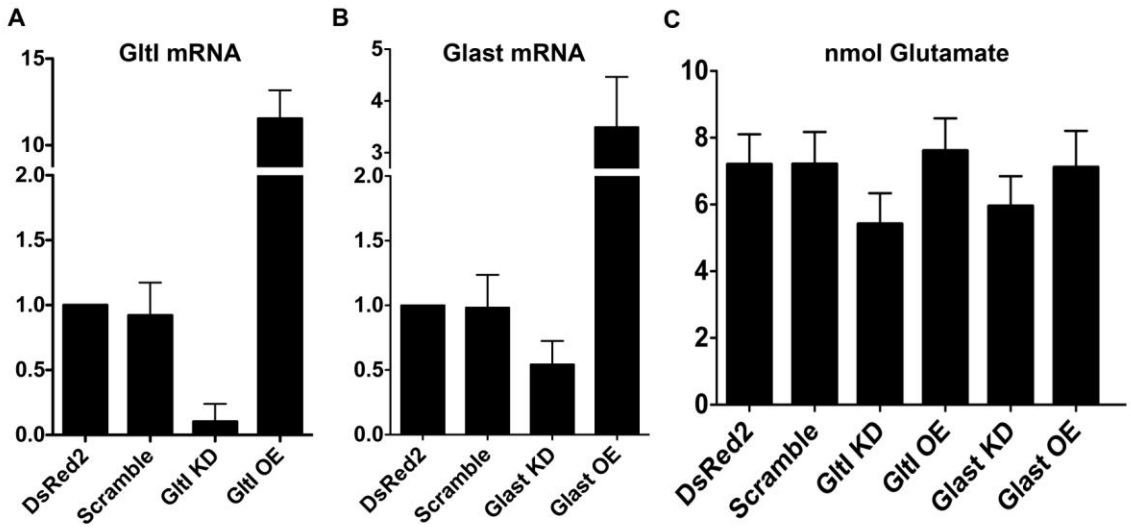

Figure 3-7: GltI and Glast knockdown results no change in intracellular glutamate levels. Neurospheres were assayed for knockdown or overexpression after being in culture for seven days (A-B). GltI (A) and Glast (B) mRNA is knocked down and overexpressed appropriately. After misexpression was confirmed, neurospheres were dissociated and assayed for intracellular glutamate concentrations. The decrease in intracellular glutamate concentrations observed under siRNA conditions was not significant. This suggests that GltI and Glast are responsible for trafficking glutamate into neural progenitor cells. Statistical analysis in (C) was performed using a One Way ANOVA and Bonferonni *post hoc* analysis. KD, knockdown; OE, overexpression.

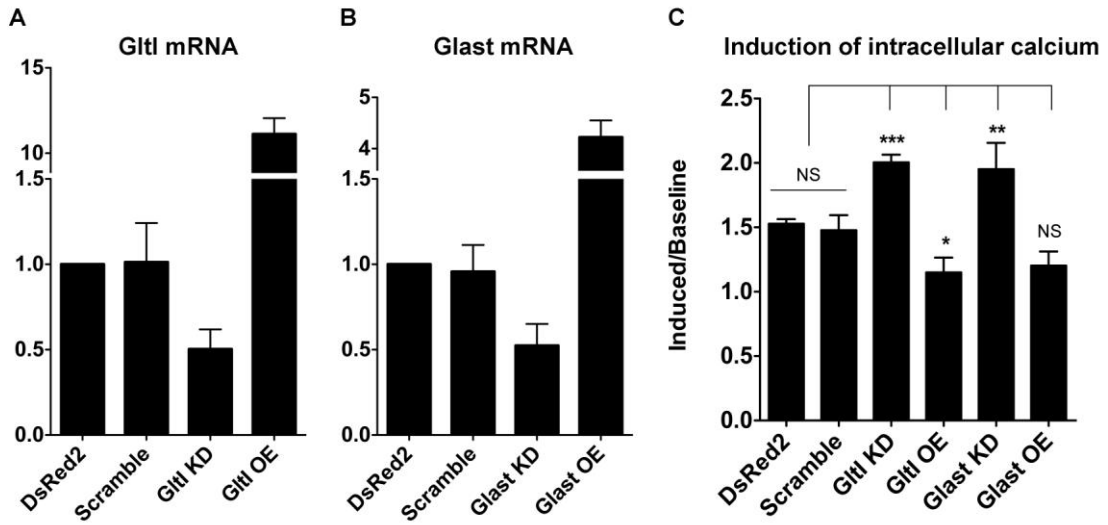

Figure 3-8: Glutamate transporters indirectly regulate intracellular calcium stores. Seven days after neurospheres were transfected, misexpression of GltI (A) and Glast (B) were confirmed using Q-PCR. Using a Fluo-4 AM calcium indicator, we were able to analyze the effect of glutamate transporter misexpression on intracellular calcium levels (C). Knockdown of GltI and Glast resulted in increased percentages of cells expressing Fluo-4 while their overexpression led to a decrease in the percentage of cells with the calcium marker. This suggests that GltI and Glast regulate the availability of glutamate to glutamate receptors. This in turn controls intracellular calcium levels which contribute to progenitor proliferation. Statistical analysis in (C) utilized a One Way ANOVA and Bonferonni *post hoc* analysis (**P*<0.05; ***P*<0.001; ****P*<0.0001). KD, knockdown; OE, overexpression.

