TWO DISTINCT PROLIFERATION EVENTS ARE INDUCED IN THE HIPPOCAMPUS BY ACUTE FOCAL INJURY

by

CARL ERNST

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Abstract

In models of global brain injury, such as stroke or epilepsy, a large increase in neurogenesis occurs in the dentate gyrus (DG) days after the damage is induced. In contrast, more focal damage in the DG produces an increase in neurogenesis within 24 hours. In order to determine if two distinct cell proliferation events can occur in the DG, focal electrolytic lesions were made and cell proliferation was examined at early (1 day) and late (5 day) time points. At the early time point, a diffuse pattern of BrdU+ cells was present ipsilateral to the lesion. When BrdU was administered at the later time point, the number of subgranular zone BrdU+ cells was significantly greater than at 24 hours. There was a four-fold increase in new neurons at the late time point while at the early time point no significant difference in neurogenesis was observed from control hemispheres. At both early and late time points, BrdU+ cells did not arise from microglia, as they rarely co-labeled with the microglia marker ED-1. These results indicate that focal injury in the dentate gyrus can activate two proliferation reactions, and that a latent period greater than 1 day is required before the injury-induced increase in new neurons is observed.

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List of abbreviations

CNS Central nervous system

BrdU 5-Bromo-2-deoxyuridine

DG Dentate gyrus

KDa Kilodalton

IL-6 Interleukin-6

IL-1B Interleukin-1beta

TNF-a Tumor necrosis factor-alpha

NGF Nerve growth factor

EGF Epidermal growth factor

TGF-b Transforming growth factor-beta

FGF2 Fibroblast growth factor 2

Preface

The background component of this thesis is an examination of the interactive effects of the brain's response to injury and the synthesis of new neurons. In the 1st chapter, I begin by establishing cornerstones of the brain's response to injury. First, I describe how the mammalian brain protects itself from the rest of the body with different types of structural barriers. Next, I describe some of the cellular and molecular responses of the brain to injury, in particular the role of astrocytes and microglia. In the 2nd chapter, I discuss neurogenesis in the adult mammalian brain. I describe the process and how it is particular to only certain regions of the brain. Finally, I review literature that has focused on the neurogenic response to injury.

The 3rd chapter of this thesis comprises a paper that I, along with my supervisor, have written and submitted for publication. The paper represents novel findings related to adult neurogenesis and neuroinflammation.

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Chapter 1: Neuroinflammation

Introduction

Inflammation is an attempt by the host to minimize damage caused by injury or infection. Injury can come in the form of any external agent causing cellular damage including impact damage or chemical damage. Infection is the result of microbial agents (i.e. bacteria or viruses) damaging cells through a multitude of different molecular pathways. The purpose of any inflammatory response in the brain or elsewhere is to eliminate infectious organisms, to initiate repair processes, or to acquire immunological information (Rothwell, 1995). In the context of the brain, these processes, together, are termed neuroinflammation. It is this definition that will be used within this thesis.

The brain has developed many barriers to protect itself against outside agents. The following section will describe some of these protective barriers as well as important cells involved in an inflammatory reaction.

Structural barriers

Barriers around the brain

The brain has a series of defenses against any form of injury. The first line of defense, and the one almost all are familiar with, is the skull. The importance of the brain to bodily function is emphasized in the thickness of the bone completely enveloping, but not touching, the brain. The thickness of the skull is approximately 6 mm, although it varies in different regions (Hwang et al., 1999)

The next protection that the brain has against injury or infection is the membranous coverings. The membranous coverings differ in their thickness and position, but all envelop the brain. The first and most exterior (closest to the skull) is the dura mater. This term, latin for hard mother, is the thickest of the three coverings. Closer to the brain is the arachnoid layer. This layer establishes a main pathway for fluid outflow from the brain via the subarachniod space to the blood capillaries and terminal lymphatics (Cserr and Berman, 1978; Cserr et al., 1981; Cserr et al., 1992). Finally, closest to the brain, is the pia mater (latin: soft mother). The pia is a soft sheet of clear tissue that encircles the brain. It is composed of extracellular matrix proteins such as collagen IV and members of the laminin family (Erickson and Couchman, 2000). One of the main functions of this layer operates during development, where it helps to organize cortical neurogenesis.

Barriers in the brain

Inflammation in the mammalian brain is distinct from other areas of the body. This distinction is due to the protective barriers in most areas of the brain that ensure that the entry of almost all molecules is regulated. These barriers maintain ionic composition in the brain, avoiding any sudden change which could have disastrous effects on cell signaling that is highly dependent on exact ratios of ions, particularly potassium and sodium. Further, these barriers ensure that metabolic substances gain access to the brain via protein carriers or receptors.

The concept of selective access to the brain was first described by Ehrlich in 1885 (for a discussion of this concept, see Ehrlich, 1900). In his experiments, he injected aniline dyes and noticed that they did not stain the CNS. Aniline dyes travel through the

bloodstream, gain access to surrounding organs, and stain local cells. The result from this experiment suggested that the brain had a protective barrier preventing the efflux of certain molecules unlike any other organ. This concept gained further ground when, in 1948, Medawar grafted skin allografts into the brain (Medawar, 1948). Unlike every other area of the body, there was no transplantation rejection response. Normally, transplantation is followed by host defense mechanisms that attack the grafted tissue. In Medawar's experiments, skin allografts persisted and this suggested that host defense molecules that normally attack transplanted tissue could not gain access to the brain. Medawar described the brain therefore as, "immunologically privileged." Today, this description is perhaps too simplistic given that brain cells express major histocompatibility complex II (a marker present on cells that distinguishes them as 'self' and not foreign) and that immune cells (B and T lymphocytes) can be found in the brain that can recognize antigens. Nevertheless, the foundation of the finding holds: The brain has protection that other organs do not have and this prevents the entry of a wide array of molecules.

Two different barriers have been described in different areas of the brain. The blood-brain barrier (BBB), and the brain-cerebral spinal fluid barrier (B-CSF). Both of these barriers explain the initial observations of Ehrlich and Medawar.

The BBB is present throughout the CNS, except in the circumventricular organs (e.g. pituitary gland, pineal gland). The structure of the blood-brain barrier was confirmed in 1967 by Reese and Karnovsky (Reese and Karnovsky, 1967). This particular barrier prevents access to the brain in two structural ways: Tight junctions between endothelial cells and astrocyte endfeet attached to capillary membranes. Astrocytes are a type of

glial cell in the brain and will be discussed later. Astrocyte endfeet cover 85-95% of the endothelial cell-derived capillary surface and function to maintain endothelial cell properties, likely through the release of diffusible factors. Endothelial cells, in this case, form the wall of the cerebral vasculature. Tight junctions are a form of cell adhesion molecule that prevents the diffusion of factors between intercellular spaces created by the symmetrical alignment of endothelial cells and allow for the quick transport of material between adjacent cells. The barrier properties of astrocytes and endothelial cells are not that of two independent events but of one interdependent function. For example, if these specialized endothelial cells are cultured without the presence of astrocytes, they lose key characteristics (tight junctions) after a few passages (Abbott et al., 1992). This suggests that a dynamic interplay between astrocytes and endothelial cells is required to form a barrier between the brain and blood (Janzer and Raff, 1987)

Almost all molecules must be specifically transported from the blood into the brain. Due to the presence of tight junctions between endothelial cells, transport from the blood into the brain is transcellular. Endothelial cells have specific properties designed for continuous transport including a large number of mitochondrion (Oldendorf et al., 1977), the organelle responsible for converting glucose into ATP.

The entry of specific molecules from the blood into the brain is determined by lipophilicity, size, and presence of endothelial cell membrane receptors. For example, the higher the lipid content of the molecule the more likely it will be to gain access. (e.g. vitamins enter via passive diffusion), and the larger the molecule the less likely it will be to gain access,

Transport systems are proteins embedded in the endothelial cell membrane whose specific function is to transport a molecule from the blood into the endothelial cell (Singer and Nicolson, 1972). These proteins are responsible for facilitated transport (e.g. D-glucose down a concentration gradient) and carrier mediated transport (e.g. monocarboxilic acids, like L-DOPA or tryptophan, bind to the carrier and are transported into the cell).

The B-CSF barrier is near the ventricles where passage of molecules from the blood to the CSF-filled ventricles occurs. Unlike the blood-brain barrier, the B-CSF barrier is made up of fenestrated capillaries at the choroid plexus (Cserr, 1971). This barrier is much less stringent in what can and cannot pass, permitting the ultrafiltration of plasma and passage of watersoluble molecules.

Cells involved in the inflammatory reaction in the brain

Once the structural barriers of the brain have been breached, numerous cells and molecules become involved in the neuroinflammatory response to injury or infection. The immediate response to injury is from microglia, neutrophils, and astrocytes Microglia rapidly migrate to a site of injury while neutrophils are first detected 12-24 hours after the insult (Keane and Hickey, 1997). Astrocytes and microglia will be discussed in more detail below. Monocyte (a type of white blood cell derived from the bone marrow) recruitment is slower. For example, phagocytes (a type of monocyte) reach peak numbers after 3-5 days (Issekutz et al., 1980; Issekutz et al., 1981). B and T lymphocytes are lymph node-derived cells and are antigen presenting cells. B lymphocytes also form the basis of antibody generation for immunohistochemical studies

(Kohler and Milstein, 1975). These cell types, although all involved in the inflammatory response, are by no means always present after CNS injury or in identical quantities. Rather, the type of injury and the species determine stoichiometric relationships between inflammatory cells and whether or not specific cell types will participate in the response to injury.

Microglia

In 1919, Rio-Hortega proposed the existence of a population of brain resident phagocytic cells, which are now termed microglia (Rezaie and Male, 2002). Microglia, which make up 10-20% of all glial cells in the brain, function to cope with invasions from any foreign source and to clean up cellular debris (Wood, 1998). In the resting state, microglia possess numerous pseudopodia that can stretch up to 50 um. In an activated state, microglial cells decrease the length of their extensions and have a circular morphology, similar to that of an amoeba (Perry et al., 1993). Intriguingly, microglia also have distinct biochemical and electrophysiological properties, including voltage gated currents (Korotzer and Cotman, 1992)

The origin of microglia is still under debate (Ling et al., 2001). The neuroectoderm, a layer formed during development that gives rise to neurons and other glia, has been proposed to give rise also to microglia. Alternately, others have proposed that microglia are monocytic cells designed to clear debris after synaptic re-modeling during development and then remain residents in the brain (Kaur et al., 2001).

Activated microglia, which include resident microglia and elicited macrophages, undergo numerous biochemical changes when damage in the brain is sensed (Streit et al.,

1988). One of these changes, which will be addressed in my submitted paper, is the up-regulation of the lysosomal membrane marker, ED-1 (Perry et al., 1993). When damage is sensed, microglia can rapidly invade a damaged area (Gehrmann et al., 1991; Nimmerjahn et al., 2005)

The initial intervention by microglia at the site of damage, before any phagocytic action takes place, is the release of many different types of diffusible factors, including cytokines. Cytokines are 26-37 KDa proteins that are synthesized, post-translationally modified, and released from microglia (Wood, 1998). Cytokines are not specific to the brain, but their synthesis there is almost solely in microglia. Cytokines function as growth factors or inflammatory mediators. Of the numerous different cytokines identified, all bind to specific receptors on cell membranes and induce biochemical reactions within a given cell, whether it be mitogenic, the induction of cell death pathways, or some other functions (Wood, 1998). Some of the better studied cytokines include IL-1b, TNF-a, and IL-6.

Microglia release other diffusible factors into the extracellular matrix. NGF, EGF, and TGF-b are some trophic factors released from microglia and their function to cell growth *in vivo* and *in vitro* cannot be overstated (Gage et al., 1995). Microglia can also secrete large amounts of glutamate, and this can contribute to NMDA induced excitotoxicity (Piani et al., 1991; Patrizio and Levi, 1994).

