ACTIVATION OF EARLY NEURAL PROGENITORS IS REQUIRED FOR TRAUMATIC BRAIN INJURY-INDUCED HIPPOCAMPAL NEUROGENESIS.

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DEDICATION

This is dedicated to my parents who have always stood beside me in all my decisions.

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by

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Traumatic brain injury (TBI) is the most common form of acquired brain injury in both children and adults in the United States. TBI causes neuronal loss and results in a variety of neurological impairments and deficits in hippocampus-dependent functions. However, cognitive recovery commonly occurs though the mechanism is unknown. Exploration of post-natal neurogenesis in the hippocampus raises the possibility that adult-born neurons may contribute to cognitive recovery from TBI.

Several studies in animal models that mimic TBI demonstrate there is enhanced generation of adult-born neurons in the dentate gyrus and those adult-born neurons may correlate with cognitive recovery. Due to the limits of current methodology in studying neurogenesis, it remains unclear what relevance injury-induced neurogenesis may have in the recovery process following TBI.

In order to explore the relevance of injury-induced neurogenesis, I have characterized a previously generated transgenic mouse line that has rtTA-IRES-eGFP expression under the control of a nestin promoter and also contains a neural progenitor-specific regulatory element. By using this line, I have demonstrated that eGFP-expressing cells represent early neural progenitors in the adult dentate gyrus. Performing unilateral controlled cortical injury (CCI) demonstrates that this injury depletes doublecoritn (Dcx)-expressing late neural progenitors while activating eGFP-expressing early neural progenitors.

To address whether the subsequent recovery of Dcx-expressing late progenitors was derived from activation of early neural progenitors, I generated a transgenic line that expresses modified herpes simplex viral thymidine kinase (delta-HSV-TK) under the control of the neural progenitor-specific regulatory element of the nestin gene. This allows for temporally regulated ablation of dividing neural progenitors by exposing the animal to ganciclovir. Using this line, I demonstrate that ablation of dividing GFP-expressing early neural progenitors in neurogenic areas occurs only in the presence of ganciclovir. CCI on these mice, reveals that no newly born Dcx-expressing late neural progenitors are observed seven days after injury when exposed to ganciclovir. However, the repopulation of Dcx-expressing cells is apparent when ganciclovir was removed one

day before injury. Four weeks after injury, those newly born Dcx-expressing cells became mature NeuN-expressing neurons. This suggests that injury-induced activation of early neural progenitors is required for the recovery of injured hippocampal neurons.

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PRIOR PUBLICATIONS

Yu TS, Dandekar M, Monteggia LM, Parada LF, Kernie SG (2005) Temporally regulated expression of Cre recombinase in neural stem cells. *Genesis* 41:147-153.

Yu TS, Zhang G, Liebl DJ, Kerine SG (2008) Traumatic brain injury-induced hippocampal neurogenesis requires activation of early nestin-expressing progenitors. *J Neuroscience* (In review).

LIST OF FIGURES

LIST OF ABBREVIATIONS

BDNF – brain-derived neurotrophic factor

bFGF – basic fibroblast growth factor

BLBP – brain lipid binding protein

BrdU – bromodeoxyuridine

CCI – controlled cortical impact

Dcx – doublecortin

EGF – epidermal growth factor

eGFP – enhanced green fluorescent protein

FACS – fluorescence-activated cell sorting

GFAP – glial fibrillary acidic protein

hGFAP – human glial fibrillary acidic protein

HSV – herpes simplex virus

LTP – long-term potentiation

MAM – methylazomethanol acetate

OPC – oligodendrocyte precursor cell

PFA – paraformaldehyde

rtTA – reverse transcriptional activator

SGL – subgranular layer

SVZ – subventricular zone

TBI – traumatic brain injury

TK – thymidine kinase

CHAPTER ONE
Introduction and Literature Review

Traumatic brain injury and Adult hippocampal neurogenesis

Traumatic Brain Injury

Acquired brain injury is damage to the brain acquired after birth. It results from traumatic and non-traumatic causes. Nontraumatic brain injury can result from stroke, hypoxia-ischemia, or infection. Traumatic brain injury (TBI) is usually caused by car accidents, falls, or assaults. Traumatic brain injury is the most common form of acquired brain injury in both children and adults. It occurs when physical trauma causes brain damage. The injury can be focal, where the injury is confined to one area, or diffuse, where the injury affects more than one brain area. Depending on whether the skull is broken, TBI can be divided into one of two categories: closed head injury or penetrating head injury. Usually, a closed head injury results from violent and sudden hits to the skull and there is no breakage of the skull. If an object pierces and enters the skull, it is defined as a penetrating TBI (Heegaard and Biros, 2007;Nortje and Menon, 2004).

Signs and Symptoms

TBI can vary in degree of severity and is classified as mild, moderate or

severe. The signs of TBI vary and depend on the extent of the damage to the brain tissues. Some symptoms are apparent immediately, yet others do not become evident until days or weeks after TBI.

Primary Insults

At the moment that TBI occurs, some damage occurs immediately, which include systemic insults, intracranial lesions, transient neuronal depolarization, and excessive release of glutamate (Mattson and Scheff, 1994). It causes robust neuronal death mediated by excitotoxicity associated with glutamate release. The alteration of ions within hours of TBI results in brain swelling, with an increase in intracranial pressure and a subsequent decrease in cerebral perfusion leading to ischemia. Due to the lack of immediate medical care, injuries often worsen and lead to secondary damages (Reilly, 2001) (Fig. 1-1).

Secondary Insults

Many secondary injuries are initiated from primary insults when effective care is not given in time. The secondary insults can be divided into systemic or intracranial damages. The symptoms of systemic secondary injuries include hypotension, hypoxia, anemia, and hyper- and hypocapnia. Intracranial damages

consist of severe intracranial hypertension, extra-axial lesions, seizures, and cerebral edema (Heegaard and Biros, 2007;Nortje and Menon, 2004;Enriquez and Bullock, 2004). For TBI patients, the degree and type of secondary insults are the principle determinants on the final neurologic consequences. Alleviation of the damages caused by secondary insults is the current interest of clinical trials attempting to provide better lives for TBI patients (Marklund et al., 2006;Hatton, 2001;Gentleman, 1999).

Long-Term Deficits caused by TBI

A variety of long-lasting outcomes are reported to be caused by TBI. They are categorized into physical deficits, cognitive deficiencies, and emotional impairments.

Physical deficits resulting from TBI often include seizures and can also be caused by Parkinson's disease brought on by the TBI. It is known that patients who suffer penetrating head injuries usually develop seizures. The risk of seizure development increases with other types of brain trauma, like cerebral contusions or hematomas. Parkinson's disease and other motor problems can also develop in TBI patients if the basal ganglia are damaged. Symptoms of Parkinson's disease include tremor, rigidity, stiffness, slow or inability to move, and shuffling walk.

Other motor disorders that could develop after TBIs are tremor or ataxia (Teasell et al., 2007;Galvan and Jin, 2007).

Generally, severe TBI patients suffer from cognitive disabilities, including the loss of some higher levels of mental skills after recovering consciousness. Memory loss is another cognitive deficit from which severe TBI patients suffer. Some of the patients may face post-traumatic amnesia, either anterograde or retrograde. Patients who suffer moderate or mild TBI also may have some level of cognitive impairment. These impairments may result in difficulty in concentration and attention in these patients. Disability in communication and language are another challenge in TBI patients. They may have difficulty in understanding language or more subtle aspects of communication (Rickards, 2006;Jellinger, 2004;Bajo and Fleminger, 2002).

Emotional or behavioral alterations are another problem that TBI patients may suffer. Emotional alterations, like irritability, insomnia, apathy, anxiety, anger, and paranoia, may last for up to two years. The specific location of injury might cause the alterations in behaviors. Problem behaviors consist of violence, emotional outbursts, impaired self-control, egocentrism, alcohol or drug abuse or addiction and social inappropriateness. TBI patients are at great risk for developing psychiatric problems (Rees et al., 2007;Moore et al., 2006;Bryant,

2001).

Current Medical Care

Generally speaking, a TBI patient does not receive any medical care until emergency medical technicians arrive on the scene or when the patient arrives at the emergency room. Unfortunately, damage begins immediately and is not reversible after injury takes place. The initial medical care focuses on stabilizing the patient and preventing further injury; this includes maintenance of proper oxygen supply, adequate blood flow and control of blood pressure. The following medical care depends highly on the severity of TBI after the vital signs of the patient are stabilized. However, the current treatments remain supportive due to the lack of knowledge in the pathology of TBI. If the patients survive, rehabilitation helps the patient to recover from a variety of neurological deficits (Heegaard and Biros, 2007; Wahlstrom et al., 2005; Royo et al., 2003; Hatton, 2001).

Future Treatments

The identification of neural progenitors in the adult brain raises hope for treatment of both neural degenerative diseases and acquired brain injuries such as

TBI by opening up the possibility for transplantation of neural progenitors or enhancing endogenous neurogenesis to rebuild the lost neuronal circuits (Bjugstad et al., 2008;Park et al., 2006;Schouten et al., 2004;Lu et al., 2003;Yang et al., 2002;Park et al., 2002;Teng et al., 2002). Although several studies have been reported in animal models, more experiments are required to understand the mechanism of new neuron formation and how new neurons integrate into existing neuronal circuits.

Adult Neurogenesis

Historic Overview

The well-known histologist, Ramon y Cajal, made the following description in the year 1913:

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

This has been a dogma in the field of neuroscience and taught for generations and

generations.

The first evidence demonstrating that new cells form in the adult dentate gyrus was published in the 1960s by Joseph Altman and his colleagues (Altman, 1962). By treating patients with radioactive thymidine to label the cells in S phase due to incorporation of thymidine, they revealed through autoradiography that dividing cells can be found in the adult dentate gyrus. However, due to the lack of specific markers to label the mature neurons, a solid conclusion was not made at that time.

In the 1970s, Michel Kaplan and his colleagues integrated autoradiography with electron microscopy. They confirmed that radioactive thymidine-labeled cells in the olfactory bulbs and the dentate gyrus were new neurons (Kaplan and Hinds, 1977).

Fernando Nottebohm and collaborators demonstrated in the 1980s that new neurons form in adult song birds and revealed their roles in learning new songs (Nottebohm, 2002). Their pioneer studies revealed that new neurons integrate into the existing circuits, and that adult neurogenesis can be affected by hormones, experience, and even circadian rhythms (Nottebohm, 2002).

Finally, in the 1990s, Brent Reynolds and his colleagues determined that the formation of neurospheres *in vitro* are derived from adult neural progenitors (Reynolds and Weiss, 1992). Furthermore, Fred Gage and his colleagues used a thymidine analogue, bromodeoxyuridine (BrdU), and specific neuronal markers. They demonstrated the formation of new neurons in the dentate gyrus in humans (Eriksson et al., 1998). The idea that new neurons do form and neural progenitors exist in the adult mammalian brain became widely accepted.

Currently, the manipulation of neural progenitors and their differentiation to neurons is studied extensively and has become a potential target of pharmaceuticals for treating neuronal degeneration diseases (Fig. 1-2).

Methodology to Study Adult Neurogenesis in vivo

Our understanding about adult neurogenesis *in vivo* highly correlates with advances in the identification of newborn neurons among the existing neurons in adult brains. Current favored methods for investigating adult neurogenesis include BrdU-incorporation, retroviral infection and cell-specific reporter gene expression in transgenic mice (Fig. 1-3).

Incorporation of Nucleotide Analogue during Mitosis

In the S phase of mitosis, DNA replicates and exogenous nucleotides, like tritium-labeled thymidine or BrdU, are incorporated into newly synthesized DNA and will be passed on to the cell's progeny. BrdU is used extensively in current studies due to its relative safety and convenience. BrdU is detected by using a specific antibody and this allows for stereological quantification and phenotypic analysis when combined with other cell specific antibodies. By using different pulsing paradigms and examining BrdU-labeled cells at different time points after pulsing, quantitative analysis of cell proliferation, differentiation, and survival of new neurons has been well established (Steiner et al., 2006;Holmes et al., 2004;Kempermann et al., 2003;Cameron and McKay, 2001;Eriksson et al., 1998;Kempermann et al., 1997b;Kempermann et al., 1997a;Kuhn et al., 1996).

Although BrdU has been comprehensively used to investigate neurogenesis, several limitations have to be considered. First, due to the fact that DNA denaturation is required to expose the BrdU epitope for the antibody to recognize it, tissue fixation is necessary. Hence, live cell imaging is not possible. Second, due to DNA denaturation, cell specific proteins might also be damaged. Not all the cell specific antibodies work after denaturation, and the conditions of DNA denaturation might vary from experiment to experiment. Third, the signal of BrdU is restricted to the nucleus, and confocal microcopy is required to identify

the colocalization with cell specific markers (Rakic, 2002). Forth, BrdU could be diluted to undetectable levels after several rounds of cell division (Hayes and Nowakowski, 2002). The most important thing is that BrdU is the indicator of DNA synthesis not mitosis. Therefore, the incorporation of BrdU might also be due to DNA repair (Selden et al., 1993). To verify the derivation of BrdU signal, another mitotic marker, such as Ki67, is usually required.

Reporter Expression with Retroviruses

Retroviruses, like the Muloney murine leukemia (MML) virus, have become a useful tool to study neurogenesis in adult brains. For the MML virus, because it lacks a nuclear import mechanism, viral integration occurs only when the nuclear membrane breaks down during mitosis (Lewis and Emerman, 1994). Expression of reporter genes by retroviral infection allows for visualization and analysis of living newborn neurons. Furthermore, combining retroviral reporters with site-specific expression or siRNA technology make this system more powerful. However, performing a stereotaxic injection is required in this system to deliver retroviruses into the brain region of interest. Damage caused by the injection should be considered a possible confounding variable in these experiments.

Expression of reporter genes in transgenic mice

With the advancements made in the understanding of neural progenitor specific genes and their regulation, several transgenic mice containing reporter genes have been generated by using early neural progenitor specific or late neural progenitor specific promoters, like nestin or proopiomelanocortin promoter, to visualize living neural progenitors (Kim et al., 2007;Yu et al., 2005;Mignone et al., 2004;Overstreet et al., 2004;Yamaguchi et al., 2000). With these transgenic mice, the physiology of early and late neural progenitors has been investigated extensively in recent years (Kronenberg et al., 2003;Fukuda et al., 2003). Even more recently, several transgenic mice that have rtTA or creERT2 expression driven by the regulatory element of nestin gene were generated (Lagace et al., 2007;Yu et al., 2005). Inducible transgenic mice provide a precise way to manipulate gene expression temporally and spatially.

Identification of Adult Neural Progenitors

To identify an "adult neural progenitor", a cell needs to be capable of self-renewal, and the ability to generate cells in all neural lineages, including neurons, astrocytes, and oligodendrocytes (Gage, 2000).

in vitro *Neurosphere Assay*

The standard method to identify, neural progenitors is to dissect the adult brain areas that have been demonstrated to have dividing cells *in vivo*. By growing dissociated cells in a defined medium that contains specific growth factors, like basic fibroblast growth factor (bFGF) or epidermal growth factor (EGF), the dissociated cells form sphere-like cellular masses if they are able to proliferate (Palmer et al., 1999;Kuhn et al., 1997). To examine the capacity for self-renewal, the dissociated cells from neurospheres are allowed to grow clonally. The ability of the cells to grow neurospheres repeatedly under clonal density represents their self-renewal capacity. With the application of serum, the dissociated renewable cells are defined as neural progenitors if they are able to develop into neurons, astrocytes, or oligodendrocytes (Fig. 1-4).

Ablation of Dividing Cells and Fate Mapping

One way to identify the adult neural progenitors *in vivo*, is to first apply mitotic inhibitors to ablate the dividing neural progenitors, then allow for repopulation of neural progenitors by removing mitotic inhibitors. The neural progenitors can then be labeled by treating animals with BrdU. In 1999, Fiona Doetsch and her colleagues identified adult neural progenitors and characterized

their development in the lateral ventricles (Alvarez-Buylla et al., 2002;Doetsch et al., 1999b). Interestingly, early neural progenitors have characteristics of mature astrocytes. Although inhibitors can be applied directly to the area of interest, all kinds of dividing cells are inhibited due to a lack of specificity. The contribution of other cell types cannot be excluded from interpretation of data coming from such methods.

The development of transgenic models provides alternative ways to determine the existence of adult neural progenitors *in vivo*. Herpes simplex virus thymidine kinase (HSV-TK) has been used to generate transgenic mice, which allows for the ablation of specific dividing cells depending on the chosen regulatory elements (Visnjic et al., 2001;Mathis et al., 2000;Rindi et al., 1999;Delaney et al., 1996;Canfield et al., 1996;Salomon et al., 1994;Minasi et al., 1993;Wallace et al., 1991). The HSV-TK expressing transgenic line driven by the human GFAP promoter was generated. The adult neural progenitors possessing the characteristics of mature astrocytes have been confirmed (Garcia et al., 2004). Furthermore, the development of creER T2 inducible system provides a way to visualize the progress of neurogenesis (Lagace et al., 2007).

Adult Neural Progenitors in the Lateral Ventricles

The subventricular zone (SVZ) consists of several different neural progenitors classified based on their stage in neurogenesis and location throughout the lateral walls of the lateral ventricles. During the formation of new neurons, the neuroblasts migrate tangentially through the rostral migratory stream (RMS) to the olfactory bulb and become mature periglomerular or glomerular neurons. The pool of neural progenitors in the SVZs is composed of type B, type C and type A cells (García-Verdugo et al., 1998;Doetsch et al., 1997). Type B cells are slowly dividing, mature astrocyte-like cells. Histologically, they possess characteristics of mature astrocytes and have glial fibrillary acidic protein (GFAP) expression in additon to nestin, a well-known neural progenitor marker, expression. They are identified as neural stem cells because by administrating anti-mitotic drug to ablate dividing cells, the remaining type B cells have been shown to re-populate neural progenitors and generate new neurons (Doetsch et al., 1999a). Type B cells give rise to type C cells. Different from type B cells, type C cells do not express GFAP protein but retain expression of nestin. By using a thymidine analogue, bromodeoxyuridine (BrdU), to label cells in S phase, type C cells have been shown to possess a high frequency of proliferation. They have been identified as transiently-amplifying neural progenitors (Doetsch et al., 1999a;Doetsch et al., 1997). Type C cells develop and become committed neuroblasts, so called type A cells. Type A cells retain the ability to migrate along the RMS to the olfactory bulb and become mature neurons (Doetsch et al.,

1999a;Doetsch et al., 1997;Lledo et al., 2006). The model of adult neurogenesis in SVZ is shown in Fig 1-5.

Adult Neurogenesis in the Dentate Gyrus

In this study, the dentate gyrus is the brain area of interest. Therefore, adult neurogenesis in the dentate gyrus is reviewed here extensively.

Progenitors and Generation of New Neurons

A well-accepted description of neural progenitors in the adult dentate gyrus is the following: type 1 early neural progenitors behave like C cells. These cells have long processes that penetrate the granular layers and protrude into the inner molecular layers. In addition to the known neural progenitor markers, like nestin, these cells also express mature astrocyte markers, such as glial fibrillary acid protein (GFAP). With the characteristics of astrocytes, transgenic mice that express HSV-TK through activation of the human GFAP (hGFAP) promoter were generated. By using this transgenic line to ablate dividing GFAP-expressing cells, GFAP-expressing type 1 cells were determined to be neural stem cells (Imura et al., 2006;Garcia et al., 2004). By performing BrdU-pulsing experiments, the type 1 cells were shown to be slow-dividing neural progenitors

(Seri et al., 2001).

Type 2 neural progenitors are generated from type 1 cells and expression of mature astrocyte markers is no longer detectable. Morphologically, these cells do not have visible long processes. Proliferation in type 2 cells is more frequent than type 1 cell, and thus is a transiently-amplifying cell (Steiner et al., 2006;Kronenberg et al., 2003;Cameron and McKay, 2001;Seri et al., 2001).

After development into type 3 cells, the expression of neural progenitor markers is down-regulated and cell fate is committed (Ming and Song, 2005). This type of cell begins a dramatic change in morphology and electrophysiology soon after its differentiation. The growth of dendrites begins in type 3 cells and cells begin to receive glutamatergic input from the entorhinal cortex (Zhao et al., 2006). Originally the excitatory stimuli from GABAergic interneurons are inhibitory in type 3 cells due to the alteration of intracellular chloride ion concentration (Ge et al., 2006). While receiving input from surrounding neurons, the growth of axons is in progress and synapses eventually form with pyramidal cells in CA3 region {Zhao 2006}. Interestingly, a recent study demonstrates that 4-6 week old new neurons have a lower threshold to trigger the formation of long-term potentiation (LTP) and have a larger LTP amplitude (Ge et al., 2006). Because the LTP formation has been correlated to memory formation (Lynch,

2004), this finding indicates that new neurons might be involved in new memory formation (Kee et al., 2007) (Fig. 1-6).

Microenvironment of Neural Progenitors in the Dentate Gyrus

In the dentate gyrus, neural progenitors are observed in the subgranular layers. Although they are able to proliferate and form neurospheres when isolated from tissues and cultured *in vitro*, it has been shown that neural progenitors *in vivo* interact with other types of cells. The neural progenitors in the subgranular layers are adjacent to endothelial cells of blood vessels (Palmer et al., 2000). This suggests a close relationship between these two cell types. In an early study in song birds, it is demonstrated that endothelial cells secrete brain-derived neurotrophic factor (BDNF) to stimulate neurogenesis during mating seasons (Louissaint et al., 2002). Later, *in vitro* experiments demonstrated that endothelial cells help sustain the self-renewal ability of neural progenitors and enhance neurogenesis (Shen et al., 2004). It is believed that endothelial cells play an important role in regulating neurogenesis.

Another important cell involved in governing neurogenesis is the astrocyte. Mature astrocytes are located in the hilus, molecular layer, and subgranular layer. Although the mechanism in regulating neurogenesis by astrocytes *in vivo* is still

not clear, by using *in vitro* co-culture system, it has been determined that mature astrocytes stimulate new neuron formation by both secreting factors and through physical contacts (Song et al., 2002;Lim and Alvarez-Buylla, 1999). Further studies suggest that interaction between astrocytes and neural progenitors may be mediated through cannonical Wnt signaling pathway (Lie et al., 2005).

Of course, it is not surprising that neurons have a role in regulating neurogenesis via neuronal activities. Neural progenitors express both GABA and glutamate NMDA receptors. In early neural progenitors their proliferation is known to be correlated with excitatory GABA stimulation from interneurons (Ge et al., 2007;Ge et al., 2006). Neurogenesis has been shown to be up-regulated by the neurotransmitter glutamate. Furthermore, this enhancement might be mediated by NMDA receptors and L-type Ca^{2+} channels (Deisseroth et al., 2004). These data suggest that neurogenesis is affected by other types of cells surrounding the neural progenitors (Fig. 1-7).

Neurogenesis, Learning and Memory

To relieve the symptoms of severe epilepsy, the hippocampi in patient H.M. was removed in 1957. Because of this radical surgery, H.M. was reported to have suffered from severe anterograde amnesia (Scoville and Milner, 2000). The role

of hippocampus in learning and memory was established by this case study.

As shown in Fig. 1-8, the hippocampus is composed of the dentate gyrus, CA3 and CA1 regions. The architecture of neuronal connections in the hippocampus is complicated. To put it simply, axons of neurons in layers 2 and 3 of the entorhinal cortex form the perforant path and synapse with granular cells in the molecular layer of the dentate gyrus. The granular cells in the dentate gyrus send out signals to the CA3 region via axon bundles named mossy fibers. Then, pyramidal cells in CA3 region transmit the information to CA1 region through Schaffer collaterals. After information is processed in CA1, it is transmitted back to neurons in layer 5 of the cortex, but not to neurons in layer 2 or 3. Based on numerous studies, many theories are proposed to explain how information is processed within this circuitry and becomes stored memory (Bird and Burgess, 2008;Aggleton et al., 2007;Ferbinteanu et al., 2006). Despite the extensive amount of effort expended on this issue, the specific roles of the sub-regions in the hippocampus remain unclear.

The identification of newborn neurons in the adult dentate gyrus has drawn a great deal of attention. Many studies have been performed to understand whether newborn neurons are involved in memory formation. Unfortunately, the data remains controversial due to the limitations of current methodologies.

Although several studies have proven that newborn neurons in the adult dentate gyrus do functionally integrate into the existing circuitry, it is not known whether those neurons encode any information. To understand whether newborn neurons are required in information processing, several methodologies have been developed to inhibit neurogenesis in adult animals. The methods can be divided into 3 categories, systemic application of mitotic inhibitors, low-dosages of irradiation, and transgenic expression of HSV-TK (Leuner et al., 2006;Wojtowicz, 2006;Winocur et al., 2006;Garcia et al., 2004). Systemic application of mitotic inhibitors would ablate all kinds of dividing cells, making the specificity the critical concern. With low-dosages of irradiation, animals require ~4 weeks to recover from the severe inflammatory reaction caused by this treatment (Wojtowicz, 2006). Another critical concern is the completeness of ablation of neural progenitors since the hippocampus resides deeply in the brain and irradiation would not likely be able to reach this area before treatment reaches lethality. Due to these limitations, conclusions on the functions of newborn neurons in memory formation made based on upon different behavioral paradigms (as shown in figure 1-9) remain controversial.

To conquer the limitations of the methods discussed previously, a transgenic line that expresses HSV-TK under the control of human GFAP promoter was generated. In this line, HSV-TK is expressed in mature astrocytes as well as

neural progenitors in adult brains and allows for the temporal ablation of dividing neural progenitors thereby inhibiting new neuron formation. By using this specific transgenic line, recent studies demonstrate that adult neurogenesis is not required for behavioral effects induced by enriched environments (Meshi et al., 2006). However, inhibition of adult neurogenesis impairs contextual fear conditioning and synaptic plasticity in the adult dentate gyrus (Saxe et al., 2006). Although these studies demonstrate that the hGFAP-HSV-TK transgenic line provides a specific way to inhibit neurogenesis, it remains uncertain whether astrocytes are affected during ablation of dividing neural progenitors since TK is also expressed in mature astrocytes. A specific transgenic line is required to ablate neural progenitors without affecting other cell types to make a firm conclusion.

Neurogenesis in Traumatically Injured Brains

The existence of adult neural progenitors inspires a new direction in treating acquired brain injuries. Transplantation of adult neural progenitors to damaged or degenerating brain areas has been tested in a variety of acquired brain injuries (Gaillard et al., 2007; Yamashima et al., 2007; Shear et al., 2004; Hallbergson et al., 2003; Parent, 2003; Peterson, 2002). Rather than transplanting exogenous neural progenitors, another aim for using neural progenitors pharmaceutically is

developing treatments to enhance endogenous neurogenesis. Many studies have reported that different kinds of acquired brain injuries promote neurogenesis, primarily in the dentate gyrus (Sun et al., 2007;Urrea et al., 2007;Rola et al., 2006;Ramaswamy et al., 2005;Chirumamilla et al., 2002;Kernie et al., 2001). Although some studies show that injury might induce neurogenesis in other brain areas, especially in the injured areas, more experiments are required to make a definitive conclusion (Gotts and Chesselet, 2005;Jin et al., 2003b;Jin et al., 2003a;Nakatomi et al., 2002). These observations are based on BrdU pulsing and expression of specific cellular markers. Relying on this method requires knowledge of whether BrdU is incorporated only by dividing cells since a recent study suggests that non-apoptotic neurons in the injured brains also exhibit the ability to synthesize DNA (Burns et al., 2007). Another concern is that astrocytes become active in injured brains and express neural progenitor markers. Some studies further suggest that even active microglia and NG2 cells in injured brains express neural progenitors markers (Bjugstad et al., 2008;Wu et al., 2005;Kruglyakova et al., 2005;Sahin Kaya et al., 1999;Duggal et al., 1997;Lin et al., 1995). The less than exclusive specificity of neural progenitor markers results in the difficulty to make a solid conclusion on whether injury-induced neurogenesis is derived from activation of neural progenitors.

Brain injury is known to result in altering the biophysiological status in the

brain. For example, extracellular potassium and glutamate concentration are known to become dramatically elevated in various forms of brain injuries. Interestingly, some of the alterations in ion concentrations are known to stimulate neurogenesis in the dentate gyrus. Although it is believed that those injury-induced newborn neurons might contribute to the recovery of cognition (Sun et al., 2007), injury-induced neurogenesis may simply be a side-effect of alternations in the biophysiological status. This issue certainly requires further investigation.

Specific Aims in this Project

1. Examine the dynamics of neural progenitors in traumatically injured brains. To accomplish this aim I analyzed three transgenic lines that express enhanced green fluorescence protein (eGFP) driven by neural progenitor-specific regulatory elements and examined whether eGFP-expressing cells represent the true distribution of neural progenitors. Whether the eGFP-expressing cells are distinguishable from other types of cells was also examined.
2. Determine whether the neural progenitors, but not other cell types, are the primary source of new neurons for injury-induced neurogenesis in the dentate gyrus. In order to achieve this goal, a transgenic line that expresses HSV-TK in neural progenitors was generated which provides an

efficient and specific way to ablate dividing neural progenitors temporally.