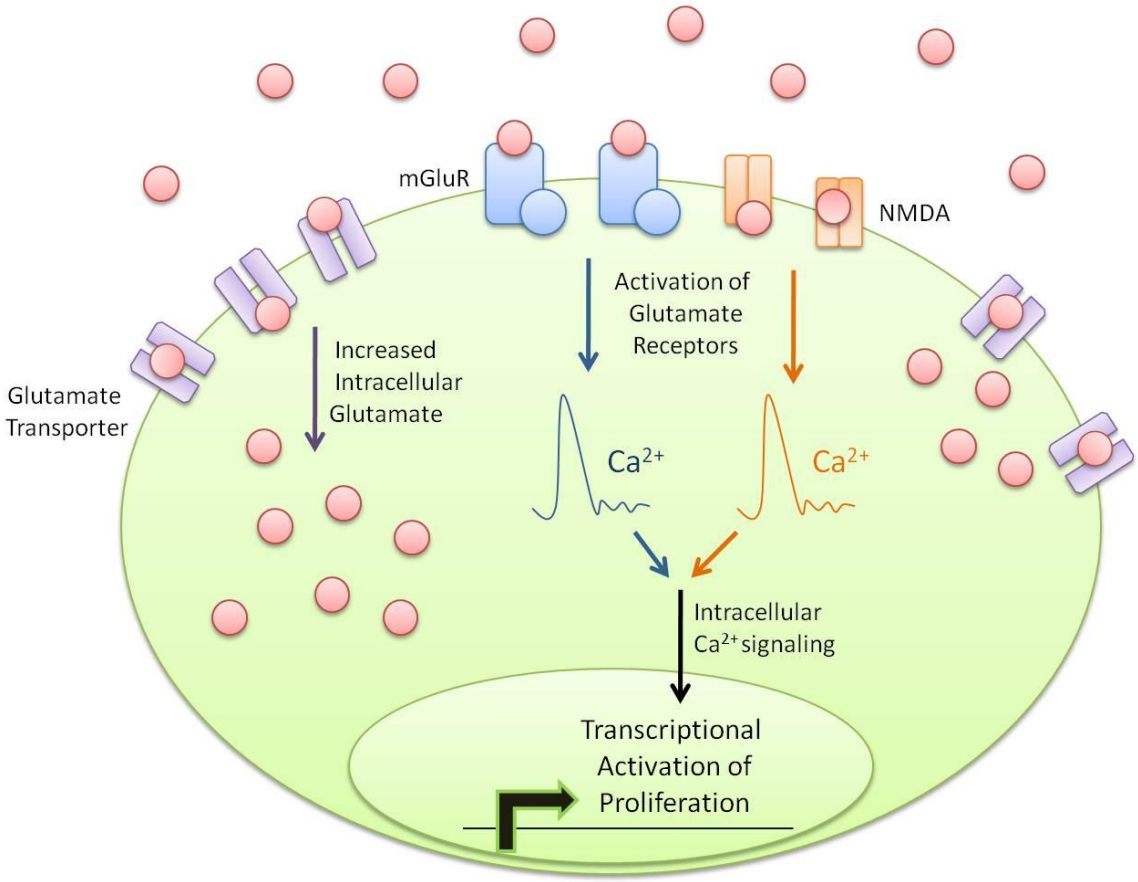

Figure 3-9: Proposed mechanism of GltI and Glast's role in regulating proliferation. Glutamate's stimulation of NMDA and mGluR receptors modulates fluxes in intracellular calcium which can lead to cellular proliferation. Glutamate transporters regulate the availability of glutamate to these receptors by trafficking extracellular glutamate into the cell. Expression of GltI and Glast therefore indirectly regulate neural progenitor proliferation.

Materials and Methods

Quantitative PCR

Using the Trizol method, RNA was extracted from either FAC-sorted cells or neurospheres before being reverse transcribed into cDNA with the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen). Afterwards, relative mRNA levels were determined using SYBR Green master mix, cDNA and proper primers. GAPDH was used as an internal control (Gilley et al., 2009).

Primers include: GAPDH forward: 5'-CTC AAC TAC ATG GTC TAC ATG TTC CA-3'; GAPDH reverse: 5'-CCA TTC TCG GCC TTG ACT GT-3'; GltI forward: 5'-GGA AGA TGG GTG AAC AGG C-3'; GltI reverse: 5'-TTC CCA CAA ATC AAG CAG G-3'; Glast forward: 5'-ACG GTC ACT GCT GTC ATT G-3'; Glast reverse: 5'-TGT GAC GAG ACT GGA GAT GA-3'.

Western Blots

Bradford assays were used to measure protein lysates concentrations and all samples consisted of 20 μg per well of total protein. After the gel had been run, proteins were transferred to a PVDF membrane for three hours at 83 volts. Membranes were then blocked in 5% non-fat dry milk for one hour and then incubated with primary and secondary antibodies for two hours at room temperature. All wash steps used 1% Tween in PBS and a Lumi-Light Western Blotting Substrate Kit (Roche) was utilized to visualize protein bands.

Analysis of GFP and PDGFRα expression was performed on dentate gyrus lysates from P14 nestin-eGFP mice and were run on 12% and 10% polyacrylamide gels, respectively. Antibodies used include rat anti-PDGFRα 1:250 (Pharmingen), rabbit anti-GFP 1:1000 (Molecular Probes) and rabbit anti-β-tubulin 1:1000 (Sigma). Secondary antibodies (Santa Cruz) used a dilution of 1:5000.

To examine GltI protein expression, whole cell lysates were isolated from the dentate gyrus of P7 and P28 transgenics and from P14 cortex, hippocampus, dentate gyrus and hippocampus without dentate gyrus. Equal amounts of protein were loaded on a 10% polyacrylamide gel, transferred and blotted with guinea pig anti-GltI 1:5000 (Chemicon) and rabbit anti-β-tubulin 1:1000 (Sigma). Horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) were used 1:5000.

Neurosphere Assays

For the glutamate neurosphere assay, all media (activated papain, neural stem cell media and semisolid media) was prepared using Neurobasal A media which lacks glutamate and aspartate. Neurospheres derived from P7 dentate gyrus were incubated with accutase for 5 minutes at 37 degrees, mechanically dissociated into a single-cell solution, mixed with semisolid media and supplemented with 0 μM or 5 μM glutamate. After seven days in culture, neurospheres over 50 μm were quantified and the diameters of twenty neurospheres from both groups were measured.

The misexpression neurosphere assays were also performed in the absence of glutamate-containing media on neurospheres that had been transfected with various combinations of GltI and Glast siRNA and overexpression plasmids (see Transient Transfections). Briefly, neurospheres derived from P7 dentate gyrus were dissociated with accutase and triturated into single cells and transiently transfected using Lipofectamine (Invitrogen) and were allowed to grow in culture for seven days in neural stem cell media. These transfected neurospheres were then dissociated again, counted and plated in semisolid media in the presence or absence of 5 μM glutamate. After seven more days in culture, neurospheres were quantified and their diameter was measured.

Immunostaining

Sections from P7 and P28 transgenic animals were stained with chicken anti-GFP 1:500 (Aves Laboratories), mouse anti-GFAP 1:100 (BD Pharmingen) or goat anti-Dcx 1:200 (Santa Cruz) and guinea pig anti-GltI 1:200 (Chemicon) or guinea pig anti-Glast 1:200.