Astrocytes

Astrocytes were first described as 'sternzellen' (star cells) by Deiters in 1865, and the idea that they were a distinct cell type from neurons was confirmed by Cajal in 1913

using the Golgi staining technique (Keane and Hickey, 1998; Sotelo, 2003). These cells occupy 1/3 the volume of the cortex and outnumber neurons 10 to 1. Their function was first thought to be that of support for neurons or for the formation of the scar after injury; However, today we know this heterogeneous population of cells to be much more than this (Wilkin et al., 1990).

Astrocytes in the uninjured brain have numerous functions. Astrocytes, which all occupy a distinct area in the brain , are connected by gap junctions (Robinson et al., 1993; Liu et al., 2002). This forms a means of long range communication and this is mediated by glutamate-induced calcium waves (Cornell-Bell et al., 1990). At the synapse, astrocytes can take-up excess neurotransmitter (spillover), and synthesize and release glutamate for neurons. Astrocytes play a fundamental role in establishing the blood brain barrier and can release numerous different types of growth factors. One of their most important identified functions though, is the response to CNS injury.

There are numerous ways that astrocytes respond to CNS injury. After the insult, the most obvious change in astrocytes is in size. Within minutes of injury, responding astrocytes enlarge and extend cytoplasmic processes (Mongin et al., 1994; Kimelberg, 1995). Responding astroytes are also thought to express more of the protein, GFAP (Dahl and Bignami, 1976). Astrocyte proliferaton is thought to begin 3 days post-injury and return to baseline levels approximately 14 days post-injury (Parent et al., 1997).

Astrocyte involvement in CNS injury involves the encasement of the injured site and the synthesis of numerous diffusible factors. The encasement of the damaged site may well be to isolate the uninjured CNS from the inflammatory focus – a focus where many damaging molecules may be released, including the excitotoxic compound

glutamate (Matsumoto et al., 1992). In this way, astrocytes around the lesion may quench any neurotoxic compounds that may further damage the CNS. Besides the uptake of molecules from the extracellular matrix, astrocytes involved in the inflammatory response can synthesize and release numerous molecules including FGF2 (Joannides et al., 2004), IL-1 (Bethea et al., 1992), and prostaglandin E (Fontana et al., 1982).

In summary, the brain has developed different ways of protecting itself from injury. These include gross structures such as the skull and membranous coverings, and cells within the brain that cope with inflammation. Some of the most important cells in the brain that cope with inflammatory cascade are microglia and astrocytes. Studies of these two cells have revealed not only their ability to quench the damaging effect of injury, but also their contributions to the proliferative response through the release of diffusible factors that have mitogenic properties.

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Chapter 2: Adult neurogenesis

Introduction

In many areas of the mammalian body, different types of cells including bone, blood, and skin cells have the ability to regenerate. This regenerative capacity can be seen after injury and as a regularly occurring process in mammals. The brain, however, differs from these regions, as it has not, until recently, been seen as an organ capable of regenerating nerve cells either as a process of normal function or after injury. The support for this concept likely came from experiments using new cell markers. Experimenters could not detect any signal in the in most brain areas. Over the past 40 years, it has become clear that the lack of a neuronal regenerative capacity is false in both the normal and injured brain

Adult neurogenesis – History and technical considerations

In 1962, Joseph Altman proposed that the growth of new neurons occurred in the brains of adult mammals, a process that is today referred to as adult neurogenesis (Altman, 1962). In this and other work done throughout the 1960's and 1970's, Altman and colleagues established that mitosis was indeed occurring in the adult mammalian brain by using a radio-labeling technique in conjunction with autoradiography (Altman and Das, 1965; Kaplan and Hinds, 1977).

The acceptance of adult neurogenesis was not readily accepted (Rakic, 1985, 1985). Long-held beliefs and scientific dogma dictated that new neurons could not grow in adult brains, and that the synthesis of new neurons was restricted to the developmental

period. Over the past 4 decades, it has become clear that adult neurogenesis does occur. The fact that it has been demonstrated in numerous species, including humans, highlights this fact (Eriksson et al., 1998; Gould et al., 1999; Zupanc, 2001).

The advent of immunohistochemical markers (5'-Bromo-2'-deoxyuuridine-BrdU), rather than a radioactive label (tritiated thymidine), helped to establish the existence of adult neurogenesis. Markers used for cell division in the brain, be they radio- or immunohitochemical labels, involve the modification of the thymidine residue normally found as a component of DNA. In S phase of the cell cycle, cells replicate their DNA semi-conservatively. In so doing, available nucleotides are incorporated into a growing strand of DNA. Modified thymidine residues then, cross the blood brain barrier and become a part of this nucleotide pool. BrdU is an extrinsic marker of neurogenesis as it must be injected into the body before it becomes part of an available pool of nucleotides. The advantage of the extrinsic marker BrdU is the temporal specificity it provides. BrdU can be injected, and within 2 hours, be almost fully incorporated into any cells undergoing DNA synthesis. This allows the experimenter to analyze DNA synthesis at a time point near the time of BrdU injection. It also allows the experimenter to co-label BrdU+ cells with other markers which may help determine what the phenotype of the cell. The disadvantage to extrinsic markers is that they label any cell undergoing DNA synthesis. These markers, therefore, are not necessarily indicative of a dividing cell. Cells undergoing DNA repair may also incorporate the thymidine analog.

Intrinsic markers have the advantage of not requiring injections, being easy to detect, and being more indicative of cell division rather than DNA repair. Some intrinsic markers used today by many experimenters are Ki-67 and proliferating cell nuclear

antigen (PCNA). Ki-67 and PCNA are nuclear proteins expressed during G1, S, G2, and M phase of the cell cycle (Scholzen and Gerdes, 2000). The advantage of these markers is that they are thought to be expressed near the time of cell division, making them a more specific marker of cell division. The disadvantage of these markers is that they have no temporal specificity; expression of these markers in a cell says nothing about where the cells are in the cell cycle. A further disadvantage of these markers is that they can not be used to assess the phenotypes of new cells. It takes a certain period of time before new cells express immunohistochemically detectable proteins that are reflective of cell fate.

My preference is to use extrinsic markers of DNA synthesis in conjunction with intrinsic markers. This does not mean using the markers in the same experiment, per se, but only to use them in a set of experiments to control for different variables. This idea will be further explored in my submitted paper (Chapter 3) where I use both BrdU and Ki-67. This approach ensures an accurate phenotypic analysis and can at least start to rule out DNA synthesis processes without cell division.

Regions of adult neurogenesis in the brain

New neurons are not created equivalently throughout the adult mammalian brain; only certain regions possess populations of active progenitor cells (Gould and Gross, 2002; Steindler and Pincus, 2002; Taupin and Gage, 2002; Alvarez-Buylla and Lim, 2004). Substantial adult neurogenesis occurs in only the dentate gyrus of the hippocampus and in the subventricular zone adjacent to the lateral ventricles (Altman, 1962; Kaplan and Hinds, 1977; Gould et al., 1999). Approximately 9000 new neurons

are created in the mature rat dentate gyrus every day (Cameron and Mckay 2001). The number of new neurons created in the subventricular zones has not yet been estimated,

The hippocampus, one major site of adult neurogenesis, is a bilateral limbic structure that plays a role in certain learning and memory processes (Milner and Penfield, 1955; Scoville and Milner, 1957; Penfield and Milner, 1958; Lisman, 1999). The dentate gyrus (DG) is a substructure of the hippocampus that contains a C-shaped layer of small, round granule cells. New neurons seem to be created from progenitor cells just below this granule cell layer in what is termed the "subgranular zone" (Figure 2.1A).

The subventricular region adjacent to the lateral ventricles is the second major source of new neurons in the adult mammalian brain (Figure 2.1B). Non-dividing ependymal cells line the ventricles (Garcia-Verdugo et al., 1998), but in one portion of the subependymal zone next to the lateral ventricles, there are numerous proliferative precursor cells that develop into neurons and glia (Doetsch et al., 1997).

The subgranular and subventricular precursor cells are close to one another in the mammalian brain - the hippocampus forms the medial wall and floor of the lateral ventricle in each hemisphere. However, these two sites of adult neurogenesis are characterized by different patterns of development (van Praag et al., 2002; Overstreet et al., 2004). Progenitor cells in the subgranular zone first divide, and the resulting daughter cells migrate the short distance (20-30 microns) into the granule cell layer. Once in the granule cell layer, dendrites grow from the cells into the area near the fissures immediately superior and inferior to the DG, and an axon grows to the CA3 region of the pyramidal cell layer. This process occurs over a period of approximately 4 weeks, and it results in the formation of new neurons that exhibit morphological and

electrophysiological characteristics typical of mature granule cells (van Praag et al., 2002; Figure 2.2). In contrast, progenitor cells in the subependymal zones divide and the resulting daughter cells migrate a substantial distance (~6mm) to the olfactory bulbs (Figure 2.1B) (Lois et al., 1996; Garcia-Verdugo et al., 1998). Once in the olfactory bulbs, these cells become local interneurons (Lois and Alvarez-Buylla, 1994).

It has recently been reported that new neurons can, under some conditions, be created in other brain regions, including the neocortex (Gould et al., 1999; Magavi et al., 2000; Dayer et al., 2005). However, it has yet to be determined if these cells become functional.

Adult neurogenesis and neuroinflammation in the dentate gyrus

Brain damage results in the up-regulation of new neurons in the subgranular zone of the DG. Models of brain damage have come in numerous different forms and these different forms can be divided into two distinct areas. The 1st area is that of global brain damage; this includes such models as stroke, and epilepsy. The term 'global' was selected because these models lead to ubiquitous amounts of damage throughout a large portion of the brain. The 2nd model of brain damage is that of acute injury to the DG. The term acute is used because models of acute injury lead to specific damage in the DG and minimally in the overlying cortex. These models include penetrating electrodes into the DG (mechanical injury) and sterotaxic infusion of a chemical into the DG (chemical lesion).

All models of global brain injury lead to increased levels of new neurons in the DG. In stroke damage models where the middle cerebral artery is occluded for 90

minutes, increased levels of neurogenesis are detected in the SGZ of the DG (Jin et al., 2001; Arvidsson et al., 2002). In the study from Jin et al. (2001), BrdU was administered 5 days after the stroke was induced and animals were sacrificed 3 weeks after this BrdU injection. BrdU+ cells co-labeled with markers for immature neurons suggesting that new cells become neurons. In this model, more new cells were detected in the DG of the damaged hemisphere, although the number of new cells was increased in both hemispheres.

Epilepsy models are also models of global brain damage, under certain conditions. All models of experimental epilepsy used in studies of adult neurogenesis have induced seizures by the intraperitoneal injection of a chemical (Parent et al., 1997; Jiang et al., 2003). In the study from Parent et al. (1997), pilocarpine was injected into rats followed by BrdU at varying time points. In this model, neurogenesis was increased bi-laterally; however, neurogenesis did not increase until 3 days after the onset of seizures. This work, replicated by Jiang et al. (2003) with a different seizure-inducing chemical, suggests that a latent period exist before neurogenesis is increased in the DG (Parent, 2003)

Models of acute injury increase neurogenesis in the DG but the temporal appearance of new cells differs from models of global brain damage. In one study, both mechanical and chemical lesions were made directly in the dentate gyrus (Gould and Tanapat, 1997). In these models, new cell growth was assessed by injecting tritiated thymidine and BrdU 24 hours after surgery. Phenotypic analysis was conducted 3 weeks after these injections. The authors reported a significant increase in new neurons specifically on the lesioned side.

Results from both global and injury models seem to indicate that brain injury increases neurogenesis in the DG. With each of these models, there is undoubtedly an inflammatory response as has been outlined in the preceding chapter. It is interesting to note then, that when studies have examined how inflammation interacts with DG neurogenesis that the opposite result was found.

Neuroinflammation is reported to be detrimental to neurogenesis in the DG (Monje et al., 2003; Ekdahl et al., 2003). In the study from Monje et al. (2003), bacterial lipopolysaccharide (LPS) was intraperitoneally injected to induce experimental autoimmune encephalomyelitis (EAE). EAE induces inflammation throughout the brain, and in particular, causes the activation of microglia. Monje et al. (2003) used indomethacin, a non-steroidal anti-inflammatory drug in attempt to block the inflammatory cascade. Before indomethacin injection there was a 35% decrease in neurogenesis after EAE. After indomethacin treatment, hippocampal neurogenesis was restored to baseline levels and the number of microglia was reduced by 240%. In the study from Ekdahl et al. (2003), LPS was again injected to cause an increase in microglia throughout the brain and to decrease levels of hippocampal neurogenesis. Neurogenesis was recovered by the injection of minocycline, a different non-steroidal anti-inflammatory drug.