Figure:

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Fig. 1-1. Description of primary insults caused by a TBI. The moderate or severe TBI could result in brain damage due to fracture of skull, midline shift, hemorrhagic contusion, or epidural hemtomas. When effective treatment is not obtained in time, secondary injuries progress and the symptoms could be long-lasting or even permanent. *Adapted from www.blackmanlaw.com*

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Fig. 1-2. A brief history in the discovery of adult neurogenesis: the identification of newborn neurons in the adult brains from the 1960s. With the advances in techniques and methodology, the existence of adult neural progenitors and generation of adult newborn neurons currently well accepted. *Adapted from Colucci-D'Amato L et al., 2006.*

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Fig. 1-3.Current methodologies used in investigating adult neurogenesis. (a) Tracking neurogenesis based on the incorporation of nucleotide analogs (e.x. BrdU) during DNA synthesis in S-phase of mitosis. Accompanied with cell-specific markers, time of birth and cell fates can be determined. (b) Retroviruses can be used to deliver genes of interest into the dividing cells. Manipulating gene expression or tracing the cell fates can be performed in the dividing cells adjacent to the injection sites. (c) Generation of transgenic mice that have expression of interesting transgenes under control of a specific regulatory element, expression of gene can be manipulated in specific types of cells. *adpated from Ming et al., 2005*

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Fig. 1-4. This scheme depicts the neurospheres growing from dissociated neural progenitors from adult brains. The cells that comprise neurospheres (a, g) possess the expression of GFAP, the marker of early neural progenitors (j) and nestin (a, g). These cells do not express a neuronal marker (d, e). However, once differentiated, expression of nestin is down-regulated in the cells (c, i), they possess the mature neuronal marker (f, m) and astrocyte marker (l, n, o), suggesting that new cells eventually become mature cell types. *Adapted from Mignone JL*

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Fig. 1-5. This scheme represents the composite of adult neural progenitors in the subventricular zones of the lateral ventricles. Type B cells are the early neural progenitor type that express astrocyte markers, like vimentin and GFAP. Type B cells enable to self-renewal and give rise to their progeny, the type C cell. Type C cells are the transiently-amplifying cells. This type of cell proliferates frequently and differentiates to become the fate committed neuroblast, type A cell. Type A cells migrates out toward the olfactory bulb along the rostral migratory stream. *Adapted from Doetsch F., 2003*

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Fig. 1-6. (a). Adult neurogenesis in the dentate gyrus. The type 1and 2 early neural progenitors reside adjacent to vasculatures and are affected by excitatory GABA stimuli. Later, neural progenitors extend their processes to the molecular layers and receive excitatory inputs from entorhinal cortex. In the meantime, GABA inputs from interneurons become inhibitory. During the progress of maturation, new neurons also grow axons also known as mossy fibers, and form synapses with pyramidal cells in the CA3 region. (b). This scheme summarizes the known properties of and time frames for development of new neurons during neurogenesis. *Adapted from Zhao et al., 2008*

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Fig. 1-7. Microenvironment of adult neural progenitors in the dentate gyrus. Adult neural progenitors are located in the subgranular layers. Surrounding them, blood vessels exist and are adjacent to the neural progenitors. Mature astrocytes are close to the neural progenitors in the subgranularlayers. GABAergic interneruons and glutamatergic neurons from entorhinal cortex have been demonstrated to interact with neural progenitors. Type B cells are called type 1 cells in the text. Type D cells are type 2 and 3 cells. G represents the newborn neuron. BV: blood vessel; Light pink cells represent the mature granular cells. Dark pink processes are the process radiating from GABAergic interneurons or glutamatergic entorhinal neurons. *Adapted from Riquelme PA et al., 2007*

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Fig 1-8 Simplified neuronal circuitry in the hippocampus. The cortex receives sensory inputs from other brain regions and sends them to the dentate gyrus via pyramidal cells in the layer 2 and 3 of the cortex. In the hippocampus, received information is transmitted from the dentate gyrus to pyramidal cells in CA3 through the mossy fibers after processing. Then, pyramidal cells in CA3 send out the information to CA1 via Schaffer collaterals. The information is conveyed to cortex again but now to neurons in layer 5. *Adapted from Neves G et al., 2008*

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Fig. 1-9. This summary table presents the controversial data on whether neurogenesis aids in different types of learning and memory paradigms. *Adapted from Leuner B et al., 2006.*

CHAPTER TWO

Generation of Transgenic Lines to Monitor Adult Neural Progenitors

Introduction

The observation of Ramon y Cajal that there is no neuron formation in adult brains has been a dogma in the field of neuroscience. However, the discovery of newborn neurons in the adult mammalian brain, including the human brain, breaks this belief and has led to excitement in this field (Eriksson et al., 1998;Alvarez-Buylla and Lois, 1995). To find out the source of the newborn neurons in adult brains, the examination of adult neural progenitors has recently become an intense area of investigation.

Several methods have been used to identify adult neural progenitors. Being a neural progenitor, the cells should proliferate. Therefore, cells should enter S-phase. To take advantage of this, BrdU or tritium-labeled thymidine have been used to identify dividing cells and trace their cell fates. It is well accepted that adult neural progenitors exist primarily in two neurogenic brain areas, known as subgranulaur layers of the dentate gyrus and subventricular zones of the lateral ventricles (Zhao et al., 2008;Lie et al., 2004;Alvarez-Buylla et al., 2002). By performing BrdU-pulsing experiments and using cell-specific markers, neural

progenitors have been identified and their development has been characterized.

In the subventricular zones of lateral ventricles, early neural progenitors, called type B cells, are believed to be a subset of specific astrocytes (García-Verdugo et al., 1998;Doetsch et al., 1997). B cells have a similar gene expression profile as mature astrocytes, expressing genes such as GFAP. vimentin, or the glutamate transporter GLAST. Different from mature astrocytes, type B cells also express markers of radial glia like nestin, RC2, or brain lipid binding protein (BLBP) (Pinto and Götz, 2007;Mori et al., 2005). Therefore, some believe that type B cells are the remenants of radial glia in the subventricular zones at the end of embryonic neurogenesis. By treating mice with systemic mitotic inhibitors, type B cells are known to divide with a low frequency (Doetsch et al., 1999a). After removal of the inhibitor, type B cells are shown to self renew and give rise to their progeny, type C cells (Doetsch et al., 1999a). Again, BrdU-pulsing experiments demonstrate that type C cells proliferate frequently (Doetsch et al., 1999a;Doetsch et al., 1997). The expression of markers of the radial glia is down-regulated in type C cells, however, some of the neural progenitor markers, like nestin, are retained. Following this, type C cells become committed type A cells, which are migratory neuroblasts. Type A cells migrate out of the subventricular zones to the olfactory bulb through the rostral migratory stream (RMS) (Lledo et al., 2006;García-Verdugo et al., 1998;Doetsch et al., 1997). Consistent with its

motility, type A cells express doublecortin (Dcx), a gene which is known to correlate with migration. In the olfactory bulb, the neuroblasts become mature glomerular cells and integrate with the existing neurons (Lledo et al., 2006).

In summary, similar populations of neural progenitors are identified in the subgranular layers of the dentate gyrus, but with different classifications. Type 1 cells are the early neural progenitor that retains the characteristics of radial glia. Type 1 cells proliferate with low frequency and are able to maintain their population. These cells generate type 2 cells. Similar to type C cells in the subventricular zone, type 2 cells retain nestin expression but not other markers of radial astrocytes. Later, type 2 cells begin to express Dcx and extend their processes to the molecular layer and to the CA3 region and become type 3 cells. Type 3 cells mature and integrate into the existing circuits (Zhao et al., 2008;Ihrie and Alvarez-Buylla, 2007;Zhao et al., 2006;Seri et al., 2004;Schinder and Gage, 2004).

These studies have provided us with the basic knowledge of adult neurogenesis. Currently, a critical concern is the lack of specificity in these studies. For example, BrdU incorporation is not restricted to neural progenitors only. In adult brains, endothelial cells, mature astrocytes, and microglial cells are known to proliferate (Goings et al., 2006;Tatsumi et al., 2005;Kernie et al., 2001).

Furthermore, the specificity of the marker is questionable. Nestin, which is the intermediate filament protein that is expressed in embryonic neural progenitors, is commonly used as a marker to identify neural progenitors in the adult brains (Zimmerman et al., 1994;Tohyama et al., 1993). However, endothelial cells and some mature astrocytes still retain the expression of nestin in the adult brain. GFAP, vimentin, and GLAST are also used as markers of adult early neural progenitors (Pinto and Götz, 2007;Mori et al., 2005;Palmer et al., 2000). Nevertheless, mature astrocytes retain expression of these genes (Pinto and Götz, 2007;Mori et al., 2005). Due to the lack of specificity in the markers used, it is difficult to demonstrate the mechanism underlying adult neurogenesis.

To overcome the limitation of using the markers to identify adult neural progenitors, the regulatory elements of the nestin gene were elucidated in 1994. A small DNA fragment (~700 bps) contains the neural progenitor-specific regulatory element in the 2nd intron of nestin gene (Zimmerman et al., 1994). Yamaguchi and his colleagues used a 2.5Kb DNA fragment of the nestin promoter and a 1.8Kb DNA fragment within the 2nd intron of nestin gene to generate a transgenic line that expresses eGFP (Yamaguchi et al., 2000). During the embryonic stage, eGFP-expressing cells are restricted to the neural tube, suggesting that eGFP-expressing cells represent the radial glia in embryos. In the adult brain, eGFP-expressing cells are found in the neurogenic areas. These

eGFP-expressing cells are able to incorporate BrdU and retain the expression of known neural progenitor markers. These data suggest that eGFP-expressing cells represent the distribution of neural progenitors in embryos and adults. The generation of this transgenic line allows researchers to visualize neural progenitors by using specific regulatory elements.

By using this transgenic reporter line, the properties of adult neural progenitors can be studied extensively. However, it is not known how specific eGFP-expression is, especially in injured brains. Many studies describe how the expression of nestin is up-regulated in several different types of cells in the brain in a variety of brain injury models. For example, expression levels of nestin are up-regulated in reactive astrocytes, active microglia, and NG-2 cells in injured brain (Ridet et al., 1997;Belachew et al., 2003;Aguirre and Gallo, 2004;Yokoyama et al., 2006). To investigate how neural progenitors respond to the brain injury, it is critical to clarify the expression pattern of the reporter genes in the transgenic mice.

To achieve this, I examined three previously generated transgenic lines that have expression of eGFP driven by the nestin promoter and neural progenitor-specific regulatory element. One specific line (PN18) was demonstrated to display a high level of eGFP expression that is restricted to neural progenitors of

adult brains. These eGFP-expressing cells represent the distribution of early neural progenitors and are distinguishable from late neural progenitors in the dentate gyrus. Different from previous studies, a small population of early neural progenitors was identified in the granular layers. These early neural progenitors are relatively quiescent under normal physiological situations.

Results

Spatial and Temporal Characteristics of eGFP-expressing Cells in the Transgenic Lines:

To express the reporter gene, eGFP, specifically in neural progenitors, a 5.4-Kb fragment of nestin promoter and a well-characterized and conserved a 0.7-Kb fragment of regulatory element in nestin's second intron were used to restrict the expression of the transgene in neural progenitors (Fig. 2-1) (Lendahl and McKay, 1990;Zimmerman et al., 1994;Panchision et al., 2001). Twenty-three independent lines were generated initially, and three of these were extensively characterized. In all examined lines, eGFP expression is identical, although each differs in the degree of eGFP intensity. In all three maintained stable lines, eGFP expression was observed to mimic that of the neural-specific form of nestin, with its expression limited to the neural tube. It was not present in the somites throughout the embryonic stages (Fig. 2-2). In the adult brain, the eGFP-expressing cells were predominantly detected in the lateral ventricles and the dentate gyrus (Fig. 2-3).

eGFP-expressing Cells in the Transgenic Lines Possess Characteristics of Neural Progenitors in vitro:

I demonstrated that the appearance and distribution of eGFP-expressing cells is consistent with previously published data on native neural progenitors in the previous section. To further demonstrate that eGFP-expressing cells in the transgenic lines represent neural progenitors, several criteria must be met. The first issue that must be addressed is to make sure that eGFP-expressing cells are able to proliferate and form neurospheres in the presence of growth factors *in vitro*. To this end, eGFP-expressing cells from the adult hippocampus and lateral ventricles were harvested via fluorescence-activated cell sorting (FACS) and were grown clonally in neural progenitor growth medium with 20ng/ml of basic fibroblast growth factor (bFGF). Neurospheres formed 14 days after culturing from single sorted cells. By performing immunofluorescence to identify the molecular markers, neurospheres were composed of cells that expressed eGFP, GFAP and nestin. This experiment indicated that eGFP-expressing cells are able to proliferate and form neurospheres. By growing cells from dissected hippocampus and the lateral ventricles, the formed neurospheres consisted of eGFP-expressing cells in the presence of bFGF. After dissociation of neurospheres, the cells were able to form neurospheres when cultured clonally, suggesting that eGFP-expressing cells possess self-renewal ability (Fig. 2-4). To demonstrate the multipotency of eGFP-expressing cells, dissociated cultured neural progenitors were allowed to differentiate in serum-containing medium. By

using antibodies to identify specific cell markers, GFAP-expressing astrocytes, NeuN-expressing neurons, and RIP-expressing oligodendrocytes were detected 21 days after differentiation, suggesting that cultured GFP-expressing cells maintain characteristics of neural progenitors *in vitro*.

eGFP-expressing Cells Represent Adult Neural Progenitors in vivo:

In these transgenic lines, eGFP expression is controlled by the nestin promoter and its neural progenitor-specific regulatory element. To examine whether eGFP-expressing cells express nestin, an antibody that identifies nestin was used. eGFP-expressing cells in neurogenic areas were shown to colocalize with nestin expression in all ages that were examined (Fig. 2-5). The expression of nestin is downregulated in the adult brain (Dahlstrand et al., 1995), not all the eGFP-expressing cells in the adult brains have nestin expression due to the difficulty in labeling nestin by the antibody. eGFP-expressing cells are detectable in these lines since transgenic mice usually possess multiple copies of this transgene. In the adult dentate gyrus, several subtypes of neural progenitors have been identified based on their molecular markers and ages. To determine which subtypes of neural progenitors eGFP-expressing cells represent, antibodies that identify several different molecular markers were chosen for the immunofluorescence and confocal microscopy. These techniques were used to

determine the colocalization of markers in eGFP-expressing cells. Noticeably, a subset of eGFP-expressing cells obtain a long process penetrating the granular layer and arborizing in the molecular layer. These eGFP-expressing cells have expression of putative neural stem cell markers, like GFAP and vimentin (Fig. 2-5 and Fig. 2-6). These properties of eGFP-expressing cells are similar to the previously described type 1 neural stem cells. These type 1-like eGFP-expressing cells were detected not only in the subgranular layers (Fig. 2-6, a-c, arrow) but also in the granular layers (Fig. 2-6, d-f, arrow). Besides GFAP and vimentin, these eGFP-expressing cells also demonstrate nestin expression (Fig. 2-5, a-c, arrow).

Another subset of eGFP-expressing cells did not have long processes. This population did not express neural stem cell markers, but retained nestin expression (Fig. 2-5, a-c, dash arrow). Different from type 1-like eGFP-expressing cells, they did not have long processes and were observed in the subgranular layers only. These features suggest that they are type 2a-like neural progenitors. As a type 2a cell differentiates, eGFP expression is down regulated and the immature neuronal marker doublecortin (Dcx) begins to be expressed. A type 2b cell represents this transition between early and late progenitors and no longer expresses eGFP but does express Dcx (Fig. 2-7). Consistent with other studies, these eGFP-expressing cells are adjacent to blood vessels and mature

astrocytes (Fig. 2-5, d-i). According to the molecular markers that eGFP-expressing cells retain, one could divide the adult neural progenitors in the adult dentate gyrus into two categories: eGFP-expressing cells represent the type 1- and type 2a-like early neural progenitors and Dcx-expressing cells are the late neural progenitors in these transgenic lines (Fig. 2-8).

Quantification of eGFP-expressing Cells and Their Proliferation in the Adult Dentate Gyrus

To further demonstrate that eGFP-expressing cells represent the neural progenitors, it is important to verify their ability to proliferate independent of their expression of molecular markers. To examine whether eGFP-expressing cells are able to divide, 8-week old mice were injected with thymidine analogue, bromodeoxyuridine (BrdU), 12- and 2-hours before being sacrificed. To quantify the number of GFP-expressing cells and BrdU-incorporated eGFP-expressing cells, serial 12^{th} sections encompassing the hippocampus were used for immunofluorescent staining and confocal microscopy. Most of eGFP-expressing cells were observed in the subgranular layer (Fig. 2-9, a: 1067+/-92 and 973+/-111 in the left and right dentate gyrus, respectively), only small number of eGFP-expressing cells were quantified in the granular layer (Fig. 2-9, a: 173+/-47 and 179+/-65 in the left and right hemispheres, respectively). Many studies

demonstrate that type 1 neural stem cells bear a low frequency of proliferation. However, type 2a cells have a high proliferation rate. Consistent with this, more BrdU-incorporated eGFP-expressing cells were counted in the subgranular layer when compared with the number in the granular layer (Fig. 2-9, b: 46+/-15 and 43+/-27 in the left and right SGLs, respectively; 1+/-0.5 and 3+/-2 in the left and right GLs, respectively). As described in the previous paragraph, type 2a-like eGFP-expressing cells were abundant in the subgranular layer. Most of the counted BrdU-incorporated eGFP-expressing cells were from this population.

Discussion

By analyzing three transgenic lines that have eGFP expression driven by the promoter of the neural progenitor-specific regulatory element of the nestin gene, eGFP-expressing cells were observed in the neural tube during the embryonic stage and in the neurogenic areas in the adult brain. By performing *in vitro* culturing assays, the eGFP-expressing cells from the adult hippocampus or lateral ventricles are able to form neurospheres and differentiate into neurons, astrocytes and oligodendrocytes. This suggests that eGFP-expressing cells are neural progenitors. The BrdU-pulsing analysis revealed that eGFP-expressing cells do proliferate, and they also retain the expression of known neural progenitor markers. These data suggest that the eGFP-expressing cells are neural progenitors *in vivo*. Interestingly, the expression level of eGFP is down-regulated and is not detectable by immunofluorescent staining in the Dcx-expressing late neural progenitors in the adult dentate gyrus. Therefore, I am able to divide the neural progenitors into two categories based on the expression of eGFP, which indentifies the early neural progenitors, and Dcx, which identifies the late neural progenitors. Furthermore, a subset of early neural progenitors is identified in the granular layers instead of subgranular layers. Depending on the quantification, those early neural progenitors are relatively quiescent compared to cells in the subgranular layers.

Distinct from the transgenic mice generated by Yamaguchi and his colleagues, the promoter fragment in our transgenic construct is around 5.5-Kb compared to 2.5-Kb in Yamaguchi's construct, and the neural progenitor-specific regulatory element in the construct is around ~0.7-Kb, compared to~1.8-Kb in Yamaguchi's construct. Although it is not known whether the extra 3-Kb in the promoter region of the nestin gene possesses any function in regulating nestin expression, it has been reported that the fragment in the third intron of nestin gene, which is present in Yamaguchi's construct, may regulate the expression of nestin in astrocytes, especially in reactive astrocytes (Johansson et al., 2002). This could explain the result from a recent study demonstrating that protoplasmic astrocytes in CA1 region display the expression of eGFP in Yamaguchi's transgenic mice (Kronenberg et al., 2007). The ectopic expression of eGFP is barely observed in the adult transgenic mice that we generated. This indicates that the eGFP expression in our transgenic mice is more specific than Yamaguchi's.

Similar to other studies, the type 1-like eGFP-expressing cells in the dentate gyrus reside along the subgranuar layers and have long processes penetrating the granular layers and arborizing in the inner molecular layers. These cells are adjacent to the blood vessels that wander along the subgranular layers and are

close to the hilar mature astrocytes (Palmer et al., 2000;Seri et al., 2004). Another type of eGFP-expressing cell in the subgranular layer is the type 2a-like neural progenitor. These cells share the same microenvironment with type 1-like eGFP-expressing cells. These cells do not have long processes and the expression levels of type 1 neural progenitor markers are down-regulated. Although some studies point out that a subset of Dcx-expressing late neural progenitors retain the expression of nestin (Seri et al., 2004), I did not observe any overlap in expression of eGFP and Dcx. This could be the result of the sensitivity of my chosen antibody, the half life of eGFP protein or the method to process brain tissues.

Different from others' observations, a small population of type 1-like eGFP-expressing cells was identified in the granular layers. These cells also have long processes and are immuno-positive to known early neural progenitor markers. Interestingly, no type 2a-like eGFP-expressing cells have been identified. This explains why a low frequency of proliferation is observed in the granular eGFP-expressing cells when compared with ones in the subgranular layers. It is not clear what the role of these type 1-like cells in the granular layers is. Whether they generate neurons or not remains unknown. Another interesting question is do granular type 1-like eGFP-expressing neural progenitors have a similar niche microenvironment as ones in the subgranular layers? This requires further study.

I have identified that eGFP-expressing cells represent the distribution of adult neural progenitors in adult brains. Due to the effectiveness of the chosen promoter and regulatory element in the construct, I decided to examine the specificity of expression in injured brains and to describe how adult neural progenitors respond to the injuries.

Figures

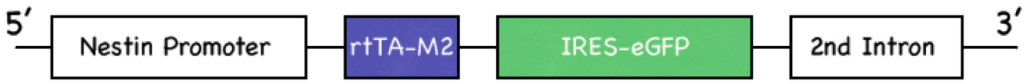

Fig. 2-1. Scheme of transgenic construct. A 5.5-Kb fragment of nestin promoter and a 0.7-Kb regulatory element in second intron of nestin gene were used to restrict the expression of the transgene.

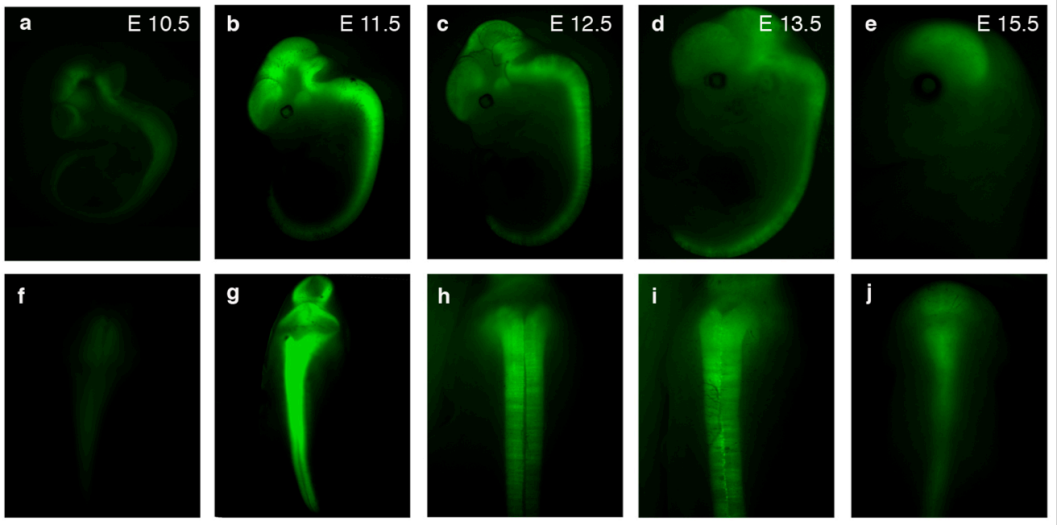

Fig. 2-2. Nestin-rtTA-M2-eGFP transgenic mice express eGFP exclusively in the developing neural tube. eGFP is detected in transgenic mice at E10.5 (a,f) and peaks at E11.5-12.5 (b,g,c,h) and then becomes downregulated towards later gestation (e,j). Dorsal views demonstrate that eGFP is expressed exclusively in the neural tube and unlike full nestin, promoter expression is absent in the somite (f-j).

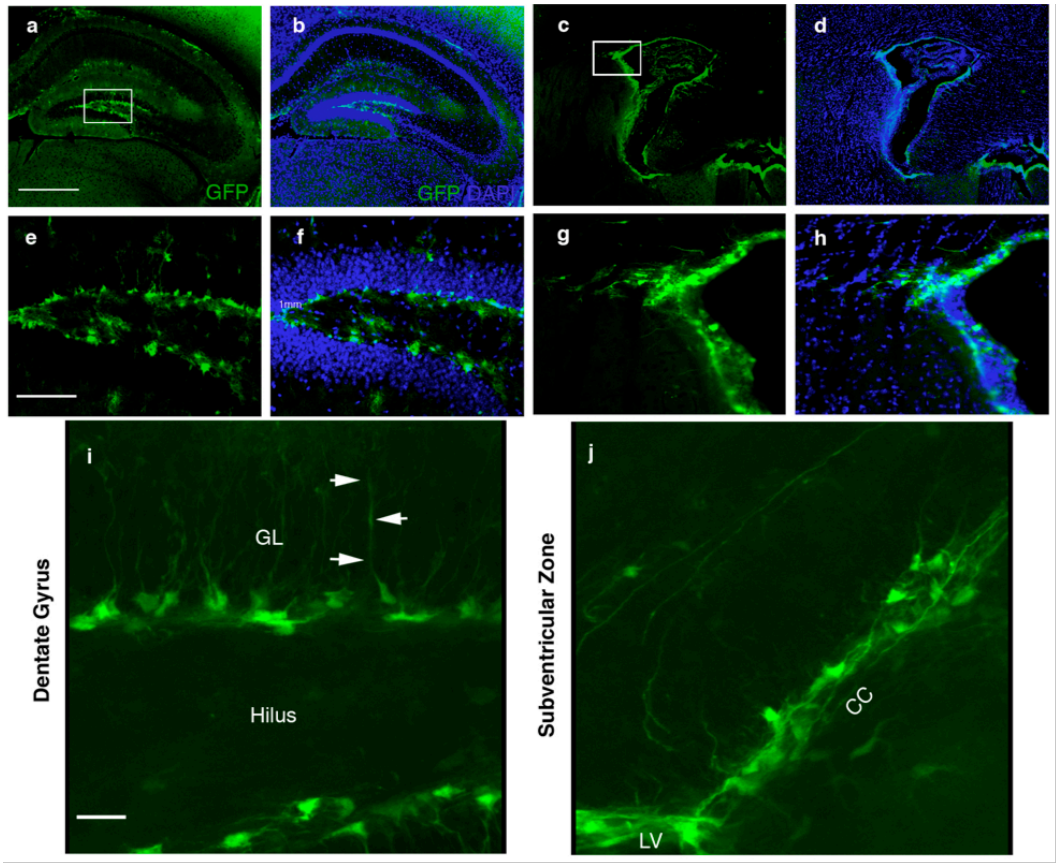

Fig. 2-3. Adult nestin-rtTA-M2-eGFP transgenic mice express eGFP exclusively in the neurogenic areas. Adult transgenic mice express eGFP exclusively in the subgranular zone of the dentate gyrus (a,b,e,f) and in the subventricular zone of the lateral ventricles (c,d,g,h). e-h: Enlargements of boxed areas from a (e,f) and c (g,h). Confocal images of endogenous GFP expression demonstrate that cells from the sub-granular zone of the dentate gyrus extend long process that penetrate through the granular layer (i, arrows). GFP-positive cells also migrate out of the subventricular zone into the corpus callosum and, like precursors in the dentate gyrus, extend GFP-positive processes (j). GL, granular layer; LV, lateral ventricle; CC, corpus callosum; Scale bars = 500 um (a), 100 um (e), 20 um (i).

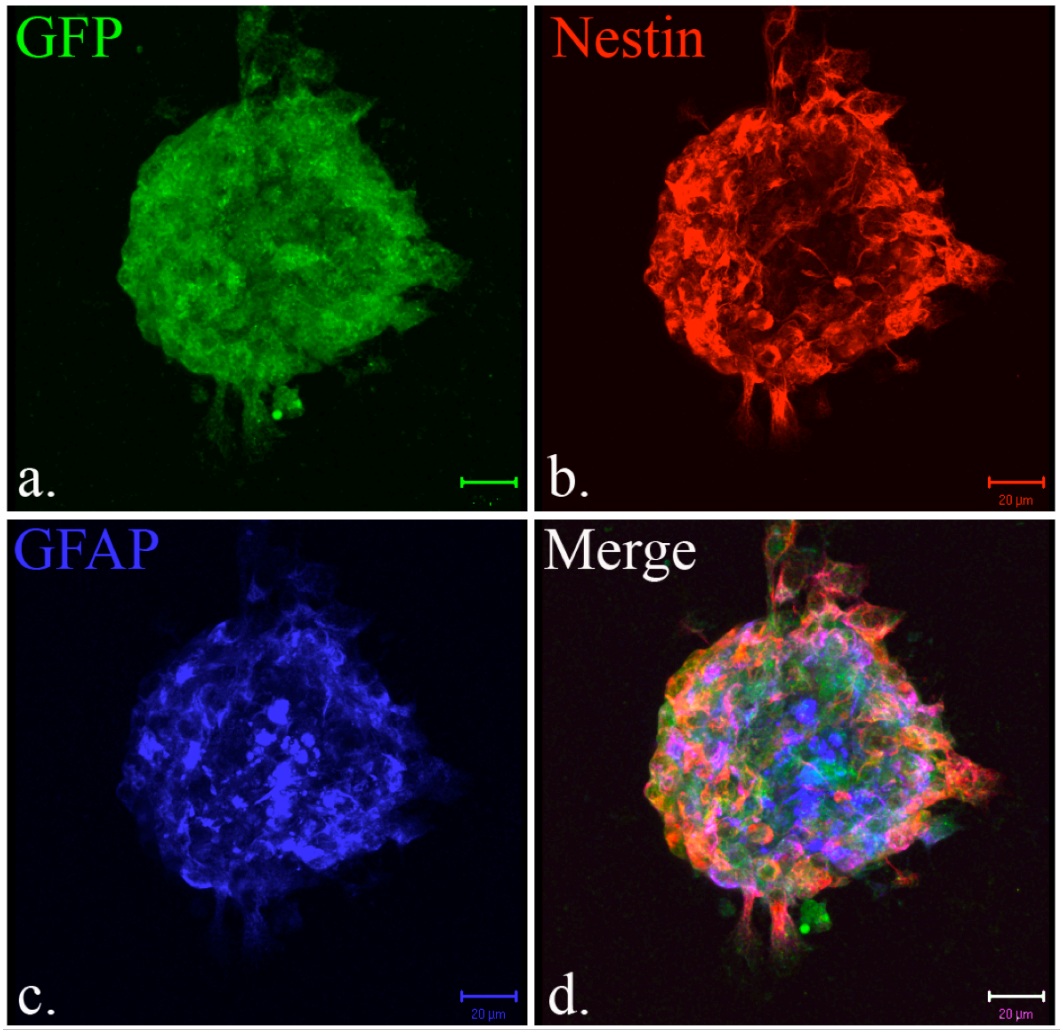

Fig. 2-4. Clonally-cultured eGFP-expressing cells proliferate and form neurospheres. (a) Neurospheres are composed of eGFP-expressing cells. (b). eGFP-expressing cells express nestin, a well-known marker of neural progenitors. (c) Some of eGFP-expressing cells in neurospheres display expression of GFAP, a marker of putative neural stem cells. (d) A representative image of neurospheres formed from a FAC sorted eGFP-expressing cells. Scale bar = 20µm.

