Kindling-Induced Changes in Neurogenesis in the Dentate Gyrus of the Rat

by

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Abstract

Dentate granule cell neurogenesis in the rat continues to occur throughout adulthood. Granule cell production is sensitive to a variety of physiological and environmental factors which suggests that the proper regulation of granule cell neurogenesis may be important to the functioning of the hippocampus. This study examined the production of new cells within the dentate gyrus in response to kindled seizures using the mitotic indicator bromodeoxyuridine. An increase in cell production was found to occur within the granule cell layer and molecular layer but not the hilus, when compared to unstimulated control animals. This study shows for the first time that the increased electrical activity of kindled seizures can stimulate the production of new neurons within the dentate gyrus. A possible role for neurogenesis in human temporal lobe epilepsy is discussed.

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Chapter 1

Introduction

1.1 The Hippocampus

The hippocampus is an area of the brain believed to be important for learning and memory. Human amnestic syndromes have been found to occur after hippocampal damage (Zola-Morgan *et al.*, 1986), and the hippocampus is metabolically activated by certain types of memory tasks (Friedman & Goldman-Rakic, 1988). Animal experiments have also shown memory impairments with hippocampal lesions (Zola-Morgan & Squire, 1986; Nilsson *et al.*, 1987), as well as the existence of "place" cells within the hippocampus, which fire when the animal is in a location within the receptive field for that cell (Eichenbaum & Cohen, 1988). The hippocampus is also believed to play a role in temporal lobe epilepsy (Sloviter, 1994). The unique plasticity of hippocampal structure and function may underlay both the learning and memory, and pathological phenomena associated with the hippocampus. The discovery that the hippocampus is also one of the few areas of the adult brain in many species in which neurogenesis persists into adulthood raises the question of how this might be involved in both the normal and pathological functioning of the hippocampus.

1.2 Anatomy and Neuronal Connections Within the Hippocampal Formation

The hippocampal formation of the rat is an elongated structure which curves in a "C" shaped fashion from the septal nuclei of the basal forebrain rostrodorsally, towards the temporal lobe caudoventrally (Paxinos & Watson, 1986). The complex can be subdivided into a number of regions which are projectionally linked. These regions are cytoarchitectonically distinct from each other and consist of the subicular complex (SC), entorhinal cortex (EC), dentate gyrus (DG) and the hippocampus proper which can be further subdivided into the CA1, CA2 and CA3 regions (Lorente de Nó, 1933; Figure 1.1A).

The connections linking the regions of the hippocampal formation are primarily unidirectional and have traditionally been described as forming a serial circuit which conducts information entering the EC as afferent input to the DG through the perforant pathway. Granule cell axons (mossy fibers) of the DG then project to the pyramidal cells of CA3 which themselves project (via the Schaffer collaterals) to those of CA1 (Figure 1.1B). The SC then completes the circuit by relaying signals from CA1 back to the EC. This scheme of information flow through the hippocampal formation has provided a convenient framework with which to logically organize it's components, but does not accurately reflect the complex intrinsic connections which exist between the regions. It is true that the information flow through the hippocampus is mainly unidirectional, but parallel

connections between the regions and local circuit interactions such as feed-back and feed-forward inhibition, make the serial circuit model inappropriate.

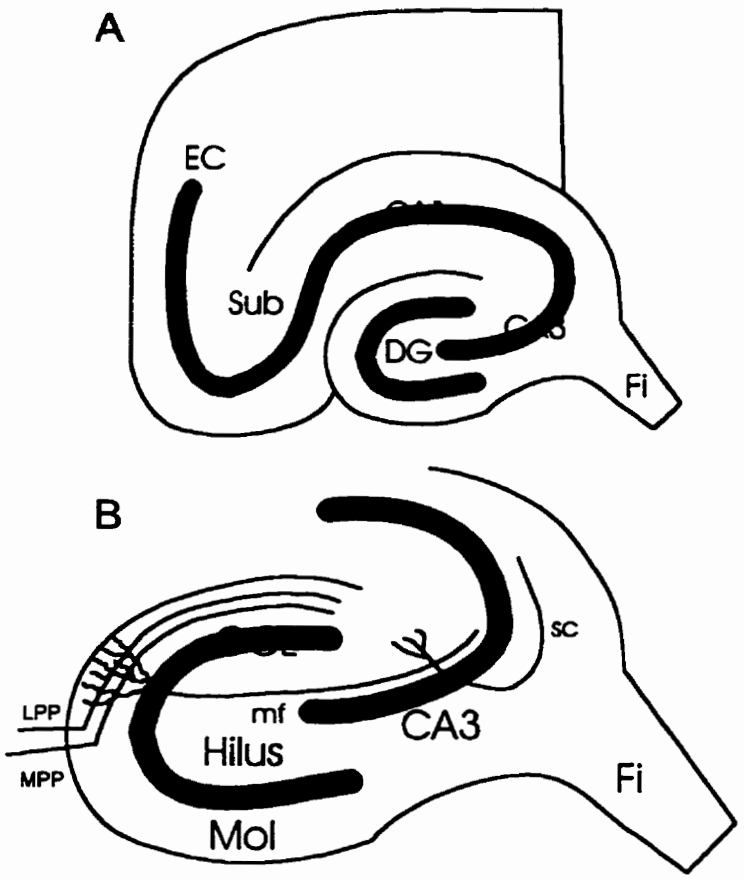


Figure 1.1: The Hippocampal Formation

A. Schematic diagram outlining the relative locations major components of the hippocampal formation. B. Schematic diagram illustrating the major anatomical components of the dentate gyrus. MPP, medial perforant path ; LPP, lateral perforant path; GCL, granule cell layer; mf, mossy fibre; Mol, molecular layer; Fi, fimbria; Sub, subiculum; EC, entorhinal cortex; DG, dentate gyrus; sc, Schaffer collateral axons. (structur.cdr)

1.1.1 Hippocampus Proper

The hippocampus proper has a laminar organization with its principle cell type being pyramidal cells located in the pyramidal cell layer. The pyramidal cells of CA1 are generally smaller than those of CA3 and do not receive any mossy fibre connections from DG. The precise location of CA2 is questionable but is generally considered to be a thin region (about 250 μm) separating CA3 and CA1, with pyramidal cells similar to those of CA3 (Amaral & Witter, 1995). In the CA3 region, the mossy fiber axons from dentate granule cells synapse on CA3 pyramidal cell dendrites in the stratum lucidum – a narrow band located just above the pyramidal cell layer. CA3 and CA2 pyramidal cells send highly collateralized axons (Schaffer collaterals) to CA1 (ipsi- and contra-lateral) as well as to a number of other cell types (e.g. basket cells) throughout the hippocampus, and to the hilus of the DG as well as subcortically to the lateral septal nucleus.