Transient Transfections

Neurospheres derived from P7 dentate gyrus were dissociated into a single-cell solution and transfected with either siRNA compounds or overexpression plasmids driven by the CMV promoter. For knockdown experiments cell were transfected using the Lipofectamine RNAi Max (Invitrogen) protocol for reverse transfections. Briefly, 10 nM (12 pmol) GltI siRNA, 10 nM (12 pmol) Glast siRNA or a combination of both GltI and Glast siRNA were used. Lipofectamine 2000

was used to transiently transfect 300 ng pCMV-GltI-DsRed2 (final concentration: 150 ng/mL), 300 ng pCMV6-Glast-Myc (final concentration: 150 ng/mL) or a combination of both overexpression plasmids. 10 nM scrambled siRNA (Invitrogen) and 300 ng of pCMV-DsRed2 (final concentration: 150 ng/ml) were transfected using Lipofectamine RNAi Max and Lipofectamine 2000, respectively and served as negative controls. After seven days in culture, some neurospheres from each transfection were used to confirm knockdown or overexpression via RNA extraction and cDNA synthesis followed by Q-PCR analysis. Only transfections with confirmed knockdown or overexpression after seven days in culture were used for further analysis.

BrdU incorporation

Using methods adapted from Yang et al (in preparation), transiently transfected neurospheres were pulsed with 10 μM BrdU for 15 minutes, dissociated with activated papain and triturated into a single-cell solution before fixation in 100% ethanol. The DNA was then denatured with 2N HCl/0.5% TritonX in PBS for 30 minutes at room temperature and afterwards the reaction was neutralized with 0.1 M NaB_4O_7. Samples were then incubated in staining solution (1.3 μl BrdU-APC, 5 μl RNase and 50 μl 0.5% Tween/1% BSA in PBS) overnight at 4 degrees. Finally, cells were washed and resuspended in PI-containing PBS and analyzed for BrdU incorporation. Only Brdu-positive and PI-negative cells were quantified. Statistical analysis was determined using a One Way ANOVA and Bon Feronni *post hoc* analysis. The experiment was repeated at least four times.

Intracellular Glutamate Measurement

After misexpression was confirmed, neurospheres were dissociated and one million cells were collected and homogenized according the protocols within the Glutamate Assay Kit (BioVision). According to the protocol, glutamate standards were prepared and their absorbance was determined and plotted on a standard curve. After cells were homogenized they were mixed with Assay Buffer, Glutamate Developer and Glutamate Enzyme Mix and incubated for 30 minutes at 37 degrees. Afterwards, intracellular glutamate was measured via optical density at 450 nm. Glutamate concentrations were then extrapolated from the standard curve with their corresponding absorbance. Statistical analysis was calculated by One Way ANOVA followed by *post hoc* analysis with Bon Feronni.

Intracellular Calcium Measurement

Seven days after transfection misexpression was confirmed using Q-PCR methods. Neurospheres were then dissociated and stained with Fluo4-AM calcium indicator according to the manufacture's protocol (Invitrogen). Afterwards, cells were analyzed using flow cytometry. Baseline intracellular calcium measurements were recorded for thirty seconds before cells were spiked with 5 μM glutamate and then readings were recorded for an additional minute and a half. The percentage of cells containing increased intracellular calcium levels (compared to baseline) is presented. The final percentage of cells expressing Fluo-4 was normalized to the percentage of positive cells at baseline

levels (% induced cells/% baseline cells). Calcium levels were documented in at least quadruplicate for each condition. Significance was calculated using a One Way ANOVA followed by Bon Feronni *post hoc* analysis.

CHAPTER FOUR

In vivo relevance with regard to injury

Introduction

Neural progenitor cells have recently been implicated in repair mechanisms as a result of various brain injuries. Different experimental models including stroke, HI, TBI and seizures have suggested that neural precursors are required for remodeling of the brain after such injuries (Parent et al., 1997, Dash et al., 2001, Kernie et al., 2001, Rola et al., 2006, Miles and Kernie, 2008, Yu et al., 2008). These studies suggest that hippocampal progenitors are activated in response to brain injury in order to produce new neurons within the granular layer of the dentate gyrus. More focused experiments have demonstrated that the early (type I and II) nestin-expressing progenitors proliferate after injury while Dcx-positive neuroblasts remain susceptible to injury (Miles and Kernie, 2008, Yu et al., 2008). Although injury-induced neurogenesis is thought to contribute to functional recovery after injury (Eriksson et al., 1998, Chen et al., 2003, Richardson et al., 2007, Yu et al., 2008), the mechanisms underlying this process are not well understood.

The Rice-Vanucci model of unilateral HI is a well-characterized model of brain injury. In this injury model the right carotid artery is ligated and the mice are allowed to recover before they are exposed to 8% oxygen for a period of time. Neither surgical ligation nor brief exposure to hypoxia alone causes significant neural damage. However, a combination of the two results in neuronal injury and ischemia to the hippocampus, striatum, cortex and thalamus

on the injured side of the brain (Rice et al., 1981, Vannucci, 1990). Although this injury has been well-characterized, various mouse strains are differentially susceptible to HI injury. Therefore we previously backcrossed our nestin-GFP transgenic into CD1 mice, an outbred strain which is thought to be more consistently injured (Sheldon et al., 1998).

Although several studies have analyzed the effect of hypoxia and TBI on the expression of astrocytic and neuronal glutamate transporters (Torp et al., 1995, Martin et al., 1997, Rao et al., 1998, Rao et al., 2001a, Rao et al., 2001b, Yi and Hazell, 2006), none have specifically looked at their expression or function in neural progenitors after injury. Previous studies in our lab have characterized GFP-expressing progenitors after HI injury and found that type I and II progenitors are activated after injury and this induced proliferation peaks three days afterwards (Miles and Kernie, 2008, Yu et al., 2008). We have also shown that type I cells continue to proliferate 45 days after injury in order to replace dying neuroblasts (Dcx-expressing type III cells). Since we have previously shown that GltI and Glast are expressed on type I progenitors, this led us to hypothesize that glutamate transporters may be expressed on activated progenitors after injury. In addition, we believe that GltI and Glast may be involved in injury-induced proliferation because of their role in regulating neurosphere propagation in vitro (see Chapter 3).

It is now well known that exposing cells or tissue to hypoxia or ischemia prior to brain injury can be neuroprotective. Several experimental models have been used to show that astrocytic upregulation of glutamate transporters in

response to hypoxic or ischemic preconditioning is neuroprotective (Cimarosti et al., 2005, Rothstein et al., 2005, Chu et al., 2007, Romera et al., 2007, Weller et al., 2008, Bigdeli et al., 2009). However, none of these studies investigated the effect of hypoxic preconditioning on subsequent injury-induced neurogenesis. In addition all of these studies have only focused on altered glutamate transporter expression in mature astrocytes in various regions of the brain. Therefore no data exist demonstrating the effects of hypoxic preconditioning on glutamate transporter function in neural progenitor cells as it relates to injury-induced neurogenesis. Based on our previous results, we therefore addressed the in vivo role of glutamate transporters in the activation of neural progenitor cells after injury.