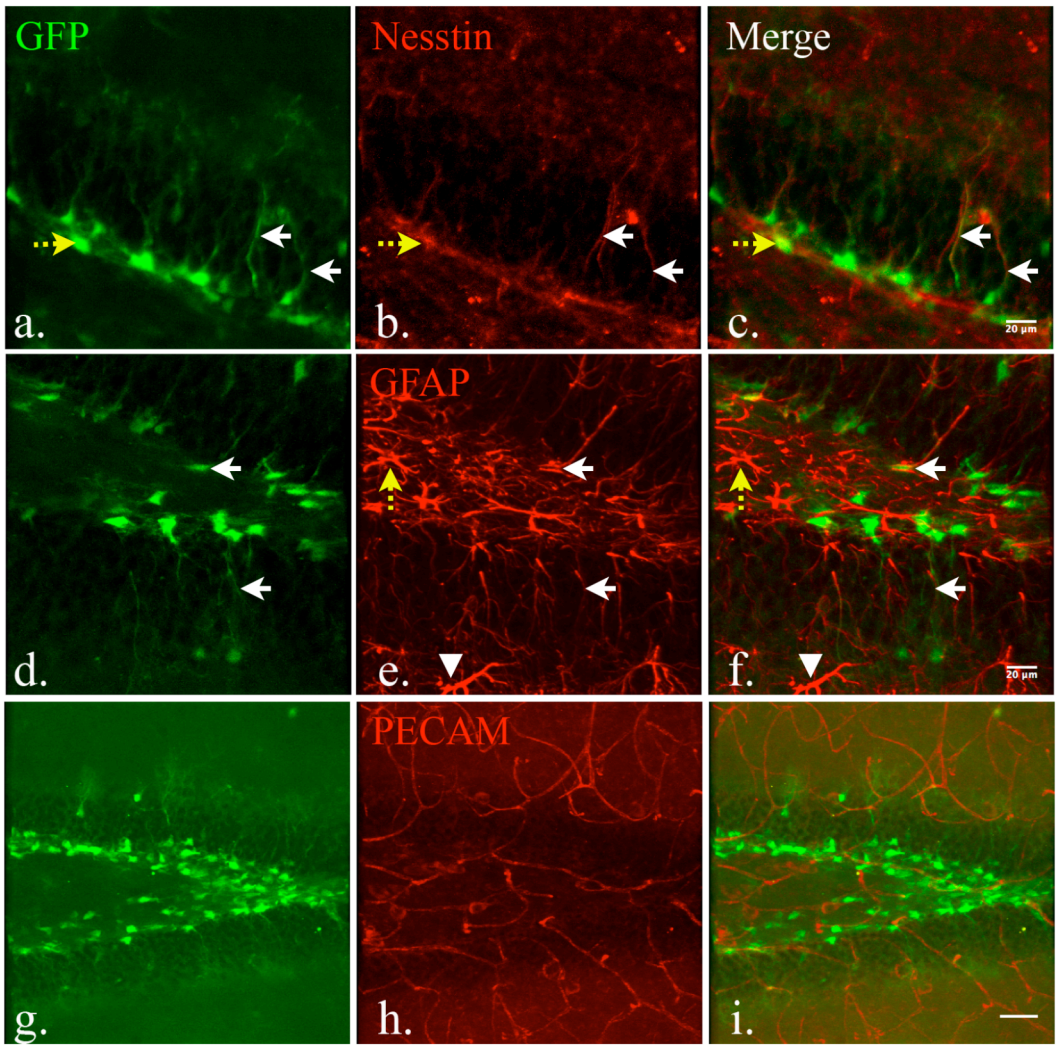

Fig. 2-5. Microenvironment in the adult dentate gyrus. (a-c) Type 1-like eGFP-expressing cells also express nestin (arrow). Another subset of eGFP-expressing cells residing in the subgranular layers do not have a long process, but they still have the expression of nestin (dash arrow). They are type 2a-like neural progenitors. (d-f) These eGFP-expressing cells possess expression of another neural stem cell marker, GFAP (arrow) These cells tend to have a long process and their morphology is consistent with type 1 neural progenitors. The eGFP-expressing cells are surrounded by the mature astrocytes in the hilus (dash arrow) and molecular layers (triangles). (g-i) The dentate gyrus is highly vascularized as revealed here by using PECAM as a marker to visualize blood vessels. eGFP-expressing cells are adjacent to the blood vessels in the subgranular layers. Scale bars represent 50um in i and 20um in c and f

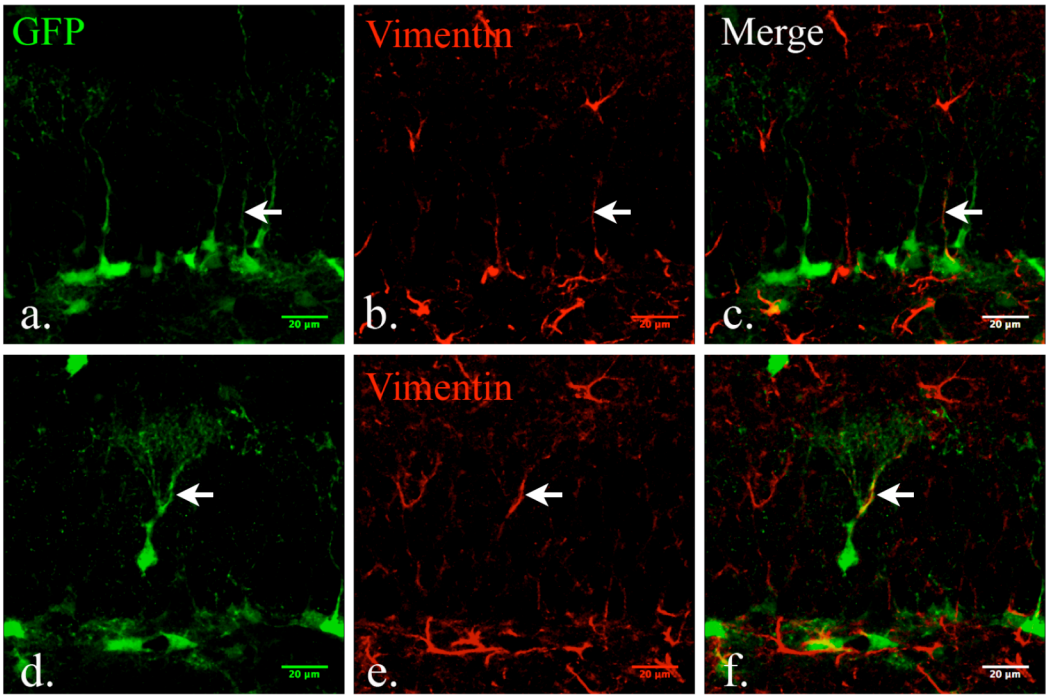

Fig. 2-6. Type 1-like eGFP-expressing cells in the dentate gyrus. (a-c) A subset of eGFP-expressing cells in the subgranular layer express one of the known neural stem cell markers, vimentin (arrow). (d-f) The type 1-like eGFP-expressing cell is detected not only in the subgranular layer but also in the granular layer (arrow). Scale Bars indicate 20µm.

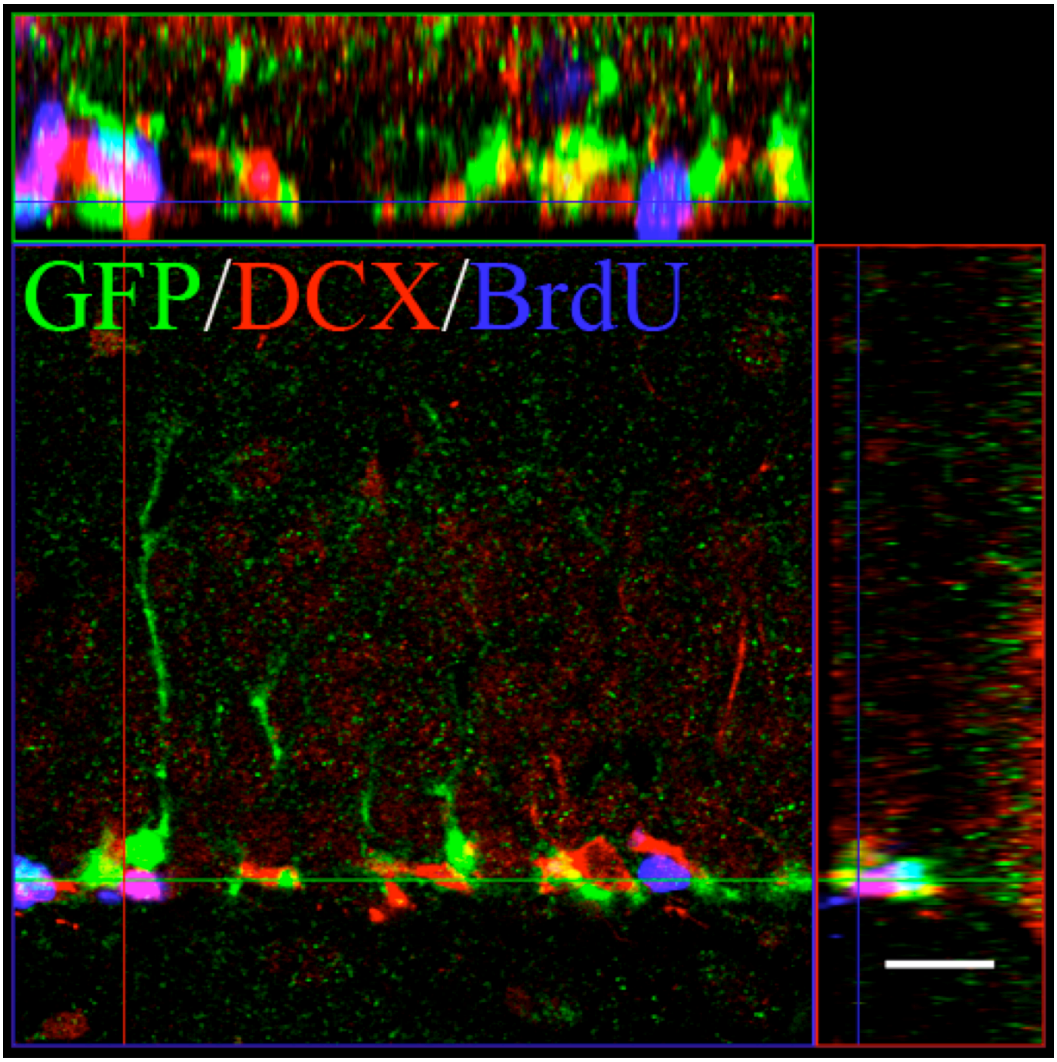

Fig. 2-7. A representative confocal image demonstrates that Dcx expression is not detectable in eGFP-expressing cells. eGFP-expressing cells and Dcx-expressing cells reside next to each other in the subgranular layers of the adult dentate gyrus. Scale bar; 20μm.

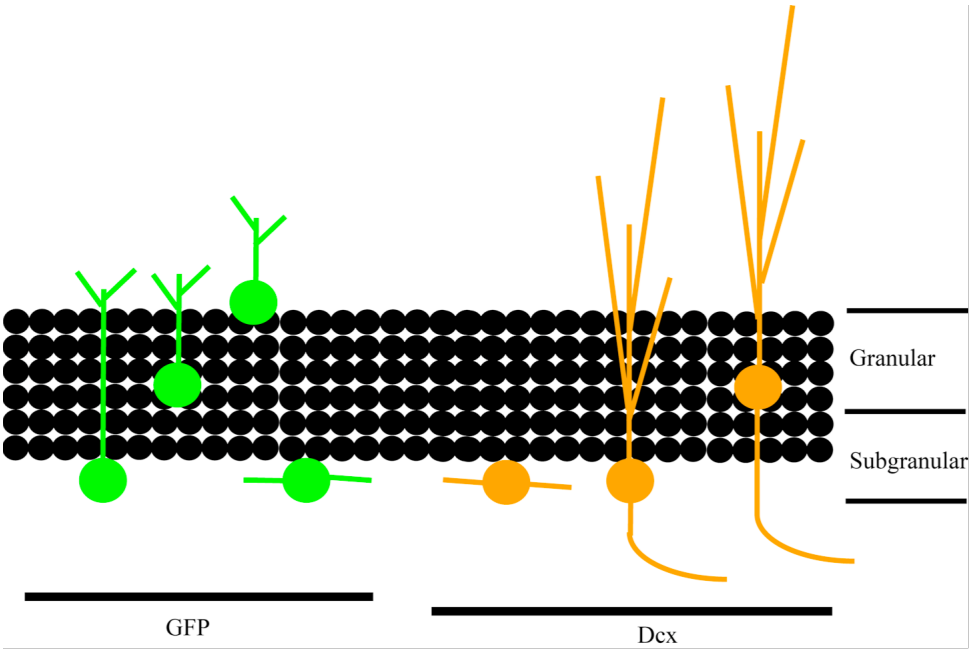

Fig. 2-8. This scheme represents the distribution of eGFP-expressing cells in the adult dentate gyrus. eGFP-expressing cells are found in both the subgranular and granular layers. The eGFP-expressing cells with long processes have expression of neural stem cell/progenitor markers, like GFAP, vimentin, and nestin. The eGFP-expressing cells without long processes are found in the subgranular layers only. They do not express neural stem cell markers except for nestin. However, eGFP-expressing cells are distinguishable from late neural progenitor markers, like Dcx and NeuroD.

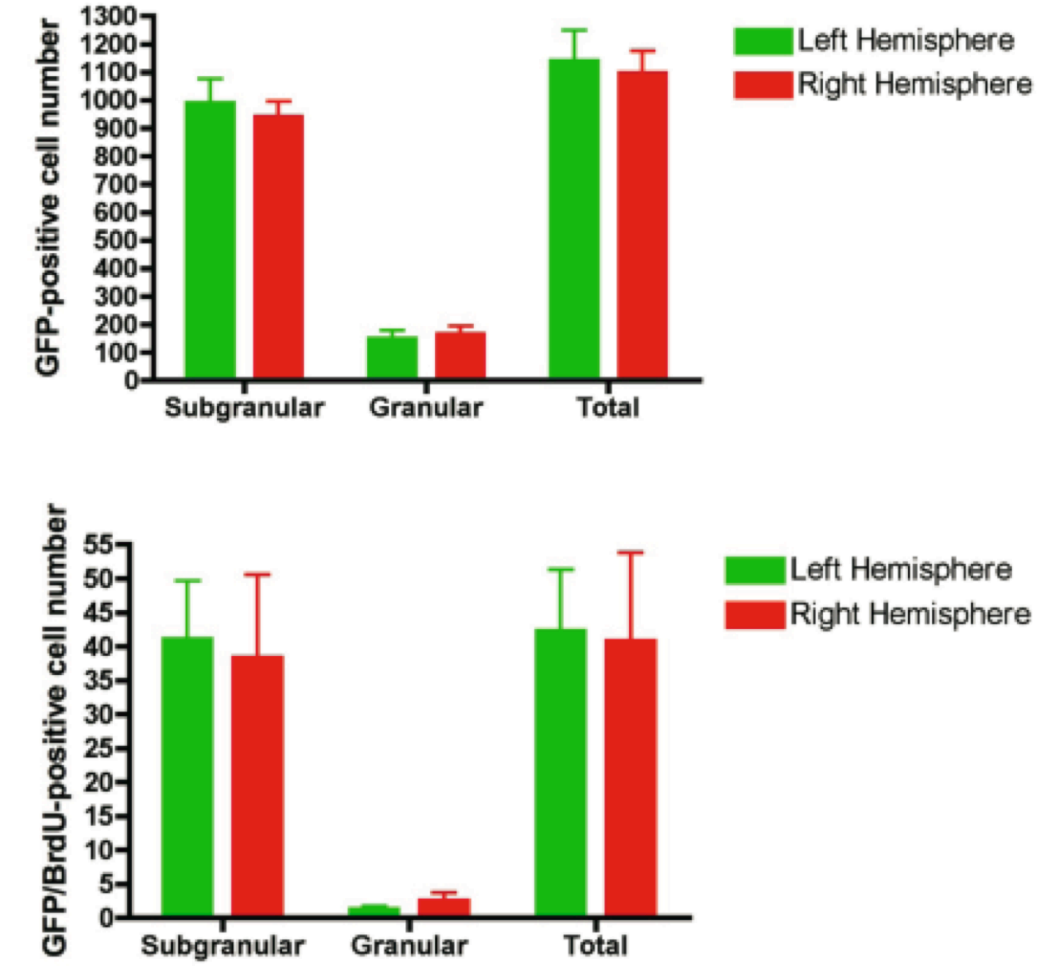

Fig. 2-9. Quantification of eGFP-expressing cells and proliferating cells in the adult dentate gyrus. (a) Most of the eGFP-expressing cells were counted in the SGLs (1067+/-92 and 973+/-111 in the left and right dentate gyrus, respectively). Only a small population of eGFP-expressing cells were detected in the GLs (173+/-47 in the left and 179+/-65 in the right dentate gyrus). (b). BrdU incorporation was used to label the cells in the S phase of mitosis. The quantification data suggests that cells in the granular layer are relatively quiescent. (N=4), *no significant difference is revealed in unpaired Student's t test*.

Materials and methods

Pronuclear Injection

All protocols involving the use of animals were approved by the Institutional Animal Care and Research Advisory Committee (IACRAC) at UT Southwestern Medical Center at Dallas. Pronuclear injection and oviduct transplantation of injected embryos was performed as described by Hogan et al. (1986) using either CD2 or B6D2F1 females. The plasmid for the nestin-rtTA-eGFP transgene was constructed by cloning a 750-bp rtTA-M2 cassette (gift of Dr. W. Hillen) next to a 1,300-bp IRES-eGFP cassette (Stratagene, La Jolla, CA). This 2-kb fragment was then cloned between the 5-kb nestin promoter and the 700-bp second intron of the nestin gene (gift of Dr. R. McKay). The DNA containing the nestin promoter, rtTA-M2, IRES-eGFP, and second nestin intron was prepared for pronulcear injection by SalI restriction enzyme digestion and then purified from 1% agarose gels with a Qiagen (Chatsworth, CA) gel purification kit. After injection, the eggs were transferred back to the oviducts of pseudopregnant CD2 female mice for development. After weaning at 3–4 weeks of age, the pups were genotyped (see below) and all transgene-positive offspring were kept to develop stable lines. Twenty-three stable transgenic lines were established by standard procedures and three of these were maintained for detailed characterization. The two lines characterized here have been designated PN9 and PN18.

Genotyping

Genotyping of embryos or adult mice for the nestin-rtTA-eGFP transgene was carried out using the following PCR conditions: 94°C, 30 s; 63°C, 30 s; 72°C, 30 s; 30 cycles. The primers for GFPPCR are: 5'-GAG CTG GAC GGC GAC GTA AAC-3' and 5'-CGT TGT GGC TGT TGT TAG TTG TAC-3'. Genotyping was confirmed with PCR to rtTA-M2 as well with the same conditions as above except for an annealing temperature of 59°C. Primer sequences for rtTA were 5'-GGA CAA GAG CAA AGT CAT AAA CGG-3' and 5'-TTC GTA CTG TTT CTC TGT TGG GC-3'. Tet-Op-Cre mice (gift of L. Monteggia) were genotyped using the Cre-specific primers 5'-CCG TTT GCC GGT CGT GGG-3' and 5'-CG TAT ATC CTG GCA GCG ATC-3' at the same conditions as above but an annealing temperature of 63°C. Stop-LacZ-ROSA26 mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and genotyped according to published protocols (Soriano, 1999).

Immunofluorescence

All mice were deeply anesthetized with a ketamine and xylazine mixture as described above before perfusion. The transcardiac perfusion was performed with 50ml of 1xPBS, followed by 50ml of 4% paraformaldehyde (PFA)/1xPBS. Following post-fixation in 4% PFA/1xPBS overnight, the whole brains were

dissected and embedded in 3% agarose/1xPBS. Serial 50-μm sections were cut with a vibratome (VT1000S, Leica). All sections encompassing the hippocampus were collected in every well sequentially in 12-well plates. Free-floating method was used for the immunohistochemistry. For BrdU staining, all sections from a single well were washed with 1xPBS 3 times and rinsed with water. Then, sections were denatured with 0.1N HCl for 1 hr in 37°C water bath. After denaturation, sections were neutralized with 0.1M Borax (Sigma), pH8.5 for 10 min. Sections were washed with 0.3% Triton X-100/1xPBS (wash buffer) for 3 times and blocked with 5% normal donkey serum (Sigma-Aldrich) containing wash buffer for 1 hr at room temperature. 1:200 rat anti-BrdU (Abcam) antibody was used to label BrdU by incubating overnight at 4°C. The following day, sections were incubated with Cy5-conjugated anti-rat antibody (all 1:200, Jackson Immunoresearch) for 3 hrs at room temperature. The sections were placed on the slides and covered with coverslips for confocal microscope observation after antibody incubation. For regular staining the protocol was as above for the BrdU except sections were not treated with HCl and Borax. Primary antibodies used in this study were as follows: rabbit anti-GFP (1:500, Molecular Biology), chicken anti-GFP (1:200, AbCam), mouse anti-GFAP (1:100, BD Pharmingen), rabbit anti-GFAP (1:500, DAKO), goat anti-doublecortin (1:100, Santa Cruz biotech), mouse anti-vimentin (1:200, 40E-C, Hybridoma Bank, Univ. Iowa), rabbit anti-Ki67 (1:200, NeoMarkers), mouse anti-NeuN (1:500, Chemicon), rabbit anti-NG2

(1:200, Chemicon), rat anti-PDGFRa (1:200, BD Pharmingen), and goat anti-Iba-1 (1:200, AbCam). Cy2-, Cy3- or Cy5-conjugated donkey anti-species antibodies were used as secondary antibodies (all 1:200, Jackson ImmunoResearch).

Quantification and Confocal Microscopy

For BrdU-positive cell quantification, a series of every 12th sections was used. BrdU-positive cells were counted under a 40x objective (Olympus) through the whole blades of the dentate gyrus. According to the location, counted cells were subdivided into two categories, in the subgranular or granular layers. The subgranular layer (SGL) was defined as the region encompassing two-cell widths above and below the boundary between the granular layer and hilus. The rest was defined as granular layer in this study.

To quantify the cell number and determine the proliferation index of early and late neural progenitors in the dentate gyrus, a Zeiss LSM510 scanning confocal microscope with Argon 488, He 543, and He 633 lasers and a Zeiss Neofluar 40x/1.3 oil DIC lens was used to determine each single cell and the colocalization of different cell markers. Cell quantification was performed from the dentate gyrus in both hemispheres in injured and control mice. Every 12th section was chosen for immnofluorescent staining to label eGFP, Dcx and BrdU. The images covering whole blades of the dentate gyrus were taken and the 50μm z-axis was scanned with a consistent 2um interval. The number of eGFP-or Dcx-expressing

cells was counted through each frame by using ImageJ (NIH) without any adjustments of color, contrast, or brightness. The colocalization of eGFP or Dcx with BrdU was determined only when BrdU signal was clearly covered or wrapped with eGFP or Dcx signal, respectively. The number of colocalized cells was counted frame by frame.

Statistics

Statistics were done using an unpaired Student's t test. Differences were deemed significant with $p<0.05$.

CHAPTER THREE
Dynamics of Neural Progenitors in the Dentate Gyrus in Injured Brains

Introduction

Adult neurogenesis is modulated by many factors under normal physiological conditions, like endogenous hormones, age, genetic background or behavior (Lledo et al., 2006). Adult neurogenesis is also affected under pathological conditions, including acquired brain injuries (Lichtenwalner and Parent, 2006;Leker, 2006;Miles and Kernie, 2006;Romanko et al., 2004;Parent, 2003;Peterson, 2002).

Acquired brain injury is a neurological condition that is defined as damage to the brain acquired after birth. The injury usually affects cognitive, emotional, physical and other functions (Rees et al., 2007;Arciniegas and Silver, 2006;Starkstein and Jorge, 2005;Nortje and Menon, 2004). Depending on the cause, acquired brain injuries can be divided into non-traumatic brain injury (such as stroke, hypoxia, ischemia) and traumatic brain injury (resulting from accidents, falls, etc). Traumatic brain injury (TBI) is the most common form of acquired brain injury in both children and adults in the United States (Chirumamilla et al., 2002; Marshall, 2000). Furthermore, TBI causes neuronal loss and results in a variety of neurological and cognitive deficits, especially in hippocampus-

dependent functionality (Levin, 1998; Bramlett et al., 1997). To study traumatic brain injury and investigate its pathology, several animal models have been utilized (Prins and Hovda, 2003).

One of the models is a unilateral controlled cortical impact (CCI) injury. Unilateral CCI injury model uses an air-driven piston to penetrate the brain of the experimental animals. It allows for control of the piston velocity and depth of penetration. By using this and other TBI models, many studies have demonstrated that the injury causes acute inflammatory reactions and immediate neuronal cell deaths around the injured areas (Simi et al., 2007; Fitch and Silver, 2007; Tatsumi et al., 2005; Sofroniew, 2005; Chen and Swanson, 2003; Morganti-Kossmann et al., 2001; Norton, 1999; Ridet et al., 2997; Kimelberg, 1992). Due to neuronal cell death, the neurotransmitter, glutamate, is released into the extracellular matrix and triggers extensive bursts and results in excitatory toxicity. Injury also leads to elevation of extracellular potassium concentration and it causes further neuronal degeneration (Yi and Hazell, 2006;Johnston, 2005;Petzold et al., 2005;Raghupathi, 2004;Arundine and Tymianski, 2004;Jensen, 2002;Akasu et al., 2002;Zhang et al., 2001;Stover and Unterberg, 2000;Mena et al., 2000;Raghupathi et al., 2000;Santhakumar et al., 2000). Besides the inflammatory response and neuronal cell deaths, another effect of TBI is that astrocytes in the brains become transcriptionally active. They have been

demonstrated to express embryonic genes, and become hypertrophic (Ridet et al., 1997). Although reactive astrocytes have long been known for their supportive roles in injured brains (Chen and Swanson, 2003;Sofroniew, 2005;Ridet et al., 1997), why developmental genes are expressed after injury remains undetermined.

Neuronal degeneration in the injured brain is progressive. The long lasting neuronal degeneration correlates with a variety of cognitive deficits (Pullela et al., 2006;Raghupathi, 2004;Arundine and Tymianski, 2004;Felderhoff-Mueser and Ikonomidou, 2000;Eldadah and Faden, 2000;Raghupathi et al., 2000). Indeed, the injury results in deficits in memory formation and retrieval in the Morris water maze test and some studies have demonstrated that injured mice display symptoms of depression and stress (Milman et al., 2005; McAllister et al., 2004; Lalonde and Strazielle, 2003; Rohling et al., 2003; Zhang and Sachdev, 2003; Walker, 2003; Andersson and Bergedalen, 2002). Along with neuronal degeneration, many studies point out that injury enhances neurogenesis, primarily in the dentate gyrus (Sgubin et al., 2007;Rola et al., 2006;Ernst and Christie, 2006;Ramaswamy et al., 2005;Rice et al., 2003;Kernie et al., 2001).

The adult neural progenitor is an attractive target to treat neuronal degeneration diseases and brain injuries. Although several animal studies have

demonstrated that transplanted adult neural progenitors could differentiate into neurons and form synaptic connections with existing neurons (Iwanami et al., 2005;Podgornyi et al., 2004;Jeong et al., 2003;Herrera et al., 1999;McDonald et al., 1999;Brüstle and McKay, 1996), the lack of ability to harvest and grow enough adult neural progenitors for transplantation is one limiting factor to this treatment. Another way to replace lost neurons is to enhance and guide endogenous neurogenesis. Before any manipulation of endogenous neural progenitors is possible, it is important to understand how neural progenitors respond to the brain injury.

Using BrdU to label dividing cells in injured brains shows a dramatic increase of BrdU-incorporated cells after brain injury (Parent et al., 2006;Rola et al., 2006;Miles and Kernie, 2006;Darsalia et al., 2005;Chirumamilla et al., 2002;Kernie et al., 2001). Combining this with mitotic markers, it has been shown that BrdU-positive cells undergo mitosis. A significant number of dividing cells are observed proximal to the injured region. The majority of the cells are active microglia and reactive astrocytes. Another region that exhibits an abundance of dividing cells is the lateral ventricles. By performing a BrdU-pulsing tracing experiment, the dividing cells in the lateral ventricles are identified as neural progenitors and it can be seen that they migrate to the injured region. While cells arrive to the injured area, neural progenitors differentiate into

astrocytes and participate in glial scar formation (Kernie et al., 2001).