1.1.2 Entorhinal Cortex

The EC is a laminated structure containing many cell types which receive afferent input from a large number of cortical and subcortical structures - especially the olfactory bulb (in rat) and the amygdaloid complex respectively - as well as portions of the hypothalamus and brain stem (Amaral & Witter, 1995). Cortical efferents from the EC are widespread throughout the limbic, paralimbic

and olfactory regions, while subcortical projections include the amygdala, striatum and septal complex, but not the brain stem (Amaral & Witter, 1995). The EC has been grossly subdivided into the lateral entorhinal area (LEA) and the medial entorhinal area (MEA), projections from which compose the lateral and medial portions of the perforant pathway respectively. The EC makes projections throughout the hippocampus but the majority are through the perforant pathway to the dendrites of dentate granule cells in the outer two thirds of the molecular layer of the dentate gyrus. Fibers from the LEA terminate in the outer third of the molecular layer, while those from the MEA terminate in the middle third (Witter, 1993).

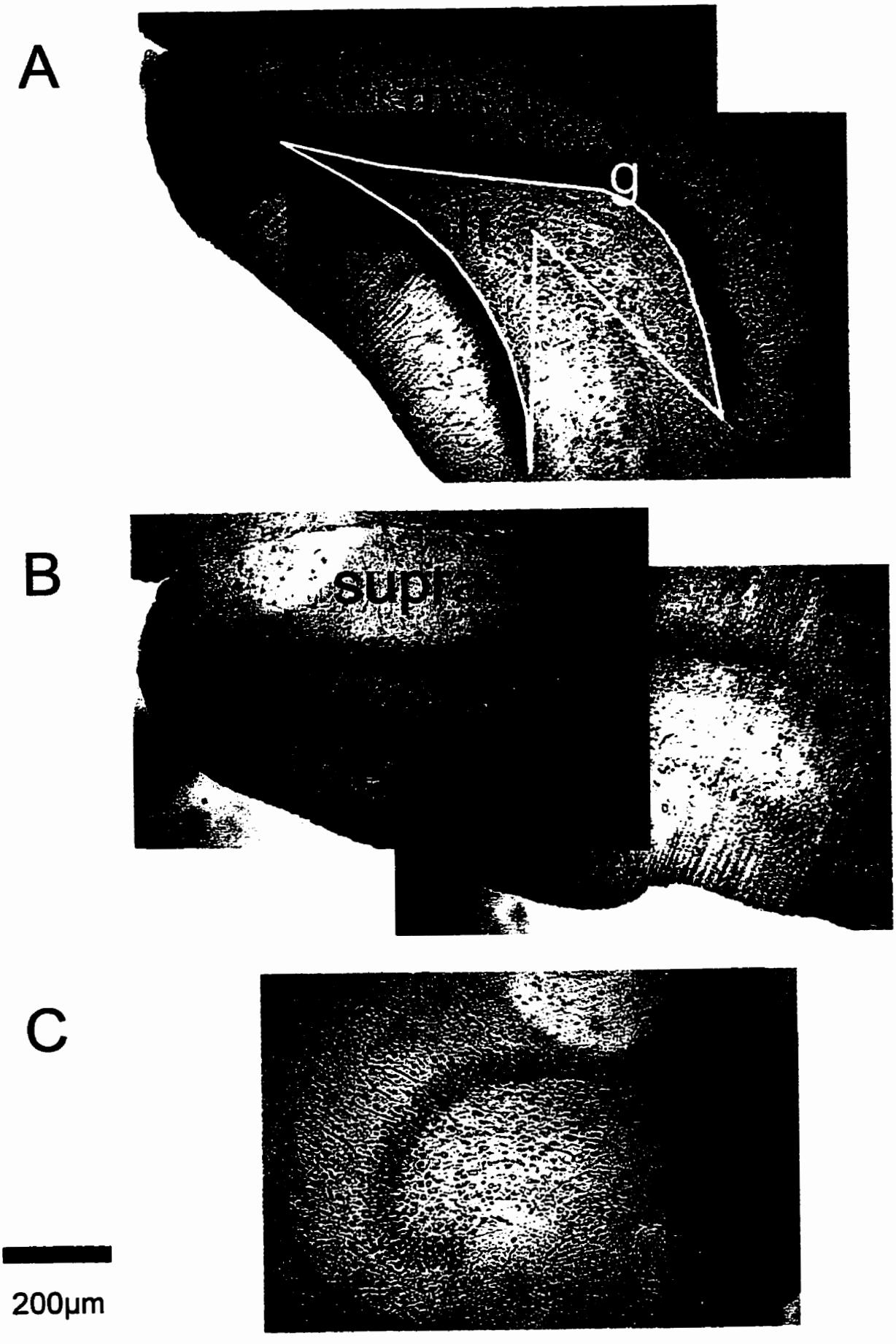
1.1.3 Dentate Gyrus

The three primary anatomical regions of the DG are the molecular layer, granule cell layer (GCL) and the hilus (Figure 1.1B). As the hippocampal formation curves from dorsomedial to ventrolateral there is a gradual change in the shape of the DG. The GCL forms a sharp apex dorsomedially when the DG is transversely sectioned, while in ventrolateral DG the GCL becomes curved (Figure 1.2). The DG does not have any extra-hippocampal projections and does not receive any direct input from cortical structures other than the EC. The DG (particularly the hilus) does receive input from subcortical structures including the septal nuclei and posterior hypothalamus as well as several nuclei in the brainstem (Gaykema *et al.*, 1990; Amaral & Kurz, 1985; Amaral & Witter, 1995).

Figure 1.2: The Shape of the Dentate Gyrus

Transverse sections of the dentate gyrus stained with methylene blue, highlighting the granule cell layer (g). A. Dentate gyrus from dorsal hippocampus with a sharp apex. B. Dentate gyrus from middle hippocampus shows changes in curvature of blades of the granule cell layer and blunting of the apex. C. Dentate gyrus from ventral hippocampus shows rounded appearance. m, molecular layer; h, hilus; g, granule cell layer; supra, suprapyramidal blade; infra, infrapyramidal blade; sgz, subgranular proliferative zone. Scale bar applies to A, B and C. (dmvshape.cdr)

Figure 1.2



The molecular layer is mainly acellular, containing mostly the dendrites of dentate granule cells and their afferent axonal inputs. Some neuronal cell types do exist there though, and are mainly GABAergic basket cells and axo-axonic cells which make inhibitory connections with dentate granule cell bodies and axon initial segments respectively (Ribak & Seress, 1983; Seress & Ribak, 1983; Soriano *et al.*, 1990).