Results

GltI and Glast are continually expressed on activated type I cells after injury

To determine the range of expression of glutamate transporters after injury, we profiled GFP-expressing progenitors one, three, seven and 45 days after injury. Beginning 24 hours after injury, both GltI and Glast are upregulated on the side ipsilateral to the injury while the contralateral side maintains baseline expression of each transporter. Almost every activated GFP-positive cell also expressed GFAP and GltI and Glast suggesting that glutamate transporters are upregulated on type I cells immediately after HI injury. We also show that GltI and Glast remain upregulated on the ipsilateral side 45 days after injury.

GltI and Glast upregulation caused by induced injury leads to decreased proliferation of early progenitors

To determine if glutamate transporters might be involved in injury-induced neurogenesis, we used a combination of the HI and TBI animal models. In animals exposed to sham HI injuries (carotid ligation with no hypoxia), there was no observed differences in the percentage of GFP- and BrdU-labeled cells. In these animals the contralateral (uninjured) and ipsilateral (injured) sides of the brain we observed an average of 14.09% and 13.29% double-labeled cells, respectively. However we did observe a significant difference among colocalized cells in the contralateral (15.05%) and ipsilateral (7.83%) side in animals exposed to both HI and TBI. These results suggest that concurrent acquired brain injuries results in decreased capacity for injury-induced neurogenesis. However, we must first confirm that GltI and Glast are actually upregulated on these progenitor cells after injury.

GltI and Glast are upregulated after HI and in combination with TBI

To ensure that preconditioning with HI resulted in increased glutamate transporter expression, we immunostained Sham+TBI and HI+TBI sections for GltI and Glast expression. In both groups of animals, GltI and Glast were upregulated on the ipsilateral side (Figure 4-4, C-D, G-H, K-L, O-P), due to the presence of at least one injury. However, the contralateral sides in both groups only exhibited background or low levels of glutamate transporter expression

(Figure 4-4, A-B, E-F, I-J, M-N). This data confirms that GltI and Glast are upregulated after TBI which is independent of HI injury (Figure 4-4, ipsilateral panel). Moreover, these results demonstrate that upregulation of GltI and Glast via HI injury leads to decreased proliferation of early progenitors after secondary injury with TBI.

Discussion

Previous studies in our lab and others have shown that BrdU-expressing neural progenitors within the SGZ are activated after HI and TBI injury (Kernie et al., 2001, Miles and Kernie, 2008, Yu et al., 2008). Results from the current study suggest that glutamate transporters are upregulated on activated type I cells after HI and TBI (Figure 4-1,2,4). Therefore, according to our model (Figure 3-9), increased expression of glutamate transporters due to HI may sequester extracellular glutamate in order to prevent activation of glutamate receptors. This finding is in accordance with studies relevant to mGluR-mediated proliferation of neural progenitor cells.

In our Sham + TBI experiment, it might therefore be expected to see an increased percentage of GFP and BrdU colocalized on the injured side of the brain (Figure 4-3, E). Although there appears to be an increase in both GFP-positive and BrdU-expressing cells on the ipsilateral side, our results show no significant difference between the two sides. This suggests that although GFP and BrdU cell numbers might increase, the ratio of double-labeled cells to total number of BrdU-expressing cells is equivalent to the ratio observed on the

contralateral side. As a result the ipsilateral and contralateral sides of the brain show similar percentages of proliferating progenitors. However, more stringent quantifications of cell number are required to confirm this.

Other studies have induced expression of glutamate transporters and investigated its effect on neuroprotection. Bigdeli et al showed that preconditioning with normobaric hyperoxia or temporary middle cerebral artery occlusion resulted in increased levels of glutamate transporters in astrocytes and demonstrated marked brain protection (Bigdeli et al., 2009). Furthermore, Cimarosti et al exposed newborn rats to 8% oxygen for three hours one day prior to unilateral hypoxic-ischemic injury and found an upregulation of GltI and decreased neuronal damage compared to control animals (Cimarosti et al., 2005). Similar results were observed in vitro when rat cortical cultures were preconditioned via glucose-oxygen deprivation. This same study also found that injection of a peroxisome proliferator-activated receptor gamma (PPARγ) agonist immediately after middle cerebral artery occlusion also resulted in elevated GltI levels and neuroprotection suggesting that GltI is a target of PPARγ signaling (Romera et al., 2007). Moreover, induction of GltI expression by various methods including β-Lactam antibiotics, transgenic mice and ceftriaxone injections has also shown to be neuroprotective in vivo (Rothstein et al., 2005, Chu et al., 2007, Weller et al., 2008). Similar to these studies, the current research demonstrated that GltI and Glast are upregulated in response to injury and might therefore also be involved in hypoxic preconditioning and neuroprotection. These results taken together suggest that GltI and Glast

function similarly in astrocytes and neural progenitors to regulate extracellular glutamate concentrations after injury. However, our data suggest a potential mechanism for the attenuation of injury-induced neurogenesis upon subsequent injuries.

There is increasing clinical evidence of cognitive dysfunction in patients who are exposed to multiple brain injuries. These findings are also apparent in experimental models of recurring brain injury. Several studies have shown that multiple insults to the brain, particularly repeated TBI, can lead to cumulative damage to hippocampal cells as indicated by increased expression of CNS damage markers including S-100 protein beta (S-100β) and neuron-specific enolase (NSE) (Slemmer et al., 2002, Willoughby et al., 2004, Engel et al., 2005, Slemmer and Weber, 2005). Furthermore, the ability to recover from an insult declines with the frequency and severity of the brain trauma (Dacey et al., 1991, Salcido et al., 1991, Spettell et al., 1991, Salcido and Costich, 1992, Finset et al., 1999, Jeremitsky et al., 2003, Slemmer and Weber, 2005). It may therefore be that decreased proliferation of progenitor cells in mice exposed to HI and TBI (Figure 4-3) may provide insight into a mechanism explaining the inability to recover from multiple brain injuries. However, more focused studies of progenitor proliferation after multiple brain insults are required to elucidate the exact mechanisms involved and whether injury-induced expression of glutamate transporters underlies this observation.

In summary, we present evidence that GltI and Glast are permanently upregulated on type I cells in response to HI injury. In addition we show that

hypoxic preconditioning prior to TBI decreases injury-induced neurogenesis. Taken together, these results suggest a novel functional role for glutamate transporters in injury-induced proliferation on hippocampal progenitor cells. Furthermore, these experiments may explain why multiple brain traumas incur impaired functional recovery.