The Hippocampus is another region that has a great number of dividing cells after injury. The dividing cells in this region have been identified as reactive astrocytes, microglia, NG2 cells, oligodendrocyte precursor cells (OPC) and adult neural progenitors (Rola et al., 2006). Although neuronal death is observed in the hippocampus (Tashlykov et al., 2007; Griesemer and Mautes, 2007; Pullela et al., 2006; Otani et al., 2006; Yoshimura et al., 2003), newborn neurons are also detected in the dentate gyrus (Kernie et al., 2001). In these studies, nestin expression is used as an indicator of the neural progenitor presence. Interpretation of data from experiments based on nestin expression has to be done cautiously, especially in injured brains. Several studies have mentioned that an upregulation of nestin expression occurs in reactive astrocytes, in microglia and even in NG2 cells (Yokoyama et al., 2006;Ridet et al., 1997). This increases the difficulty of identifying the source of newborn neurons in the injured brains.

By performing unilateral CCI injury in this study, I used previously characterized transgenic mice to determine whether eGFP expression in the dentate gyrus remains restricted to the early neural progenitors after brain injury. Using Dcx as the late neural progenitor marker, I further describe the response of neural progenitors to unilateral CCI injury.

Results

eGFP-expressing Cells Are Distinguishable from Other Cell Types Following Injury:

Nestin itself is known to be expressed in several cell types, such as NG2-expressing oligodendrocyte progenitor cells, reactive astrocytes, and endothelial cells -- especially in injured brains (Yokoyama et al., 2006;Belachew et al., 2003;Ridet et al., 1997). Although the promoter and regulatory element of the nestin gene we used are specific to neural progenitors, it is not known whether those fragments are involved in nestin expression in other cell types. To clarify whether eGFP was expressed in other cells after injury, CCI injury was performed on 8-week old male transgenic animals. In mice sacrificed three days after CCI, eGFP-expressing cells in the dentate gyrus were distinguishable from reactive astrocytes that were defined as cells with upregulated expression of GFAP and hypertrophic (Fig. 3-2, a). Likewise, we demonstrate that eGFP-expressing cells did not express NG-2, PDGFRα, or Iba-1 (Fig. 3-2, b-d). Therefore, we conclude that eGFP-expressing cells in the dentate gyrus were distinguishable from NG2-expressing oligodendrocyte precursor cells, reactive astrocytes, and active microglia and thus represent early neural progenitors.

Early and Late Neural Progenitors Demonstrate Opposing Responses Following Unilateral CCI Injury:

To describe the dynamics of adult neural progenitors in the dentate gyrus after CCI injury, we used 8-week old male eGFP-expressing transgenic animals and performed unilateral CCI injury and sacrificed them 1, 3, and 7 days after injury and compared them to uninjured littermates. 12 and 2 hrs before sacrifice, a BrdU dose (100mg/kg) was given to label the dividing cells. The injured site was restricted to the cortex overlying the hippocampus.

In 8-week old transgenic mice, the distribution of eGFP-expressing and Dcx-expressing cells in the dentate gyrus are as described in the prior chapter (Fig. 3-3, a-b). The number of BrdU-positive cells is small (Fig. 3-3, c). Following injury, these two adjacent progenitor populations behave with distinct differences. At 72 hrs following CCI injury, the injured side demonstrates upregulation of eGFP-expressing progenitors whereas the Dcx-expressing late progenitors are significantly ablated, and this occurs in a highly proliferative environment as evidenced by BrdU pulsing (Fig. 3-3, e-h). At 7 days post-injury, eGFP-expressing cells increase further on the ipsilateral side and the Dcx-expressing population reestablishes itself while the proliferative environment is maintained (Fig. 3-3, i-l). Interestingly, the contralateral side also demonstrates

activation of eGFP-expressing progenitors, but unlike the injured side, it has enhanced numbers of Dcx-expressing late progenitors as early as 7 days following injury (Fig 3-3, m-p).

In order to determine the amount and location of eGFP- and Dcx-expressing progenitors following injury, we quantified their distribution within the dentate gyrus. In the subgranular layer, there is no change in the number of eGFP-expressing cells at 1, 3, or 7 days following injury (Fig. 3-4, a). In the granular layer, however, there is a clear increase that occurred 3 days after injury and persisted for at least 7 days (Fig. 3-4, a). Doublecortin-expressing cells decrease at 72 hrs but then rebound by 7 days within the subgranular layer and actually increase above baseline in the granular layer (Fig. 3-4, c). The decrease in the number of Dcx-expressing cells is due to the initiation of cell degeneration 24 hours after injury as revealed by performing Fluoro Jade C staining (Fig. 3-6, a-c). On the contralateral side, where the decrease in cell number in the hippocampus is not observed, the increase in eGFP and doublecortin expression is even more dramatic, whereby there is an increase of both eGFP-expressing cells in the granular layer and Dcx-expressing cells after 7 days in both the subgranular and granular layers (Fig. 3-4, b, d).

Proliferation of eGFP-expressing Cells and Degeneration of Dcx-expressing Cells:

Next, we used BrdU incorporation as a marker to determine what types of cells were proliferating at the time of sacrifice (1, 3, and 7 days post injury). Since BrdU incorporation after injury can, at least in theory, occur during DNA repair (Bauer and Patterson, 2005), we used this method to examine whether BrdU-positive cells coexpress a marker of cell division, Ki67. We determined that all cells that express BrdU also express Ki67 though also as expected, not all Ki67-expressing cells express BrdU (Fig. 3-6, g-i). In addition, in the subgranular zone BrdU-positive cells co-localized largely, but not exclusively, with eGFP-expressing progenitors (Fig. 3-6, d-f). When quantified, we demonstrate that the number of eGFP-expressing cells that are dividing at the time of sacrifice increase most significantly in the ipsilateral granular layer at 3 and 7 days after injury (Fig. 3-5, a). Interestingly, on the contralateral side, this did not occur until 7 day after injury in both the subgranular and granular layers (Fig. 3-5, b). We also quantitatively examined Dcx-expressing late progenitors in which we observed essentially no Dcx expression in dividing cells in the granular layer at any point after injury, though we observed increased numbers in the subgranular layer on both the ipsilateral and contralateral sides 7 days after injury (Fig. 3-5, c-d). Together, these findings suggest a vulnerability of Dcx-expressing type 2b neural

progenitors and a proliferative response of nestin-expressing type 1 and/or type 2a neural progenitors due to injury.

Replenishment of Lost Dcx-expressing Cells Might Be Derived From Activation of eGFP-expressing Cells

I have observed that CCI causes the loss of Dcx-expressing late neural progenitors at the same time that eGFP-expressing early neural progenitors become active. Because the time when the replenishment of lost Dcx-expressing cells occurs is correlated with their development from eGFP-expressing cells (Zhao et al., 2006;Seri et al., 2001), it indicates that replenishment of Dcx-expressing cells might be derived from eGFP-expressing early neural progenitors. To find out whether the restoration of Dcx-expressing cells is generated by eGFP-expressing cells, one injection of BrdU was given 3 days after injury to label the dividing eGFP-expressing cells. Mice were sacrificed 7 days after injury to trace the fate of BrdU-incorporated eGFP-expressing cells. In the ipsilateral dentate gyrus, BrdU/Dcx-positive cells were observed and their distribution was not restricted in the subgranular layers but they were also located in the granular layers (Fig. 3-7, a-c). Because the time BrdU injected was the peak of eGFP-expressing early neural progenitor activation, the detection of BrdU/Dcx-positive

cells in the dentate gyrus suggests that the restoration was derived from eGFP-expressing early neural progenitors.

Discussion

In this study, I have determined that in our transgenic mice the expression of eGFP in the dentate gyrus of the injured brain remains restricted to the neural progenitors, and eGFP expression is not observed in other examined reactive astrocytes, NG-2 cells, oligodentrocyte precursor cells, nor active microglia. Therefore, eGFP-expressing cells are believed to represent early neural progenitors in the dentate gyrus of the injured brain. By using a unilateral CCI injury model, Dcx-expressing late neural progenitors are relatively vulnerable and degenerate in the ipsilateral dentate gyrus due to injury. Nevertheless, eGFP-expressing early neural progenitors become actively proliferative, especially for granular eGFP-expressing cells. Depending on BrdU-pulsing observation and quantification, the replacement of degenerating Dcx-expressing cells is observed 7 days after injury and may be derived from eGFP-expressing cells.

An unexpected result is that the activation of eGFP-expressing early neural progenitors is also observed in the contralateral dentate gyrus, though with a delayed timecourse. The activation of contralateral eGFP-expressing cells results in more Dcx-expressing cells. However, whether these Dcx-expressing cells form mature neurons is not examined in this study.

Contrary to other reported transgenic mice, the specificity in expression of eGFP in our transgenic mice is further demonstrated in the injured brains according to observations using immunofluorescence. This specificity might result from the chosen DNA genomic fragments in this construct. It contains sufficient information to restrict the expression of the transgene. Another possibility is that there is a lower copy number of transgene in our transgenic lines. However, it is not clear since the copy number of transgene in our transgenic mice has not been determined. Nevertheless, the increased specificity of the eGFP expression allows me to examine the dynamics of neural progenitors in the dentate gyrus in the injured brains..

Consistent with other studies, Dcx-expressing late neural progenitors in the ipsilateral dentate gyrus are relatively vulnerable (Miles and Kernie, 2008), when compared to early neural progenitors. A significant decrease in number of Dcx-expressing cells is observed 3 days after injury. Different to the previous study using hypoxia-ishemia (HI) model, the loss of Dcx-expressing cells is not a result of apoptosis because no cleaved caspase 3 signal, a marker for apoptosis, is detected (Miles and Kernie, 2008). By performing Fluoro Jade C histochemistry staining, which is a method to identify degenerating neurons, it was determined that Dcx-expressing cells begin to degenerate 24 hours after injury. This suggests that injury caused reduction of Dcx-expressing cells occurs via another

mechanism rather than capase-3-dependent apoptosis as seen in the hypoxia-ischemic model.

Unexpectedly, the number of Dcx-expressing cells in the ipsilateral dentate gyrus is not only recovered, but surpasses the number seen in uninjured mice. The increase in the Dcx-expressing cell population might be generated from eGFP-expressing cells according to the BrdU-pulsing experiment. It is not known from this study if the increase is transient or these Dcx-expressing cells become mature neurons. However, a recent study demonstrated that there is a significant decrease in newborn neurons one month after CCI injury (Rola et al., 2006). This indicates that the increase in Dcx-expressing cells in our study might be transient. However, this is not conclusive because of the differences in surgery protocol and genetic background.

The activation of eGFP-expressing early neural progenitors in the contralateral dentate gyrus is one of the most unexpected observations. Unlike what occurs in the ipsilateral dentate gyrus, there is no change in the size of the Dcx-expressing cell population and the activation occurs 7 days after injury. By using Ki67, a marker for cells in S-phase, to identify dividing cells, a recent study demonstrates an increase in dividing cells in the contralateral subgranular layers (Rola et al., 2006). This suggests that there is a delayed mechanism triggering the

proliferation of the early neural progenitors. At the same time, a significant increase in number and proliferation of Dcx-expressing cells is also observed in the contralateral dentate gyrus. This observation is contrast to Rola's study. However, it might be due to the differences in surgery protocol and genetic background. These differences suggest that different levels of injuries may cause different responses in neural progenitors in the contralateral dentate gyrus.

The identification of granular type 1-like early neural progenitors is specific in the previous study though their function is not known. Their response in injured brains is unexpected. They become active and proliferate in the dentate gyrus in both hemispheres at different time points after CCI injury. By performing BrdU-pulsing experiments, we see that BrdU-incorporated Dcx-expressing cells can be observed in the ipsilateral granular layers. A recent study demonstrates that a localized neural progenitor in the retina may undergo asymmetric mitosis and become mature neurons locally (Godinho et al., 2007). This study supports the idea that the localized neural progenitors may contribute in compensating for the alteration of neural progenitor population size.

The expression of eGFP is restricted to the early neural progenitors in the dentate gyrus of the injured brain. The dynamics of eGFP-expressing cells indicates that replenishment of Dcx-expressing cells is derived from the early

neural progenitors. However, the possibility of de-differentiation from reactive astrocytes, active microglia, or OPCs is not excluded in this study. These cell types have expression of neural progenitor markers in the injured brains and are able to generate neurons *in vitro* (Yokoyama et al., 2006;Yokoyama et al., 2004;Diers-Fenger et al., 2001;Kondo and Raff, 2000). Some studies demonstrate that these cells do proliferate in the dentate gyrus after injury (Yokoyama et al., 2004;Belachew et al., 2003;Diers-Fenger et al., 2001;Levine et al., 2001;Kondo and Raff, 2000).

Ablation of dividing early neural progenitors will provide direct evidence in determining the requirement of early neural progenitors for the replenishment of Dcx-expressing cells after injury. In the next chapter, I describe the generation of transgenic mice that express a modified herpes simplex viral thymidine kinase (delta HSV-TK) gene in early neural progenitors in order to inducibly ablate dividing progenitors.

Figures:

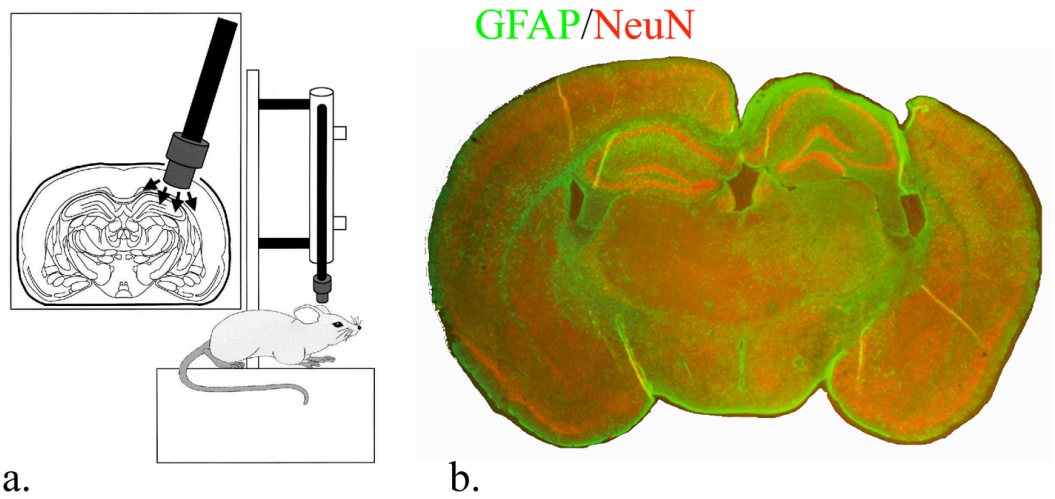

Fig. 3-1. (a.) This scheme depicts the CCI injury operation. The mouse is fixed and the brain is exposed by removing a small piece of skull. The injury is generated by allowing the piston to hit the brain with consistent speed and deflection. (b.) The picture represents the brains one month after CCI injury. As shown in the picture, the cortex in the injured area is gone and the structure of hippocampus is altered. CA1 region is shrunk indicating the decrease in cell number. However, dentate gyrus is hypertrophic, suggesting an increase in cell number. *Adapted from Salman et al.*

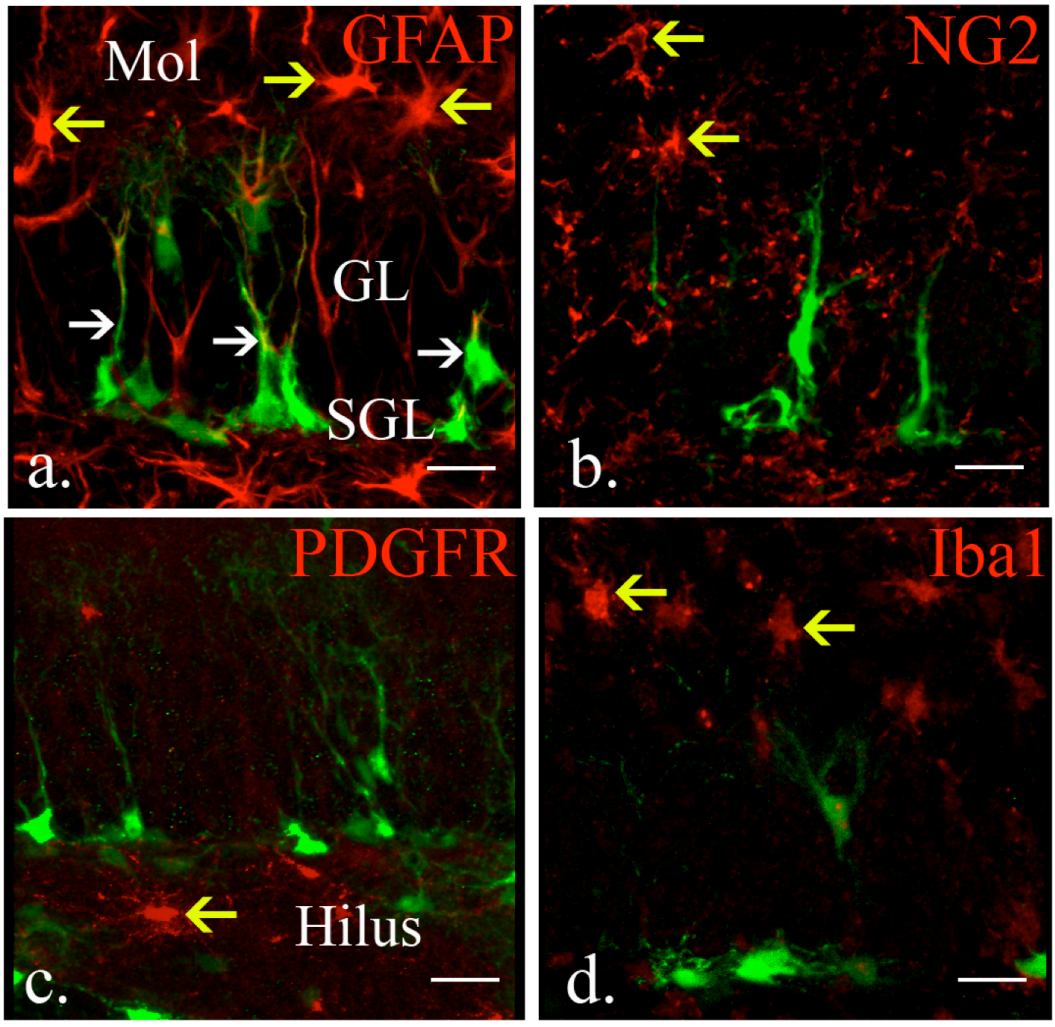

Fig. 3-2. eGFP-expressing cells were distinguishable from other cell types after injury. (*a*) eGFP-expressing type I cells in the dentate gyrus were not reactive astrocytes (*arrow: eGFP-expressing type I cells, yellow head: reactive astrocytes*). The eGFP-expressing cells in the dentate gyrus were not either NG2 cells (*b, yellow arrow*), PDGFR-expressing OPCs (*c, yellow head*), or Iba-1-expressing active microglia (*d, yellow head*). Scale bars indicate 20μm. Abbreviation: Mol -- molecular layer, SGL -- subgranular layer, GL -- granular layer.

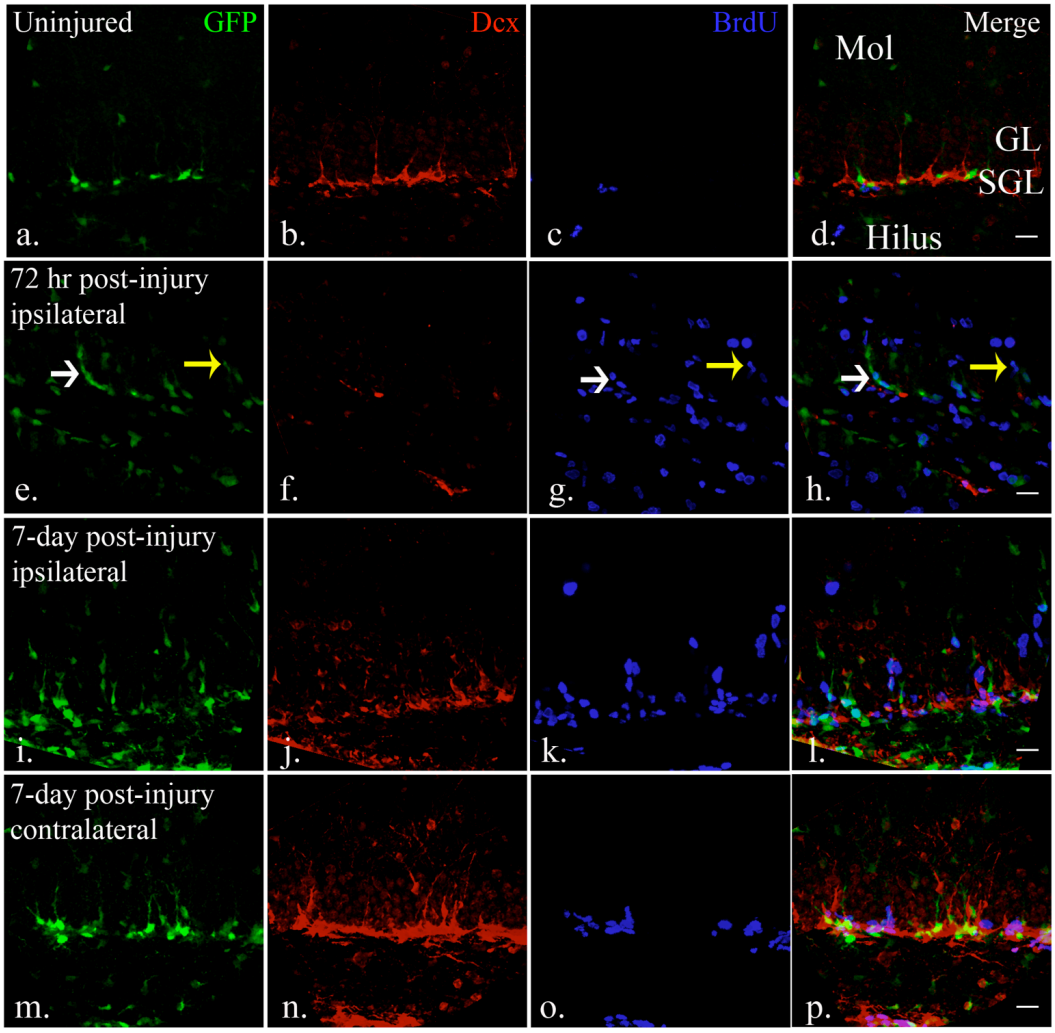

Fig. 3-3. The distribution and responses of neural progenitors in adult dentate gyrus after CCI injury. (*a to d*) eGFP- and DCX-expressing cells were found in the subgranular and granular layers of the dentate gyrus. BrdU-labeled cells were barely seen in the dentate gyrus of uninjured mice. (*e to h*) 72 hours after injury, eGFP-expressing early neural progenitors were detected in the subgranular layers (*arrow*), as well as granular layers (*yellow arrow*). The apparent decrease of DCX-expressing late neural progenitors in the dentate gyrus in the ipsilateral hemispheres indicates vulnerability. An abundant of BrdU-labeled cells were found in the dentate gyrus. This suggests the peak of cell proliferation. (*i to l*) A robust number of eGFP-expressing cells persist in the ipsilateral dentate gyrus 7 days after injury. A certain level of recovery in DCX-expressing late neural progenitors was found at this time point. (*m to p*) Interestingly, the trend of increasing in cell number and proliferation was also observed in the contralateral dentate gyrus 7 days after injury. Scale bars indicate 20μm. Abbreviation: Mol -- molecular layer, SGL -- subgranular layer, GL -- granular layer.

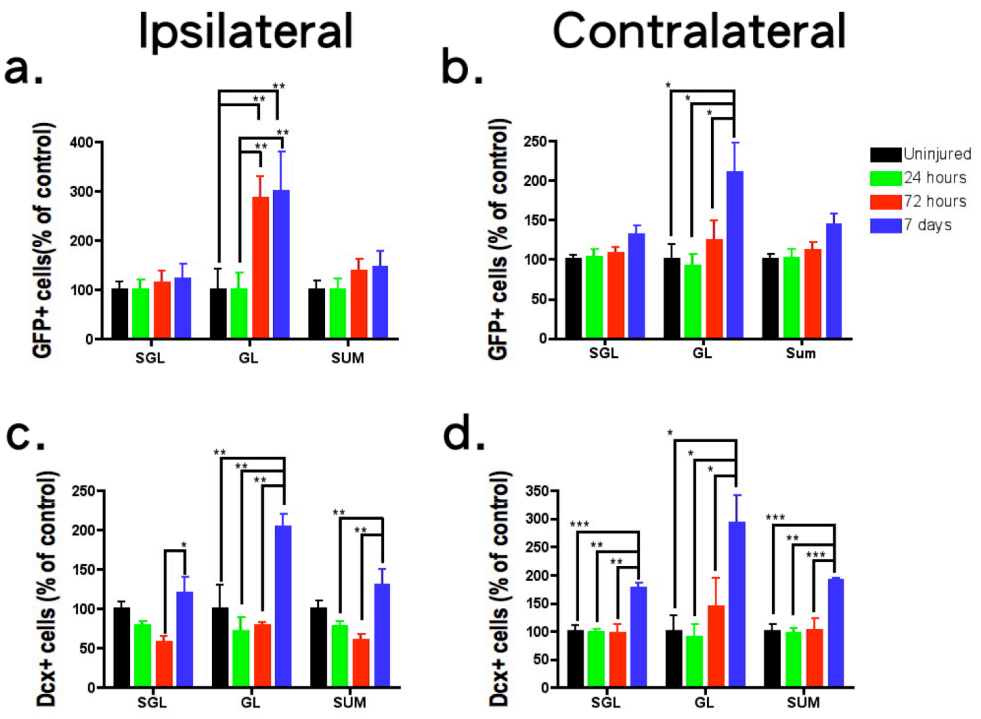

Fig. 3-4. Quantification of early and late neural progenitors in the dentate gyrus after injury. (a) In the ipsilateral dentate gyrus, no significant change was observed in the early neural progenitors in the subgranular layers (SGL). A significant increase in the number of early neural progenitors was observed in the granular layers (GL) (*p=0.0001*). Post hoc analysis revealed a significant increase of early neural progenitors 72 hrs and 7 days after CCI (*p<0.01*) when compared with uninjured samples and 24 hrs after injury. (b) A similar phenomenon was noted in the contralateral dentate gyrus, with no significant change in the subgranular layers but increases in the granular layers. (c) The opposite result was seen in the DCX-expressing late neural progenitor population where in the ipsilateral dentate gyrus, there was a significant difference in both layers examined. In the subgranular layer, there was a significant decrease in late neural progenitors 72 hrs after the injury compared with 7 days after the injury, when the cell population had returned to baseline ($p<0.05$). The granular layer revealed a similar increase 7 days after the injury when compared with uninjured group, 24 hrs and 72 hrs after injury ($p<0.01$). (d) In the contralateral dentate gyrus, the DCX-expressing cell number remained unchanged 72 hrs after injury, but increased significantly 7 days after injury in both the subgranular and granular layers (** p<0.05, ** p<0.01, *** p<0.0001 in post hoc Newman-Keuls multiple comparison test*). N=4 in each groups and error bars indicate SEM.

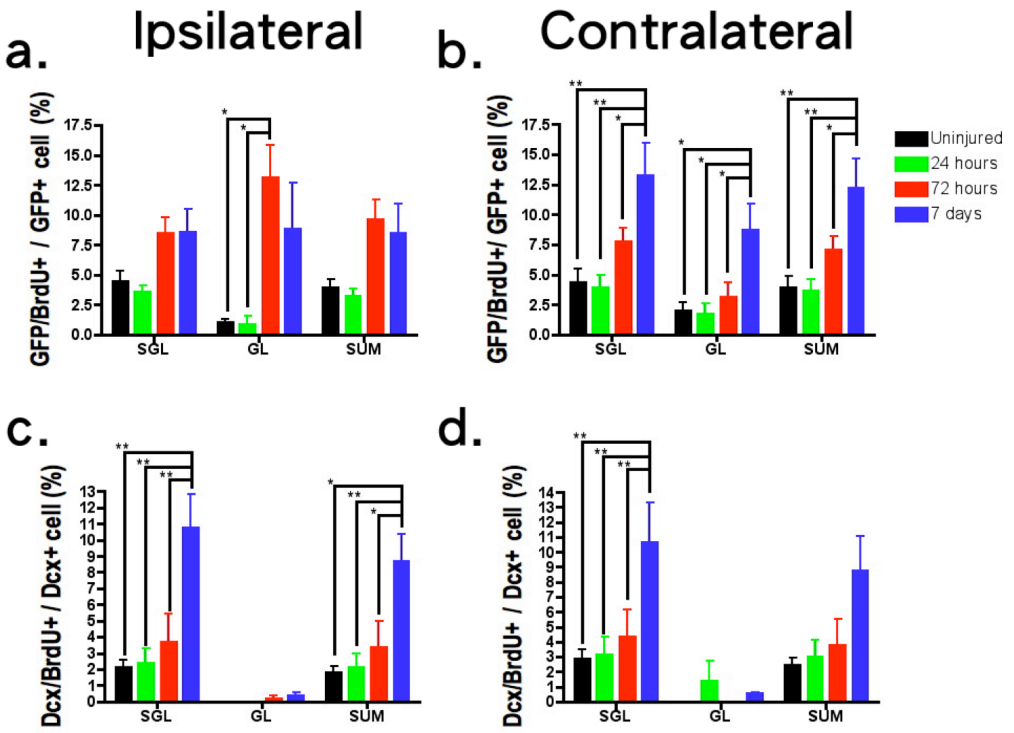

Fig. 3-5. The proliferation index of early and late neural progenitors in the dentate gyrus after injury. (a) In the ipsilateral dentate gyrus, there is significant CCI-stimulated proliferation of eGFP-expressing early neural progenitors ($p<0.05$). A significant increase of proliferative early neural progenitors was observed in the granular layers 3 days after injury compared to both uninjured controls and 24 hours after injury. ($p<0.05$). (b) On the contralateral side, there was a significant change in the number of proliferative early neural progenitors 7 days after injury when examined in the subgranular, granular layers, and total. Post hoc comparison revealed a significant increase when comparing 7 days after injury to all other time points examined. (c) For dividing DCX-expressing late neural progenitors in the ipsilateral dentate gyrus, a significant increase was seen in the subgranular layers and total (SUM), but not in the granular layers. A significant increase of proliferative late neural progenitors was observed 7 days after injury compared to the other time points. (d) A similar trend was seen in the contralateral dentate gyrus, but did not reach statistical significance in the summation group. N=4 in each group. Error bars indicate SEM. (* $p<0.05$, ** $p<0.01$).