The GCL contains tightly packed granule cells whose somata are elliptical in shape and approximately 10 µm high and 18 µm wide, and forms a "V" shape with one blade (supra-pyramidal) located closer to CA1 while the other (infra-pyramidal) is located ventrally (Amaral & Witter, 1995). The spine covered dendrites of dentate granule cells extend into the molecular layer toward the hippocampal fissure where they receive glutamatergic projections from the EC via the perforant pathway (outer two thirds of the molecular layer) as well as ipsi- and contra-lateral input from excitatory hilar mossy cells (inner third of molecular layer) (Laurberg & Sorensen, 1981; Swanson *et al.*, 1981; Soriano, & Frotscher, 1994).

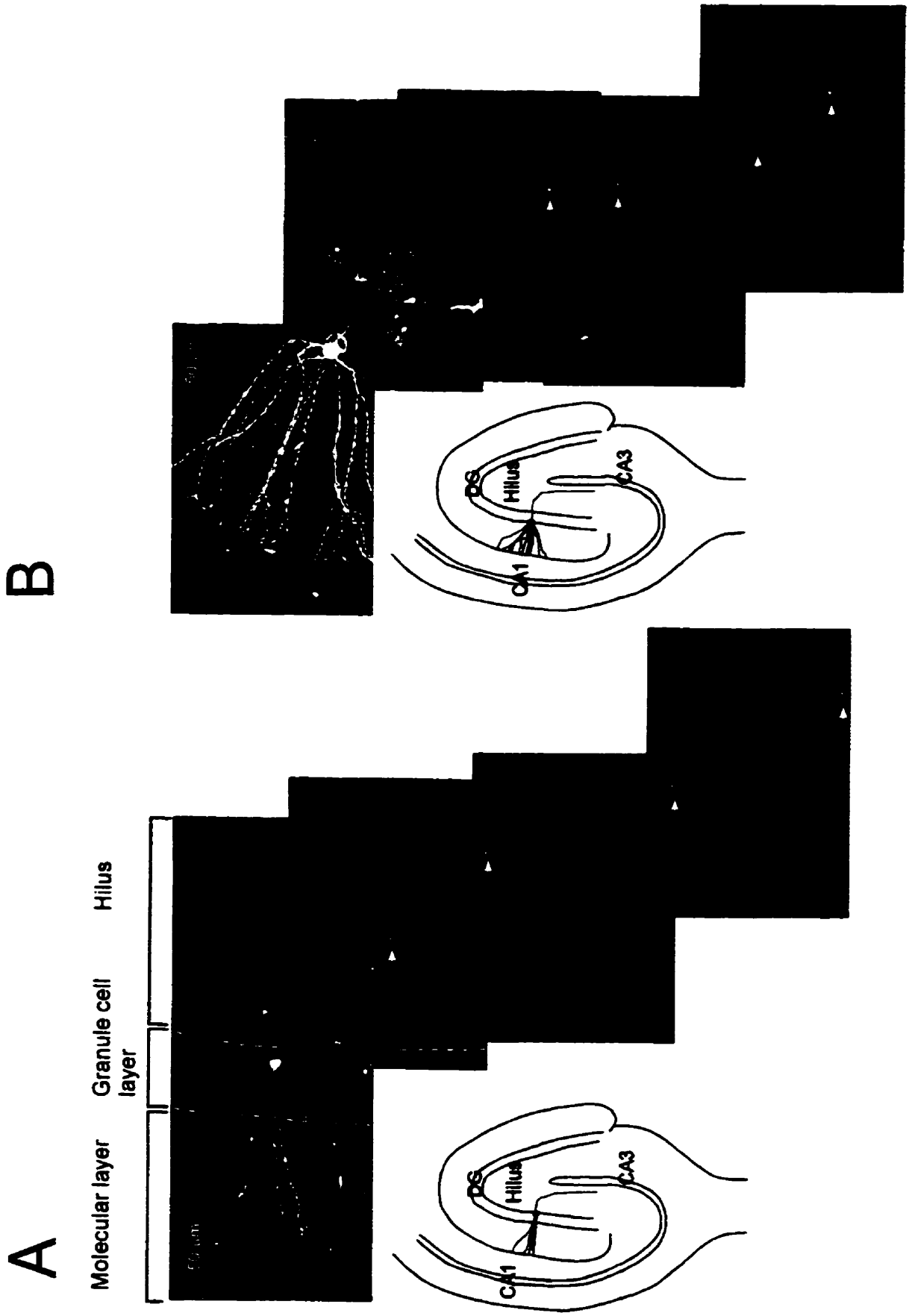
The dendritic tree shape of granule cells has been shown to be correlated with the position of the cell body within the GCL (Claiborne *et al.*, 1990). Cells located closer to the molecular layer generally have a greater number of primary

dendrites then those located closer to the hilus. These superficially located granule cells also typically have a "bell" shaped dendritic tree with a larger transverse spread compared to those located closer to the hilus, which have a more conical shape and a smaller transverse spread. Preliminary data from our laboratory has largely confirmed those of Claiborne *et al.* (1990) by examining the dendritic trees of cells filled with Lucifer Yellow while conducting whole-cell patch recordings (Scott *et al.*, 1997; Figure 1.3). A number of functional differences between the two cell types have been described, for example, granule cells located closer to the hilus have been found to have a greater amount of GABA-ergic inhibition than those located more superficially (Wojtowicz *et al.*, 1997; Scott *et al.*, 1997). This may indicate a mechanism to prevent excessive excitation of the younger cells or perhaps a different contribution to the normal functioning of the dentate gyrus than those located more superficially.

The axons of dentate granule cells (mossy fibres) project through the hilus where they collateralize on basket and mossy cells before continuing on to synapse on dendrites of CA3 pyramidal cells. In CA3, the mossy fibres terminate on the apical dendrites of CA3 pyramidal cells in the stratum lucidum, with the primary neurotransmitter thought to be glutamate (Storm-Mathisen & Fonnum, 1972). Each granule cell is believed to contact between 14 and 28 pyramidal cells, while each pyramidal cells receives input from about 50 granule cells (Claiborne *et al.*, 1986; Amaral & Witter, 1995).