Figures

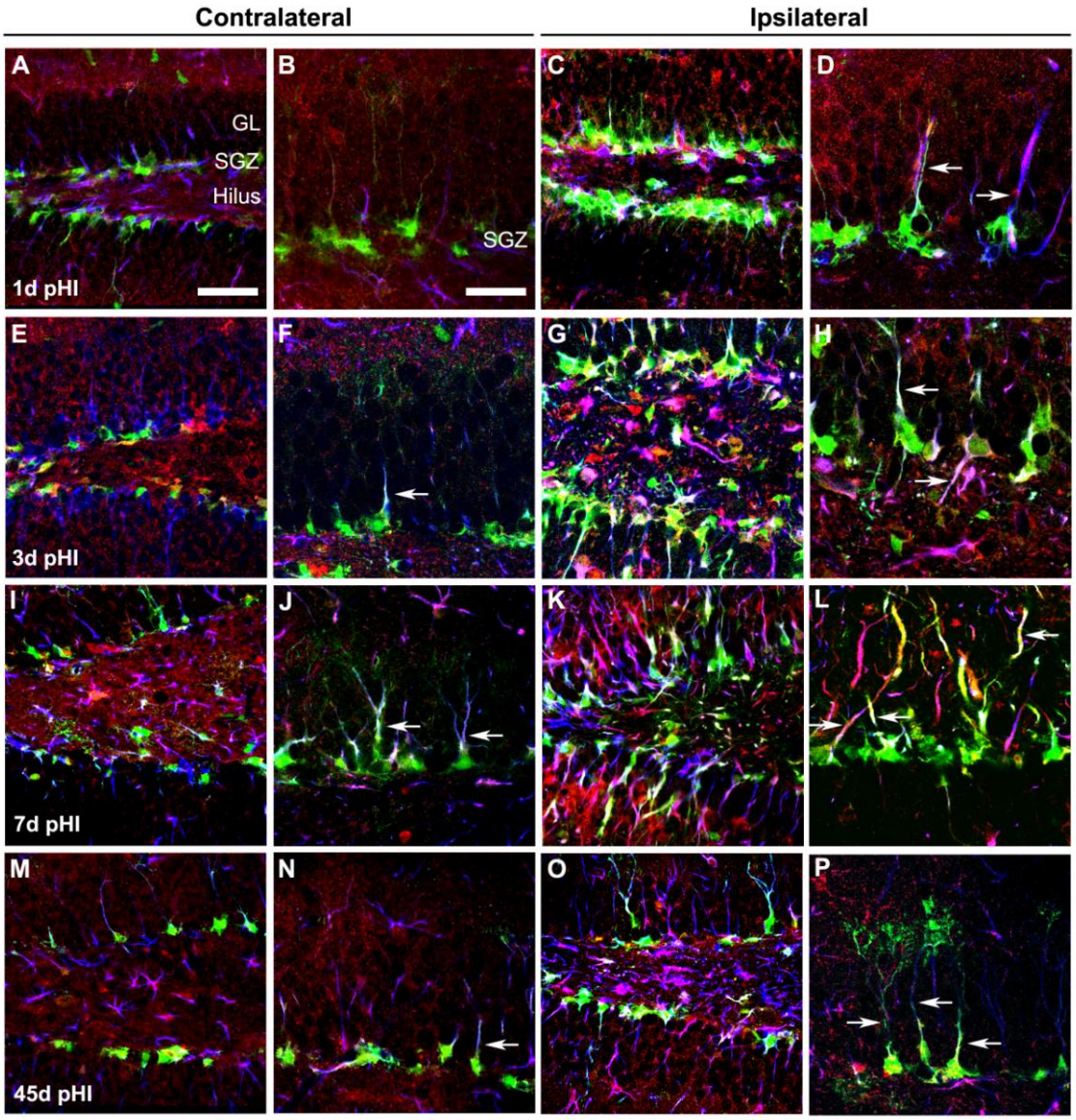

Figure 4-1: GltI is permanently upregulated after hypoxic-ischemic injury. After HI injury mice were sacrificed one day (1d) (A-D), 3d (E-H), 7d (I-L) and 45d (M-P) post-HI (pHI) and immunostained for GFP (green), GltI (red) and GFAP (blue). The contralateral (uninjured) side of the brain is shown in the left panels (A-B, E-F, I-J and M-N) while the ipsilateral (injured) side are in the right panels (C-D, G-H, K-L, O-P). Magnified pictures on the ipsilateral side show activation of GltI-expressing type I cells (arrows) 1d (D), 3d (H), 7d (L) and 45d (P) after injury compared to the contralateral side (B, F, J, N). The scale bar in (A) is 50 μm and the one in (B) is 35 μm. GL, granular layer; SGZ, subgranular zone; GL, granular layer; pHI, post hypoxic-ischemia.