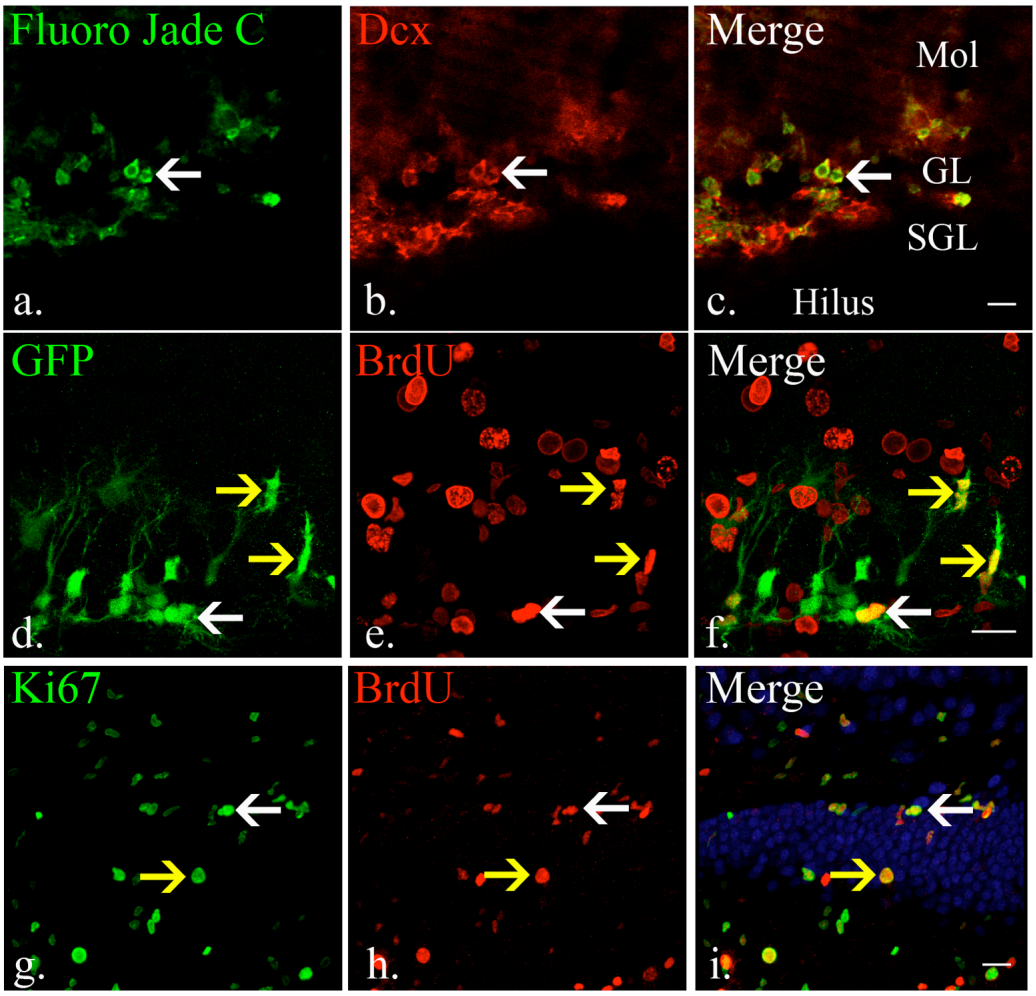

Fig. 3-6. eGFP-expressing cells in the injured brains are proliferative. *(a to c)* By using Fluoro Jade C to label degenerating cells, the colocalization of DCX-expressing cells with Fluoro Jade C signals demonstrates that DCX-expressing cells degenerate 24 hours after injury (*arrow*). (*d to f*) By using BrdU-pulsing, eGFP-expressing cells in the subgranular and granular layers were seen labeled with BrdU 3 days after injury (*white arrow: subgranular layer, yellow arrow: granular layer*). (*g to i*) BrdU-positive cells in the dentate gyrus express the cell cycle marker Ki67 (*white arrow: sugbranular layer, yellow arrow: granular layer*). Scale bars = 20μm. Abbreviations: Mol - molecular layer, SGL --subgranular layer, GL - granular layer.

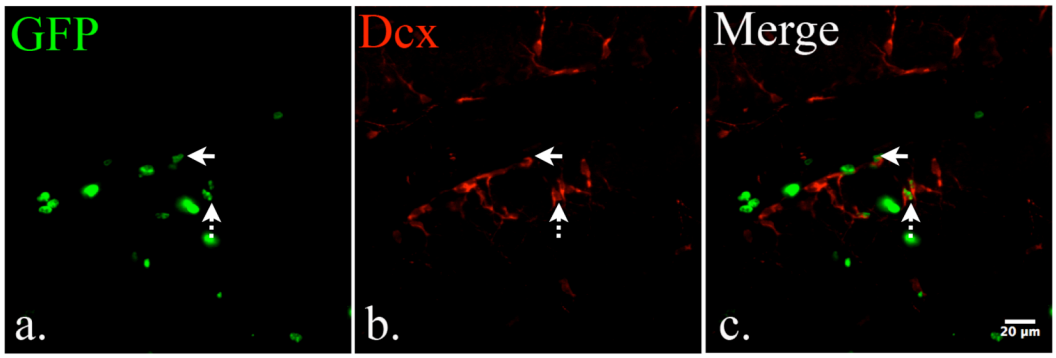

Fig. 3-7. The recovery of Dcx-expressing cells occurs in the subgranular and granular layers. By injecting BrdU to labels the dividing cells three days after injury, mice are sacrificed seven days after injury. Consistent to quantification data, BrdU-incorporated Dcx-expressing cells are identified in both subgranular (arrow) and granular layers (dash arrow). Scale bars = 20µm. Abbreviations: Mol - molecular layer, SGL --subgranular layer, GL - granular layer.

Materials and methods

Animals

Experimental animals were housed and cared for in the Animal Resource Center (ARC) at UT Southwestern Medical Center (UTSWMC), which is certified by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All animal experiments were conducted with prior approval of the Institutional Animal Use and Care Committee (IACUC) at UTSWMC in compliance with the highest standard for the humane and compassionate use of animals in biomedical research.

Controlled cortical impact (CCI) injury

To perform CCI, the standard protocol and a controlled cortical impact device used to generate brain injuries were used as previously described (Kernie et al., 2001). 8-week old male wild-type or transgenic mice were anesthetized with 0.15ml of ketamine (100mg/ml):xyzaline (20mg/ml) 10:1 mixture. Mice were placed in a stereotactic frame. The midline incision was made, the soft tissues were reflected, and a 5mm*5mm craniectomy was made between bregma and lambda and 1mm lateral to the midline. The injury was generated with a 3mm stainless steel tipped impact device with deformation of 0.7mm and constant speed of 4.4 m/s. After injury, the scalp was fastened with staples and the mice

were allowed to recover. Following injury, BrdU (100mg/Kg, Sigma) was injected intraperitoneally at 12 and 2 hrs prior to sacrifice.

Immunofluorescence

All mice were deeply anesthetized with ketamine and xylazine mixture as described above before perfusion. The trans-cardiac perfusion was performed with 50ml of 1xPBS, followed by 50ml of 4% paraformaldehyde (PFA)/1xPBS. Following post-fixation in 4% PFA/1xPBS overnight, the whole brains were dissected and embedded in 3% agarose/1xPBS. Serial 50-μm sections were cut with a vibratome (VT1000S, Leica). All sections encompassing the hippocampus were collected in every well sequentially in 12-well plates. Free-floating method was used for the immunohistochemistry. For BrdU staining, all sections from a single well were washed with 1xPBS 3 times and rinsed with water. Then, sections were denatured with 0.1N HCl for 1 hr in 37°C water bath. After denaturation, sections were neutralized with 0.1M Borax, pH8.5 (Sigma) for 10 min. Sections were washed with 0.3% Triton X-100/1xPBS (wash buffer) for 3 times and blocked with 5% normal donkey serum (Sigma-Aldrich) containing wash buffer for 1 hr at room temperature. 1:200 rat-anti BrdU (Abcam) antibody was used to label BrdU overnight at 4°C. The following day, sections were incubated with Cy5-conjugated anti-rat antibody (all 1:200, Jackson Immunoresearch) for 3 hrs at room temperature. The sections were placed on the

slides and covered with coverslips for confocal microscope observation after antibody incubation. For regular staining the protocol was as above for the BrdU except sections were not treated with HCl and Borax. Primary antibodies used in this study were as follows: rabbit anti-GFP (1:500, Molecular Biology), chicken anti-GFP (1:200, AbCam), mouse anti-GFAP (1:100, BD Pharmingen), rabbit anti-GFAP (1:500, DAKO), goat anti-doublecortin (1:100, Santa Cruz biotech), mouse anti-vimentin (1:200, 40E-C, Hybridoma Bank, Univ. Iowa), rabbit anti-Ki67 (1:200, NeoMarkers), mouse anti-NeuN (1:500, Chemicon), rabbit anti-NG2 (1:200, Chemicon), rat anti-PDGFRa (1:200, BD Pharmingen), and goat anti-Iba-1 (1:200, AbCam). Cy2-, Cy3- or Cy5-conjugated donkey anti-species antibodies were used as secondary antibodies (all 1:200, Jackson ImmunoResearch).

Fluoro-Jade C staining

To determine whether the decrease in Dcx-positive cell number was due to cell death, Fluoro-Jade C (Chemicon) staining was used. Because the signal is visualized by using a Cy2/FITC filter, wild-type mice were used to avoid any interference from eGFP signals in transgenic mice. Mice were sacrificed and perfused with 4% PFA/1xPBS at 24 and 48 hrs after CCI injury (N=4 each time points). Vibratome sections (50 μm) encompassing the hippocampus were collected and mounted on slides. Sections were first immunostained by using goat anti-doublecortin antibody (1:100, N-19, Santa Cruz biotech) and donkey anti-

goat IgG (1:200, Jackson ImmunoResearch) and then mounted on slides (described as above). The sections were allowed to dry at room temperature for 20 min in the dark. Sections were washed with water for 5 min, then transferred directly to the freshly prepared 0.06% potassium permanganate solution for 5 min, stained with 0.0004% Fluoro-Jade C for 15 min, washed twice with water for 5 min and coverslipped for confocal microscopy.

Quantification and confocal microscopy

For BrdU-positive cell quantification, a series of every 12th sections was used. BrdU-positive cells were counted under a 40x objective (Olympus) through the whole blades of the dentate gyrus. According to the location, counted cells were subdivided into 2 categories, in the subgranular or granular layers. The subgranular layer (SGL) was defined as a region encompassing 2-cell widths above and below the boundary between granular layer and hilus. The rest was defined as granular layer in this study.

To quantify the cell number and determine the proliferation index of early and late neural progenitors in the dentate gyrus, a Zeiss LSM510 scanning confocal microscope with Argon 488, He 543, and He 633 lasers and a Zeiss Neofluar 40x/1.3 oil DIC lens was used to determine each single cell and the colocalization of different cell markers. Cell quantification was performed from the dentate gyrus in both hemispheres in injured and control mice. Every 12th sections was

chosen for immnofluorescent staining to label eGFP, Dcx and BrdU. The images covering the whole blades of the dentate gyrus were taken and the 50 μm z-axis was scanned with a consistent 2 μm interval. The number of eGFP-or Dcx-expressing cells was counted through each frame by using ImageJ (NIH) without any adjustments of color, contrast, or brightness. The colocalization of eGFP or Dcx with BrdU was determined only when BrdU signal was clearly covered or wrapped with eGFP or Dcx signal, respectively. The number of colocalized cells was counted frame by frame.

Statistics

Statistics were done using a one-way analysis of variance (ANOVA) with a Newman-Keuls multiple comparison test, or unpaired Student's t test. Differences were deemed significant with $p<0.05$.

CHAPTER FOUR
Generation of Nestin-delta-HSV-TK Transgenic Mice and Requirement of Activation of Neural Progenitors in the Injured Brains

Introduction

Why are adult newborn neurons present in the dentate gyrus? This has been an unsolved question since adult neurogenesis was identified there. Because of its role in learning and memory, many hypotheses have been proposed to explain the role of new neurons in new memory formation (Zhao et al., 2008;Becker and Wojtowicz, 2007;Leuner et al., 2006;Aimone et al., 2006). However, its true function remains unknown. One direct way to answer this question is to inhibit adult neurogenesis.

Several methods have been developed to inhibit neurogenesis. Systemic application of the antimitotic agent, methylazomethanol acetate (MAM), has been used to inhibit neurogenesis in adult rats (Shors et al., 2002;Shors et al., 2001). By performing classic eyeblink trace conditioning, a hippocampal-dependent test in which an animal must associate stimuli that are separated in time, a substantial reduction in the number of newborn neurons is observed and impairment in this task is displayed. However, inhibition of neurogenesis does not affect the performance of animals in hippocampal-independent trace conditioning learning,

a task in which stimuli are not separated in time. Later on, more behavioral paradigms were used to examine the role of adult newborn neurons. Only some types of hippocampal neurons are affected due to the reduction of newborn neurons in adult dentate gyrus. The critical concern in this methodology is that the application of anti-mitotic agent inhibits all kinds of dividing cells, not specifically dividing neural progenitors. Conclusions from these experiments can only determine what the role of all dividing cells in the brain is. We cannot exclude the possible roles of other dividing cells, like endothelial cells or astrocytes, in these behavioral paradigms (Leuner et al., 2006).

Low-dosages of irradiation treatment have been demonstrated to diminish neural progenitors and inhibit adult neurogenesis in adult brains (Wojtowicz, 2006;Mizumatsu et al., 2003;Monje and Palmer, 2003). Different from systemic applications of antimitotic agents, irradiation can be limited to a small area of a brain region. Again, several hippocampal-dependent learning paradigms have been used to examine the role of adult newborn neurons, and it has been demonstrated that not all types of hippocampal-dependent behaviors are affected (Wojtowicz et al., 2008;Saxe et al., 2007;Winocur et al., 2006;Saxe et al., 2006;Rola et al., 2004;Raber et al., 2004). The caveat in this methodology is that irradiation results in a severe inflammatory response that can last for ~1 month. The treated animals usually need ~4 weeks to recover. One should also keep in

mind that the ablation of adult neural progenitors is not 100%. Due to the structure of hippocampus and its location in the brain, the efficiency of ablation in the ventral part of hippocampus is relatively low. One must be cautious in studying the role of adult neurogenesis in behaviors related to stress or depression because the ventral part of hippocampus has been related to emotional behaviors (Sahay and Hen, 2007).

In order to overcome the limitations of current methodologies for inhibiting neurogenesis, several temporal induction models of ablation in dividing cells have been developed. One of the systems takes advantage of a well-known kinase, herpes simplex viral thymidine kinase (HSV-TK), to ablate dividing cells (Fig.1) (St Clair et al., 1987). By generating a transgenic line that has expression of HSV-TK gene driven by a cell specific promoter, temporal and spatial ablation of dividing specific cells is possible depending on when the substrate is applied (Bush et al., 1998).

In 1998, Sofroniew and colleagues generated a transgenic line that expresses the HSV-TK gene when activated by human GFAP promoter. The expression pattern of HSV-TK is similar to that of endogenous GFAP. The astrocytes *in vitro* and after CNS injury *in vivo* are vulnerable in the presence of ganciclovir (Bush et al., 1998). Later, GFAP expression was identified in the

embryonic and adult neural progenitors. By treating transgenic mice with ganciclovir, GFAP-expressing progenitors are the principal source of constitutive neurogenesis in the adult mouse forebrain (Garcia et al., 2004;Morshead et al., 2003). Expectedly, not all types of hippocampal-dependent behavioral paradigms are affected when using this transgenic line to inhibit adult neurogenesis (Saxe et al., 2007;Saxe et al., 2006). The primary concern with using this transgenic line is that, although neurogenesis is indeed inhibited, dividing astrocytes in CNS are also ablated in the presence of ganciclovir. Because frequent proliferation of reactive astrocytes occurs in the injured brain, this transgenic line is not an ideal model for studying the role of neural progenitors in injured brains.

As mentioned in the prior chapter, the lack of specificity of current markers used to identify neural progenitors makes it difficult to determine the source of new neurons generated in injured brains. Reactive astrocytes, active microglia, and OPCs are reported to generate neurons *in vitro*, and they all have expression of nestin gene in the injured brains (Yokoyama et al., 2006;Yokoyama et al., 2004;Diers-Fenger et al., 2001;Kondo and Raff, 2000).

To determine whether the adult neural progenitor is the primary source of newborn neurons in the injured brains, specific ablation of neural progenitors is a direct way to prove it. However, Sofroniew's transgenic mice are not suitable to

achieve this task. Our prior studies demonstrate that the chosen regulatory element DNA fragment in our transgenic mice is active specifically in neural progenitors. Therefore, I can take advantage of this and generate a transgenic line that has expression of HSV-TK to allow temporal ablation of dividing neural progenitors. By doing so, I would be able to effectively determine the requirement of neural progenitors for injury-induced neurogenesis.

In this study, I describe the generation of transgenic lines that have HSV-TK expression driven by the well-characterized nestin regulatory DNA fragments. By using this transgenic line, I demonstrate that activation of neural progenitors in the injured brain is required to replenish the lost neurons in the dentate gyrus.

Results

Delta-HSV-TK Transgenic Mice Were Generated to Temporally Inhibit Neurogenesis:

The major problem with using BrdU to trace cell fate is that many different types of cells divide, especially after injury. For example, the number of microglial cells and astrocytes increase after brain injury and the notion that these dividing cells may serve as potential sources for neurogenesis remains speculative (Yokoyama et al., 2006;Mori et al., 2005). To determine whether early type 1 neural progenitors were the main source for recovery of the Dcx-expressing cells after injury, and to investigate how early neural progenitors repopulate lost dentate gyrus neurons, a modified herpes simplex virus thymidine kinase (delta-HSV-TK) and eGFP-expressing transgenic line was generated. Ganciclovir is specifically phosphorylated by HSV-TK and kills dividing cells by acting as a toxic thymidine analogue (St Clair et al., 1987). Expression of HSV-TK has been successfully used in the central nervous system to inducibly ablate GFAP-expressing astrocytes as well as neural stem cells (Garcia et al., 2004;Morshead et al., 2003).

We first generated four transgenic lines using the full length HSV-TK. Though we were able to demonstrate ganciclovir-mediated cellular ablation specifically in neural progenitors, we found a significant percentage of animals had aberrant brain development and male sterility. Previously published data demonstrate that there is a testis-specific enhancer fragment in the HSV-TK gene between the 1st and 2nd ATG start codons, which renders male mice infertile and may cause other developmental defects (Salomon et al., 1995). Deletion of this enhancer fragment has been demonstrated to recover the fertility defect and reduce toxicity due to overexpression (Salomon et al., 1995). We therefore generated delta-HSV-TK transgenic with a deletion of this enhancer element.

We used the nestin promoter and its 2nd intron to direct expression of delta-HSV-TK and restrict its expression to neural progenitors (Fig. 4-2, a). Following pronuclear injection, thirty-one offspring were screened using PCR and three lines were identified that carried the transgene. One of these (delta-TK-145) was fertile and transmitted the transgene according to Mendelian ratios. This has subsequently been characterized in detail. Although RT-PCR analysis revealed the expression of delta-HSV-TK in testes and brain in this line (Fig. 4-2, b), both male and female mice were fertile.

Similar to our rtTA-M2-IRES-eGFP line, endogenous eGFP expression was visible only in the neural tube during embryonic stages (Fig. 4-2, c). In the adult brain, eGFP-expressing cells were restricted to known neurogenic areas, namely the SVZ and SGL. We used antibody staining specific to HSV-TK to confirm its expression in early neural progenitors (Fig. 4-2, d). Thus, HSV-TK expression in this transgenic line is coincident with early hippocampal progenitors.

Delta-HSV-TK is Functional in vivo*:*

To examine the functionality of delta-HSV-TK *in vivo*, 6-week old male delta-HSV-TK mice were treated with vehicle or ganciclovir (200mg/kg•day) for 2 weeks via implanted osmotic mini-pumps. A single injection of BrdU (100mg/kg) was given three days after the beginning of drug administration to monitor the number of dividing cells. Mice were sacrificed immediately after 2-weeks of treatment for further investigation. In mice with vehicle treatment, BrdU-positive cells were observed in the subventricular zones and subgranular layers. As expected, those BrdU-positive cells were Dcx-expressing cells. However, BrdU-positive cells were scarcely seen in neurogenic areas in mice with ganciclovir treatment (Fig. 4-3, a-d). However, BrdU-positive cells remained apparent outside neurogenic areas, such as the striatum, cortex, the hilus and

molecular layers of the dentate gyrus. This suggests that there is inhibition of adult neurogenesis in the neurogenic areas.

Unlike Dcx-expressing cells in the subventricular zones, Dcx-expressing cells were still observed in the subgranular layers of the dentate gyrus. In order to verify whether complete inhibition of neurogenesis in the dentate gyrus is achievable, a 4-week treatment of ganciclovir or vehicle was performed in wild-type and transgenic mice. Mice were sacrificed at the end of treatment. Both Dcx- and NeuroD-expressing cells were barely detected in the subgranular layers of the dentate gyrus after 4-weeks of treatment with ganciclovir (Fig. 4-4, g, h). At the same time, a number of Tuj-1-expressing immature neurons and NeuN-expressing mature neurons were not affected (Fig. 4-4. i, j). This suggests that no early neural progenitors develop into Dcx-expressing cells if the existing Dcx-expressing cells develop into immature neurons after 4-weeks of treatment with ganciclovir.

The Existing Dcx-Expressing Cells are not Affected by Ganciclovir Treatment:

To demonstrate that the reduction of Dcx-expressing cells was due to the maturation of existing Dcx-expressing cells and ablation of dividing early neural progenitors, three injections of BrdU (100mg/kg, one injection per day for 3

consecutive days) were given to 6-week old nestin-delta-HSV-TK mice. After BrdU injections, the mice were treated with vehicle or ganciclovir via mini-pumps for 4 weeks. In theory, only cells that incorporate BrdU and become Dcx-expressing cells before ganciclovir treatment would be observed after 4 weeks of treatment and that most of the BrdU-positive cells would die due to ganciclovir treatment. As expected, the Dcx-expressing cells were not visible in the dentate gyrus after ganciclovir treatment (Fig. 4-5, b), but remained abundant in the control (Fig. 4-5, a). The BrdU-positive cells express NeuN in the dentate gyrus (Fig. 4-5, c), meanwhile there was approximately a 50% decrease in number of eGFP-expressing cells in the dentate after ganciclovir treatment suggesting an efficient ablation in this line (Fig. 4-6. a, d, g). These data demonstrate that the development of existing Dcx-expressing late neural progenitors is not affected by ganciclovir treatment and eGFP-expressing cells represent all early neural progenitors in this line since no newly-generated Dcx-expressing late neural progenitors are observed after ganciclovir treatment.

The Remaining eGFP-Expressing Cells Are Able to Proliferate and Generate New Late Neural Progenitors and Neurons:

One of the advantages in this inducible ablation transgenic model is that only the dividing cells are ablated when ganciclovir is applied. Therefore, the

remaining cells should be able to proliferate and develop after the treatment is discontinued. To determine whether the remaining eGFP-expressing cells were proliferative, one injection of BrdU was given 2 hours before sacrifice after a 4-week treatment of ganciclovir or vehicle. Compared to the percentage of BrdU incorporation in eGFP-expressing cells after vehicle treatment approximately a 50% increase was observed in transgenic mice that received ganciclovir treatment (Fig. 4-6, b, e, h). This demonstrates that the remaining eGFP-expressing cells were able to proliferate. This enhancement in proliferation indicates that the remaining cells try to maintain homeostasis in neural progenitor populations.

After demonstrating the capability of proliferation in the remaining eGFP-expressing early neural progenitors, a 4-week treatment of ganciclovir was performed again in 6-week old transgenic mice to allow for the development of existing late neural progenitors and to further inhibit neurogenesis. Three days after treatment, mice were given BrdU (100mg/kg•Day) for 3 consecutive days (one injection per day). Mice were sacrificed one or four weeks after the discontinuation of treatment. As expected, BrdU/Dcx-positive cells were identified in the subgranular layers of the dentate gyrus one week after discontinuation of ganciclovir treatment (Fig. 4-7). BrdU/NeuN-positive cells became visible 4 weeks after the removal of treatment (Fig. 4-8). Therefore, the

remaining eGFP-expressing early neural progenitors are able to undergo neurogenesis and generate neurons in this inducible ablation model.

The Dynamics of eGFP-Expressing Early Neural Progenitors in Delta HSV-TK Mice Resembles that Observed in Other nestin-eGFP Lines:

In order to determine whether activation of early neural progenitors is required for injury-induced neurogenesis, the dynamics of eGFP-expressing cells in injured delta-TK transgenic mice was examined. Again, unilateral CCI injury was performed in 8-week old transgenic mice and animals were sacrificed at 3- and 7-days after injury. Consistent with the prior observations in injured nestin-rtTA transgenic mice, a decrease in the number of Dcx-expressing late neural progenitors was observed in the ipsilateral dentate gyrus and an increase in the number of eGFP-expressing cells was seen 3 days after injury (Fig. 4-9, e-h). Seven days after injury, regeneration and compensation of Dcx-expressing cells in the ipsilateral and contralateral dentate gyrus, respectively, was observed. At the same time, activation of eGFP-expressing cells remained (Fig. 4-9, i-l). The dynamics of early and late neural progenitors in the dentate gyrus after injury are parallel to what I observed using nestin-rtTA transgeneic mice. Furthermore, eGFP-expressing cells were also distinguishable from other cell types, like NG-2 cells, reactive astrocytes, and oligodendrocyte progenitor cells in injured delta

HSV-TK transgenic mice. Hence, this transgenic line is a beneficial tool for studying the requirement of early neural progenitors in injury-induced neurogenesis.

Ablation of Neural Progenitors Inhibits the Recovery of Dcx-expressing *Cells After Injury:*

To examine whether early neural progenitors are required for the recovery of neurogenesis after injury, 6-week old male nestin-delta-HSV-TK mice were treated with ganciclovir for 4 weeks to allow for the maturation of existing Dcx-expressing cells. Unilateral CCI injury was performed and ganciclovir treatment continued for 7 days afterwards to ablate injury-activated eGFP-expressing early neural progenitors. Three injections of BrdU (100mg/Kg, one injection per day) were given at 3-, 4- and 5-day post-injury to label the dividing cells. A few eGFP-expressing cells incorporated with BrdU, which suggests that a small portion of dividing eGFP-expressing cells might escape the ablation (Fig. 4-10). However, later developing Dcx-expressing cells were not observed in the dentate gyrus 7 days after injury with ganciclovir treatment (Fig. 4-10). BrdU-positive cells were apparent in other brain regions outside the dentate gyrus (Fig. 4-10, a), while within the dentate gyrus dividing reactive astrocytes, NG-2 cells and OPCs were not affected in their incorporation of BrdU (Fig. 4-11). These data

demonstrate the specificity of ablation in this line following injury and suggests that injury-activated early neural progenitors are required for the recovery of later Dcx-expressing cells after injury.

Injury-Induced Activation of Early Neural Progenitors is Required to Repopulate Damaged Late Progenitors:

The prior observations suggest that early nestin-expressing progenitors repopulate late Dcx-expressing progenitors in both the subgranular and granular layers. To demonstrate this, nestin-delta-HSV-TK mice were treated continuously with ganciclovir from 6 to 10 weeks of age. Then, unilateral CCI injury was performed one day after the removal of ganciclovir. Again, three BrdU injections were given at days 3, 4 and 5 post-injury to determine the fate of proliferating cells. Consistent with prior observations, eGFP-expressing early neural progenitors were spread throughout the granular layers. BrdU/Dcx-positive cells were seen in both the subgranular and granular layers in the dentate gyrus (Fig. 4-13). These BrdU-positive Dcx-expressing cells matured and became NeuN-expressing cells four weeks after injury (Fig. 4-14, a-c). Without injury, re-population of Dcx-expressing cells and their subsequent maturation into NeuN-expressing neurons was seen primarily in the subgranular layers (Fig. 4-8). Consistent with recovery and previous data showing an increase of Dcx-

expressing cells after injury, a 100% increase in NeuN-expressing neurons was observed in the dentate gyrus 4 weeks after injury when compared to ones in uninjured brains (Fig. 4-14, d). We conclude that activation of early neural progenitors is required for injury-induced neurogenesis and that these neural progenitors are able to develop to mature neurons in the dentate gyrus.