Figure 1.3: Shapes of Dentate Granule Cells

Representative granule cells from the inner (A) and outer (B) edges of the granule cell layer, filled with Lucifer Yellow. Notice the "bell" shape of the dendritic tree of the outer cell with multiple primary dendrites, while the inner cell has a triangular shaped dendritic tree with a single primary dendrite. Both cell types project to the CA3 area and form typical mossy fibre terminals. Arrows follow mossy fibre from each cell through the hilus. DG, dentate gyrus. Photos provided by Sabrina Wang. (lucifer.cdr)


A
Molecular layer
Granule cell layer
Hilus
DG
Hilus
CA3
CA1
B
DG
Hilus
CA3
CA1

The hilus contains a number of cell types with the most prominent being the large (25-35 μm) mossy cells which receive collateral input from granule cell mossy fibres as they pass through the hilus to CA3. These cells stain positive for glutamate but not GABA and make synapses on granule cell dendrites in the inner third of the molecular layer which are likely excitatory (Soriano, & Frotscher, 1994). A number of pyramid-shaped basket cells are located immediately below the GCL which send a thick apical dendrite through the GCL to the molecular layer, and a number of smaller basal dendrites which extend deeper into the hilus. The axons of these cells form a large multicellular basket plexus which surrounds dentate granule cell somata, and are largely GABAergic and are therefore likely inhibitory to granule cells (Ribak & Seress, 1983; Seress & Ribak, 1983).

1.3 Development of the Granule Cell Layer

The GCL of the rat is unusual when compared to most areas of the brain in that it is formed primarily post-natally, with only about 15% of granule neurons being generated before birth (Schlessinger *et al.*, 1975). Recent evidence suggests that granule cells continue to be produced until late in the animal's lifetime (Kuhn *et al.*, 1996). This unusual property of the DG makes it a convenient area in

which to study the factors influencing neurogenesis at a time when it has stopped in most other regions of the brain.

During gestation, cells which will eventually form the GCL arise from a proliferative zone in a portion of neuroepithelium lining the walls of the lateral ventricle, near cells which will form the hippocampus proper (Altman & Bayer, 1990; Figure 1.4). These precursor cells migrate to the immature DG region in a stream across the portion of the marginal zone which will become the fimbria (Cowan *et al.*, 1980). Here they form a secondary proliferative zone from which dentate granule cells will be generated. This proliferative zone exists in the hilus until the end of the second post-natal week in the hilus, with the peak of cell production occurring near the end of the first post-natal week and the rate of production estimated as up to 50,000 cells per day (Schlessinger *et al.*, 1975). The hilar proliferative zone gradually disappears as cells migrate into the GCL, with proliferating cells persisting within an area known as the subgranular zone (SGZ; see Figure 1.2) at the base of the GCL, bordering the hilus. The rate of new granule cell production then falls to a minimum by the end of the third post-natal week.

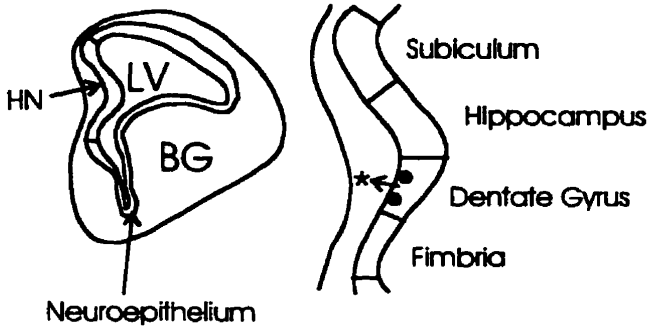


Figure 1.4: Origin of Granule Cell Precursors

During embryonic development (embryonic days 16 to 19), granule cell precursors migrate (black-filled dots, right figure) from the hippocampal neuroepithelium to form a second proliferative zone in what will become the dentate gyrus (*). LV, lateral ventricle; BG, basal ganglia. Adapted from Gould & Cameron, 1996. (develop.cdr)

During the development of the GCL, gradients of granule cell production exist whereby the ventral GCL is formed earlier than the dorsal GCL and the suprapyramidal blade forms slightly earlier than the infrapyramidal blade (Schlessinger *et al.*, 1975; Cowan *et al.*, 1980). The suprapyramidal blade is clearly distinguished by the second post-natal day while the infrapyramidal blade is not recognizable until near the end of the first postnatal week (Cowan *et al.*, 1980). Within each blade too, a gradient exists in that new granule cells are added to the inner aspect of the GCL such that those closer to the molecular layer are older than those located closer to the hilus.

1.4 Evidence for Adult Neurogenesis and Functional Incorporation of New Granule Cells In the Dentate Gyrus

1.4.1 Proliferation in the Dentate Gyrus

Neurogenesis in the adult rat has been found to occur in only two regions - the olfactory bulb and the dentate gyrus. Those olfactory neurons produced in adulthood arise from a progenitor population located in the subependyma of the rostral lateral ventricle which migrate and differentiate along a pathway to the olfactory bulbs (Louis & Alvarez-Buylla, 1994). The subependymal progenitor cells are derived from a population of multipotent stem cells also located there (Weiss *et al.*, 1996). Together, these cells in the subependyma compose the largest population of proliferating cells in the adult rodent brain. Unlike the newly generated olfactory neurons, dentate gyrus granule cells are generated from a population of precursor cells located within the adult dentate gyrus itself.

A number of lines of evidence indicate that the production of new granule cells in the rodent dentate gyrus continues into adulthood and possibly for the entire lifespan of the animal. Adult rats (age 60 - 63 days) rats injected with radiolabelled thymidine (^{3}H-Thymidine) were found to have cells at the base of the granule cell layer proliferating, with their offspring surviving for at least 200 days (Bayer, 1982). In that same study, Bayer found a linear increase in the total number of granule cells from 30 to 200 days of age of up to 43%. A

simultaneous increase in cell density and a decrease in cell volume was also noted, suggesting that there might not be a substantial turnover of cells but instead granule cells are being packed into the GCL tightly as more are produced, adding to the total number and not merely replacing those which have died. Crespo *et al.* (1986) have shown that new granule cells are added to the inner aspect of the GCL between 40 and 450 days of age, just as in pre- and early post-natal development of the GCL. Recent experiments examining proliferation in the DG with advancing age using the thymidine analog 5-bromo-2'-deoxyuridine (BrdU), suggests that neurogenesis in the dentate continues for over 2 years with a significant decline in the rate of neurogenesis occurring between 12 and 27 months old (Kuhn *et al.*, 1996).

1.4.2 Identity of New Cells in the Dentate Gyrus

A number of attempts have been made to firmly establish the granule cell identity of cells in the dentate labelled with the mitotic indicators ^{3}H-Thymidine or BrdU. Kaplan and Hinds (1977) found cells in the dentate gyrus of 3 month old rats labelled with ^{3}H-Thymidine 30 days earlier and examined with electron microscopy, had morphology typical of dentate granule cells and not glia. Round cell bodies with large round nuclei (10 μm diameter) were found, as well as dendritic processes extending parallel to that of surrounding granule cells. Synapses on the cell body and proximal processes of ^{3}H-Thymidine labelled

cells 20 days after injection have also been demonstrated (Kaplan & Bell, 1984). Double labelling of ^{3}H-Thymidine labelled cells after injection of retrograde tracer Fast Blue or Diamidino Yellow dihydrochloride into the mossy fiber pathway has shown that these cells extend projections as dentate granule cells normally do (Stanfield & Trice, 1988).