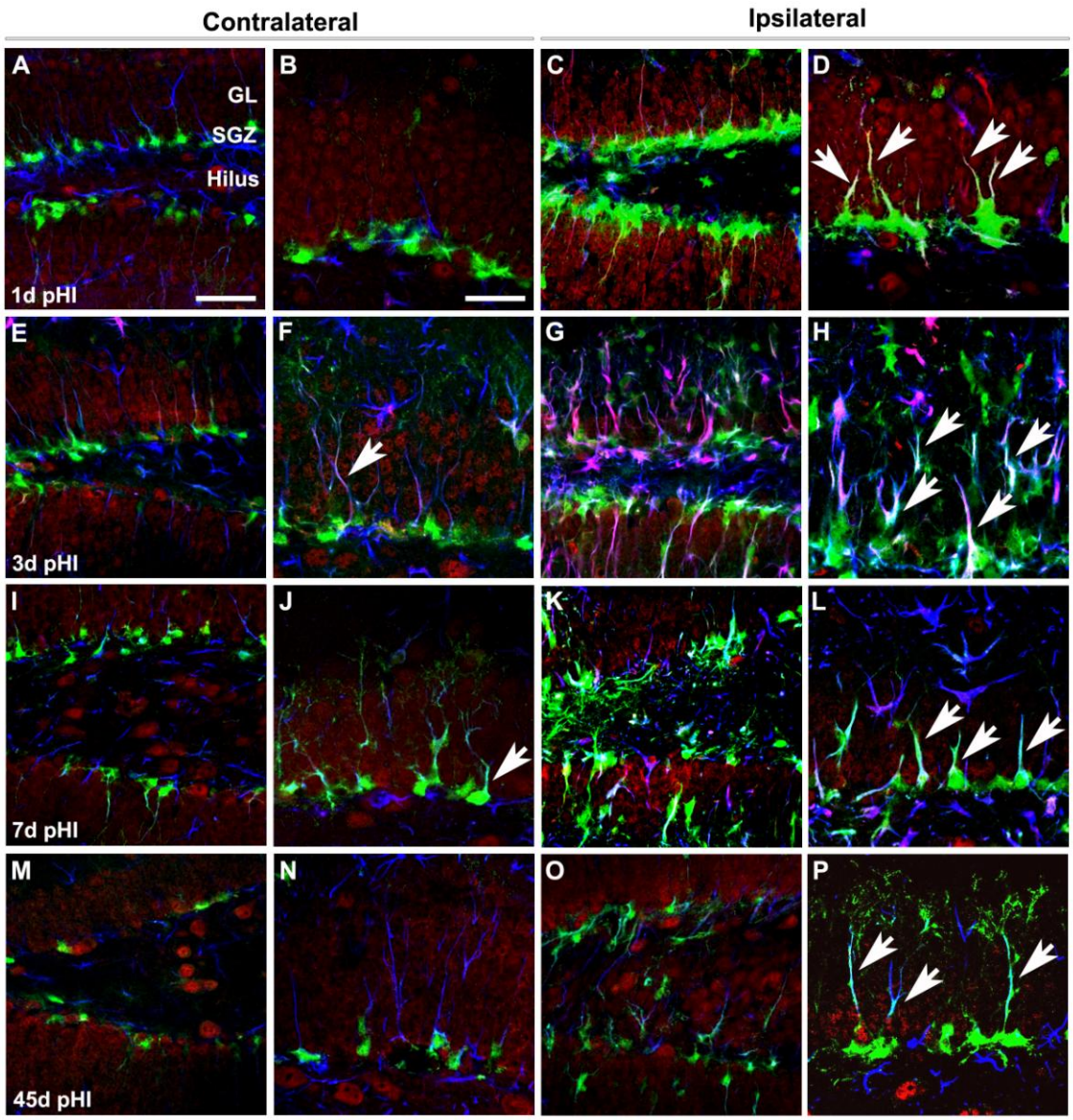

Figure 4-2: **Glast is permanently upregulated after HI injury**. After HI injury transgenic mice were sacrificed one day (1d) (A-D), 3d (E-H), 7d (I-L) and 45d (M-P) and stained for GFP (green), Glast (red) and GFAP (blue). Similar to GltI, Glast is upregulated on the ipsilateral side of the brain at least 45d pHI (D, H, L and P) compared to the contralateral side (B, F, J and N). The scale bar in (A) is 50 μm and the one in (B) is 35 μm. pHI, post hypoxic-ischemia.

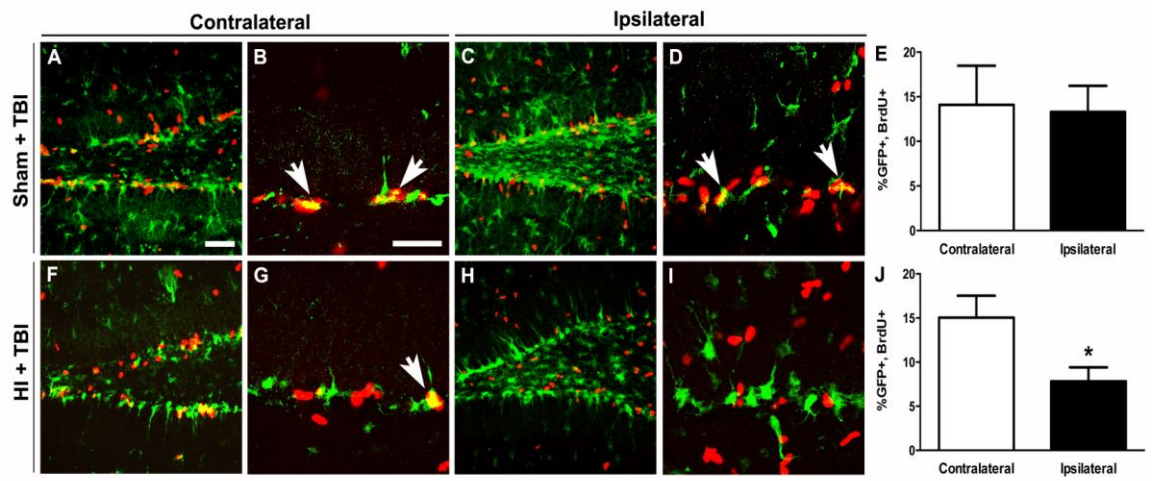

Figure 4-3: **Dentate gyrus progenitors in mice exposed to HI and TBI are more proliferative than those in mice exposed to TBI alone**. P28 Transgenic mice were exposed to either HI followed by TBI (32d pHI) (F-J) or sham HI followed by TBI (A-E). GFP (green) and BrdU (red) double-labeled cells on the contralateral and ipsilateral side of the brain were quantified and are shown in (E) and (J). In the Sham + TBI animals, there was no difference in the percentage of proliferative progenitors (E) between the contralateral (A-B) and ipsilateral side (C-D). Animals exposed to HI + TBI show decreased proliferation on the injured side (H-I) compared to the uninjured side (F-G) of the brain (J). The scale bar in (A) is 1 mm and the scale bar in (B) is 35 μm. Statistical significance was calculated using a One Way ANOVA and Bonferonni *post hoc* analysis (*P<0.05). HI, hypoxic-ischemia; TBI, traumatic brain injury.