Discussion

In this study, the expression of modified HSV-TK is demonstrated to exist in the neural progenitors specifically and its functionality is determined by treating with ganciclovir. This study also demonstrates that the development of the existing Dcx-expressing cells is not affected and that 4 weeks are required for the expression of Dcx to become down-regulated. The requirement of neural progenitors was determined in injury-induced neurogenesis by treating mice with ganciclovir. No newborn Dcx-expressing cells and neurons were observed, however, the dividing reactive astrocytes, NG-2 cells, and OPCs were not affected. The discontinuation of ganciclovir treatment allowed the regeneration of Dcx-expressing cells and neurons in injured brains. This suggests that adult neural progenitors are the primary source of neurons developed through injury-induced neurogenesis.

The identification of modified HSV-TK in diminishing its toxicity and leakiness provides a good way to generate a healthy transgenic mouse in which the neural progenitors are ablated specifically. To ablate neural progenitors in the adult brains, 200mg/Kg/day of ganciclovir has to be used in this transgenic line. The working concentration of ganciclovir in these experiments is high when compared to other studies (Garcia et al., 2004;Morshead et al., 2003;Bush et al.,

1998). However, the body weights in those treated mice are not different from those treated with vehicle or wild-type mice. Therefore, the high concentration of ganciclovir does not seem to affect the health of the mice.

The expression of modified HSV-TK and eGFP is demonstrated in the early neural progenitors. By using BrdU to label the dividing cells before ganciclovir treatment allows early neural progenitors to become Dcx-expressing cells, I demonstrated that the development of existing Dcx-expressing cells is not affected. It also indicates that the remaining modified HSV-TK (if any) is not sensitive to the ganciclovir.

An interesting observation made during this study was there was a significant increase in BrdU incorporation in the remaining early neural progenitors. This suggests that remaining neural progenitors are able to detect the size of population and try to maintain its homeostasis. This finding also demonstrates that remaining neural progenitors are still able to generate new late neural progenitors (Doetsch et al., 1999a). This is demonstrated by removal of ganciclovir. The newly generated Dcx-expressing late neural progenitors and neurons are observed at different time points. This property provides a useful tool to study the role of new neurons in a variety of different contexts during both the inhibition and reconstitution of neurogenesis.

Re-expression of nestin is reported in different types of cells in the injured brain. These cells are also able to proliferate in injured brains. It results in a difficulty in the identification of the source in injury-induced neurogenesis. In this transgenic line, I demonstrated that the transgene is not expressed in other cell types, even in the injured brains. By treating mice with ganciclovir, non-neural-progenitor dividing cells are not affected even in the injured brain. This further confirms the specificity of the expression of the transgene.

The caveat of this study is that the role of the Dcx-expressing cells in the injured brain is not determined because the mice are pretreated with ganciclovir, which allows the existing Dcx-expressing cells to still become mature neurons (Steiner et al., 2006). However, I have demonstrated that injury does not wipe out the whole population of Dcx-expressing cells in the ipsilateral dentate gyrus of the injured brains. It has been shown that upregulation of epidermal growth factor (EGF) is able to convert the transiently-amplifying neural progenitors to early neural progenitors (Doetsch et al., 2002). Hence, it is possible that the recovery of Dcx-expressing cells is derived from the remaining Dcx-expressing cells though it is not known whether the expression level of EGF is upregulated in the injured brain.

In the prior studies, whether the injury-induced increase in Dcx-expressing cells become mature or not has not been determined. In this study, injury-induced newborn Dcx-expressing cells become mature neurons 4 weeks after injury. The increase in the amount of newborn neurons is observed in both dentate gyri. It would be interesting to understand whether or not these newborn neurons are functional.

Figures:

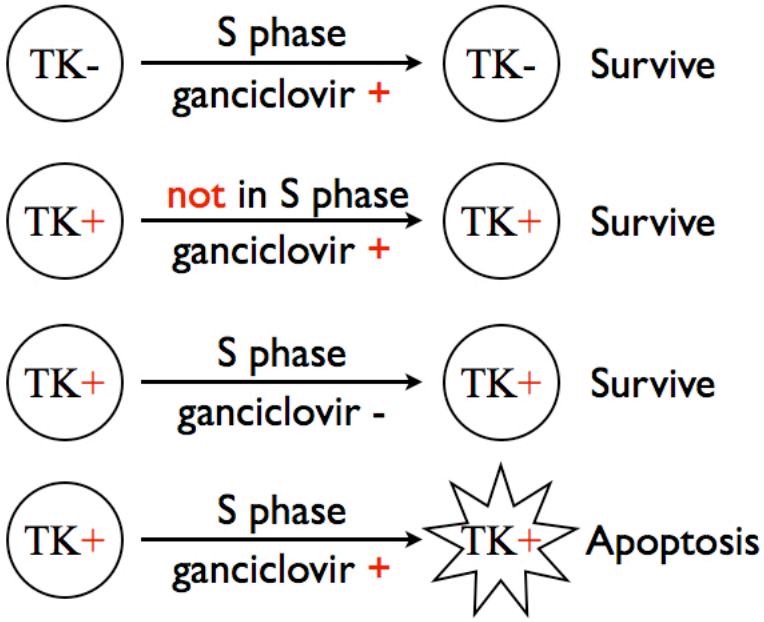

Fig. 4-1. This scheme depicts the effects of HSV-TK and ganciclovir on cells. The cell does not undergo apoptosis unless it is in S phase, expresses TK protein and is in the presence of ganciclovir. The metabolite of ganciclovir can be incorporated into DNA when cells enter S phase. DNA synthesis is blocked when HSV-TK metabolite is incorporated and cells will then undergo apoptosis. If the cells do not enter mitosis, the metabolite has no any effect in these cells.

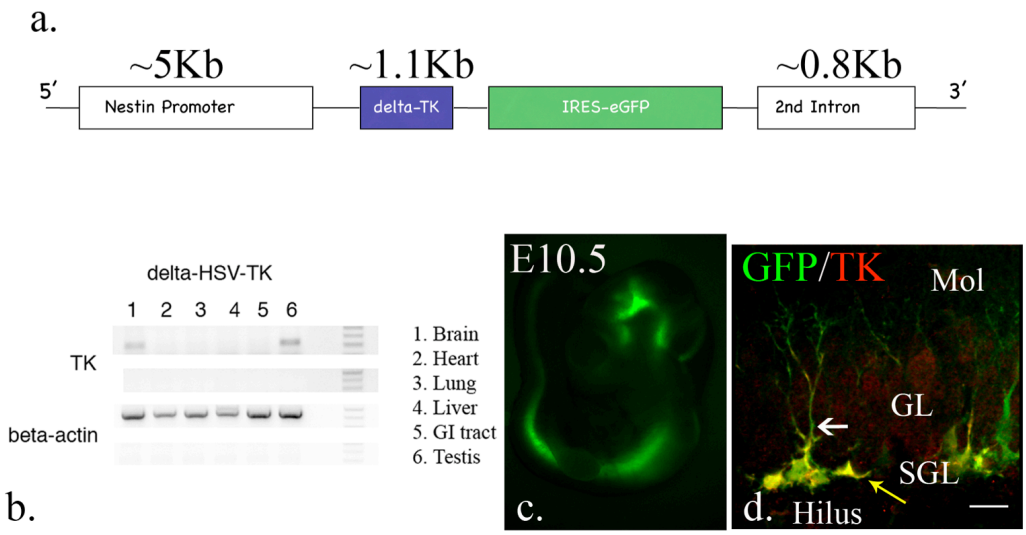

Fig 4-2. Analysis of the delta-HSV TK transgenic mice. (a). This scheme depicts the construct of the transgene (b). The total RNA was extracted from different tissues from delta-HSV-TK mice. By using primers to verify the TK expression, RT-PCR data demonstrates that testes and brains have TK expression (c). In the embryos, the endogenous eGFP was visible from E10.5, the earliest examined stage, and expression was restricted to the neural tube In the adult stage, the expression of eGFP was restricted in the neurogenic areas. Only eGFP-expressing cells possess expression of TK in the neurogenic areas, even after brain injury (d, arrow: type1-like cells, yellow arrow: type2-like cells, scale bar: 20μm). Abbreviation: Mol -- molecular layer, SGL -- subgranular layer, GL -- granular layer.

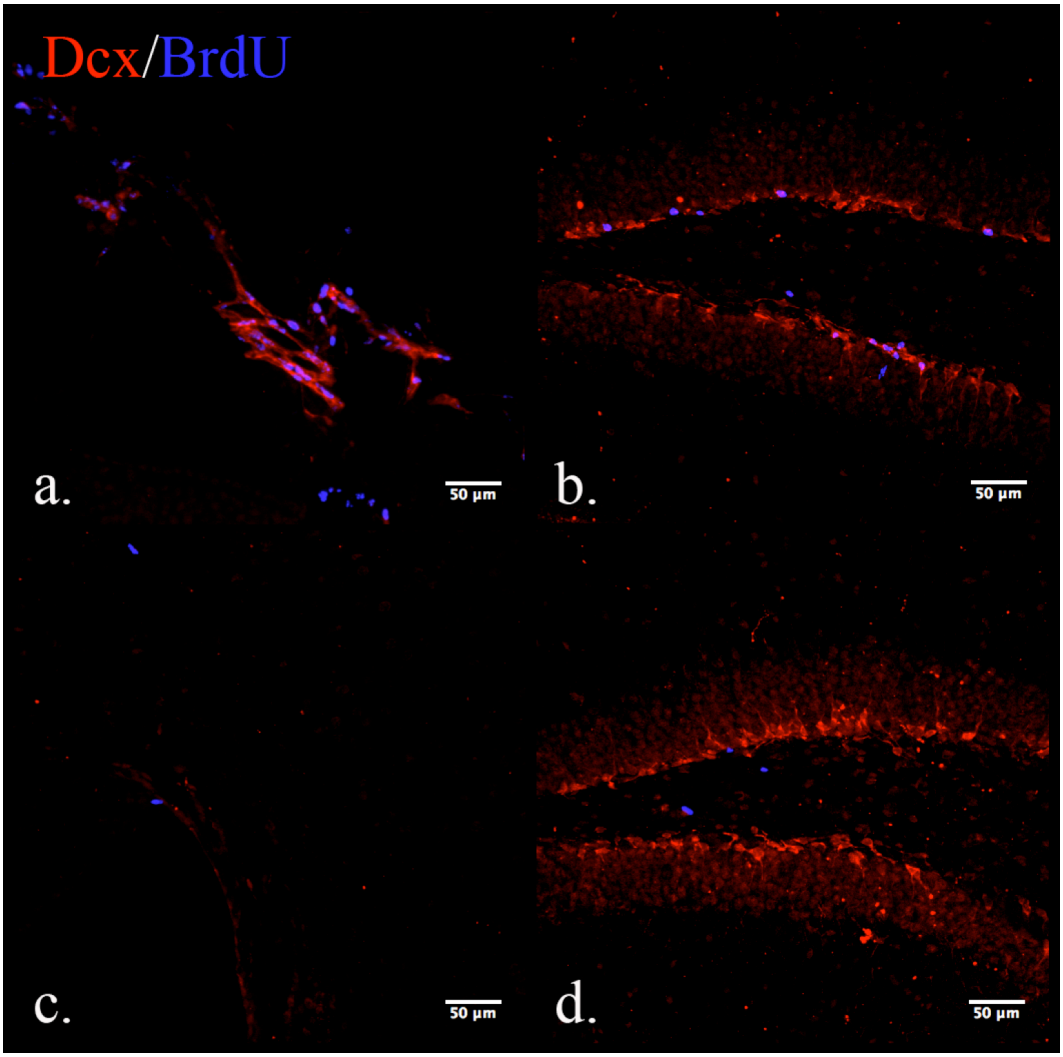

Fig 4-3. delta-HSV-TK is functional *in vivo*. To test whether delta-HSV-TK was functional, 6-week old transgenic mice were treated with vehicle or ganciclovir via osmotic mini-pumps for different periods of time. (a, b) With vehicle treatment, a robust number of BrdU-positive cells were visible in the subventricular zones of the lateral ventricles and the subgranular layers in the dentate gyrus. (c, d) After 2-week ganciclovir treatment, a dramatic decrease of BrdU-positive cells was observed in these neurogenic areas. However, BrdU-positive cells were still visible outside the neurogenic areas (*arrows*). DCX-expressing cells remained visible after 2-weeks of treatment. Scale bars = 50 µm

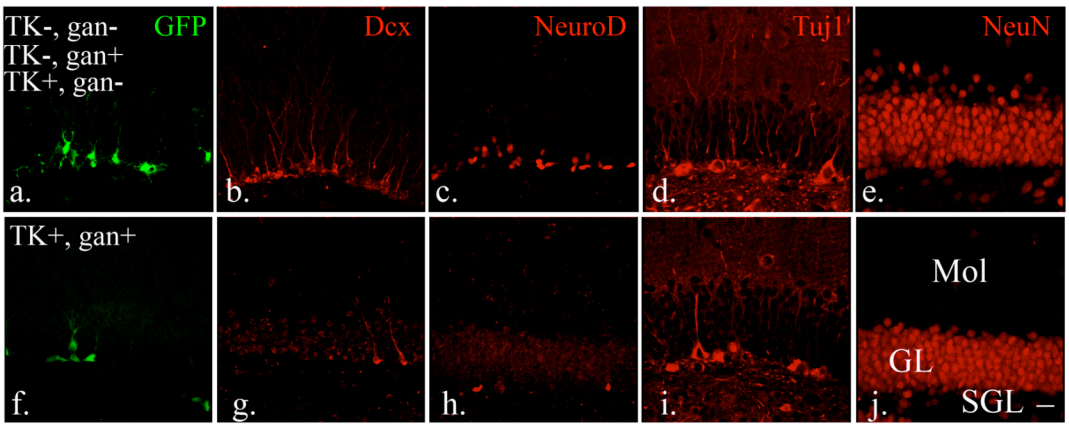

Fig 4-4. Inhibition of neurogenesis in the dentate gyrus. (a-e). The distribution of neural progenitors, immature and mature neurons in the wild type with or without ganciclovir treatment, or transgenic mice with vehicle treatment are similar. (f-j). After 4 weeks of ganciclovir treatment in transgenic mice, the number of eGFP-, Dcx-, and NeuroD-expressing cells decreases. However, the distribution of immature and mature neurons is not affected. Scale bar: 20μm. Abbreviation: Mol -- molecular layer, SGL -- subgranular layer, GL -- granular layer.

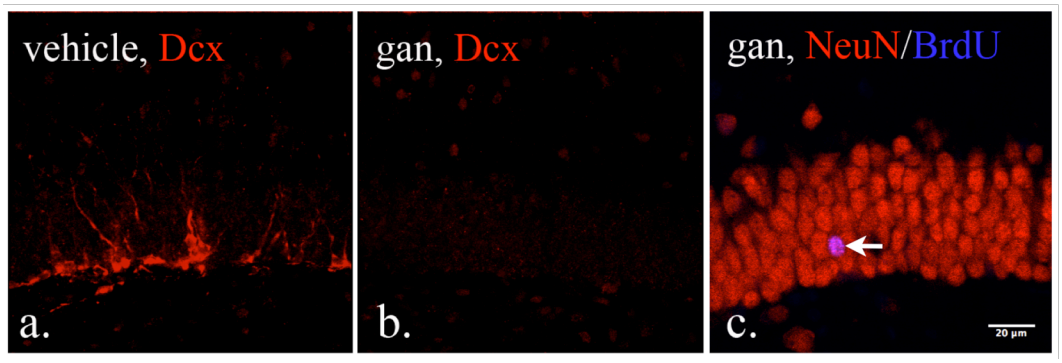

Fig 4-5. Development of the existing Dcx-expressing cells is not affected. 3 injections of BrdU were given 3 days before ganciclovir treatment (one injection each day) to prelabel dividing cells followed by 4-weeks of ganciclovir treatment. (a). With vehicle treatment, the distribution of Dcx-expressing cells is as usual. (b). With 4-week treatment of ganciclovir, Dcx-expressing cell is barely observed. (c). NeuN/BrdU-positive cells were detected in the dentate gyrus (*arrow*), suggesting that the treatment did not affect the differentiation and maturation of existing late neural progenitors. Scale bars = 50 µm (a to d), and 20 µm (e to f). Abbreviations: LV - lateral ventricle, SVZ - subventricular zone, Mol - molecular layer, SGL - subgranular layer, GL - granular layer.

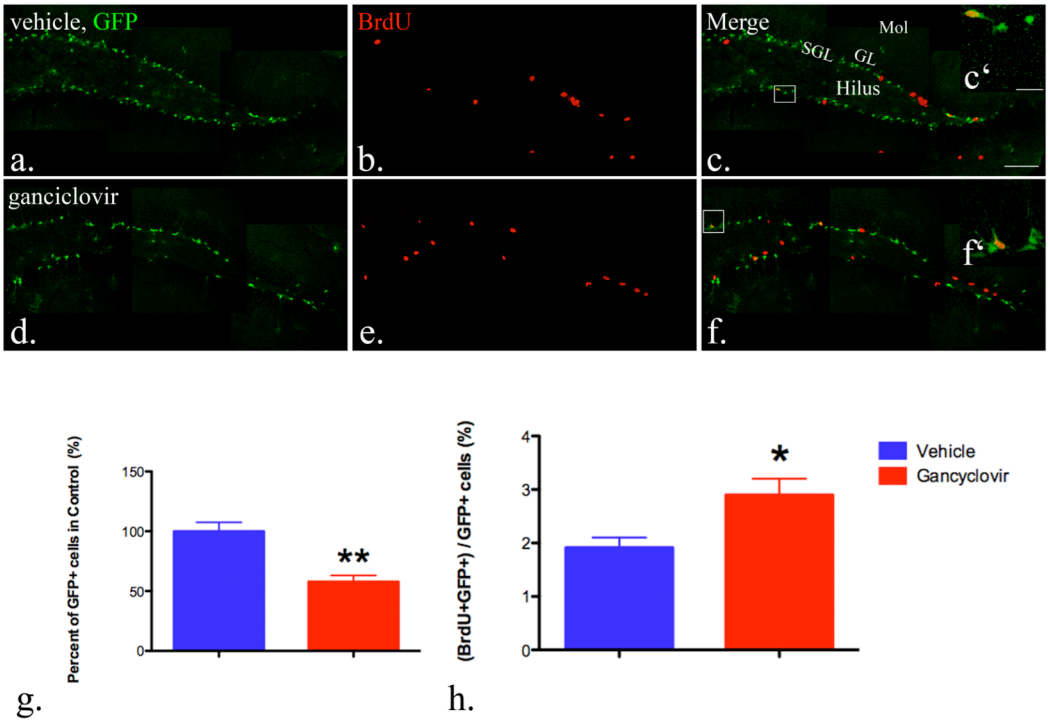

Fig 4-6. The effects of ganciclovir treatment in eGFP-expressing early neural progenitors. (a-c) After 4 weeks of vehicle treatment, the distribution of eGFP-expressing type 1 and 2a cells remained the same in the dentate gyrus. (d-f) After 4 weeks of ganciclovir treatment, the number of eGFP-expressing cells decreased significantly in the dentate gyrus. (c' and f') Images magnified from boxed areas in (c) and (f), respectively, demonstrate that the morphology of residual eGFP-expressing cells in ganciclovir-treated animals resembles type 1 early progenitors. (g) Compared to vehicle-treated mice, the number of eGFP-expressing cells in the dentate gyrus decreased significantly after 4-weeks of treatment with ganciclovir (*vehicle vs ganciclovir: 100+/-7 vs 57+/-5, data presented as mean+/-SD; **: p<0.01 in unpaired Student's t test*). (h) To determine the progenitor proliferative rate, one injection of BrdU was given 2 hrs before sacrifice and a significant increase in BrdU incorporation in eGFP-expressing cells was observed after 4 weeks of ganciclovir treatment when compared to vehicle treatment. (*vehicle vs ganciclovir: 1.9+/-0.1 vs 2.8+/-0.3; * p<0.05, in unpaired Student's t test*). N=4 in each group and error bars indicate SEM. Scale bar = 100μm (c) and 20μm (c'). Abbreviations: Mol - molecular layer, SGL --subgranular layer, GL - granular layer.

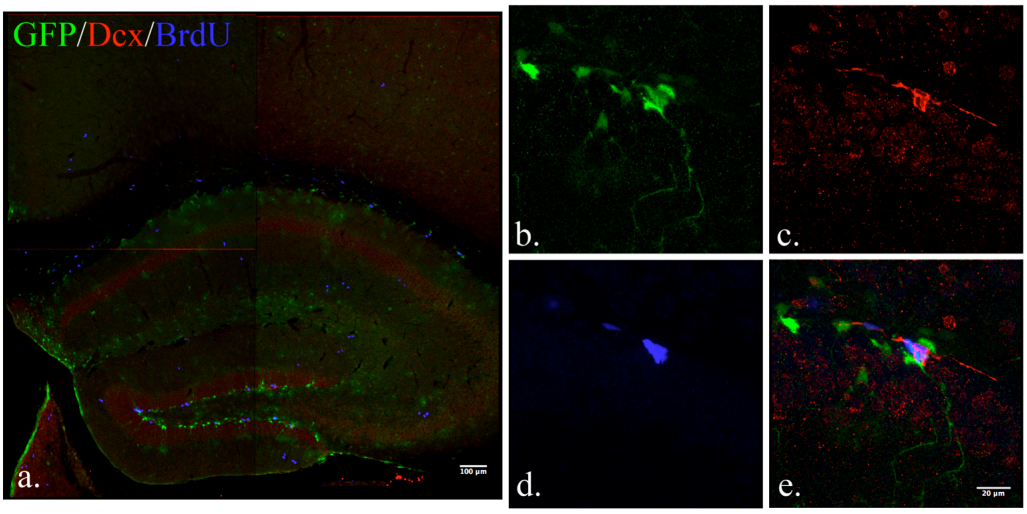

Fig. 4-7. Remaining eGFP-expressing cells are able to generate new late neural progenitors. To demonstrate whether the remaining eGFP-expressing cells regenerate neural progenitor populations in the injured brain after the removal of ganciclovir, 6-week old mice were treated with ganciclovir for 4 weeks to allow for the maturation of the existing DCX-expressing cells. A total of 3 injections of BrdU were given 3, 4 and 5 days after removal of ganciclovir treatment. 7 days or 4 weeks after removal, mice were sacrificed for examination of regeneration of DCX-expressing late neural progenitors or NeuN-expressing neurons. (a) 7 days after the removal of ganciclovir, BrdU/DCX-positive cells were observed in the subgranular layers in the uninjured brains (c-e). Scale bars in a indicate 100µm and 20µm in e.

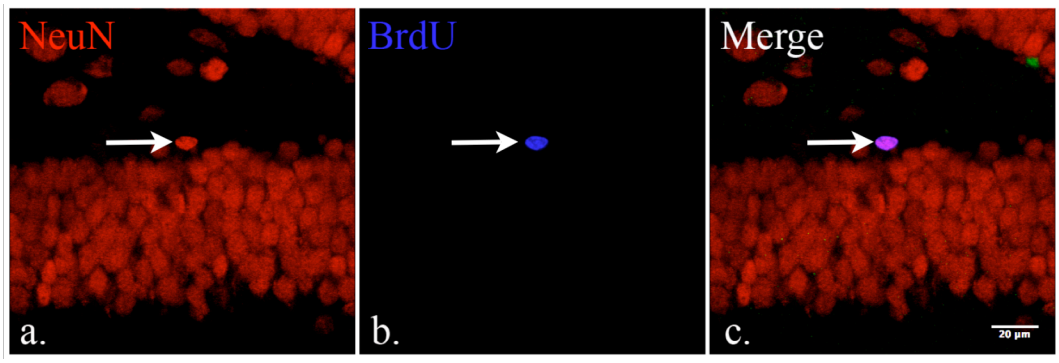

Fig 4-8 Remaining eGFP-expressing cells are able to generate new neurons. Four weeks after the removal of ganciclovir, BrdU/NeuN-positive cells were observed in the subgranular layers in the uninjured brains (*arrow*). Scale bar indicates 20µm.

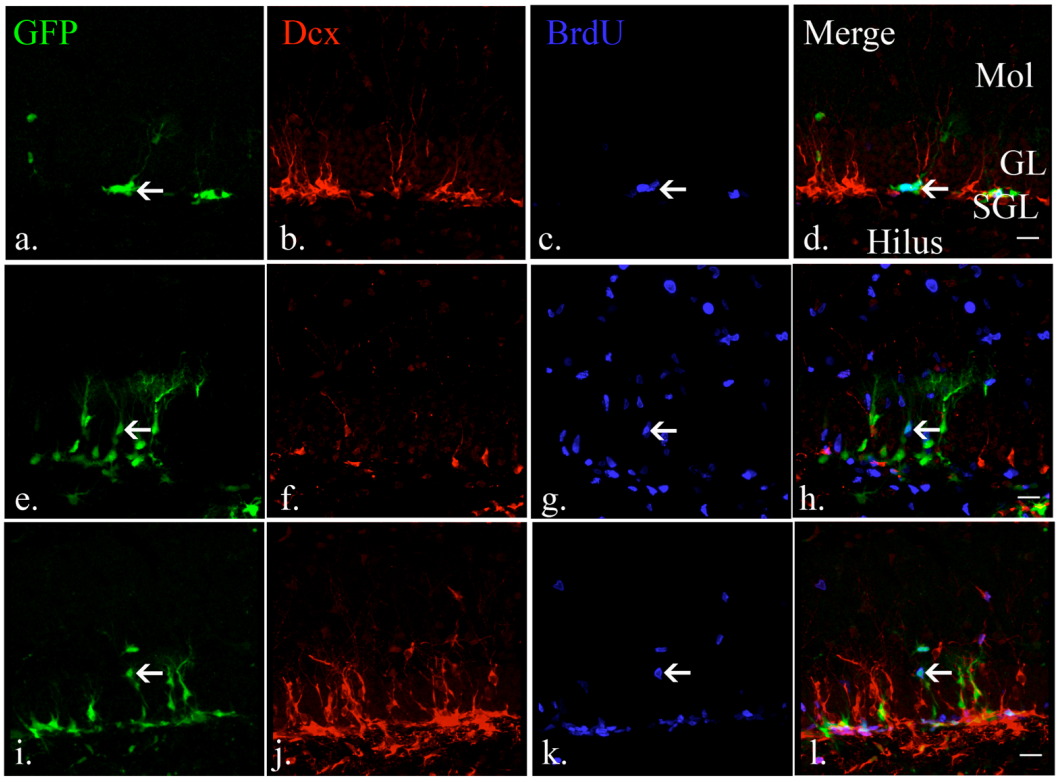

Fig 4-9 The dynamics of eGFP-expressing early neural progenitors in nestin HSV-TK mice resembles that observed in other nestin-eGFP lines. (a to d) BrdU/eGFP-positive cells (arrow) were observed in the subgranular layers in the uninjured adult brains whereas DCX-expressing cells were seen in the subgranular and granular layers. (e to h) Three days after injury, the number of DCX-expressing cells is decreased in the ipsilateral dentate gyrus proximal to the injured areas. The number of BrdU/eGFP-positive early neural progenitors increased, especially the ones in the granular layers (*arrow*). (i to l) 7 days after injury, lost DCX-expressing cells are replaced and BrdU/eGFP-positive early neural progenitors remained active (*arrow*). Scale bars = 20 μm. Abbreviations: Mol - molecular layer, SGL --subgranular layer, GL - granular layer.

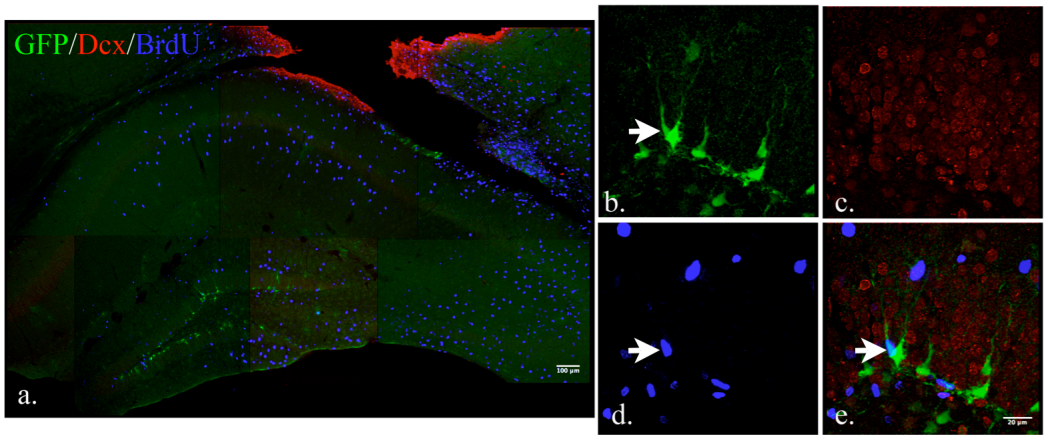

Fig 4-10. Activation of eGFP-expressing cells is required for injury-induced neurogenesis. 10-week old nestin-HSV-TK transgenic mice were given ganciclovir continuously starting at 6 weeks of age. No DCX-expressing cells were observed in the subgranular zone (a, c) although eGFP-expressing cells were still observed in the subgranular layers (b) and BrdU-positive cells were detected in the molecular layer and hilus in the dentate gyrus and other brain regions (a, d). Only occasional BrdU+ cells expressed GFP (arrow). Scale bars indicate 100µm in a and 20µm in e.