More recent studies have examined mitotically labelled cells for the expression of cell specific markers. Cameron *et al.* (1993) found that ^{3}H-Thymidine cells begin to express the neuron-specific marker Neuron Specific Enolase (NSE) by 2 weeks post-injection, and that by 3 weeks over 80% of cells labelled with ^{3}H-Thymidine were also NSE positive. A small percentage of ^{3}H-Thymidine labelled cells were also found to be positive for the glial marker Glial Fibrillary Acidic Protein (GFAP) and had the morphology of radial glia. Kuhn *et al.* (1996) have found that mitotically labelled cells in the SGZ of the dentate gyrus also express the neuronal marker Neuronal Nucleus (NeuN) as well as calbindin-D_{28k}, which is found in mature dentate granule cells (Sloviter, 1989). Newly generated cells within the SGZ therefore not only appear to show neuronal morphology, but also express proteins considered indicative of neuronal cell types. Whether or not the newly generated neurons and glia arise from a single multipotent precursor or from separate precursor cell lineages is not known.

1.4.3 Radial Glia and Neuronal Migration

Radial glia are thought to serve as substrates for neuronal migration during development (Rakic, 1981). Migrating neurons in the bird have been found to be associated with the processes of radial glia, which are thought to guide the young neurons or neuronal precursors to their final destination (Alvarez-Buylla & Nottebohm, 1988). When brain development ceases, the majority of radial glia are believed to change into mature astrocytes (Pixley & de Vellis, 1984). The cell bodies of radial glia are found in the dentate and are primarily located within the SGZ (Schmidt-Kastner & Szymas, 1990; Cameron *et al.*, 1993). The persistence of radial glia in the adult dentate gyrus - especially within the SGZ - may indicate the migration of newly generated granule cells into the GCL along radial glial substrates.

The migration of newly born granule neurons from the SGZ into the GCL proper, if it does occur, would seem to occur very slowly. Newly generated granule cells which have been previously pulsed with an injection ^{3}H-Thymidine at post-natal day 10, appear to only progress about half-way through the width of the GCL (about 4 or 5 cell widths) by post-natal day 450 (Crespo *et al.*, 1986). Crespo *et al.* suggest instead that new-born granule neurons are deposited along the inner aspect of the GCL, increasing the width of the GCL in this way over time as new neurons are added behind those already formed.

The highly polysialylated form of Neural Cell Adhesion Molecule (NCAM-H) has been shown to be transiently expressed by cells with features characteristic of granule neurons in the SGZ labelled with a thymidine analog (Seki & Arai, 1993). NCAM-H expression in neurons is mainly confined to the developmental period (Sunshine *et al.*, 1987), and is thought to be involved in neuronal migration (Heckmat *et al.*, 1990), neurite extension (Doherty *et al.*, 1990), axon pathfinding (Tang *et al.*, 1992) and synaptic remodelling (Jørgensen, 1995). NCAM-H might therefore be involved in the development of young granule neurons as they migrate and/or extend processes forming synaptic connections (Seki & Arai, 1996).

1.5 Granule Cell Neurogenesis in Other Species

The persistence of neurogenesis in the dentate gyrus into adulthood with the continual addition of new granule cells without any significant turnover implies that they may play a unique and important role in the functioning of the dentate gyrus. Adult granule cell neurogenesis has been reported in a number of rodent species including rats, mice, guinea pigs (Altman & Das, 1967), meadow voles (Galea & McEwen, 1995), and more recently in the non-rodent insectivore tree shrew (Gould *et al.*, 1997). Studies of rhesus monkeys suggest that granule cell production tapers off in juveniles and does not occur in adulthood (Rakic, 1985; Eckenhoff and Rakic, 1988). On the other hand, some limited observations of

adult neurogenesis in the DG have been reported in the primate species marmoset and cynomolgus monkey (Gould *et al.*, 1997). Although the existence of dentate granule cell proliferation in humans is unknown, the presence of adult granule cell neurogenesis in a number of species including some primates, plus evidence that neuronal precursors may exist in the adult human subependymal layer (Kirschenbaum *et al.*, 1994) suggests that the possibility of granule cell neurogenesis in humans should be examined more carefully.

1.6 Modulation of Granule Cell Proliferation in the Adult Rat

A number of factors have been found which modulate the production and/or survival of granule cells in the dentate gyrus. With advancing age, a selective decrease in the production of granule cells has been found to occur through the decreased proliferative activity of granule cell precursors (Kuhn *et al.*, 1996). This reduction in proliferation was not found to be generalized to other proliferating cell types in other regions of the brain (e.g. wall of the lateral ventricle). The age-related reduction in proliferation was therefore thought to be specific to dentate granule precursors. Recent evidence though suggests that the production of olfactory sensory neurons from the olfactory epithelium may also show an age-related decrease (Weiler & Farbman, 1997).

1.6.1 Steroid Hormones

Levels of adrenal steroid hormones are believed to play a role in the regulation of proliferation of dentate granule cells (Gould & Cameron, 1996). During the development of the dentate gyrus, levels of adrenal steroids have been found to be negatively correlated with the rate of granule cell production (Sapolsky & Meaney, 1986). Experimental manipulation of adrenal steroid levels in young and adult rats has been found to dramatically influence both granule cell birth and death. Injections of corticosterone or aldosterone in intact young and adult rats reduces the number of proliferating cells in the dentate, while adrenalectomy has been found to increase both neurogenesis and cell death simultaneously within the dentate gyrus. (Gould *et al.*, 1991; Cameron & Gould, 1994). Massive cell death has also been found to occur primarily in the outer (closer to molecular layer) regions of the GCL and not other areas of the hippocampus with the removal of the adrenal glands, as evidenced by the number of pyknotic cells and silver impregnation (Gould *et al.*, 1990; Sloviter *et al.*, 1993). Replacement of corticosterone to adrenalectomized rats does not alter the increased rate of neurogenesis associated with adrenalectomy, but does appear to protect cells from adrenalectomy-induced cell death (Gould *et al.*, 1990; Cameron & Gould, 1994). Therefore, adrenalectomy increases both cell genesis and cell death in the GCL, but it is not the newly generated cells which are dying. Older granule neurons which are generated in early post-natal development are primarily responsible for the increase in cell death in the GCL

while adult generated granule neurons do not die, but in fact are produced in greater numbers (Cameron & Gould, 1996). Adrenal steroids therefore appear to regulate the birth and death of different populations granule cells in the dentate gyrus which might be differentiated on the basis of age.