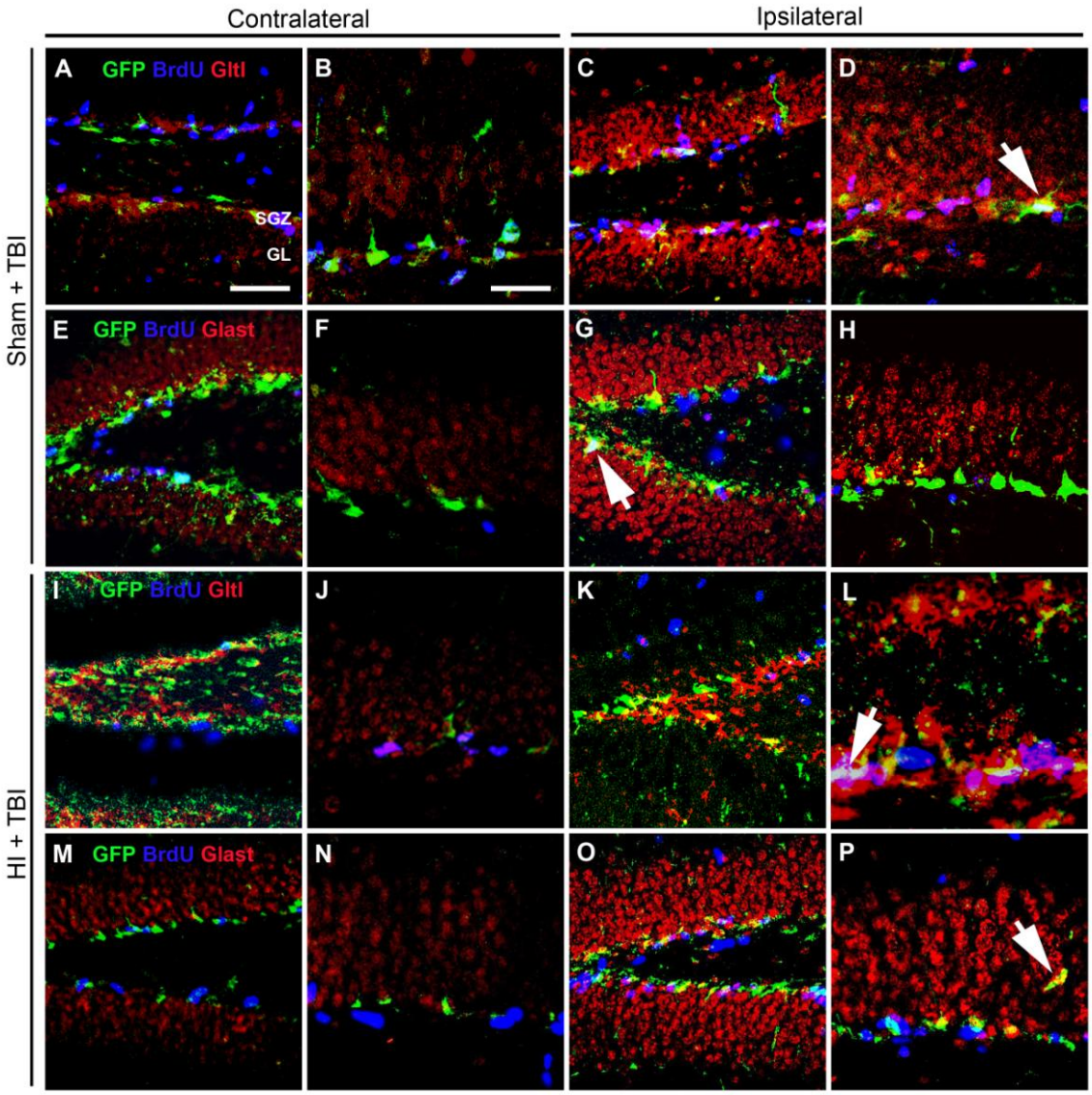

Figure 4-4: GltI and Glast are upregulated after TBI independent of HI injury. Sham+TBI and HI+TBI sections were immunostained with GFP (green), BrdU (blue) and either GltI or Glast (red) to confirm glutamate transporter expression on activated progenitors after injury. The left panel (contralateral) serves at the uninjured control while the right panel (ipsilateral) shows the injured side of the brain. (C-D) and (G-H) respectively demonstrate that GltI and Glast are upregulated on the injured side of Sham+TBI animals when compared to the uninjured controls (A-B) and (E-F) (arrows). In animals exposed to HI and TBI, both glutamate transporters are highly expressed on GFP-positive progenitors within the ipsilateral side of the brain (K-L) and (O-P) (arrows). These results indicate that GltI and Glast are upregulated on the injured side of the brain in Sham+TBI and HI+TBI animals. Scale bar in (A) is 50 μm and 35 μm in (B). SGZ, subgranular zone; GL, granular layer.

Materials and Methods

Mice

Nestin transgenics were divided into two groups: sham HI with TBI (Sham+TBI) and HI with TBI (HI+TBI). At p28 both groups of animals were anesthetized, had the right carotid artery ligated and were allowed to recover for 30 minutes to one hour (see below). Mice in the HI+TBI group were then placed in a chamber and exposed to 8% oxygen for one hour prior to recovery at room air. Sham+TBI animals were not treated with hypoxia. 32 days after ligation (P60) animals from both groups were injured using control cortical impact (CCI) to induce TBI. Animals were then intraperitonealy (IP) injected with a single daily dose of 100 mg/kg BrdU from P64-P67. P67 animals were sacrificed and perfused two hours after BrdU injection.

Hypoxic- Ischemic Injury

The Rice-Vanucci animal model was used on P28 transgenic mice to emulate unilateral HI brain injury. Mice were initially anesthetized in a clear chamber containing 4% isoflurane for one minute. Mice were then placed supine and maintained in a surgical plane of anesthesia with 2% isoflurane mixed with balanced 30% oxygen and 70% nitrogen. A small animal anesthesia machine was used to monitor the flow rate of 1 L/min through a nose cone adaptor (Summitt Medical Equipment, Bend, OR). Betadine solution was then swabbed onto the ventral area of the neck before a 1 cm incision was made at the midline. After glandular tissue was retracted, the right carotid artery was located and

isolated before being ligated with a one inch suture. Sham injured animals had the artery exposed but not ligated. The incision was closed with surgical clips and swabbed with antibiotic ointment. Animals which fully recovered righting reflex and spontaneous motor activity were used for further experimentation. The small percentage of mice that did not recover normally were excluded and sacrificed. Mice were then allowed to recover for one hour before being placed in a Plexiglas chamber (24x16x8 inches) mounted with an oxygen sensor (Teledyne Technologies, Thousand Oaks, CA) and equipped with a heating pad set to 37 degrees Celsius. To induce injury a premixed gas of 8% oxygen and 92% nitrogen was introduced into the chamber at a rate of 1 L/min for one hour. Afterwards mice were returned to their cage and allowed to recover.

Traumatic Brain Injury

To induce TBI a CCI devise was used as described elsewhere (Kernie et al., 2001). P60 transgenic mice previously exposed to HI or sham injuries 32 days earlier were anesthetized with isoflurane (see above) before being placed in a stereotactic frame. After a midline incision was made, a 5 mm by 5 mm craniectomy was performed between bregma and λ and 1 mm lateral to the midline. Using a constant speed of 4.4 m/s, a 3 mm stainless steel tipped impact device with a 0.77 mm deformation was used to generate the injury. The incision was then closed with staples and the mice were allowed to recover. Beginning four days after injury (P64), mice were injected daily with BrdU (100 mg/kg) for

four days. Mice were then sacrificed two hours after BrdU injection on the fourth day (P67).