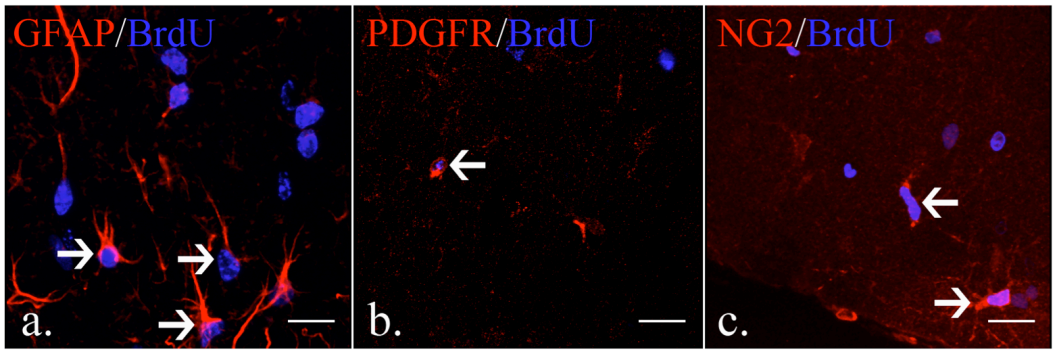

Fig 4-11. The treatment of ganciclovir in injured trangenic mice did not affect the activation of astrocytes and OPCs in the dentate gyrus. To determine whether ganciclovir treatment affects dividing reactive astrocytes OPCs after injury, a total of 3 injections of BrdU were given 3, 4 and 5 days after injury while ganciclovir treatment persisted. (a). BrdU/GFAP-positive and hypertrophic reactive astrocytes were observed in the molecular layers and hilus in the dentate gyrus (arrow). (b and c) By using alpha subunit of PDGF receptor and NG2 as markers for OPCs, BrdU-positive OPCs were observed in the dentate gyrus in the injured brains. The observations demonstrated that ganciclovir treatment did not affect the dividing reactive astrocytes and OPCs in the dentate gyrus of the injured brains. Scale bar indicates 20µm.

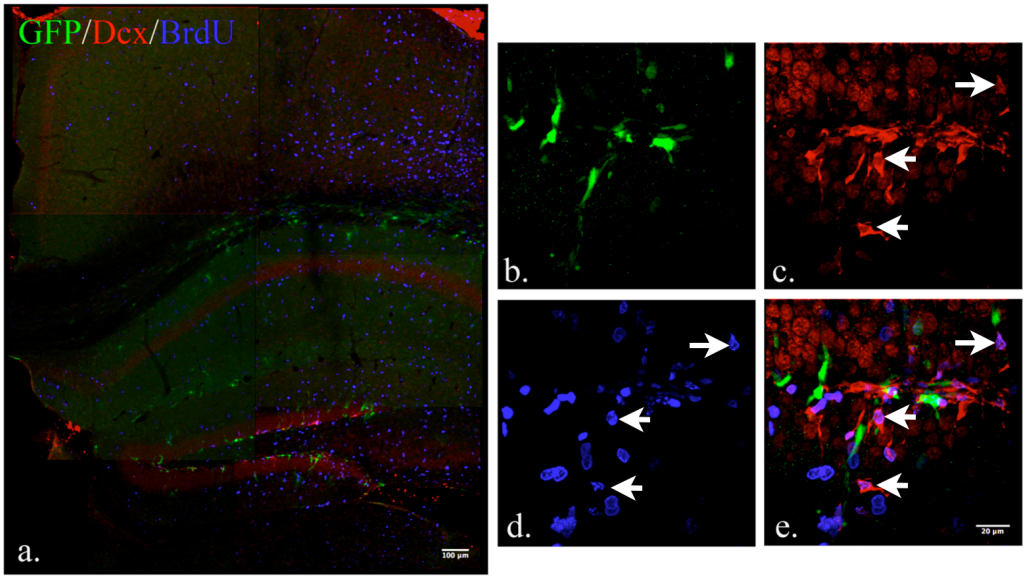

Fig 4-13. To demonstrate whether the remaining eGFP-expressing cells regenerate neural progenitor populations in the injured brain after the removal of ganciclovir, 6-week old mice were treated with ganciclovir for 4 weeks to allow for the maturation of the existing DCX-expressing cells. A total of 3 injections of BrdU were given 3, 4 and 5 days after removal of ganciclovir treatment. (a-e). In the injured brain, BrdU/DCX-positive cells are observed in the granular layers as well as subgranular layers (*arrows*). Scale bars indicate 100 µm in a and 20 µm in e.

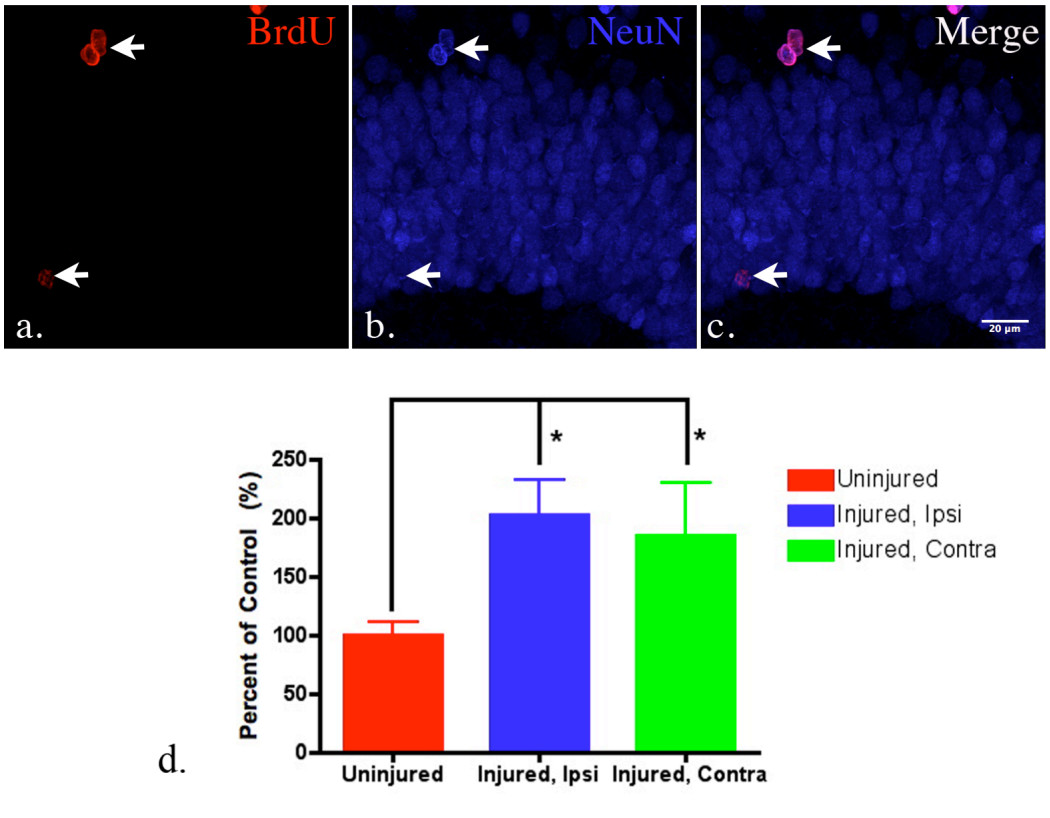

Fig 4-14. To demonstrate whether the remaining eGFP-expressing cells regenerate neural progenitor populations in the injured brain after the removal of ganciclovir, 6-week old mice were treated with ganciclovir for 4 weeks to allow for the maturation of the existing DCX-expressing cells.A total of 3 injections of BrdU were given 3, 4 and 5 days after removal of ganciclovir treatment. (a-c). Four weeks after ganciclovir removal, the distribution of BrdU/NeuN-positive mature neurons were observed in the subgranular and granular layers (*arrows*) 4 weeks after CCI. (d) More neurons were quantified in the dentate gyrus in both hemispheres after injury when compared with ones in the uninjured brains. (*control: 100+/-29, ipsilateral: 201+/-60, and contralateral: 184+/-89, data presented as mean+/-SD; * $p<0.05$*). N=4 in each group and error bars indicate SEM. Scale bars = 20 µm.

Materials and methods

Animals

Experimental animals were housed and cared for in the Animal Resource Center (ARC) at UT Southwestern Medical Center (UTSWMC), which is certified by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All animal experiments were conducted with prior approval of the Institutional Animal Use and Care Committee (IACUC) at UTSWMC in compliance with the highest standard for the humane and compassionate use of animals in biomedical research.

Generation of modified herpes simple virus (delta-HSV-TK) transgenic mice

A construct containing fl-HSV-TK is a generous gift from Dr. Graff (Dept. Dev Biol., UT Southwestern Medical Center). A pair of primers was designed to amplify the ~1.1Kb delta-HSV-TK fragments with EcoRI and BamHI sites on the 5' and 3' end, respectively. The sequences of the primers were as follows. The forward primer: *5'-ccg aat tca agc tta tgc cca cgc tac tgc gg-3',* and the reverse primer*: 5'-cgg gat ccc gtc agt tag cct ccc cca tct c-3'*. The PCR conditions were one cycle of 94°C, 1 min, followed by 35 cycles of 94°C for 1 min, 60°C for 2 min, 72°C for 3 min. The final extension was one cycle of 72°C for 10 min. The amplified fragment was used to replace the rtTA-M2 fragment on pNERV/rtTA-

M2/IRES-eGFP construct with EcoRI (New England Labs) and BamHI (New England Labs) sites (Yu et al., 2005). The linearized plasmid was cut with SalI to remove the pUC fragment and purified with EluTip D (Whatman). The transgenic mice were generated in C57BL/6 genetic background. To verify whether the transgene was transmitted, genomic DNA was extracted from tails at P21 to perform PCR to amplify the eGFP fragment. The primers used to verify eGFP were the forward primer: *5'-gag ctg gac ggc gac gta aac-3'* and the reverse primer: *5'-cgt tgt ggc tgt tgt tag ttg tac-3'*. The PCR condition was one cycle of 94°C for 2 min, followed by 35 cycles of 94°C, 1 min, 59°C, 1 min, and 72°C for 2 min, and one cycle of 72°C for 10 min.

Total RNA extraction and reverse transcribed PCR

Total RNA was extracted from tissues and reverse transcribed (Invitrogen). PCR primers were: delta-HSV-TK (forward: *5'-gcc ttg acc agg gtg aga ta-3'*; reverse: *5'-atg ctg ccc ata agg tat cg-3'*) and GAPDH (forward: *5'-acc aca gtc cat gcc atc ac-3'*; reverse: *5'-tcc acc acc ctg ttg ctg ta-3'*). Primers were used at a concentration of 100μg/reaction. PCR amplification of cDNA was performed in PCR buffer containing 0.5mM dNTPs and 0.5U Taq polymerase (Invitrogen) on a thermal cycler (Thermo Electron Corp.). PCR products were separated on agarose gel and stained with ethidium bromide.

Controlled cortical impact (CCI) injury

To perform CCI, the standard protocol and a controlled cortical impact device used to generate brain injuries were used as previously described (Kernie et al., 2001). 8-week old male wild-type or transgenic mice were anesthetized with 0.15ml of ketamine (100mg/ml):xyzaline (20mg/ml) 10:1 mixture. Mice were placed in a stereotactic frame. The midline incision was made, the soft tissues were reflected, and a 5mm*5mm craniectomy was made between bregma and lambda and 1mm lateral to the midline. The injury was generated with a 3mm stainless steel tipped impact device with deformation of 0.7mm and constant speed of 4.4 m/s. After injury, the scalp was fastened with staples and the mice were allowed to recover. Following injury, BrdU (100mg/kg, Sigma) was injected intraperitoneally at 12 and 2 hrs prior to sacrifice.

Immunofluorescence

All mice were deeply anesthetized with ketamine and xylazine mixture as described above before perfusion. The trans-cardiac perfusion was performed with 50ml of 1xPBS, followed by 50ml of 4% paraformaldehyde (PFA)/1xPBS. Following post-fixation in 4% PFA/1xPBS overnight, the whole brains were dissected and embedded in 3% agarose/1xPBS. Serial 50-μm sections were cut with a vibratome (VT1000S, Leica). All sections encompassing the hippocampus were collected in every well sequentially in 12-well plates. Free-floating method

was used for the immunohistochemistry. For BrdU staining, all sections from a single well were washed with 1xPBS 3 times and rinsed with water. Then, sections were denatured with 0.1N HCl for 1 hr in 37°C water bath. After denaturation, sections were neutralized with 0.1M Borax, pH8.5 (Sigma) for 10 min. Sections were washed with 0.3% Triton X-100/1xPBS (wash buffer) for 3 times and blocked with 5% normal donkey serum (Sigma-Aldrich) contained wash buffer for 1 hour at room temperature. 1:200 rat-anti BrdU (Abcam) antibody was used to label BrdU overnight at 4°C. The following day, sections were incubated with Cy5-conjugated anti-rat antibody (all 1:200, Jackson Immunoresearch) for 3 hrs at room temperature. The sections were placed on the slides and covered with coverslips for confocal microscope observation after antibody incubation. For regular staining the protocol was as above for the BrdU except sections were not treated with HCl and Borax. Primary antibodies used in this study were as follows: rabbit anti-GFP (1:500, Molecular Biology), chicken anti-GFP (1:200, AbCam), mouse anti-GFAP (1:100, BD Pharmingen), rabbit anti-GFAP (1:500, DAKO), goat anti-doublecortin (1:100, Santa Cruz biotech), mouse anti-vimentin (1:200, 40E-C, Hybridoma Bank, Univ. Iowa), rabbit anti-Ki67 (1:200, NeoMarkers), mouse anti-NeuN (1:500, Chemicon), rabbit anti-NG2 (1:200, Chemicon), rat anti-PDGFRa (1:200, BD Pharmingen), goat anti-Iba-1 (1:200, AbCam), and rabbit anti-TK serum (1:10, from Dr. William Summers, Yale University). Cy2-, Cy3- or Cy5-conjugated donkey anti-species antibodies

were used as secondary antibodies (all 1:200, Jackson ImmunoResearch).

Quantification and confocal microscopy

For BrdU-positive cells quantification, a series of every 12th sections were used. BrdU-positive cells were counted under a 40x objective (Olympus) through the whole blades of the dentate gyrus. Based on location, counted cells were subdivided into 2 categories, in the subgranular or granular layers. The subgranular layer (SGL) was defined as a region encompassing 2-cell widths above and below the boundary between granular layer and hilus. The rest was defined as granular layer in this study.

To quantify the cell number and determine the proliferation index of early and late neural progenitors in the dentate gyrus, a Zeiss LSM510 scanning confocal microscope with Argon 488, He 543, and He 633 lasers and a Zeiss Neofluar 40x/1.3 oil DIC lens was used to determine each single cell and the colocalization of different cell markers. Cell quantification was performed from the dentate gyrus in both hemispheres in injured and control mice. Every 12th sections was chosen for immnofluorescent staining to label eGFP, Dcx and BrdU. The images covering the whole blades of the dentate gyrus were taken and the 50μm z-axis was scanned with a consistent 2μm interval. The number of eGFP-or Dcx-expressing cells was counted through each frame by using ImageJ (NIH) without any adjustments of color, contrast, or brightness. The colocalization of eGFP or

Dcx with BrdU was determined only when BrdU signal was clearly covered or wrapped with eGFP or Dcx signal, respectively. The number of colocalized cells was counted frame by frame.

Ganciclovir administration

Ganciclovir (200mg/kg•day, Cytovene-IV, Roche Pharmaceuticals) or vehicle (dH_2O) was delivered via osmotic minipums (model 2002, Alzet). The pumps were replaced every 2 weeks based on the requirements of each experiment. The implantation of pumps was as outlined in the instruction from Alzet and is briefly described below: 6-week old mice were anesthetized by using isofluorane. A small incision was made in the skin between the scapulae. A small pocket was formed by using a hemostat to spread the subcutaneous connective tissues apart. The pumps were inserted into the pocket with the opening of pumps pointing away from the incision. The skin incision was closed with staples.

Statistics

Statistics were done using a one-way analysis of variance (ANOVA) with a Newman-Keuls multiple comparison test, or unpaired Student's t test. Differences were deemed significant with $p<0.05$.

CHAPTER FIVE

Conclusions and Future Directions

Conclusions

By using the well-characterized regulatory elements of the nestin gene, the expression of transgenes and reporters are restricted to the neurogenic areas in the CNS from embryonic stages to adulthood in the generated transgenic mice. In injured brains, eGFP expression, at least in the adult dentate gyrus, is not detectable in reactive astrocytes, active microglia, or OPCs as determined by immunofluorescence. Therefore, it is concluded that the expression of eGFP is restricted to the neural progenitors in our transgenic mice.

As a result of further investigation, eGFP-expressing cells in the dentate gyrus were divided into two categories based on their morphology. eGFP-expressing cells with long processes are type 1-like early neural progenitors. These types of eGFP-expressing cells are known to express early neural progenitor markers, like GFAP, vimentin, RC2, and nestin. The other type of cell has no visible long process and no detectable expression of early neural progenitor markers, however, it does display nestin expression. Interestingly, the expression of eGFP is not detectable in the late neural progenitors, i.e., Dcx-

expressing late neural progenitors. Therefore, eGFP expression is restricted to type 1 and type 2a early neural progenitors and is distinguishable from Dcx-expressing type 2b and 3 late neural progenitors. The restriction of expression of eGFP still remains in early neural progenitors.

By using specific antibody-labeled endothelial cells to visualize blood vessels, subgranular eGFP-expressing type 1- and 2a-like neural progenitors in the dentate gyrus have been demonstrated to reside next to blood vessels. Some of the long processes from type 1-like eGFP-expressing cells physically interact with vessels in the molecular layers. Besides contacting blood vessels, eGFP-expressing cells in the dentate gyrus are also surrounded by mature astrocytes in the hilus and in the molecular layers. These observations are consistent with others' studies, and suggest that eGFP-expressing cells correctly represent the distribution of adult early neural progenitors and the expression of transgenes do not affect the microenvironment in the dentate gyrus (Zhao et al., 2006;Seri et al., 2004;Palmer et al., 2000). These transgenic lines provide a useful tool to monitor the dynamics of early neural progenitors under physiological and pathological conditions.

Unexpectedly, the eGFP-expressing cells with a long processes are also visible in the granular layer. The granular eGFP-expressing cells express all

tested early neural progenitor markers. By using BrdU to label dividing cells, granular eGFP-expressing cells display a low frequency of proliferation under physiological conditions. This suggests that granular eGFP-expressing cells are early neural progenitors. However, no type 2a-like neural progenitors are observed in the granular layers under physiological conditions. Interestingly, granular eGFP-expressing type 1-like cells become active in proliferation and appear to generate new neurons in injured brains. This indicates that these granular eGFP-expressing type 1-like neural progenitors stay quiescent under physiological conditions, but become active in pathological situations.

Another unexpected observation is that the activation is not confined to the ipsilateral dentate gyrus only. In the contralateral dentate gyrus, eGFP-expressing neural progenitors become active seven days after injury. These injury-induced newborn Dcx-expressing cells become mature neurons in the dentate gyrus in both hemispheres.

The generation of modified HSV-TK mice in this project has been validated as a useful tool for specifically inhibiting neurogenesis. The expression of modified HSV-TK and reporter genes is consistent with early neural progenitors, even in the injured brains. Treatment with ganciclovir causes an efficient ablation of dividing neural progenitors. Interestingly, the dividing cells in

other brain regions, like the cortex, thalamus, and cerebellum, are not affected. The ablation is unique to the dividing early neural progenitors in the dentate gyrus of the injured brains. Dividing reactive astrocytes, active microglia, or OPCs are not affected in injured brains with ganciclovir treatment. The requirement of activation of early neural progenitors in the injured brains is determined by treating mice with ganciclovir to ablate dividing neural progenitors and discontinuing ganciclovir treatment to allow regeneration of newborn neurons. This transgenic line is a useful tool in studying the role of adult neurogenesis in a variety of physiological aspects.

Future Directions

1. The role of newborn neurons in the adult dentate gyrus remains controversial due to the limitations of current methodologies (Leuner et al., 2006). By using this transgenic line, the ablation is specific to dividing neural progenitors only and the remaining neural progenitors are still able to generate progeny after drug discontinuation. It will be interesting to investigate the role of newborn neurons in hippocampus-dependent tasks by manipulating neurogenesis.
2. Although more newborn neurons are observed in the dentate gyrus in the injured brains, whether they contribute to recovery is not known. A recent

study demonstrates that the time when injured rats recover from cognitive deficits is correlated with the time in which new neurons are generated after injury (Sun et al., 2007). By using this transgenic line, one will be able to provide direct evidence to determine whether injury-induced newborn neurons are required for recovery.

3. Enriched environment (EE) is known to stimulate neurogenesis and enhance learning and memory (Bruel-Jungerman et al., 2005;Frick et al., 2003;Frick and Fernandez, 2003;Teather et al., 2002). However, whether EE improves the recovery from cognitive deficits after TBI or whether EE-enhanced neurogenesis is critical in the recovery are not known. By treating transgenic mice with ganciclovir or vehicle and housing them in a normal cage or under EE after injury, the role of EE-induced newborn neurons in improving recovery after TBI can be determined.

BIBLIOGRAPHY

Aggleton JP, Sanderson DJ, Pearce JM (2007) Structural learning and the hippocampus. Hippocampus 17:723-734.

Aguirre A, Gallo V (2004) Postnatal neurogenesis and gliogenesis in the olfactory bulb from NG2-expressing progenitors of the subventricular zone. J Neurosci 24:10530-10541.

Aimone JB, Wiles J, Gage FH (2006) Potential role for adult neurogenesis in the encoding of time in new memories. Nat Neurosci 9:723-727.

Akasu T, Muraoka N, Hasuo H (2002) Hyperexcitability of hippocampal CA1 neurons after fluid percussion injury of the rat cerebral cortex. Neurosci Lett 329:305-308.

ALTMAN J (1962) Are new neurons formed in the brains of adult mammals? Science 135:1127-1128.

Alvarez-Buylla A, Lois C (1995) Neuronal stem cells in the brain of adult vertebrates. Stem Cells 13:263-272.

Alvarez-Buylla A, Seri B, Doetsch F (2002) Identification of neural stem cells in the adult vertebrate brain. Brain Res Bull 57:751-758.

Arciniegas DB, Silver JM (2006) Pharmacotherapy of posttraumatic cognitive impairments. Behav Neurol 17:25-42.

Arundine M, Tymianski M (2004) Molecular mechanisms of glutamate-dependent neurodegeneration in ischemia and traumatic brain injury. Cell Mol Life Sci 61:657-668.

Bajo A, Fleminger S (2002) Brain injury rehabilitation: what works for whom and when? Brain Inj 16:385-395.

Bauer S, Patterson PH (2005) The cell cycle-apoptosis connection revisited in the adult brain. J Cell Biol 171:641-650.

Becker S, Wojtowicz JM (2007) A model of hippocampal neurogenesis in memory and mood disorders. Trends Cogn Sci 11:70-76.

Belachew S, Chittajallu R, Aguirre AA, Yuan X, Kirby M, Anderson S, Gallo V (2003) Postnatal NG2 proteoglycan-expressing progenitor cells are intrinsically multipotent and generate functional neurons. J Cell Biol 161:169-186.

Bird CM, Burgess N (2008) The hippocampus and memory: insights from spatial processing. Nat Rev Neurosci 9:182-194.

Bjugstad KB, Teng YD, Redmond DE, Elsworth JD, Roth RH, Cornelius SK, Snyder

EY, Sladek JR (2008) Human neural stem cells migrate along the nigrostriatal pathway in a primate model of Parkinson's disease. Exp Neurol.

Bruel-Jungerman E, Laroche S, Rampon C (2005) New neurons in the dentate gyrus are involved in the expression of enhanced long-term memory following environmental enrichment. Eur J Neurosci 21:513-521.

Brüstle O, McKay RD (1996) Neuronal progenitors as tools for cell replacement in the nervous system. Curr Opin Neurobiol 6:688-695.

Bryant RA (2001) Posttraumatic stress disorder and traumatic brain injury: can they co-exist? Clin Psychol Rev 21:931-948.

Burns KA, Ayoub AE, Breunig JJ, Adhami F, Weng WL, Colbert MC, Rakic P, Kuan CY (2007) Nestin-CreER Mice Reveal DNA Synthesis by Nonapoptotic Neurons following Cerebral Ischemia Hypoxia. Cereb Cortex 17:2585-2592.

Bush TG, Savidge TC, Freeman TC, Cox HJ, Campbell EA, Mucke L, Johnson MH, Sofroniew MV (1998) Fulminant jejuno-ileitis following ablation of enteric glia in adult transgenic mice. Cell 93:189-201.

Cameron HA, McKay RD (2001) Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. J Comp Neurol 435:406-417.

Canfield V, West AB, Goldenring JR, Levenson R (1996) Genetic ablation of parietal cells in transgenic mice: a new model for analyzing cell lineage relationships in the gastric mucosa. Proc Natl Acad Sci U S A 93:2431-2435.

Chen Y, Swanson RA (2003) Astrocytes and brain injury. J Cereb Blood Flow Metab 23:137-149.

Chirumamilla S, Sun D, Bullock MR, Colello RJ (2002) Traumatic brain injury induced cell proliferation in the adult mammalian central nervous system. J Neurotrauma 19:693-703.

Dahlstrand J, Lardelli M, Lendahl U (1995) Nestin mRNA expression correlates with the central nervous system progenitor cell state in many, but not all, regions of developing central nervous system. Brain Res Dev Brain Res 84:109-129.

Darsalia V, Heldmann U, Lindvall O, Kokaia Z (2005) Stroke-induced neurogenesis in aged brain. Stroke 36:1790-1795.

Deisseroth K, Singla S, Toda H, Monje M, Palmer TD, Malenka RC (2004) Excitation-neurogenesis coupling in adult neural stem/progenitor cells. Neuron 42:535-552.

Delaney CL, Brenner M, Messing A (1996) Conditional ablation of cerebellar astrocytes

in postnatal transgenic mice. J Neurosci 16:6908-6918.

Diers-Fenger M, Kirchhoff F, Kettenmann H, Levine JM, Trotter J (2001) AN2/NG2 protein-expressing glial progenitor cells in the murine CNS: isolation, differentiation, and association with radial glia. Glia 34:213-228.

Doetsch F, Caillé I, Lim DA, García-Verdugo JM, Alvarez-Buylla A (1999a) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97:703-716.

Doetsch F, García-Verdugo JM, Alvarez-Buylla A (1997) Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. J Neurosci 17:5046-5061.

Doetsch F, García-Verdugo JM, Alvarez-Buylla A (1999b) Regeneration of a germinal layer in the adult mammalian brain. Proc Natl Acad Sci U S A 96:11619-11624.

Doetsch F, Petreanu L, Caille I, Garcia-Verdugo JM, Alvarez-Buylla A (2002) EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cells. Neuron 36:1021-1034.

Duggal N, Schmidt-Kastner R, Hakim AM (1997) Nestin expression in reactive astrocytes following focal cerebral ischemia in rats. Brain Res 768:1-9.

Eldadah BA, Faden AI (2000) Caspase pathways, neuronal apoptosis, and CNS injury. J Neurotrauma 17:811-829.

Enriquez P, Bullock R (2004) Molecular and cellular mechanisms in the pathophysiology of severe head injury. Curr Pharm Des 10:2131-2143.

Eriksson PS, Perfilieva E, Björk-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH (1998) Neurogenesis in the adult human hippocampus. Nat Med 4:1313-1317.

Ernst C, Christie BR (2006) Temporally specific proliferation events are induced in the hippocampus following acute focal injury. J Neurosci Res 83:349-361.

Felderhoff-Mueser U, Ikonomidou C (2000) Mechanisms of neurodegeneration after paediatric brain injury. Curr Opin Neurol 13:141-145.

Ferbinteanu J, Kennedy PJ, Shapiro ML (2006) Episodic memory--from brain to mind. Hippocampus 16:691-703.

Fitch MT, Silver J (2007) CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. Exp Neurol.

Frick KM, Fernandez SM (2003) Enrichment enhances spatial memory and increases

synaptophysin levels in aged female mice. Neurobiol Aging 24:615-626.

Frick KM, Stearns NA, Pan JY, Berger-Sweeney J (2003) Effects of environmental enrichment on spatial memory and neurochemistry in middle-aged mice. Learn Mem 10:187-198.

Fukuda S, Kato F, Tozuka Y, Yamaguchi M, Miyamoto Y, Hisatsune T (2003) Two distinct subpopulations of nestin-positive cells in adult mouse dentate gyrus. J Neurosci 23:9357-9366.

Gage FH (2000) Mammalian neural stem cells. Science 287:1433-1438.

Gaillard A, Prestoz L, Dumartin B, Cantereau A, Morel F, Roger M, Jaber M (2007) Reestablishment of damaged adult motor pathways by grafted embryonic cortical neurons. Nat Neurosci 10:1294-1299.

Galvan V, Jin K (2007) Neurogenesis in the aging brain. Clin Interv Aging 2:605-610.

Garcia AD, Doan NB, Imura T, Bush TG, Sofroniew MV (2004) GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain. Nat Neurosci 7:1233-1241.

García-Verdugo JM, Doetsch F, Wichterle H, Lim DA, Alvarez-Buylla A (1998)

Architecture and cell types of the adult subventricular zone: in search of the stem cells. J Neurobiol 36:234-248.