1.6.2 Glutamate and Afferent Input

Dentate granule cells receive their primary input from glutamatergic synapses by perforant path axons from the EC. Glutamate NMDA receptor activation has been shown to regulate the production of dentate granule cells in the adult. Systemic treatments with the NMDA receptor antagonists MK 801 or CGP 37849 increase the production of granule cells and the overall density of neurons in the GCL (Cameron *et al.*, 1995). Removal of the afferent input to the dentate by lesions of the EC also results in an increase of granule cell production. In contrast, activation of NMDA receptors with the agonist NMDA results in a decrease in granule cell production (Cameron *et al.*, 1995). The influence of NMDA receptors and removal of excitatory input to the dentate gyrus suggest that afferent input may normally regulate the production of new granule cells, but in a negative fashion with increased afferent input causing a reduction of granule cell genesis.

1.6.3 Environment

The production of granule cells in the adult rat has recently been shown to be sensitive to environmental factors. Mice raised in an enriched environment were found to have a greater number of new granule cells produced as evidenced by an increase in BrdU labelling of cells with granule cell morphology, compared to control animals in standard housing (Kempermann *et al.*, 1997). Adult tree shrews exposed to psychosocial stress were found to show a decrease in the number of BrdU labelled granule neurons, while those systemically injected with the NMDA antagonist MK 801 showed an increase similar to that found in rat (Gould *et al.*, 1997). Even the odor of a predator has been found to suppress granule cell production in the rat (Galea *et al.*, 1996).

The affects of both adrenal steroids and excitatory input as well as those of indirect external environmental influences on the production and death of dentate granule cells serve to highlight the potential importance of newly produced granule cells to the functioning and plasticity of the dentate gyrus. The sensitivity of granule cell production to alterations in afferent input and environmental influences also raises the question of how dentate granule cell production is affected in pathological states as well as the role it might play. Forty percent of human seizure disorders are associated with the temporal lobe, especially the hippocampus (Bradford, 1995). The animal models of epilepsy

can be used to examine alterations in the production of granule cells in response to seizure activity.

1.7 The Kindling Model of Epileptogenesis

Since 1967 (Goddard, 1967), repeated brain stimulation by either electrical or chemical means has been recognized to lead to seizure activity. The term "kindling" refers to the way seizure activity can result from low-level stimulation just as small burning twigs can result in a large fire. The kindling phenomenon can be produced via electrical stimulation through surgically implanted electrodes (Racine, 1972a,b), or by pharmacological means with the application (e.g. systemic or intra-cranial injection) of agents such as bicuculline, lidocaine or pilocarpine, which result in increased glutamatergic neuronal transmission (Uemura & Kimura, 1988; Post *et al.*, 1984; Parent *et al.*, 1997). The kindling technique produces a long-lasting reduction in seizure thresholds and the development of kindled seizure behaviour is dependent upon the presence of after-discharges (Racine, 1972a,b). The rapidity of kindling will vary depending on the brain area stimulated, with the amygdala being the most commonly used due to the speed and ease with which kindling can be accomplished there (Abel & McCandless, 1992). Both electrical and chemical kindling produce results which can mimic many clinical seizure syndromes and each has its advantages and disadvantages. Electrical kindling allows precise control over stimulation

parameters as well as the location from which afterdischarge will start, but may involve specialized equipment and animal surgery. Chemical kindling, although often less expensive and more straightforward, can be influenced by factors such as drug solubility, regional brain blood flow and properties of the blood-brain barrier (Abel & McCandless, 1992).

A number of changes in the hippocampus of rats have been found to be associated with seizures and appear similar to those found in human epilepsy, including cell death, gliosis and synaptic remodeling. Sprouting and reorganization of mossy fibres has been found to occur with seizure induction, whereby new aberrant mossy fibre synapses are formed in both CA3 and molecular layer (Sutula *et al.*, 1988; Represa & Ben-Ari, 1992; Parent *et al.*, 1997). Neuronal loss within CA3 and the hilus has also been found to occur with both electrically and chemically induced seizures (Cavazos & Sutula, 1990; Bertram & Lothman, 1993) and has been suggested as a possible mechanism for the initiation of mossy fibre sprouting (Cavazos & Sutula, 1990). The loss of mossy fibre target cells may initiate the sprouting of new mossy fibres in a compensatory mechanism. There is some evidence though that cell death might not be necessary for synaptic remodelling to occur during kindling, where sprouting has been found in the absence of identifiable loss of cells (Bertram & Lothman, 1992; Stringer *et al.*, 1997). In this case, excessive activity alone might be enough to cause sprouting.

Along with changes involving neuronal populations during artificially induced seizures, glial proliferation and activation has been found to occur. Proliferation of astrocytes has been demonstrated in the hilus and CA3 of kainic acid treated rats (Niquet *et al.*, 1994). Glial hypertrophy and activation, as well as alterations in the glial cytoskeleton has been found in kindled rats (Khurgel *et al.*, 1992). It has been suggested that glial cells may contribute to mossy fibre reorganization by acting as substrates for new axon growth, with Represa *et al.* (1994) reporting growth cone-like structures being frequently associated with astrocyte projections and the areas of contact being rich in NCAM expression in kainic acid treated rats.

1.8 Kindling and Granule Cell Neurogenesis

Since afferent input to the dentate gyrus has been shown to alter granule cell production, and the excessive neuronal activity associated with seizures has been found to be correlated with the sprouting of aberrant mossy fiber synapses, it is reasonable to ask whether the two phenomena could be related. Competition among neurons for target sites and afferent input is thought to be involved in brain development (Gordon, 1995). Young differentiating neurons in the dentate gyrus would be sprouting axons and dendrites in an environment of pathological activity which may influence their development. Parent *et al.* (1997)

have addressed the question of whether pilocarpine induced status epilepticus in rat can alter the production and development of new granule cells. Granule cell production, as evidenced by BrdU immunoreactivity, was found to be increased, and newly produced granule cells formed abnormal projections to the molecular layer and CA3. Electrical stimulation of the perforant path causing focal hippocampal seizures but no hilar or pyramidal neuron injury, was also found to produce an increase in mitotic activity within the SGZ of the dentate gyrus. Furthermore, newly generated cells with granule cell-like morphology were found in ectopic locations within the hilus and molecular layer and appeared similar to the abnormalities found in human temporal lobe epilepsy.

In the study by Parent *et al.* (1997), rats were subjected to status epilepticus, which involved 3 to 5 hours of continuous seizure activity after a single systemic injection of pilocarpine. There is some evidence that the changes in the hippocampus associated with seizure activity may differ between the chemically induced status epilepticus and the electrical kindling models, as Bertram & Lothman (1993) found hilar neuronal loss after chemical status epilepticus but not after electrical kindling. Traditional electrical kindling models may also more closely mimic the sporadic brief seizures of human epilepsy, than the single intense and prolonged seizures of chemically induced status epilepticus.