Immunostaining

GltI and Glast immunostaining was performed on 50 μM vibratome sections obtained from animals one day, three days, seven days and 45 days after injury. Sections were blocked with 5% normal donkey serum in 0.3% Triton X/PBS for one hour at room temperature before being stained with chicken anti-GFP (1:500), mouse anti-GFAP (1:400) and guinea pig anti-GltI (1:200) or –Glast (1:200) for two hours at room temperature. Fluorescent secondary antibodies were incubated with sections for two hours and were used at a concentration of 1:200. Sections were thoroughly washed and then mounted on glass slides.

Vibratome sections from animals exposed to HI+TBI or Sham+TBI were blocked with normal donkey serum and stained with rat anti-BrdU (1:300), chicken anti-GFP (1:500) and guinea pig anti-GltI or guinea pig anti-Glast (both 1:200) for two hours at room temperature. Sections were then washed and incubated with fluorescent secondary antibodies (1:200) for two hours at room temperature before being mounted on slides.

Confocal Microscopy

A Zeiss LSM 510 confocal microscope was used to take all immunohistochemical photos involving GltI and Glast. Images were obtained using Zeiss Neofluar 40x

and 63x lens. In the experiments with TBI, images were taken using the 25x and 63x lens.

CHAPTER FIVE

Conclusions and future directions

Using the well-characterized nestin promoter and its enhancer elements in transgenic mouse, we were able to characterize different progenitor populations within the dentate gyrus over the course of postnatal development. Using immunofluorescence we created a developmental profile which broadly defined early (type I and II) and late (type III) progenitors as well as mature neurons in the dentate gyrus. To more adequately quantify different cell types present in the hippocampal niche, we used unbiased stereology to quantify dividing cells and early and late progenitors at developmentally relevant time points. From these studies we were able to conclude that changes within the progenitor pools were age-dependent.

To investigate the functional differences between progenitors from different aged animals, we used various in vitro techniques to analyze their proliferative and differentiation potential. We found that early progenitors from P7 mice were more neurogenic than those from P28: P7 progenitors preferred to differentiate into neurons while those from P28 mice primarily became neurons. Using neurosphere assays we also determined that P7 progenitors maintained a proliferative advantage over P28 progenitors and discovered that this proliferative phenotype was controlled by cell-autonomous factors.

Using a stem cell-specific microarray, we sought to identify potential regulators of the hippocampal neurogenic niche that might be responsible for these observed phenotypes. By isolating GFP-expressing cells from P7 and P28

transgenic mice, we were able to compare differentially expressed genes in vivo. Through strict selection processes, we identified nine candidate genes which were significantly upregulated or downregulated compared to P7 progenitors. Of these nine we chose to focus on GltI, a glutamate transporter which was upregulated 10 fold in P28 progenitors.

To characterize GltI expression on our GFP-positive progenitors we used Q-PCR, Western blot analysis and immunofluorescence. Using Q-PCR we confirmed that GltI mRNA levels were upregulated about 10 fold in vivo. In addition we showed that GltI protein levels were also higher in the dentate gyrus of P28 animals compared to P7. Finally, we determined that GltI and its partner Glast were specific to type I and IIa cells and were absent from later type III progenitors.

We utilized a number of in vitro experiments to investigate the function of GltI and Glast in early neural progenitor cells in glutamate-free conditions. In multiple experiments we were able to alter GltI and Glast expression levels via gene knockdown and overexpression in neurospheres derived from P7 dentate gyrus. Using BrdU incorporation and neurosphere assays we showed that knockdown of GltI or Glast resulted in increased proliferation suggesting a negative regulatory function for GltI and Glast. In addition we showed that this proliferative phenotype was calcium dependent using flow cytometry methods. We also found no difference in intracellular glutamate concentrations which can be attributed to extremely low levels of pericellular glutamate. From this we can conclude that glutamate transporters indirectly regulate calcium-dependent

progenitor proliferation in vitro by controlling glutamate receptor's access to extracellular glutamate.

We next wanted to see if this proliferative phenotype was relevant in vivo so we chose to examine adult neurogenesis in the context of brain injury. Using a well-studied model of unilateral HI, we characterized glutamate transporter expression on progenitor cells after injury. Compared to the uninjured side of the brain, both GltI and Glast were upregulated 1d, 3d, 7d and 45d after injury suggesting they might be involved in injury-induced neurogenesis. To test this hypothesis, we introduced a second injury model, TBI. Mice were exposed to either HI injury or Sham injury before they were subsequently injured with TBI. After pulsing with BrdU, we found that animals with HI and TBI had fewer proliferating progenitors (on the injured side of the brain) when compared to animals with the Sham injury and TBI. We next confirmed that GltI and Glast were still upregulated on the injured side of the brain. From this we can conclude that animals injured with Sham+TBI and HI+TVI resulted in induced expression of glutamate transporters on GFP-expressing cells. This experiment suggests that the upregulation of GltI and Glast (due to HI) might be responsible for the decreased proliferation observed in animals with HI and TBI. This correlates with our in vitro results and infers a negative regulatory function for glutamate transporters on progenitor proliferation. This result is also clinically relevant and might explain the mechanism underlying a reduced capacity for recovery in patients exposed to multiple brain injuries.

Future Experiments

(1) Although we were able to identify a novel function for GltI and Glast in vitro, we did it in the absence of exogenous glutamate. Because glutamate is present within the neurogenic niche during development and after injury, we need to confirm the function of glutamate transporters in media containing glutamate. However the addition of glutamate will result in activation of glutamate receptors which will ultimately lead to calcium-induced proliferation. To determine which glutamate receptor is specifically responsible for this calcium response will require pharmacological inhibition of NMDARs, AMPARs or mGluRs. Furthermore, to confirm that calcium is responsible for progenitor proliferation, it would be ideal to examine progenitor proliferation while inhibiting the calcium response. However, due to the complexities of intracellular calcium release, this might prove difficult.

(2) To further characterize the decreased proliferation observed after HI and TBI, we need to quantify the different cell populations present. To achieve this we will utilize unbiased stereological techniques to determine absolute cell number of GFP-expressing cells and BrdU-positive cells. This will allow us to better understand the results shown in this thesis which are presented as the percentage of cells coexpressing GFP and BrdU.

(3) Although we show that preconditioning with HI results in increased expression of GltI and Glast, we do not show that their overexpression is directly responsible for the decrease in proliferative capacity after TBI. To address this we need to generate a mouse which overexpresses GltI or Glast without the side effects and consequences of acquired brain injuries. To do this we will stereotactially inject a retrovirus overexpressing GltI or Glast into the dentate gyrus so that the transporter upregulation is specific to dividing cells in the area. We will then analyze the effect on progenitor proliferation during development and after injury. This experiment will allow us to more specifically study how glutamate transporter overexpression effects neural progenitor proliferation in vivo.

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