How is it that global and acute models of brain injury like stroke or discrete lesions to the DG increase neurogenesis, while a model of EAE decrease neurogenesis? Clearly different events must be occurring in the brain even if there is partial overlap between these models. Without any doubt, there are microglia in the models of brain injury that have reported an increase in neurogenesis in the DG. Therefore, there must be

some other factor operating in non-EAE models of brain injury that not only increase neurogenesis in the hippocampus, but counteracts the negative effect of microglia on adult neurogenesis. We hypothesize that some other inflammatory cell, such as astrocytes, mitigates the increase of new neurons in the acutely injured adult hippocampus.

Figure descriptions

Figure 2.1. Sites of neurogenesis in the adult rat brain

A) Coronal section through the adult rat brain demonstrating the lateral ventricles (red shading) and the hippocampus (green box). **A2)** Magnified view of the hippocampus demonstrating the dentate gyrus (blue) and Ammon's horn (black). Dentate granule cells extend axons from the inner granule zone that project onto dendrites of pyramidal cells in the CA3 layer of Ammon's horn (pink). CA3 pyramidal neurons extend axons to CA1 dendrites (black); CA1 cells then extend axons to multiple neural areas, in particular to the cortex. **(A3-A5)** Neurogenesis in the subgranular zone of the dentate gytus. **A3)** Progenitor cells (blue) give way to immature neurons (blue w/ extensions) in the subgranular zone which extend a proboscis (primary dendrite) through the granular cell layer and an axon to find CA3 cell dendrites. **A4)** Dendritic complexity increase as neuron matures and migrates into the granule cell layer. **(A5)** Some new neurons migrate to the outer granular zone and have dendrites that no longer possess a primary dendrite but have a more bush-like appearance. **B)** Mid-saggital view of the adult rat brain. Neurons derived from the sub-ventricular zone migrate via the rostral migratory stream to the olfactory bulbs.

Figure 2.2. Adult neurogenesis in adult rats

A) Granule cell neurons (green) of the dentate gyrus, 42-day old cells (red), and astrocytes (blue) of the hippocampus in rats. **B)** Contralateral hemisphere to l. **C)** Confocal image of red boxed area from (B). For new cells to be considered neurons they must stain red (BrdU, a marker of new cells) and green (NeuN, a mature neuronal marker), but not blue

(GFAP, a marker of mature astrocytes). Cell at the centre of the white crosshairs is flipped 90 degrees in both the x and y planes to ensure that the cell is co-labeled for both BrdU and NeuN (vertical and horizontal rectangles bordering figure C).

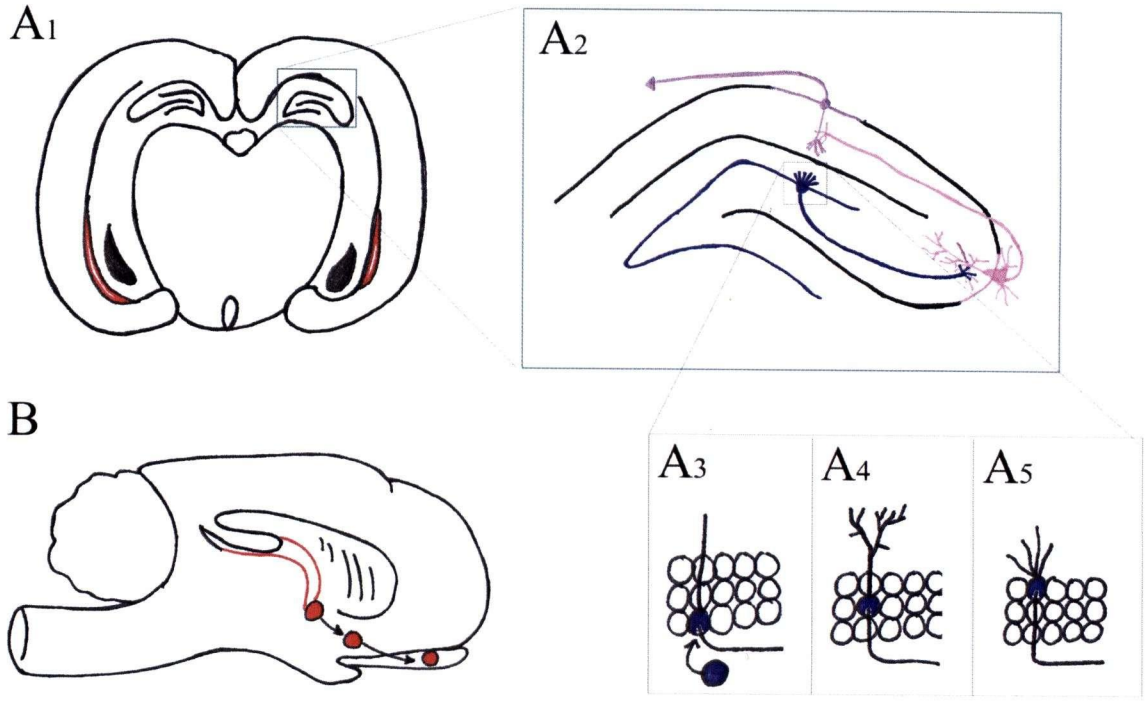

Figure 2.1

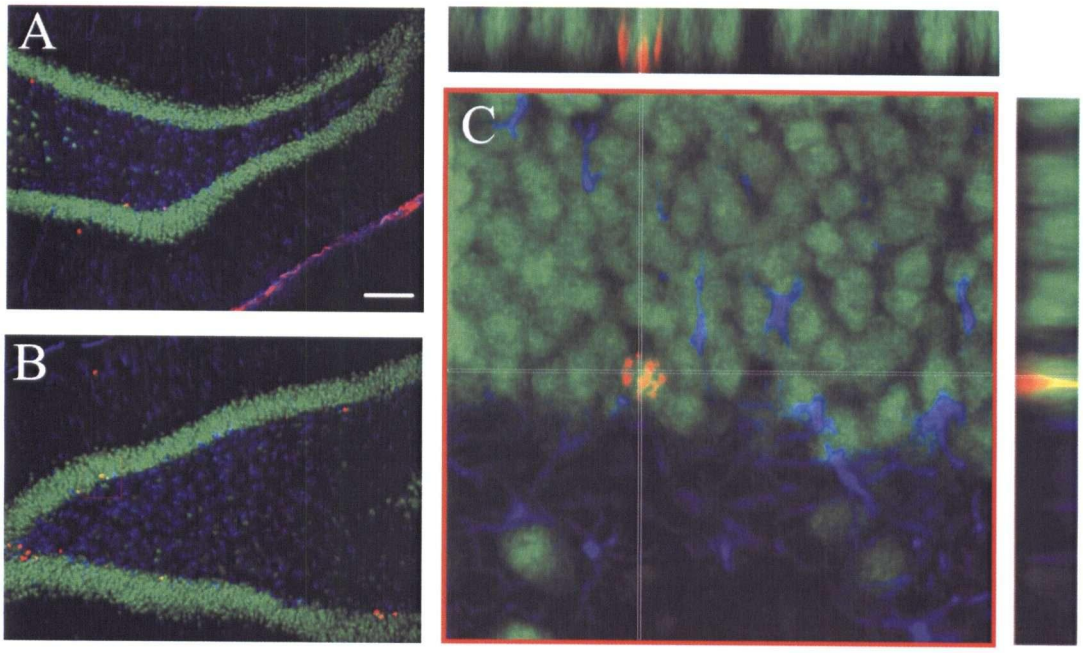

Figure 2.2

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Chapter 3: Two distinct proliferation events are induced in the hippocampus by focal acute injury[1]

Introduction

Adult neurogenesis is the process by which new neurons are created in the mature central nervous system. This phenomenon has been shown to occur in the dentate gyrus (DG) of the hippocampal formation in a wide variety of species that includes: fish (Zupanc, 2001); rats (Altman and Das, 1965; Kaplan and Hinds, 1977); mice (Kaplan and Bell, 1984); birds (Goldman and Nottebohm, 1983); and humans (Eriksson et al., 1998). The conservation of this process in these different species may be indicative of its importance to normal brain function, and it also highlights the fact that the brain possesses a limited ability to exhibit neuronal regeneration. It is hoped that a better understanding of the mechanisms involved in the process of neurogenesis will enable it to be harnessed as a therapeutic tool for use in a wide variety of neurological disorders, including brain trauma (Gage, 2002).

The process of neurogenesis can be divided into several sequential steps. Initially, cells must engage in mitotic activity, and it is during this process that thymidine analogues such as BrdU or ^{3}H-thymidine can be introduced into the DNA during synthesis phase. The initial phase is referred to as cellular proliferation, referring to the fact that it produces a large number of new cells without a distinct phenotype. The term neurogenesis is generally used to denote those cells that survive and, over a period of 3-5 weeks, assume a mature neuronal phenotype that is functionally indistinguishable from other young neurons in the DG (van Praag et al., 2002; Overstreet et al., 2004)

[1] "Two distinct proliferation events are induced in the hippocampus by focal acute injury." Carl Ernst and Brian Christie, submitted to *The Journal of Comparative Neurology*

An increase in the rate of neurogenesis has been shown in the DG with a number of models of brain injury, including following global ischemia (Jin et al., 2001; Kee et al., 2001); electroconvulsive seizures (Parent et al., 1997; Scott et al., 2000; Ferland et al., 2002); kainic and ibotenic acid injections (Gould and Tanapat, 1997; Gray and Sundstrom, 1998); trimethyltin injections (Harry et al., 2004), and blunt trauma (Dash et al., 2001; Rice et al., 2003). With global models of brain damage, an increase in the rate of neurogenesis in the DG is seen only after a latent period that lasts more than 24 hours (for review see Parent, 2003). In contrast, when an acute focal injury is made in the DG, neurogenesis is increased within 24 hours of injury (Gould and Tanapat, 1997). These findings indicate that increased neurogenesis after global brain injury requires a latent period of at least 24 hours, while increased neurogenesis after acute focal injury to the DG does not.

It is unclear whether there is a fundamental difference in the proliferation reactions that occur at early and late time points following focal acute hippocampal injury. In the present experiments we use Fluorojade-B, a marker of dying neurons, to show that electrolytic lesions can be used to generate small discrete lesions in the brain. We use this procedure to demonstrate that focal injuries that induce restricted granule cell death can instigate two distinct cell proliferation events in the DG.

Materials and Methods

Subjects and Surgical Procedures.

Seventy-five adult male Sprague-Dawley rats (250-300g) were obtained from the UBC Animal Care Center and anesthetized with sodium pentobarbital (65 mg/kg; Somnotol) before being placed in a stereotaxic frame. A trephine hole was then drilled 3.0 mm posterior and 1.2 mm lateral from Bregma to allow access to the brain, in which a 31.5 μm diameter stainless steel electrode (A-M systems, Carlsbourg, WA) was lowered stereotaxically to the level just dorsal to the DG (3.8 mm ventral from skull surface). Some animals underwent the identical injury procedure except that the electrode was placed in the frontal cortex. After animals were placed in a stereotaxic frame a trephine hole was drilled 3.0 mm anterior and 0.7 mm lateral from Bregma to allow access to the brain. The electrode was lowered stereotaxically 2.5 mm ventral from skull surface. The electrode was connected to a Grass Instruments Direct Current Constant Current Lesion Maker (D.C. LM5A) and animals were administered 1 mA of DC current for 2 seconds, after which the electrode was immediately withdrawn. The wound-point was then sutured closed, and the animals were placed in a surgical recovery chamber. All procedures were performed in compliance with the guidelines set out by the Canadian Council on Animal Care.