1.9 Hypothesis and Rationale

As described above, granule cell neurogenesis has been found to be responsive to a wide variety of internal and external influences. This suggests that the proper regulation of granule cell production might be very important to the normal functioning of the dentate gyrus and the animal as a whole. Excitatory afferent input to the dentate gyrus has been suggested to be a natural regulator of granule cell production (Cameron *et al.*, 1993), though the effects of seizures on granule cell production and development are not yet understood. The aim of my study was therefore to examine what effect the increased afferent activity associated with kindling evoked seizures might have on granule cell production. Since granule cell production is very sensitive to a number of factors, and afferent input is known to influence neural development in general, I hypothesize that granule cell production and thus the continuing development of the dentate gyrus in adulthood will be altered by the pathological state of kindled seizures.

In order to examine this hypothesis, I have used bromodeoxyuridine immunolabelling to identify cell production during kindled seizures. To determine the extent of afferent activity required for stimulation of granule cell production, I measured the density of BrdU+ cells at early and later stages of the kindling procedure. Since there is also some evidence of inherent regional differences in granule cell production (Kaplan & Hinds, 1977; Cameron *et al*, 1993) and reports of proliferation of other cell types in the dentate gyrus

(Cameron *et al.*, 1993), cell production in the hilus and molecular layer as well as the granule cell layer of dorsal, middle and ventral hippocampus were also examined.

Chapter 2

Methods

2.1 Animals and Surgery

Male Wistar rats (Charles River) were 38 or 39 days old at the time of surgery. Surgeries were performed by Antonio Mendonca. Animals were anesthetized with sodium pentobarbitol (54 mg/Kg) and placed in a stereotaxic frame. The surface of the skull was exposed and four holes were drilled, through which a wire frame would be anchored; two just anterior to each frontal suture and medial to the lateral ridge, and two anterior to each lambdoidal suture and medial to the lateral ridge. Four more holes were drilled on the sides of the skull just below the lateral ridge, with each one corresponding to a hole on the skull surface. Stainless-steel wire (0.005" diameter, Teflon coated) was threaded through each of the paired holes and fashioned into a wire-frame anchor for cement. Another hole was drilled over the proposed electrode site with respect to interaural zero: +4.9 mm antero-posterior, -4.8 mm medio-lateral (Sherwood & Timiras, 1970). A stainless-steel twisted bipolar stimulating electrode (0.35 mm diameter wires, Teflon coated; Plastics-One #M5303-1) was lowered until the dura mater was touched. Dura was then pierced with a needle and the electrode lowered to -7.0 mm from the surface, aimed at the left Nucleus lateralis, pars posterior (ALP) of the amygdala. The electrode was then cemented in place with

Cranioplastic Cement (Plastics-One) over the wire-frame and the animal was allowed to recover for 7 days.

2.2 Kindling Stimulations

Animals were stimulated once per day with a 400 μA peak-to-peak biphasic current pulse at 60 Hz starting at 46 days of age until they were killed. The positive and negative components of the stimulating pulse were 1 ms in duration and separated by 0.5 ms. Electrical activity within the amygdala before and after stimulation was recorded through the stimulating electrode. The electrode of each animal was checked for proper functioning and only animals which displayed an afterdischarge were used in this study. Control animals underwent surgery but did not receive any subsequent kindling stimulations.

Motor seizures were quantified using a scale developed by Racine (1972b) and consisted of 5 stages of increasing severity: 1) mouth and facial movements (chewing-like and facial twitches), 2) head nodding, 3) forelimb clonus (jerky movements), 4) rearing up on hindlimbs, 5) rearing on hindlimbs followed by a loss of postural control and falling over. Higher stages of seizure may include some characteristics of lower stages, for example stage 4 rearing may also include forelimb clonus. Figure 2.1 shows rats at various stages of seizure within the stimulation arena, while Figure 2.2 shows samples of EEG traces of afterdischarge following kindling stimulation in one animal. Waveform

characteristics do not correlate well with behavioural seizure level (M. Burnham, personal communication). Animals were not monitored for the presence of spontaneous seizures between stimulation sessions.