Ge S, Goh EL, Sailor KA, Kitabatake Y, Ming GL, Song H (2006) GABA regulates synaptic integration of newly generated neurons in the adult brain. Nature 439:589-593.

Ge S, Pradhan DA, Ming GL, Song H (2007) GABA sets the tempo for activity-dependent adult neurogenesis. Trends Neurosci 30:1-8.

Gentleman D (1999) Improving outcome after traumatic brain injury--progress and challenges. Br Med Bull 55:910-926.

Godinho L, Williams PR, Claassen Y, Provost E, Leach SD, Kamermans M, Wong RO (2007) Nonapical symmetric divisions underlie horizontal cell layer formation in the developing retina in vivo. Neuron 56:597-603.

Goings GE, Kozlowski DA, Szele FG (2006) Differential activation of microglia in neurogenic versus non-neurogenic regions of the forebrain. Glia 54:329-342.

Gotts JE, Chesselet MF (2005) Migration and fate of newly born cells after focal cortical ischemia in adult rats. J Neurosci Res 80:160-171.

Griesemer D, Mautes AM (2007) Closed Head Injury Causes Hyperexcitability in Rat

Hippocampal CA1 but not in CA3 Pyramidal Cells. J Neurotrauma 24:1823-1832.

Hatton J (2001) Pharmacological treatment of traumatic brain injury: a review of agents in development. CNS Drugs 15:553-581.

Hayes NL, Nowakowski RS (2002) Dynamics of cell proliferation in the adult dentate gyrus of two inbred strains of mice. Brain Res Dev Brain Res 134:77-85.

Heegaard W, Biros M (2007) Traumatic brain injury. Emerg Med Clin North Am 25:655-78, viii.

Herrera DG, Garcia-Verdugo JM, Alvarez-Buylla A (1999) Adult-derived neural precursors transplanted into multiple regions in the adult brain. Ann Neurol 46:867-877.

Holmes MM, Galea LA, Mistlberger RE, Kempermann G (2004) Adult hippocampal neurogenesis and voluntary running activity: circadian and dose-dependent effects. J Neurosci Res 76:216-222.

Ihrie RA, Alvarez-Buylla A (2007) Cells in the astroglial lineage are neural stem cells. Cell Tissue Res.

Imura T, Nakano I, Kornblum HI, Sofroniew MV (2006) Phenotypic and functional heterogeneity of GFAP-expressing cells in vitro: differential expression of LeX/CD15 by

GFAP-expressing multipotent neural stem cells and non-neurogenic astrocytes. Glia 53:277-293.

Iwanami A, Kaneko S, Nakamura M, Kanemura Y, Mori H, Kobayashi S, Yamasaki M, Momoshima S, Ishii H, Ando K, Tanioka Y, Tamaoki N, Nomura T, Toyama Y, Okano H (2005) Transplantation of human neural stem cells for spinal cord injury in primates. J Neurosci Res 80:182-190.

Jellinger KA (2004) Head injury and dementia. Curr Opin Neurol 17:719-723.

Jensen FE (2002) The role of glutamate receptor maturation in perinatal seizures and brain injury. Int J Dev Neurosci 20:339-347.

Jeong SW, Chu K, Jung KH, Kim SU, Kim M, Roh JK (2003) Human neural stem cell transplantation promotes functional recovery in rats with experimental intracerebral hemorrhage. Stroke 34:2258-2263.

Jin K, Sun Y, Xie L, Peel A, Mao XO, Batteur S, Greenberg DA (2003a) Directed migration of neuronal precursors into the ischemic cerebral cortex and striatum. Mol Cell Neurosci 24:171-189.

Jin K, Xie L, Childs J, Sun Y, Mao XO, Logvinova A, Greenberg DA (2003b) Cerebral neurogenesis is induced by intranasal administration of growth factors. Ann Neurol

53:405-409.

Johansson CB, Lothian C, Molin M, Okano H, Lendahl U (2002) Nestin enhancer requirements for expression in normal and injured adult CNS. J Neurosci Res 69:784-794.

Johnston MV (2005) Excitotoxicity in perinatal brain injury. Brain Pathol 15:234-240.

Kaplan MS, Hinds JW (1977) Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science 197:1092-1094.

Kee N, Teixeira CM, Wang AH, Frankland PW (2007) Imaging activation of adult-generated granule cells in spatial memory. Nat Protoc 2:3033-3044.

Kempermann G, Gast D, Kronenberg G, Yamaguchi M, Gage FH (2003) Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice. Development 130:391-399.

Kempermann G, Kuhn HG, Gage FH (1997a) Genetic influence on neurogenesis in the dentate gyrus of adult mice. Proc Natl Acad Sci U S A 94:10409-10414.

Kempermann G, Kuhn HG, Gage FH (1997b) More hippocampal neurons in adult mice living in an enriched environment. Nature 386:493-495.

Kernie SG, Erwin TM, Parada LF (2001) Brain remodeling due to neuronal and astrocytic proliferation after controlled cortical injury in mice. J Neurosci Res 66:317-326.

Kim EJ, Leung CT, Reed RR, Johnson JE (2007) In Vivo Analysis of Ascl1 Defined Progenitors Reveals Distinct Developmental Dynamics during Adult Neurogenesis and Gliogenesis. J Neurosci 27:12764-12774.

Kimelberg HK (1992) Astrocytic edema in CNS trauma. J Neurotrauma 9 Suppl 1:S71-S81.

Kondo T, Raff M (2000) Oligodendrocyte precursor cells reprogrammed to become multipotential CNS stem cells. Science 289:1754-1757.

Kronenberg G, Reuter K, Steiner B, Brandt MD, Jessberger S, Yamaguchi M, Kempermann G (2003) Subpopulations of proliferating cells of the adult hippocampus respond differently to physiologic neurogenic stimuli. J Comp Neurol 467:455-463.

Kronenberg G, Wang LP, Geraerts M, Babu H, Synowitz M, Vicens P, Lutsch G, Glass R, Yamaguchi M, Baekelandt V, Debyser Z, Kettenmann H, Kempermann G (2007) Local origin and activity-dependent generation of nestin-expressing protoplasmic astrocytes in CA1. Brain Struct Funct 212:19-35.

Kruglyakova EP, Khovryakov AV, Shikhanov NP, Maccann GM, Vael' I, Kruglyakov PP, Sosunov AA (2005) Nestin-expressing cells in the human hippocampus. Neurosci Behav Physiol 35:891-897.

Kuhn HG, Dickinson-Anson H, Gage FH (1996) Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. J Neurosci 16:2027-2033.

Kuhn HG, Winkler J, Kempermann G, Thal LJ, Gage FH (1997) Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. J Neurosci 17:5820-5829.

Lagace DC, Whitman MC, Noonan MA, Ables JL, Decarolis NA, Arguello AA, Donovan MH, Fischer SJ, Farnbauch LA, Beech RD, Dileone RJ, Greer CA, Mandyam CD, Eisch AJ (2007) Dynamic Contribution of Nestin-Expressing Stem Cells to Adult Neurogenesis. J Neurosci 27:12623-12629.

Lalonde R, Strazielle C (2003) The effects of cerebellar damage on maze learning in animals. Cerebellum 2:300-309.

Leker RR (2006) Manipulation of endogenous neural stem cells following ischemic brain injury. Pathophysiol Haemost Thromb 35:58-62.

Lendahl U, McKay RD (1990) The use of cell lines in neurobiology. Trends Neurosci 13:132-137.

Leuner B, Gould E, Shors TJ (2006) Is there a link between adult neurogenesis and learning? Hippocampus 16:216-224.

Levine JM, Reynolds R, Fawcett JW (2001) The oligodendrocyte precursor cell in health and disease. Trends Neurosci 24:39-47.

Lewis PF, Emerman M (1994) Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. J Virol 68:510-516.

Lichtenwalner RJ, Parent JM (2006) Adult neurogenesis and the ischemic forebrain. J Cereb Blood Flow Metab 26:1-20.

Lie DC, Colamarino SA, Song HJ, Désiré L, Mira H, Consiglio A, Lein ES, Jessberger S, Lansford H, Dearie AR, Gage FH (2005) Wnt signalling regulates adult hippocampal neurogenesis. Nature 437:1370-1375.

Lie DC, Song H, Colamarino SA, Ming GL, Gage FH (2004) Neurogenesis in the adult brain: new strategies for central nervous system diseases. Annu Rev Pharmacol Toxicol 44:399-421.

Lim DA, Alvarez-Buylla A (1999) Interaction between astrocytes and adult subventricular zone precursors stimulates neurogenesis. Proc Natl Acad Sci U S A 96:7526-7531.

Lin RC, Matesic DF, Marvin M, McKay RD, Brüstle O (1995) Re-expression of the intermediate filament nestin in reactive astrocytes. Neurobiol Dis 2:79-85.

Lledo PM, Alonso M, Grubb MS (2006) Adult neurogenesis and functional plasticity in neuronal circuits. Nat Rev Neurosci 7:179-193.

Louissaint A, Rao S, Leventhal C, Goldman SA (2002) Coordinated interaction of neurogenesis and angiogenesis in the adult songbird brain. Neuron 34:945-960.

Lu L, Bao G, Chen H, Xia P, Fan X, Zhang J, Pei G, Ma L (2003) Modification of hippocampal neurogenesis and neuroplasticity by social environments. Exp Neurol 183:600-609.

Lynch MA (2004) Long-term potentiation and memory. Physiol Rev 84:87-136.

Marklund N, Salci K, Ronquist G, Hillered L (2006) Energy metabolic changes in the early post-injury period following traumatic brain injury in rats. Neurochem Res 31:1085-1093.

Mathis C, Hindelang C, LeMeur M, Borrelli E (2000) A transgenic mouse model for inducible and reversible dysmyelination. J Neurosci 20:7698-7705.

Mattson MP, Scheff SW (1994) Endogenous neuroprotection factors and traumatic brain injury: mechanisms of action and implications for therapy. J Neurotrauma 11:3-33.

McAllister TW, Flashman LA, Sparling MB, Saykin AJ (2004) Working memory deficits after traumatic brain injury: catecholaminergic mechanisms and prospects for treatment -- a review. Brain Inj 18:331-350.

McDonald JW, Liu XZ, Qu Y, Liu S, Mickey SK, Turetsky D, Gottlieb DI, Choi DW (1999) Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. Nat Med 5:1410-1412.

Mena FV, Baab PJ, Zielke CL, Zielke HR (2000) In vivo glutamine hydrolysis in the formation of extracellular glutamate in the injured rat brain. J Neurosci Res 60:632-641.

Meshi D, Drew MR, Saxe M, Ansorge MS, David D, Santarelli L, Malapani C, Moore H, Hen R (2006) Hippocampal neurogenesis is not required for behavioral effects of environmental enrichment. Nat Neurosci 9:729-731.

Mignone JL, Kukekov V, Chiang AS, Steindler D, Enikolopov G (2004) Neural stem and

progenitor cells in nestin-GFP transgenic mice. J Comp Neurol 469:311-324.

Miles DK, Kernie SG (2006) Activation of neural stem and progenitor cells after brain injury. Prog Brain Res 157:187-197.

Miles DK, Kernie SG (2008) Hypoxic-ischemic brain injury activates early hippocampal stem/progenitor cells to replace vulnerable neuroblasts. Hippocampus.

Milman A, Rosenberg A, Weizman R, Pick CG (2005) Mild traumatic brain injury induces persistent cognitive deficits and behavioral disturbances in mice. J Neurotrauma 22:1003-1010.

Minasi LE, Kamogawa Y, Carding S, Bottomly K, Flavell RA (1993) The selective ablation of interleukin 2-producing cells isolated from transgenic mice. J Exp Med 177:1451-1459.

Ming GL, Song H (2005) Adult neurogenesis in the mammalian central nervous system. Annu Rev Neurosci 28:223-250.

Mizumatsu S, Monje ML, Morhardt DR, Rola R, Palmer TD, Fike JR (2003) Extreme sensitivity of adult neurogenesis to low doses of X-irradiation. Cancer Res 63:4021-4027.

Monje ML, Palmer T (2003) Radiation injury and neurogenesis. Curr Opin Neurol

16:129-134.

Moore EL, Terryberry-Spohr L, Hope DA (2006) Mild traumatic brain injury and anxiety sequelae: a review of the literature. Brain Inj 20:117-132.

Mori T, Buffo A, Götz M (2005) The novel roles of glial cells revisited: the contribution of radial glia and astrocytes to neurogenesis. Curr Top Dev Biol 69:67-99.

Morshead CM, Garcia AD, Sofroniew MV, van Der Kooy D (2003) The ablation of glial fibrillary acidic protein-positive cells from the adult central nervous system results in the loss of forebrain neural stem cells but not retinal stem cells. Eur J Neurosci 18:76-84.

Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, Tamura A, Kirino T, Nakafuku M (2002) Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. Cell 110:429-441.

Nortje J, Menon DK (2004) Traumatic brain injury: physiology, mechanisms, and outcome. Curr Opin Neurol 17:711-718.

Norton WT (1999) Cell reactions following acute brain injury: a review. Neurochem Res 24:213-218.

Nottebohm F (2002) Why are some neurons replaced in adult brain? J Neurosci 22:624-

628.

Overstreet LS, Hentges ST, Bumaschny VF, de Souza FS, Smart JL, Santangelo AM, Low MJ, Westbrook GL, Rubinstein M (2004) A transgenic marker for newly born granule cells in dentate gyrus. J Neurosci 24:3251-3259.

Palmer TD, Markakis EA, Willhoite AR, Safar F, Gage FH (1999) Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. J Neurosci 19:8487-8497.

Palmer TD, Willhoite AR, Gage FH (2000) Vascular niche for adult hippocampal neurogenesis. J Comp Neurol 425:479-494.

Panchision DM, Pickel JM, Studer L, Lee SH, Turner PA, Hazel TG, McKay RD (2001) Sequential actions of BMP receptors control neural precursor cell production and fate. Genes Dev 15:2094-2110.

Parent JM (2003) Injury-induced neurogenesis in the adult mammalian brain. Neuroscientist 9:261-272.

Parent JM, Elliott RC, Pleasure SJ, Barbaro NM, Lowenstein DH (2006) Aberrant seizure-induced neurogenesis in experimental temporal lobe epilepsy. Ann Neurol 59:81-91.

Park KI, Hack MA, Ourednik J, Yandava B, Flax JD, Stieg PE, Gullans S, Jensen FE, Sidman RL, Ourednik V, Snyder EY (2006) Acute injury directs the migration, proliferation, and differentiation of solid organ stem cells: evidence from the effect of hypoxia-ischemia in the CNS on clonal "reporter" neural stem cells. Exp Neurol 199:156-178.

Park KI, Teng YD, Snyder EY (2002) The injured brain interacts reciprocally with neural stem cells supported by scaffolds to reconstitute lost tissue. Nat Biotechnol 20:1111-1117.

Peterson DA (2002) Stem cells in brain plasticity and repair. Curr Opin Pharmacol 2:34-42.

Petzold GC, Windmüller O, Haack S, Major S, Buchheim K, Megow D, Gabriel S, Lehmann TN, Drenckhahn C, Peters O, Meierkord H, Heinemann U, Dirnagl U, Dreier JP (2005) Increased extracellular K+ concentration reduces the efficacy of N-methyl-D-aspartate receptor antagonists to block spreading depression-like depolarizations and spreading ischemia. Stroke 36:1270-1277.

Pinto L, Götz M (2007) Radial glial cell heterogeneity-The source of diverse progeny in the CNS. Prog Neurobiol 83:2-23.

Podgornyi OV, Kheifets IV, Aleksandrova MA, Loseva EV, Revishchin AV, Poltavtseva RA, Marey MV, Korochkin LI, Sukhikh GT (2004) Human neural stem cells normalize rat behavior after hypoxia. Bull Exp Biol Med 137:348-351.

Prins ML, Hovda DA (2003) Developing experimental models to address traumatic brain injury in children. J Neurotrauma 20:123-137.

Pullela R, Raber J, Pfankuch T, Ferriero DM, Claus CP, Koh SE, Yamauchi T, Rola R, Fike JR, Noble-Haeusslein LJ (2006) Traumatic injury to the immature brain results in progressive neuronal loss, hyperactivity and delayed cognitive impairments. Dev Neurosci 28:396-409.

Raber J, Rola R, LeFevour A, Morhardt D, Curley J, Mizumatsu S, VandenBerg SR, Fike JR (2004) Radiation-induced cognitive impairments are associated with changes in indicators of hippocampal neurogenesis. Radiat Res 162:39-47.

Raghupathi R (2004) Cell death mechanisms following traumatic brain injury. Brain Pathol 14:215-222.

Raghupathi R, Graham DI, McIntosh TK (2000) Apoptosis after traumatic brain injury. J Neurotrauma 17:927-938.

Rakic P (2002) Neurogenesis in adult primate neocortex: an evaluation of the evidence.

Nat Rev Neurosci 3:65-71.

Ramaswamy S, Goings GE, Soderstrom KE, Szele FG, Kozlowski DA (2005) Cellular proliferation and migration following a controlled cortical impact in the mouse. Brain Res 1053:38-53.

Rees L, Marshall S, Hartridge C, Mackie D, Weiser M, Erabi Group (2007) Cognitive interventions post acquired brain injury. Brain Inj 21:161-200.

Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255:1707-1710.

Rice AC, Khaldi A, Harvey HB, Salman NJ, White F, Fillmore H, Bullock MR (2003) Proliferation and neuronal differentiation of mitotically active cells following traumatic brain injury. Exp Neurol 183:406-417.

Rickards H (2006) Depression in neurological disorders: an update. Curr Opin Psychiatry 19:294-298.

Ridet JL, Malhotra SK, Privat A, Gage FH (1997) Reactive astrocytes: cellular and molecular cues to biological function. Trends Neurosci 20:570-577.

Rindi G, Ratineau C, Ronco A, Candusso ME, Tsai M, Leiter AB (1999) Targeted

ablation of secretin-producing cells in transgenic mice reveals a common differentiation pathway with multiple enteroendocrine cell lineages in the small intestine. Development 126:4149-4156.

Rohling ML, Meyers JE, Millis SR (2003) Neuropsychological impairment following traumatic brain injury: a dose-response analysis. Clin Neuropsychol 17:289-302.

Rola R, Mizumatsu S, Otsuka S, Morhardt DR, Noble-Haeusslein LJ, Fishman K, Potts MB, Fike JR (2006) Alterations in hippocampal neurogenesis following traumatic brain injury in mice. Exp Neurol 202:189-199.

Rola R, Raber J, Rizk A, Otsuka S, VandenBerg SR, Morhardt DR, Fike JR (2004) Radiation-induced impairment of hippocampal neurogenesis is associated with cognitive deficits in young mice. Exp Neurol 188:316-330.

Romanko MJ, Rola R, Fike JR, Szele FG, Dizon ML, Felling RJ, Brazel CY, Levison SW (2004) Roles of the mammalian subventricular zone in cell replacement after brain injury. Prog Neurobiol 74:77-99.

Royo NC, Shimizu S, Schouten JW, Stover JF, McIntosh TK (2003) Pharmacology of traumatic brain injury. Curr Opin Pharmacol 3:27-32.

Sahay A, Hen R (2007) Adult hippocampal neurogenesis in depression. Nat Neurosci

10:1110-1115.

Sahin Kaya S, Mahmood A, Li Y, Yavuz E, Chopp M (1999) Expression of nestin after traumatic brain injury in rat brain. Brain Res 840:153-157.

Salomon B, Lorès P, Pioche C, Racz P, Jami J, Klatzmann D (1994) Conditional ablation of dendritic cells in transgenic mice. J Immunol 152:537-548.

Salomon B, Maury S, Loubière L, Caruso M, Onclercq R, Klatzmann D (1995) A truncated herpes simplex virus thymidine kinase phosphorylates thymidine and nucleoside analogs and does not cause sterility in transgenic mice. Mol Cell Biol 15:5322-5328.

Santhakumar V, Bender R, Frotscher M, Ross ST, Hollrigel GS, Toth Z, Soltesz I (2000) Granule cell hyperexcitability in the early post-traumatic rat dentate gyrus: the 'irritable mossy cell' hypothesis. J Physiol 524 Pt 1:117-134.

Saxe MD, Battaglia F, Wang JW, Malleret G, David DJ, Monckton JE, Garcia AD, Sofroniew MV, Kandel ER, Santarelli L, Hen R, Drew MR (2006) Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. Proc Natl Acad Sci U S A 103:17501-17506.

Saxe MD, Malleret G, Vronskaya S, Mendez I, Garcia AD, Sofroniew MV, Kandel ER,

Hen R (2007) Paradoxical influence of hippocampal neurogenesis on working memory. Proc Natl Acad Sci U S A 104:4642-4646.

Schinder AF, Gage FH (2004) A hypothesis about the role of adult neurogenesis in hippocampal function. Physiology (Bethesda) 19:253-261.

Schouten JW, Fulp CT, Royo NC, Saatman KE, Watson DJ, Snyder EY, Trojanowski JQ, Prockop DJ, Maas AI, McIntosh TK (2004) A review and rationale for the use of cellular transplantation as a therapeutic strategy for traumatic brain injury. J Neurotrauma 21:1501-1538.

Scoville WB, Milner B (2000) Loss of recent memory after bilateral hippocampal lesions. 1957. J Neuropsychiatry Clin Neurosci 12:103-113.

Selden JR, Dolbeare F, Clair JH, Nichols WW, Miller JE, Kleemeyer KM, Hyland RJ, DeLuca JG (1993) Statistical confirmation that immunofluorescent detection of DNA repair in human fibroblasts by measurement of bromodeoxyuridine incorporation is stoichiometric and sensitive. Cytometry 14:154-167.

Seri B, García-Verdugo JM, Collado-Morente L, McEwen BS, Alvarez-Buylla A (2004) Cell types, lineage, and architecture of the germinal zone in the adult dentate gyrus. J Comp Neurol 478:359-378.

Seri B, García-Verdugo JM, McEwen BS, Alvarez-Buylla A (2001) Astrocytes give rise to new neurons in the adult mammalian hippocampus. J Neurosci 21:7153-7160.

Sgubin D, Aztiria E, Perin A, Longatti P, Leanza G (2007) Activation of endogenous neural stem cells in the adult human brain following subarachnoid hemorrhage. J Neurosci Res 85:1647-1655.

Shear DA, Tate MC, Archer DR, Hoffman SW, Hulce VD, Laplaca MC, Stein DG (2004) Neural progenitor cell transplants promote long-term functional recovery after traumatic brain injury. Brain Res 1026:11-22.

Shen Q, Goderie SK, Jin L, Karanth N, Sun Y, Abramova N, Vincent P, Pumiglia K, Temple S (2004) Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. Science 304:1338-1340.

Shors TJ, Miesegaes G, Beylin A, Zhao M, Rydel T, Gould E (2001) Neurogenesis in the adult is involved in the formation of trace memories. Nature 410:372-376.

Shors TJ, Townsend DA, Zhao M, Kozorovitskiy Y, Gould E (2002) Neurogenesis may relate to some but not all types of hippocampal-dependent learning. Hippocampus 12:578-584.

Sofroniew MV (2005) Reactive astrocytes in neural repair and protection. Neuroscientist

11:400-407.

Song H, Stevens CF, Gage FH (2002) Astroglia induce neurogenesis from adult neural stem cells. Nature 417:39-44.

St Clair MH, Lambe CU, Furman PA (1987) Inhibition by ganciclovir of cell growth and DNA synthesis of cells biochemically transformed with herpesvirus genetic information. Antimicrob Agents Chemother 31:844-849.

Starkstein SE, Jorge R (2005) Dementia after traumatic brain injury. Int Psychogeriatr 17 Suppl 1:S93-107.

Steiner B, Klempin F, Wang L, Kott M, Kettenmann H, Kempermann G (2006) Type-2 cells as link between glial and neuronal lineage in adult hippocampal neurogenesis. Glia 54:805-814.

Stover JF, Unterberg AW (2000) Increased cerebrospinal fluid glutamate and taurine concentrations are associated with traumatic brain edema formation in rats. Brain Res 875:51-55.

Sun D, McGinn MJ, Zhou Z, Harvey HB, Bullock MR, Colello RJ (2007) Anatomical integration of newly generated dentate granule neurons following traumatic brain injury in adult rats and its association to cognitive recovery. Exp Neurol 204:264-272.

Tashlykov V, Katz Y, Gazit V, Zohar O, Schreiber S, Pick CG (2007) Apoptotic changes in the cortex and hippocampus following minimal brain trauma in mice. Brain Res 1130:197-205.

Tatsumi K, Haga S, Matsuyoshi H, Inoue M, Manabe T, Makinodan M, Wanaka A (2005) Characterization of cells with proliferative activity after a brain injury. Neurochem Int 46:381-389.

Teasell R, Bayona N, Lippert C, Villamere J, Hellings C (2007) Post-traumatic seizure disorder following acquired brain injury. Brain Inj 21:201-214.

Teather LA, Magnusson JE, Chow CM, Wurtman RJ (2002) Environmental conditions influence hippocampus-dependent behaviours and brain levels of amyloid precursor protein in rats. Eur J Neurosci 16:2405-2415.

Teng YD, Lavik EB, Qu X, Park KI, Ourednik J, Zurakowski D, Langer R, Snyder EY (2002) Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold seeded with neural stem cells. Proc Natl Acad Sci U S A 99:3024-3029.

Tohyama T, Lee VM, Rorke LB, Marvin M, McKay RD, Trojanowski JQ (1993) Monoclonal antibodies to a rat nestin fusion protein recognize a 220-kDa polypeptide in subsets of fetal and adult human central nervous system neurons and in primitive

neuroectodermal tumor cells. Am J Pathol 143:258-268.

Urrea C, Castellanos DA, Sagen J, Tsoulfas P, Bramlett HM, Dietrich WD (2007) Widespread cellular proliferation and focal neurogenesis after traumatic brain injury in the rat. Restor Neurol Neurosci 25:65-76.

Visnjic D, Kalajzic I, Gronowicz G, Aguila HL, Clark SH, Lichtler AC, Rowe DW (2001) Conditional ablation of the osteoblast lineage in Col2.3deltatk transgenic mice. J Bone Miner Res 16:2222-2231.

Walker JP (2002) Functional outcome: a case for mild traumatic brain injury. Brain Inj 16:611-625.

Wallace H, Ledent C, Vassart G, Bishop JO, al-Shawi R (1991) Specific ablation of thyroid follicle cells in adult transgenic mice. Endocrinology 129:3217-3226.

Winocur G, Wojtowicz JM, Sekeres M, Snyder JS, Wang S (2006) Inhibition of neurogenesis interferes with hippocampus-dependent memory function. Hippocampus 16:296-304.

Wojtowicz JM (2006) Irradiation as an experimental tool in studies of adult neurogenesis. Hippocampus 16:261-266.

Wojtowicz JM, Askew ML, Winocur G (2008) The effects of running and of inhibiting adult neurogenesis on learning and memory in rats. Eur J Neurosci 27:1494-1502.

Wu D, Shibuya S, Miyamoto O, Itano T, Yamamoto T (2005) Increase of NG2-positive cells associated with radial glia following traumatic spinal cord injury in adult rats. J Neurocytol 34:459-469.

Yamaguchi M, Saito H, Suzuki M, Mori K (2000) Visualization of neurogenesis in the central nervous system using nestin promoter-GFP transgenic mice. Neuroreport 11:1991-1996.

Yamashima T, Tonchev AB, Yukie M (2007) Adult hippocampal neurogenesis in rodents and primates: endogenous, enhanced, and engrafted. Rev Neurosci 18:67-82.

Yang M, Stull ND, Berk MA, Snyder EY, Iacovitti L (2002) Neural stem cells spontaneously express dopaminergic traits after transplantation into the intact or 6-hydroxydopamine-lesioned rat. Exp Neurol 177:50-60.

Yi JH, Hazell AS (2006) Excitotoxic mechanisms and the role of astrocytic glutamate transporters in traumatic brain injury. Neurochem Int 48:394-403.

Yokoyama A, Sakamoto A, Kameda K, Imai Y, Tanaka J (2006) NG2 proteoglycan-expressing microglia as multipotent neural progenitors in normal and pathologic brains.

Glia 53:754-768.

Yokoyama A, Yang L, Itoh S, Mori K, Tanaka J (2004) Microglia, a potential source of neurons, astrocytes, and oligodendrocytes. Glia 45:96-104.

Yoshimura Y, Ohmura T, Komatsu Y (2003) Two forms of synaptic plasticity with distinct dependence on age, experience, and NMDA receptor subtype in rat visual cortex. J Neurosci 23:6557-6566.

Yu TS, Dandekar M, Monteggia LM, Parada LF, Kernie SG (2005) Temporally regulated expression of Cre recombinase in neural stem cells. Genesis 41:147-153.

Zhang Q, Sachdev PS (2003) Psychotic disorder and traumatic brain injury. Curr Psychiatry Rep 5:197-201.

Zhang SC, Goetz BD, Carré JL, Duncan ID (2001) Reactive microglia in dysmyelination and demyelination. Glia 34:101-109.

Zhao C, Deng W, Gage FH (2008) Mechanisms and functional implications of adult neurogenesis. Cell 132:645-660.

Zhao C, Teng EM, Summers RG, Ming GL, Gage FH (2006) Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus. J Neurosci 26:3-11.

Zimmerman L, Parr B, Lendahl U, Cunningham M, McKay R, Gavin B, Mann J, Vassileva G, McMahon A (1994) Independent regulatory elements in the nestin gene direct transgene expression to neural stem cells or muscle precursors. Neuron 12:11-24.