BrdU Administration

Mitotic activity in the CNS was assayed by administering peripheral injections of 5-bromo-2-deoxyuridine (BrdU; Sigma; 200 mg/kg, i.p.) as we have previously described

(Eadie et al., 2005). To quantify cellular proliferation, animals were sacrificed 2 hours after BrdU was administered (24 hour proliferation group: BrdU at 22 hours; 120 hour proliferation group: BrdU at 118 hours). To quantify cellular differentiation, animals were sacrificed 42 days following the administration of BrdU at these same time points. As an alternative to BrdU, we also assayed an intrinsic marker of cell cycle activity (Ki-67) in these same animals. This allowed us to undertake two distinct measures of mitotic activity to ensure that any differences in BrdU labeling did not reflect an alteration in the capacity of BrdU to enter the CNS following focal injury or DNA repair mechanisms.

Fluorojade B staining

To determine the extent of damage induced by the electrolytic lesion, we used the fluorescent marker Fluorojade B (FJB; Chemicon, CA) to identify damaged neurons in paraformaldehyde-fixed tissue sections (Schmued and Hopkins, 2000; Obernier et al., 2002). Animals were sacrificed with an overdose of sodium pentobarbital and then transcardially injected with 0.9% saline and 4% formaldehyde before the brain was carefully removed. Brains were then stored in 4% formaldehyde for two days to facilitate tissue fixation before being transferred to a cryoprotectant (30% sucrose) for 5 days. Brains were sectioned at 40 μm using a cryostat at -15C (Microm, 500 OM, Heidelberg, Germany) and placed individually in well plates containing Tris buffered saline (TBS, trizma hydrochloride, trizma base, dodium hydrochloride, dH2O; Fisher). We kept all tissue sections from the dorsal hippocampus (-2.00 to -4.60 mm from Bregma) and not all tissue sections had obvious DG damage. Our criterion for selection was the presence of identifiable damage in at least one of the tissue sections. Damage was defined as the presence of FJB positive cells in the DG granular layer. Tissue sections were mounted on

gelatin coated slides, allowed to air dry over night, and then placed in a solution containing 1% sodium hydroxide and 80% ethanol solution for five minutes followed by a 70% ethanol solution and deionized water for 2 minutes each. Next, slides were transferred to a 0.6% potassium permanganate solution and agitated for seven minutes to oxidize the tissue before being cleaned in deionized water for two minutes. Finally, slides were transferred to a FJB solution (Chemicon – 4 mL FJB stock in 96 mL 0.1% acetic acid) for eight minutes and then washed three times in separate deionized water baths for two minutes each. Slides were then air dried, coverslipped, and sealed with DPX, a mounting medium (Fluka, Switzerland). All samples were examined using either an Olympus upright fluorescent microscope (BX51) with a motorized Z-axis and coupled to a Photometrics CoolSNAP CCD camera, or with a Bio-Rad Radiance 2000 confocal microscope (Zeiss, Germany) at the UBC Bioimaging facility. All image processing was performed using ImagePro Plus (Mediacybernetics, MD) or ImageJ (http://rsb.info.nih.gov/ij/)

Immunohistohemistry

Brains were processed for BrdU and Ki-67 as we have described previously (Farmer et al., 2004; Eadie et al., 2005). Tissue sections were initially heated in formamide for 2 hours to denature DNA, and then washed intermittently in TBS. Next, to block non-specific binding and to allow entry of antibodies, tissue sections were saturated with a solution containing 96% TBS, 1% TritonX, and 3% donkey serum (TBS ++) before being incubated in primary antibodies. The specific antibodies used in each experiment were the thymidine analog, BrdU, a marker of DNA synthesis (1:300, Serotec, Raleigh, NC, rat anti-BrdU); the mature neuronal nuclear protein NeuN (1:25,

Chemicon, Temecula, CA, mouse anti-NeuN); the glial intermediate filament protein, GFAP, a marker for mature astrocytes (1:1000, Chemicon, rabbit anti-GFAP); The G1, S, G2, and M phase marker (Scholzen and Gerdes, 2000), Ki67 (1:200, NeoMarkers, Fremont CA, rabbit anti-Ki67); and microglia (1:200,mouse anti-ED-1, Research Diagnostics, NJ). Tissues were incubated for 48 hours at 4 degrees and then processed for respective secondary antibodies (Donkey anti-mouse, FITC; Donkey anti-rabbit, Cy5; Donkey anti-rat, Cy3; 1:250; Chemicon, Temecula, CA); secondary antibodies were added and incubated in the dark for four hours. The excitation (x) and emission (e) spectra for these fluorochromes are as follows: FJB x = 480 nm, e = 525 nm; FITC x = 480 nm, e = 525 nm; Cy3 x = 550 nm, e = 570 nm, Cy5 x = 650 nm, e = 680 nm. After staining, tissue sections were mounted on 2% gel coated slides and dried for 1 hour at room temperature before being cover-slipped with DABCO, an anti-fade agent.

Cell Counting Procedures

For tissues taken from rats sacrificed at either 1 or 5 days, we counted BrdU+ cells in the subgranular zone (SGZ) and the hilus of tissues taken from the dorsal hippocampus. We defined the SGZ as the zone directly adjacent to granule cells in the infra-and supra- blades of the DG granule layer and the area within two cell bodies of this towards both the helical region and inner granule zone. This area was chosen because most new neurons tend to originate from this area. We defined the hilus as the area outside of two cell bodies of the infra- and supra- blades of the DG granule cell layer and extending to the lateral extent of the infra-and supra-blades. For tissues taken from rats sacrificed 42 days after surgery, we counted only those cells appearing within two cell

bodies of the infra- and supra- blades of the granule cell layer on the hilus side and any cell that appeared within the DG granule cell layer itself. This was selected because of the possible migration of cells from the SGZ to the inner and outer granule zones. All counts were performed using a profile count as described previously (Kempermann et al., 1997; van Praag et al., 2002). To evaluate cellular proliferation in the DG, we required that all cells have an identifiable, complete cell body and exhibited a distinct fluorescent signal when 550 nm light, but not 480 nm or 650 nm light was projected onto the tissue section. All BrdU labeled cells were counted, at 40-100x magnification, provided they were in the appropriate position as described above. Cells throughout the extent of the tissue were counted. This method is appropriate when all cell types are identical and non-uniformly distributed, as is the case with BrdU labeled neurons in the DG (Crews et al., 2004).

Each DG was examined using the AOI (Area of interest) function in ImagePro Plus (Mediacybernetics, MD). The AOI function allows one to hand draw a perimeter around any structure of interest (in this case, the DG), enabling the computer to calculate the total area of the perimeter in μm^2. Volume measurements were accomplished by multiplying the area of the lesion by the thickness of the tissue slice. Individual BrdU+ cells were viewed with a 100x objective and every cell that was whole, possessed an intact cell membrane, and was the appropriate size (7-10 microns) was included in the data set. Cells were counted using ImagePro Plus and a CoolSnap CCD camera (Photometrics,Tucson, AZ) coupled to an Olympus BX51 microscope with a motorized z-axis. To verify that BrdU+ positive cells were truly co-labeled with NeuN or GFAP, individual cells were randomly selected for analysis with confocal microscopy (Bio-Rad Radiance 2000, Zeiss, Germany). In each condition, at least 50 cells were assessed in

multiple planes at 100-200x using 1 μm steps in the z-axis. No differences in cell co-labeling estimates were observed when the confocal microscope and upright fluorescence microscope.

All data are reported as mean ± standard error and analyses were performed using SPSSv10. At least three animals were used per group with at least eight tissue samples from each animal in all analyses performed.

Results

FJB can be used to identify localized sites of neuronal damage.

Lesioned areas were characterized by clear tissue damage that was highlighted by FJB staining (Figure 3.1). FJB+ cells were observed mainly ipsilateral to the lesion, though some isolated cells could be observed in the contralateral hemisphere. In general, FJB+ cells found contralateral to the lesion in the DG were difficult to observe and did not cluster together, while FJB+ cells ipsilateral to the lesion clustered together and were easily discernable.

To determine the extent of damage that 1mA of current produced when administered to the hippocampus for 2 seconds (Figure 3.2A), coronal sections (n=43 sections) from 6 animals were analyzed for the extent of damage. Area measurements were summated from all sections and revealed a mean lesion area of 32 333 $\mu m^2 \pm 5$ 215 μm^2. To calculate an approximate volume of the lesion, 3-D reconstructions were made of the hippocampus following a lesion. All hippocampal sections (40 μm) from 1 animal were retained and examined for the presence of FJB and obvious signs of electrolytically-induced damage. To calculate the total volume of the lesion, we

determined the area of damage in each section and multiplied each area by 40 μm. Twenty-two sections throughout the rostral-caudal extent of the lesion were then summated to determine that the procedure resulted in a total damaged volume of 0.049 mm^3. There was no significant difference between lesion areas from the animal whose entire hippocampus underwent a volume analysis and those animals that had only the lesion area assessed (data not shown).

Electrolytic lesions induce highly localized changes in cellular morphology and site-specific reactive astrocytosis

Electrolytic lesions induce highly specific alterations to the dentate gyrus. Immunohistochemical analysis revealed cells expressing the mature astrocyte marker, GFAP, extending end feet-like processes directly from the glia cells to the small, rounded granule cells in the damaged DG blade (Figure 3.2B). Further, neurons proximal to the area of damage exhibited a condensed, spheroid appearance (Figure 3.2C). This cell shrinkage is characteristic of cells undergoing apoptosis (Schreiber and Baudry, 1995; Rosenblum, 1997). Those neurons distal, but adjacent to the lesion area, appeared normal and did not have any glial processes near their soma.

Electrolytic lesions induce reactive astrocytosis at the area of damage, as with other models of brain injury (Ridet et al., 1997). To characterize this process after electrolytic lesions to the DG (Figure 3.2D), antibodies to the intermediate filament protein, nestin, were used. Nestin is expressed in reactive astrocytes after injury (Clarke et al., 1994; Geloso et al., 2004). Using nestin antibody in this context is a method to distinguish between non-reactive astrocytes and reactive astrocytes. In this way we could

demonstrate how the process of reactive astrocytosis was specific to the DG 1 day after electrolytic injury, and was not a ubiquitous reaction (Figure 3.2D).

The spatial localization of BrdU+ cells is different at early and late time points following electrolytic lesions.

An increase of BrdU+ cells was always observed in the brain following electrode penetration. To determine if the increase in the number of BrdU+ cells observed adjacent to the electrode track at the early time point reflected either non-specific brain damage and/or the breaking of the blood-brain barrier, control surgeries were performed where an electrode was lowered 1 mm from the brain surface to cortical layer 6 (Layer 6/NoDC). This reliably placed the electrode tip just dorsal to the corpus callosum. In a separate set of animals an electrode was lowered into the DG, without inducing electrical damage (DG/NoDC). The third group had DC current administered in the DG to induce a localized lesion (DG/DC). Analysis of variance revealed that significantly different patterns of BrdU staining were present in these three groups (Figure 3.3A. SGZ ipsi: $F_{(78)}=23.5$, $p<0.05$). As is depicted in Figure 3, Tukey post-hoc analysis showed that significantly more BrdU+ cells were observed in the DG/NoDC group, than in the Layer 6/NoDC group ($p<0.05$). Significantly more BrdU+ cells were observed in the DG/DC group than in either of the control groups ($p<0.05$). When the ipsilateral and contralateral hemispheres are compared for each group, cell proliferation in the SGZ was always greater ipsilateral to the electrode in both the DG/NoDC group ($t_{50} = 2.7$, $p<0.05$) and the DG/DC group ($t_{48}=7.3$, $p <0.05$).

In a similar analyses, differences in cell proliferation were also apparent in the hilus (Figure 3.3B; $F_{(78)}=32.3$, $P<0.05$). A Tukey post hoc analysis revealed there to be significant differences between all three groups. Significantly more BrdU+ were found in the hilus of the DG/NoDC group than in the Layer 6/NoDC group ($p<0.05$). In turn, more BrdU+ cells were observed in the DG/DC group animals, than in either set of comparison animals ($p<0.05$). Although there was no difference in the number of BrdU+ cells in the ipsilateral and contralateral hemispheres of the Layer 6/NoDC group, cell proliferation was significantly greater in both the DG/NoDC group ($t_{50}= 10.5$, $p<0.05$) and the DG/DC group ($t_{48}= 5.042$, $p<0.05$). These results imply that neither breaking the blood brain barrier, nor inserting electrodes into cortical regions immediately superior to the hippocampus, lead to an increase in mitotic activity in the DG, per se, but that direct and discrete trauma to the DG can significantly increase mitotic activity in the DG.

To determine if early and late time points after acute focal injury differ in any way, cellular proliferation in the DG/DC group was also assessed at a later time point (120 hours). This time point was chosen as increased neurogenesis is known to occur within this time frame in global models of brain injury (Parent et al., 1997; Jin et al., 2001). As is shown in Figure 3.4B, there was a marked increase in the number of BrdU+ cells at 120 hours over that seen at 24 hours in the SGZ ($t_{(52)} = 7.95$, $p<0.05$). Conversely, there were fewer BrdU+ cells present in the Hilus of the 120 hour group than in the 24 hour group ($t_{(52)} = 8.05$, $p < 0.05$). To further illustrate the differences at the two time points, we also examined the pattern of BrdU+ cells in the cortex at both 24 hour and 120 hour time points. As is shown in Figure 3.4C, new cells are clearly more dispersed at the 24 hour time point while they are restricted to the area immediately

adjacent to the electrode tract at the 120 hour time period. These results imply that a different spatial pattern of proliferation was present at early and late time points after a lesion. Interestingly, cell proliferation appears to be robust at the early time point and restricted at the later time point, in all areas except the SGZ of the DG where the inverse was true.

BrdU incorporation is not indicative of DNA repair following an electrolytic lesion.

BrdU can be incorporated into the DNA of any cell undergoing DNA synthesis, so it is possible that damaged cells undergoing DNA repair following an electrolytic lesion might also be BrdU+ (Miller and Nowakowski, 1988). To examine this issue we first used an anti-body against Ki-67, a protein that is expressed during the G1, S, G2, and M phase cell cycle marker(Scholzen and Gerdes, 2000; Kee et al., 2002). We reasoned if BrdU-labeling and Ki67-labeling show similar dispersal patterns at 24 and 120 hours, that the BrdU signal is likely detecting mitotic activity. As is evident in Figure 3.5, Ki67 staining is very similar to that observed with BrdU.

To further confirm that BrdU-labeling was not indicative of DNA repair, tissue from the 24 hour proliferation group was labeled with antibodies against BrdU and the marker of mature neurons, NeuN. This test could detect whether mature neurons incorporate BrdU, presumably while undergoing DNA repair. We were never able to observe NeuN and BrdU co-labeling in any cells from samples taken at the early time points (data not shown). These two tests indicate that the BrdU+ cells observed at the early time point were not representative of cells undergoing DNA repair following an electrolytic lesion.

Microglia were present in the granule cell layer at both 24 hour and 120 hour time points, but almost none co-labeled with BrdU

It is possible that the newly divided cells observed after the lesion represent a population of inflammatory cells (Riva-Depaty et al., 1994). Inflammatory responses in the nervous system are known to involve both astrocytes and microglia, and these cells can divide in response to injury (Coffey et al., 1990; Bush et al., 1999). To determine if the new cells arising in the DG after injury could be microglia, we labeled tissue samples with BrdU and ED-1, a marker for activated microglia (Figure 3.6). Microglia are known to invade the prenumbra region within 20 minutes after ischemic damage, therefore it was possible that BrdU+ cells in the DG could be this inflammatory cell (Gehrmann et al., 1991; Gehrmann et al., 1992; Gehrmann et al., 1995; Ivacko et al., 1996). We failed to detect cells that co-stained for both BrdU and ED-1 in the DG at either the early or the late time points. This implies that ED-1+ microglia observed in the damaged DG did not divide at the time that BrdU was present in the brain. ED-1+ cells were observed in the ipsilateral, but not contralateral, granule cell layer; however we could only observe cells that co-labeled for both ED-1 and BrdU within the ventricular walls of the contralateral hemisphere. Of over one hundred ED-1+ cells in the granule cell layer, only one co-labeled with BrdU. These results suggest that microglia do not account for a significant number of dividing cells at these two time-points.

Focal injuries can increase neurogenesis in the DG after a temporal delay

To determine whether cells from both the 24 hour and 120 hour groups assume a neuronal fate, tissue samples were also probed for the mature neuronal marker, NeuN, 42 days after BrdU was injected. As is shown in Figure 7 and Table 1, there was nearly a four-fold increase in the number of BrdU+ cells that co-labeled with NeuN in the granule cell layer ipsilateral to the lesion in the 120 hour differentiation group ($F_{(57)} = 37.2$, $p < 0.05$, Table 1). This was not observed in the 24 hour differentiation group, where ipsilateral cell counts were not significantly different from those in control hemispheres ($p > 0.05$; Table 1). Stacks of images were taken with a confocal microscope to further illustrate this point (Figure 3.7). Astrocytes are known to be involved in inflammatory cascades, therefore we also assessed the number of cells co-labeling for both GFAP and BrdU in these animals. We did not detect a significant increase in the number of new astrocytes in the DG cell layer in either the ipsilateral or contralateral hemispheres from the 24 hour differentiation group. There was, however, a small but significant increase in the number of new astrocytes in the granule cell layer ipsilateral to the lesion in the 120 hour differentiation group ($F_{(57)} = 11.54$, $p<0.05$; Table 1). These BrdU+/GFAP+ cells were almost always observed directly at the site of damage. Finally, we assessed the number of cells in these triple-labeled tissues that did not stain for GFAP, NeuN, or GFAP - cells that we will refer to as "undetermined" for clarity. As is shown in Table 1, we found a significant number of undetermined BrdU+ cells in the granule cell layer ipsilateral to the lesion in the 24 hour differentiation group in comparison to the 120 hour differentiation group and the contralateral hemisphere ($F_{(57)} = 33.2$, $p<0.05$).

To determine if neurogenesis was specific to the DG, or if rates of neurogenesis differed between regions at the late time-point, rats (n=3) were injected with BrdU 118 hours after acute injury to the frontal cortex. One hundred BrdU+ cells underwent phenotypic analysis by confocal microscopy. Tissue sections were stained with NeuN, GFAP, and BrdU. In no cases were we ever able to detect NeuN and BrdU co-labeling in the same cell (Figure 7G). We were able to detect co-labeling with GFAP in 31 of the 100 cells assessed.

Discussion

In these experiments, we used Fluorojade-B to label damaged neurons and to demonstrate that electrolytic lesions could be used to examine proliferative events following acute and focal injury in the hippocampus. The induction of this localized injury in the DG was followed by two distinct occurrences of cellular proliferation. Initially, there is an increase in cellular proliferation that results in the diffuse appearance of newly divided cells throughout the hippocampus after the lesion is induced. This increase in mitotic activity does not appear to be the result of DNA repair mechanisms being engaged in neurons, as none of the cells immunoreactive for BrdU co-labeled with neuronal markers. This early increase in cellular proliferation also did not result in an increase in the rate of neurogenesis, as assessed at six weeks following the lesion. In addition, the pattern of neurogenesis was similar to that normally observed in the DG of control hemispheres; not the diffuse pattern that would be expected given the pattern of proliferation observed at 24 hours. This finding was surprising given that the only other study of acute injury to the DG reported increased neurogenesis within this 24 hour time period (Gould and Tanapat, 1997).

In contrast to the early time point, a second occurrence of proliferation occurred at a later time (5 days) distinct from that observed at 24 hours. This later proliferation event was characterized by an even larger increase in mitotic activity primarily restricted to the subgranular zone of the DG. When cells were labeled with BrdU at 5 days post-lesion, and then examined six weeks later, the majority of the cells immunoreactive for BrdU were also immunoreactive for the mature neuronal marker, NeuN. In addition, there was a small but significant increase in the generation of new astrocytes. These results imply,

in accordance with models of more global brain injury, that a latent period greater than 24 hours is required before the injury-induced increase of new neurons is observed after focal electrolytic injury to the DG.

In virtually all models of brain trauma, with the exception of focal injury (Gould and Tanapat, 1997), increases in neurogenesis in the DG are only observed following a latent period greater than 24 hours (Gould and Tanapat, 1997; Parent et al., 1997; Ferland et al., 2002; Parent, 2003; Harry et al., 2004). This suggests that focal injury to the DG initiates a rapid increase in neurogenesis, while more global (and diffuse) injuries do not. Although we expected to induce a rapid neurogenic response like that produced by excitotoxic or mechanical lesions (Gould and Tanapat, 1997), the damage induced by an electrolytic lesion is more akin to that observed following the induction of seizure activity (Parent et al., 1997; Jiang et al., 2003). Although the animals were anaesthetized using sodium pentobarbital, a powerful anti-epileptogenic agent, we can not rule out the possibility that electrolytic lesions may have induced focal epileptic activity around the lesion site. Indeed, given the pattern of results observed, this seems highly likely, and would place the lesion model of brain injury in the same class as seizure or stroke induced models of damage and indicate that granule cell death per se is not the trigger that induces a neurogenic response, although it can induce cellular proliferation.

The increased levels of neurogenesis observed after 5 days in comparison to baseline levels of neurogenesis observed 1 day after injury may be due to the changing molecular environment after brain injury. For example, microglia can invade the damaged area quickly, (Nimmerjahn et al., 2005) and these inflammatory cells are known to release different factors over time including nerve growth factor and epidermal growth factor as

well as numerous cytokines. The varying release of these factors and the temporal sequence of their release may partially explain why neurogenesis is not increased at the 1 day time-point. Intriguingly, microglia have been demonstrated to be detrimental to hippocampal neurogenesis *in vivo* and *in vitro*, therefore things are clearly more complicated (Ekdahl et al., 2003; Monje et al., 2003). Astrocytes also likely play a role in the changing molecular environment in the neurogenic niche of the DG. A key role of astrocytes is the formation of a 'glial scar' which functions to encase a damaged area and protect the rest of the brain from potentially damaging secondary effects of injury (Mathewson and Berry, 1985), such as increased glutamate release (Choi 1988). Astrocytes are also known to release diffusible factors such as FGF2 (Joannides et al., 2004), IL-1 (Bethea et al., 1992), and prostaglandin E (Fontana et al., 1982), and have been previously demonstrated to stimulate neurogenesis in culture (Song et al., 2002; Horner and Palmer, 2003; Joannides et al., 2004). A dynamic interplay between microglia and astrocytes could explain the varying molecular environment in the DG after injury as well as the differing levels of neurogenesis observed at early and late time points.

A majority of cells that incorporated BrdU 1 day after injury did not stain with either mature neuronal or glial markers. While a molecular environment argument seems plausible to explain why BrdU+ cells did not become NeuN-expressing neurons, the same may not be true for why BrdU+ cells did not become GFAP- or ED-1-expressing cells. Intriguingly, all groups to date that have quantified neurogenesis in the non-injured DG and cortex using the identical markers used in this study (BrdU, NeuN, GFAP), also report cells that only label with BrdU (Kempermann et al., 1997; Gould et

al., 1999). BrdU+ cells from the electrolytic injury model to the DG that do not co-label with mature glial or neuronal markers are therefore not out-of-the-ordinary.

This study reports novel findings in the area of brain injury and hippocampal adult neurogenesis. First, a method of acute injury never before utilized in this context was carefully characterized. This method, which has been applied in many contexts, has never been used to study hippocampal neurogenesis. The method is replicable, reliable, discrete, and importantly, variable. The amount of current injected or the latency of DC current injection can be used to vary the size of a DG lesion. Lesions to the DG were characterized by morphological changes to dentate granule neurons, astrocytes, and dying, FJB+ cells. The proliferation of microglia in the DG was also assessed. Second, this study reports a surprising and interesting concept in relation to injury-induced neurogenesis in the DG: Acute injury to the DG, at least by electrolytic lesion, does not increase neurogenesis until after a latent period of greater than 24 hours. This is interesting because in models of chemical or mechanical injury to the DG, neurogenesis is increased within this 24 hour time period (Gould and Tanapat, 1997). This key finding, along with this method, may help elucidate some of the mechanisms underlying the injury-induced increase of new neurons in the adult mammalian brain.

Figure and table descriptions

Table 3.1 Cell numbers and phenotypes from co-labeling experiments in rats from the 24 hour and 120 hour differentiation groups. Cell counts are presented as number of cells per volume for the combined granule cell layer and subgranular zone (number of cells per 10^{-7} μm^3 volume). All analysis was done at 100x magnification with any cell within the SGZ or granular cell layer assessed. Ipsilateral and contralateral refer to the dentate granule cell layers ipsilateral and contralateral to the lesion. Note that percentages can only be compared within columns and not between rows.

	24 Hours		**120 hours**	
	Ipsilateral	**Contralateral**	**Ipsilateral**	**Contralateral**
NeuN/BrdU % Mean ± SEM	**33%** 9.9 ± 0.8	**62%** 9.2 ± 0.8	**83%** 34.6 ± 3.8*	**67%** 9.7 ± 1.3
GFAP/BrdU % Mean ± SEM	**0.9%** 0.25 ± 0.2	**0.7%** 0.1 ± 0.1	**4.4%** 1.8 ± 0.4*	**1.7%** 0.2 ± 0.2
BrdU % Mean ± SEM	**65%** 18.7 ± 1.8*	**37%** 5.4 ± 0.9	**12.3%** 5.1 ± 1.0	**31%** 4.5 ± 0.5

***Tukey post hoc comparison: $p<0.001$**

Figure 3.1. FluorojadeB staining reveals discrete neuronal damage after an electrolytic lesion to the dentate gyrus. (A*i*) This photomicrograph shows FJB+ cells in the dentate gyrus adjacent to a lesion site. Notice the tight clustering of FJB+ cells, indicating the specificity of this lesion. Scale is 100 μm. **(A*ii*)** Magnification of the damaged granule cell neurons shown in (A*i*). Scale is 25 μm. **(B*i*)** A sparse, non-clustered FJB staining pattern can also be observed in the dentate gyrus contralateral to the acutely injured hemisphere. This pattern of staining may be indicative of the general cell death

that is believed to occur constitutively in the dentate gyrus. **(B*ii*)** Magnification of the damaged granule cell neurons from (B*i*). Scale is 25 μm. GCL = granule cell layer, H = Hilus.

Figure 3.2. Electrolytic lesions to the DG lead to highly localized changes in cell morphology and reactive astrocytosis. (A) Representative example of the effects of an electrolytic lesion in the dorsal DG both ipsilateral (A*i*) and contralateral (A*ii*) to where the electrode was positioned. The size of the lesion (LA) at the electrode tip and the extent of damage elicited in the dentate granule cell layer (GCL) and hilus (H) can easily be seen in A*i*. Markers are NeuN (blue) and FJB (green). Scale is 100 μm. LA = Lesion area, GCL = granule cell layer, H = hilus. **(B*i*)** Photomicrograph showing the extent of damage that an electrolytic lesion caused in the DG. Granule cells and glia are indicated with NeuN (blue) and GFAP (red), respectively. The FJB+ (green) area indicates the extent of the damage in the DG 24 hours after surgery. Scale is 100 μm. **(B*ii*)** Magnification of area in B*i* showing astrocytes that extend end feet-like processes to small granule cells (arrows) and a FJB positive cell in the same region (yellow arrowhead) Scale is 10 μm. **(C*i*)** Electrolytic lesions can alter the morphology of the dentate gyrus at 24 hrs. Note the physical disruption of the upper granular cell layer (blue) and the appearance of damaged neurons (green) in the hilus and the infra and supra blades of the DG. Scale bar is 100 μm. **(C*ii*)** Neurons proximal to the damaged area were smaller and more spheroid in comparison to neurons located more distal to the damaged area **(C*iii*)**. Scale bar is 10 μm for C*ii* and C*iii*. **(D)** All cells that express nestin and have the morphological characteristics of astrocytes also express GFAP. Note that nestin

reactivity is also present at the vasculature. Scale is 15 μm. **(D*i*)** Nestin, **(D*ii*)** GFAP, **(D*iii*)** overlay. Arrow indicates a reactive astrocyte co-labeling for both GFAP and nestin. **(D*iv*)** reactive astrocytes (nestin:red) in the DG (white trace) 1 day after acute injury. Green cells are dying neurons (FJB+). Scale is 100 μm.

Figure 3.3. Examination of the proliferative response in the dentate gyrus after brain damage. (A) Significantly more BrdU+ cells were observed following lesions than in control conditions. Significant differences were observed in the ipsilateral hemisphere when the electrode penetrated the DG, but no lesion was made (DG/No DC), and when a lesion of the DG was made (DG/DC). Both of these conditions resulted in more BrdU+ cells than were observed when an electrode was just lowered into cortical layer 6. No differences were observed between the contralateral hemispheres of any of the groups **(B)** Similar results were observed for BrdU+ cells in the hilus in these three conditions, although the overall level of staining in this region was much lower than that observed in the SGZ.

Figure 3. 4. Discrete electrolytic lesions induce hemisphere-specific patterns of cell proliferation in the dentate gyrus of rats assessed at 24 and 120 hour time points. **(A)** Representative examples of the DG ipsilateral and contralateral to a lesion at 24 and 120 hours. Scale=100 μm for Contra and Ipsi. NeuN (blue); BrdU (red); FJB (green). Upper right panel shows magnified view of BrdU+ cells and FJB+ cells. Scale=10 μm. Lower right panel shows low resolution view of the lesion site at 120 hours. **(B)** Temporal dissociation of proliferating cells at 24 and 120 hours. Number of BrdU

labeled cells in the SGZ at early (BrdU injected at 22 hrs, animal sacrificed at 24 hrs) and late (BrdU injected at 118 hrs, animal sacrificed at 120 hrs) time points. **(C)** Representative example of the diffuse proliferation response at 24 hours and the more focal response observed at 120 hours. BrdU positive cells are white. Scale = 200 μm

Figure 3.5. Electrolytic lesions induce a proliferative response as evidenced by the endogenous marker of cell division, Ki67. From left to right, DAPI (blue) and Ki67 staining (red) are shown in both the ipsilateral and contralateral hemispheres. At the 24 hour time point Ki67 positive cells are widely dispersed throughout the hippocampus, while at the 120 hour time point Ki67, as with BrdU labeling, is restricted to the SGZ of the DG in both lesion and control hemispheres. Staining is stereotypically more intense ipsilateral to the lesion. Scale bar: 100 μm for 10x; 10 μm for 100x panels.

Figure 3.6. Appearance of microglia in the damaged dentate gyrus at both 24 and 120 hour time-points. (A) 24 hour proliferation group: In the contralateral hemisphere, activated microglia tended to remain around the ventricles. Arrowhead depicts a microglia that appears to co-label for both BrdU (red) and ED-1 (green). Inset in the overlay is a 10-fold magnification of this cell. In the ipsilateral hemisphere activated microglia were evident throughout the DG and hilus. Arrowhead depicts a microglia within the granule cell layer that, as is shown in the bottom row, co-labels for both BrdU (red) and ED-1 (green). Scale: Top two rows = 100 μm; Bottom row = 10 μm. **(B)** 120 hour proliferation group. In the contralateral hemisphere, activated microglia were present only in the ependymal zone. In the ipsilateral hemisphere, ED-1+ microglia

appeared more tightly adjacent to a wound point, but did not co-label with BrdU. Scales for panels are identical to those in A.

Figure 3.7. Adult neurogenesis is increased at 120 hours, but not 24 hours after focal damage to the dentate gyrus. (A*i*) Representative example of electrolytic damage and the new cell growth that occurs at 24 hours. BrdU labeled cells (red) are spread diffusely through the DG and rarely co-label with NeuN (green) or GFAP (blue). Scale is 200 μm. **(A*ii*)** The same tissue as A*i*, contralateral to the lesioned side. Scale is 200 μm. **(A*iii*)** Magnification of the boxed area from A*i* (Scale is 100 μm). **(A*iv*)** White boxed area from A*iii*. Scale is 10 μm **(B)** 3D reconstruction of the red boxed area from A*iii*. A BrdU+ cell neither NeuN nor GFAP positive cell is the focal point of the cross hairs (x and y axes). Cells like this were common in the granule cell layer of rats injected with BrdU 22 hours after surgery, despite the proximity to the lesion. Scale is 7 μm. **C)** Confocal picture of the DG at 24 hrs showing numerous BrdU+ cells that are neither GFAP nor NeuN positive. Scale is 40 μm. **(D*i*)** Representative example of electrolytic damage and the new cell growth that occurs at 120 hours. The gliogenic reaction is pronounced throughout the brain but close to the electrode tract. Scale =A1. **(D*ii*)** Representative example of the hemisphere contralateral to a lesion. This picture is from the same tissue as D*i*. Scale = A1. **(D*iii*)** Magnification of white boxed area from D*i*. Scale = 100 μm. **(D*iv*)** White boxed area from D*iii*. Almost all BrdU labeled cells are NeuN positive. Scale = 10 μm **(E)** 3D reconstruction of the red boxed area from D*iii*. A BrdU cell co-labeling for NeuN is at the focal point of the cross hairs (x and y axes). In contrast to the 24 hour group, new cells appearing at the lesion site in the 120 hour group

mostly became NeuN positive. Scale = 7 μm. **(F)** Confocal picture of the DG demonstrating numerous cells co-labeling for both NeuN and BrdU, but not GFAP. Scale = 40 μm. **(G)** No neurogenesis in the frontal cortex after acute electrolytic injury. **(G*i*)** GFAP, **(G*ii*)** BrdU, **(G*iii*)** NeuN, **(G*iv*)** Overlay. Numerous BrdU+ cells were tightly associated with neurons (satellite cells). A satellite cell in close proximity to a NeuN + cell is the focal point of the cross hairs. The white arrow indicates a cell co-labeling for both GFAP and BrdU. Scale = 20 μm.

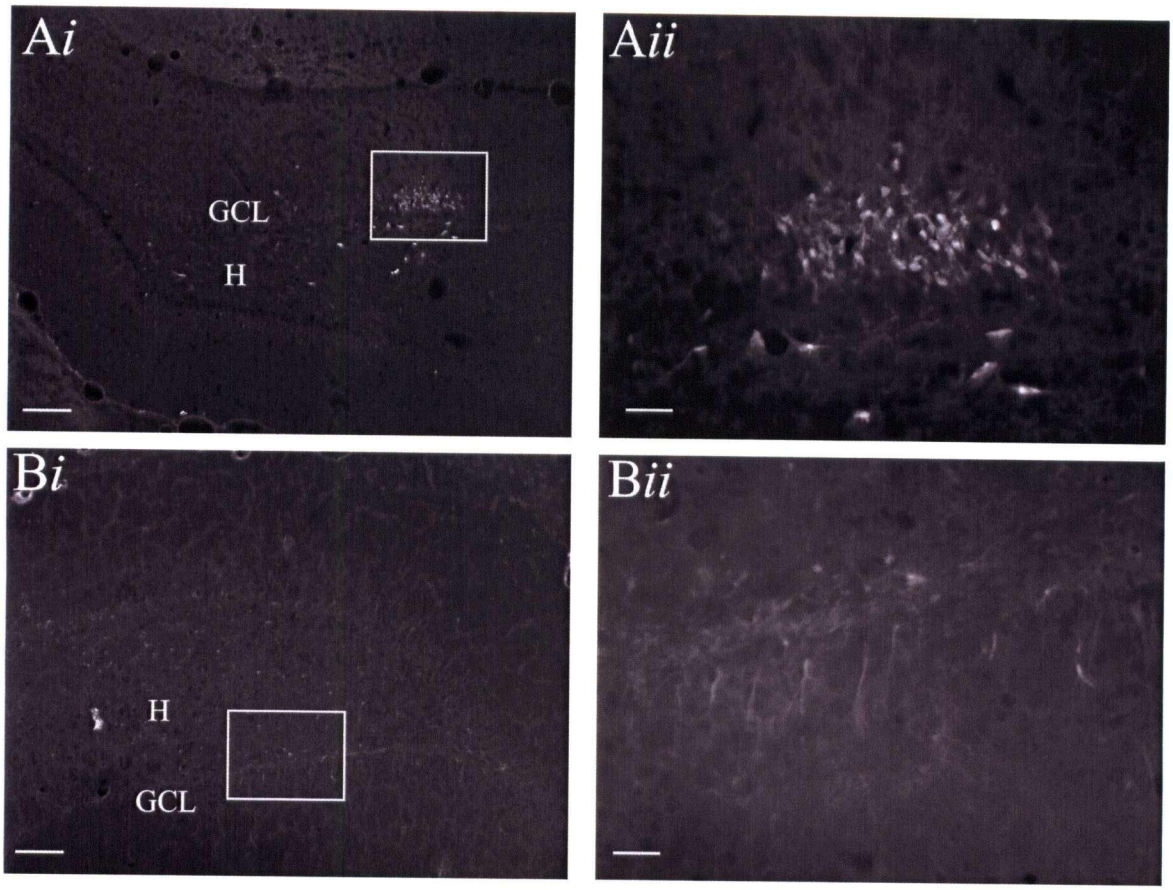

Figure 3.1

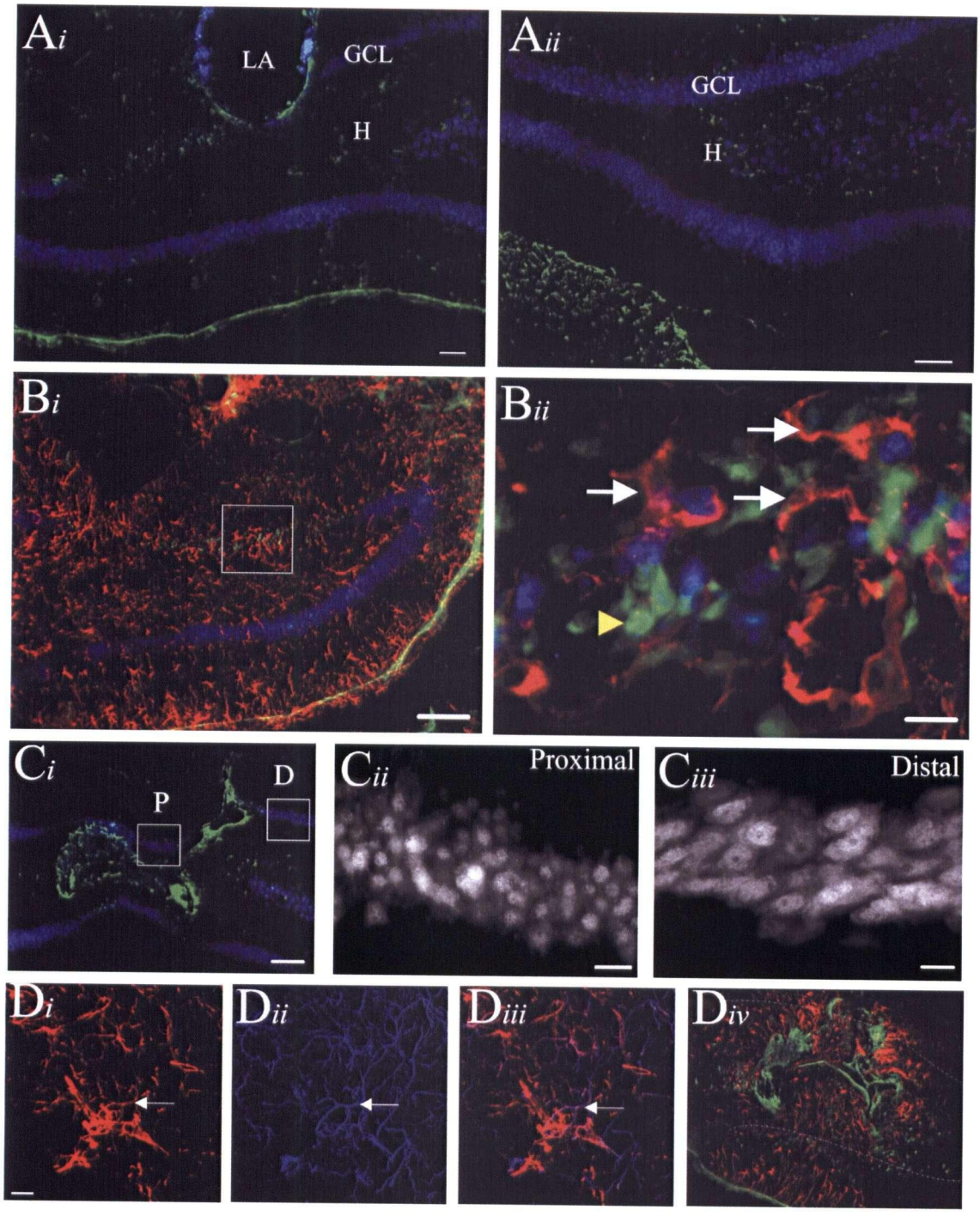
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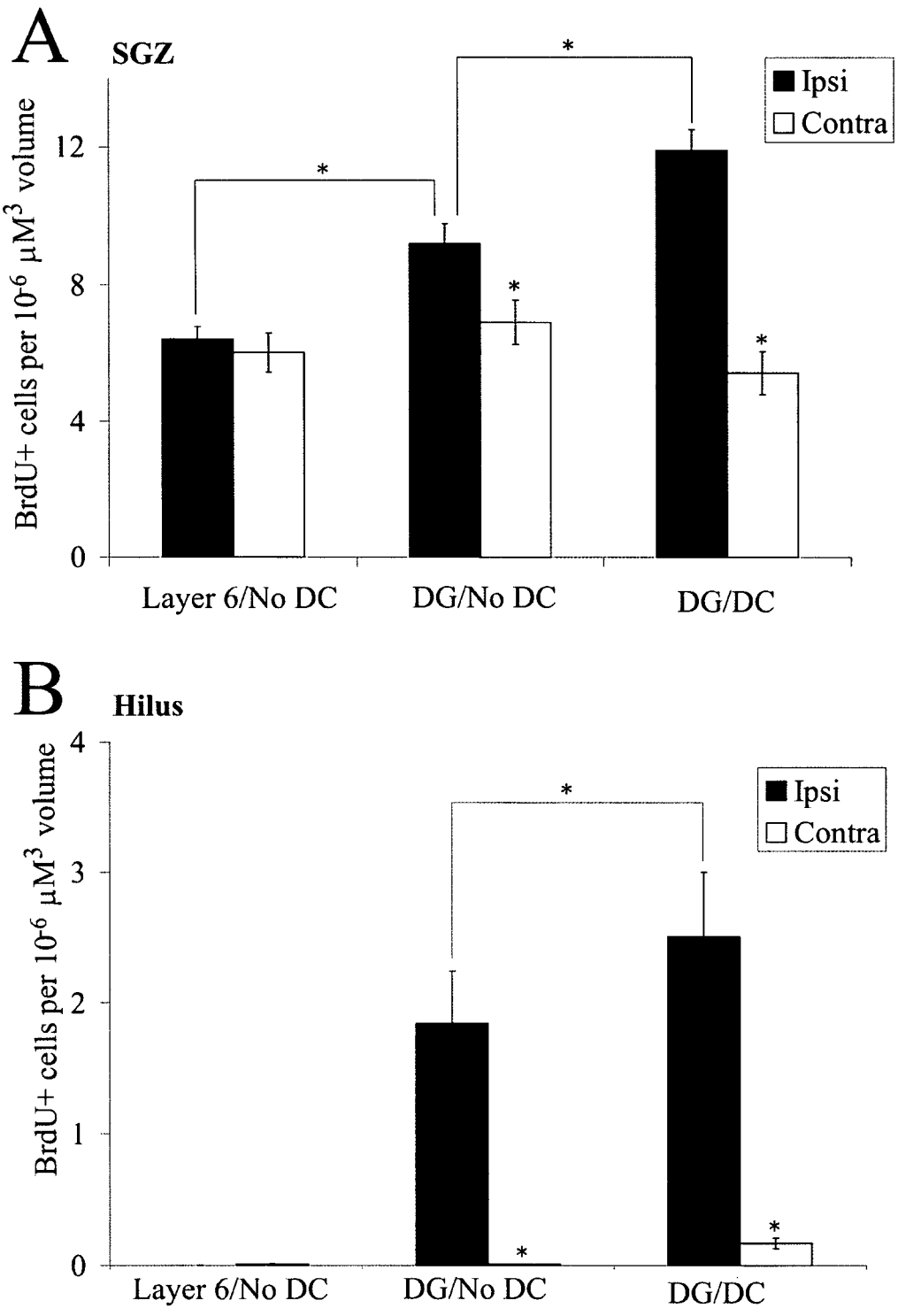
Figure 3.2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SGZ
Ipsi
Contra
BrdU+ cells per 10^{-6} μM^3 volume
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4
8
12
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Layer 6/No DC
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Contra
BrdU+ cells per 10^{-6} μM^3 volume
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DG/DC

Figure 3.3

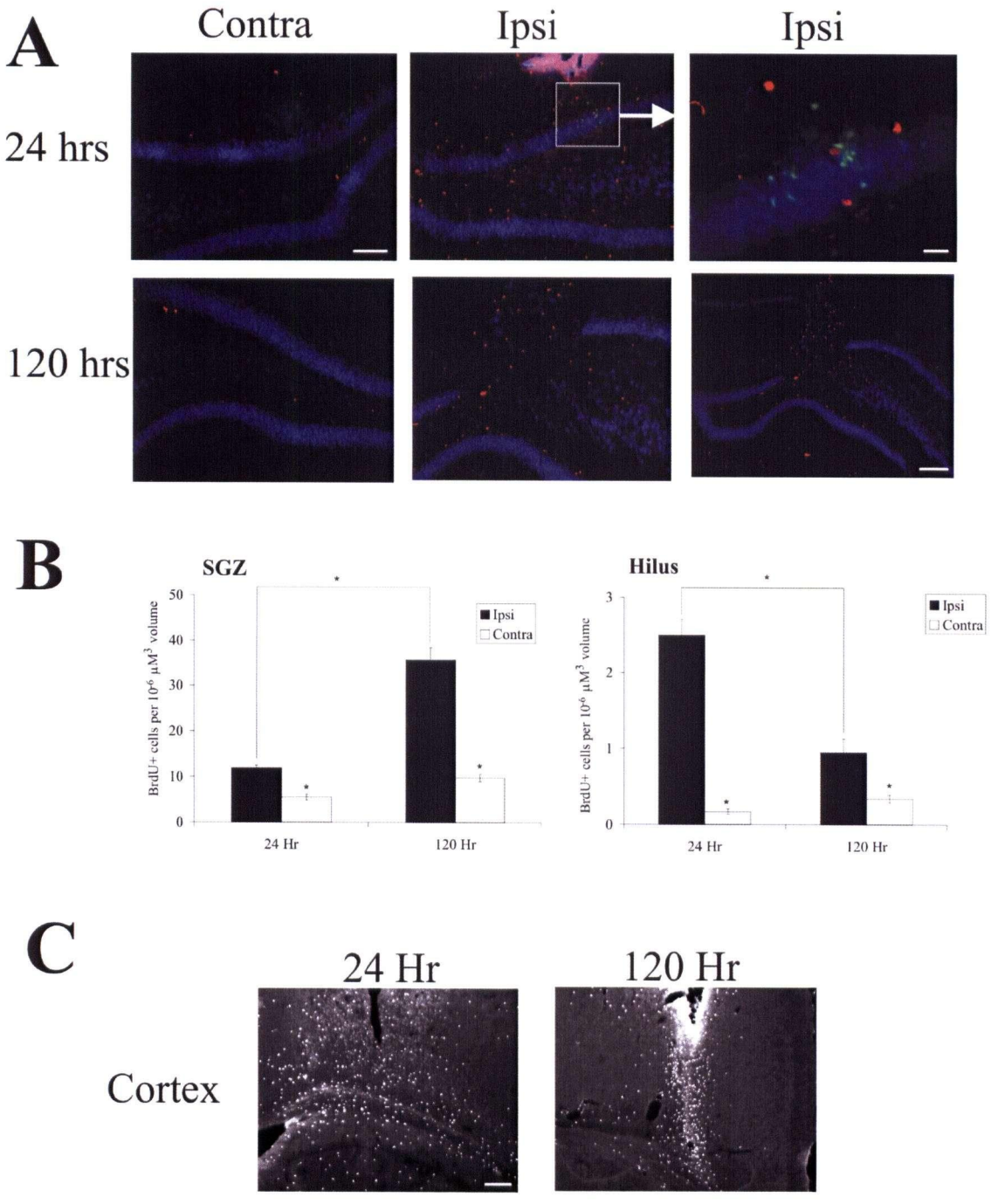
A
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Figure 3.4

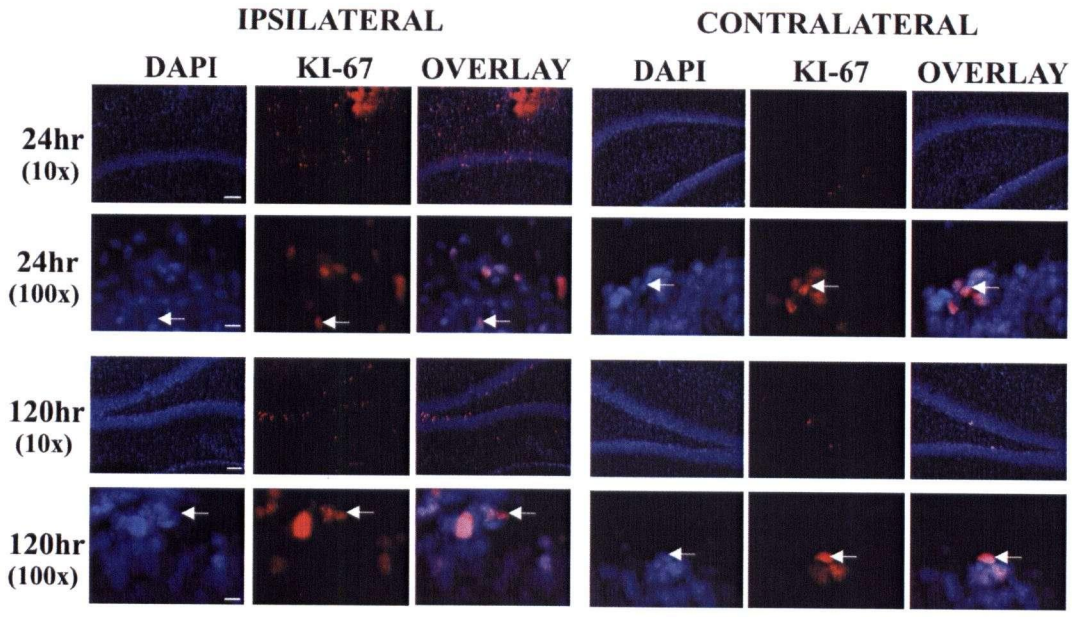
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Figure 3.5

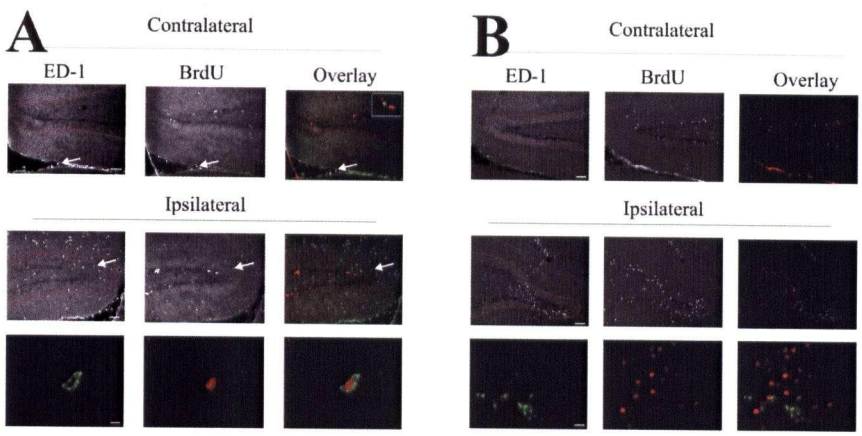
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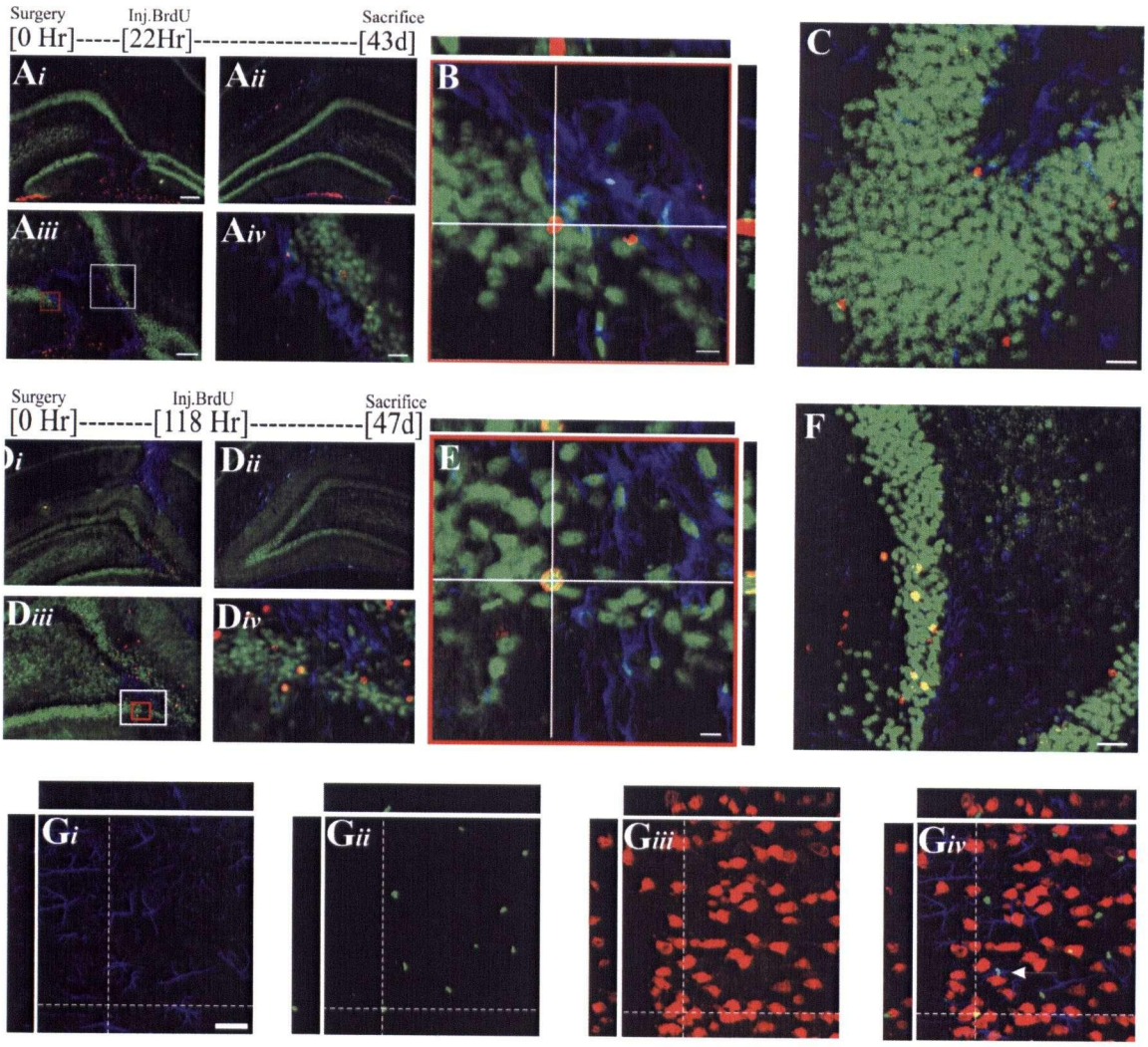
Figure 3.6


Surgery
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Sacrifice
[0 Hr]-----[22Hr]----------[43d]
Ai
Aii
Aiii
Aiv
B
C
Surgery
Inj.BrdU
Sacrifice
[0 Hr]--------[118 Hr]----------[47d]
Dii
Diii
Div
E
F
Gi
Gii
Giii
Giv

Figure 3.7

Reference:

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Chapter 4. Conclusions

Hypothesis and future directions

In virtually all models of brain trauma, with the exception of focal injury (Gould and Tanapat, 1997), increases in neurogenesis in the DG are only observed following a latent period greater than 24 hours (Gould and Tanapat, 1997; Parent et al., 1997; Ferland et al., 2002; Harry et al., 2004). This suggests that focal injury to the DG initiates a rapid increase in neurogenesis, while more global (and diffuse) injuries do not. Although we expected to induce a rapid neurogenic response like that produced by excitotoxic or mechanical lesions (Gould and Tanapat, 1997), the damage induced by an electrolytic lesion is more akin to that observed following the induction of seizure activity (Parent et al., 1997; Jiang et al., 2003). Although our animals were anaesthetized using sodium pentobarbital, a powerful anti-epileptogenic agent, we can not rule out the possibility that electrolytic lesions may have induced focal epileptic activity around the lesion site. Indeed, given the pattern of results observed, this seems highly likely, and would place the lesion model of brain injury in the same class as seizure or stroke induced models of damage and indicate that granule cell death per se is not the trigger that induces a neurogenic response, although it can induce cellular proliferation.

We hypothesize that if granule cell death alone does not normally induce neurogenesis, then some mitigating factor must be induced in surviving cells, following cellular damage that facilitates neurogenesis. The first piece of evidence for this hypothesis comes from the existence of the latent period that occurs before neurogenesis is observed in the DG following extensive brain injury (i.e. stroke, epilepsy). One

possibility is that glutamate-induced cell toxicity and calcium overload produce a change in the local environment that triggers mitotic activity (Choi, 1988, 1995). This, however, seems unlikely given that NMDA antagonists actually increase the number of new neurons in the DG, while NMDA itself decreases it (Cameron et al., 1995). The second possibility, and the one we favor, is that a glial mediated response is required for neurogenesis to be induced. In support of this hypothesis, we have shown that a large increase in reactive astrocytes can be observed 5 days after focal injury is induced in the DG. In contrast, very few reactive astrocytes were observed 1 day after acute focal injury to the DG. We hypothesize that nestin+ cells in the DG with the morphology or reactive astrocytes stimulate the neurogenic niche in the DG to promote the increase in new neurons observed 5 days after injury. This also implies that a temporal window exists for the promotion of neurogenesis, and if new undifferentiated daughter cells are not exposed to some glial mediated factor within a certain time period after being generated, they do not progress to a mature neuronal phenotype.

Although other types of glia may actually inhibit neurogenesis (Ekdahl et al., 2003; Monje et al., 2003) there is substantial evidence for certain subtypes of astrocytes being involved in both an injury response and neurogenesis. First, astrocytes are known to respond to damage and disease states producing "astrogliosis" or the presence of a "glial response" (Mathewson and Barry, 1985). GFAP antibodies are widely used to detect the reactive astrocytes that are part of this response, since reactive astrocytes stain much more strongly with GFAP antibodies than normal astrocytes (Mathewson and Berry, 1985). Second, nestin-containing cells that also express GFAP can be observed in the regions of CNS that show high rates of proliferation in both developing and adult mice

(Wei et al., 2002). Third, astrocytes have been shown to promote neurogenesis in cell culture experiments (Song et al., 2002; Horner and Palmer, 2003; Joannides et al., 2004). The neurogenesis/survival promoting capacity of astrocytes may come from their capacity to produce FGF2, and astrocytes have in fact been shown to produce FGF2 in response to brain injury (Clarke et al., 2001). This may be important for promoting neurogenesis, as FGF2 infused directly into the DG is necessary and sufficient to induce the formation of new neurons (Yoshimura et al., 2001).

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