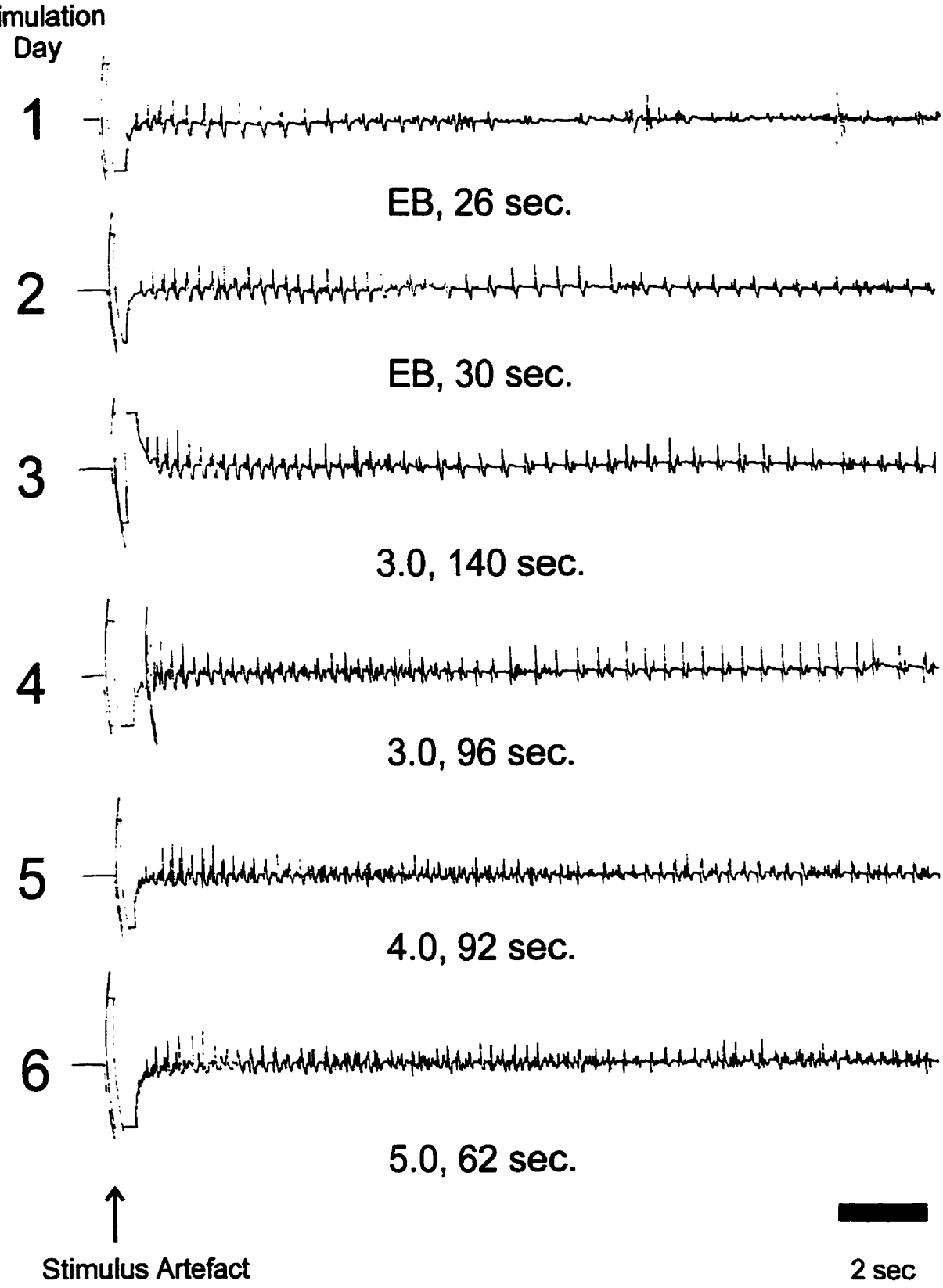
Figure 2.1: Kindled Seizures

Examples of rats experiencing various levels of kindled seizure within the stimulation arena (upper photo = stage 3, lower photo = stage 4). Stimulating electrodes are implanted in the amygdala and afterdischarges from the amygdala are recorded. (rats.cdr)

Figure 2.1

Figure 2.2: Afterdischarge in Amygdala

EEG traces of afterdischarge in the amygdala after a kindling stimulation in one animal over 6 days of stimulation. The maximum stage of behavioural seizure is shown below each trace, with the total duration of afterdischarge that session. EEG characteristics are not strongly correlated with stage of behavioural seizure. Population spikes occur at regular intervals with lower stages of seizure but EEG waveform becomes more complex as seizure level increases. EB, eye blink. (eeg.cdr)


Stimulation Day
1
EB, 26 sec.
2
EB, 30 sec.
3
3.0, 140 sec.
4
3.0, 96 sec.
5
4.0, 92 sec.
6
5.0, 62 sec.
Stimulus Artefact
2 sec

2.3 5-bromo-2'-deoxyuridine Injections

The thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) is incorporated into DNA during the DNA replication phase of the cell cycle. Single-stranded DNA containing BrdU can be immunohistochemically detected, thus indicating the progeny of cells which were undergoing mitosis at the time of BrdU exposure (Miller & Nowakowski, 1988).

Stimulated and control animals were placed into Early or Late groups which indicated when BrdU injections would be given (Figure 2.3). On the second day of kindling stimulations, BrdU injections were started with the Early group animals, while injections for the Late group animals began the day after the animal demonstrated its first stage 5 seizure, with a corresponding control animal injected at the same time in both cases. BrdU (100 mg/Kg in 0.9% saline and 0.01 N NaOH) was injected (i.p.) 5 to 10 minutes before stimulation once per day for three days. Animals were then killed 7 days after the last BrdU injection and the brain processed for BrdU immunolabelling.

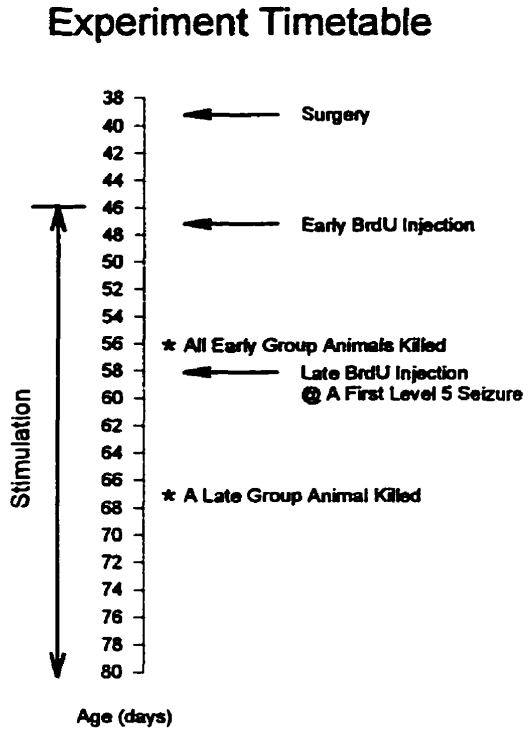


Figure 2.3: Experimental Timetable

Timetable showing the ages at which surgery and BrdU injections were performed, as well as when kindling stimulations began. Early BrdU group injections began one week after surgery while Late BrdU group injections began one day after the animal experienced his first level 5 seizure. An example animal is indicated with an *. All animals were killed one week after the third and final BrdU injection. (time.spw)

2.4 Tissue Processing and BrdU Immunolabelling

Animals were anesthetized with halothane and then decapitated. Brains were removed and the hippocampus ipsilateral to the electrode was dissected out and immersed in modified Carnoy's Fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid) overnight, while the contralateral hippocampus of some animals was used in a parallel study involving whole-cell patch clamp recording of granule cells in living tissue. After fixation, hippocampi were then transferred to phosphate buffered saline (PBS; 8.5 mM Na_2HPO_4, 1.9 mM $NaH_2PO_4 \cdot H_2O$, 137 mM NaCl, 2.7 mM KCl) overnight. Each hippocampus was cut into dorsal, middle and ventral thirds and vibratome sectioned at 15 μm in PBS at 4°C.

Six random sections from each hippocampal third were treated with 1 N HCl at 55°C for 30 minutes to denature the DNA and then washed for 15 minutes in room temperature PBS. Free-floating sections were then incubated overnight with a mouse monoclonal anti-BrdU primary antibody (Becton-Dickenson, #347580) at 1:100 dilution in PBS and 0.3% Triton-X-100 at 4°C. Sections were then washed in room temperature PBS for 15 minutes followed by incubation with a sheep biotinylated-anti-mouse secondary antibody (Amersham, #RPN1001) at 1:200 dilution in PBS and 0.3% Triton-X-100 for 2 hours at 37°C. Sections were then washed for 15 minutes in room temperature PBS followed by incubation with FITC conjugated streptavidin (Amersham, #RPN1233) at 1:100 dilution in PBS for 1 hour at 37°C. Finally, sections were washed in PBS for 10

minutes three times and then mounted on slides with PermaFluor Aqueous Mounting Medium (Immunon) under coverslip.

Methylene Blue staining was performed on representative sections from dorsal, middle and ventral hippocampus of control animals (See Figure 1.2). Sections were incubated for 5 minutes in a solution of Methylene Blue N (2 mg/ml H_2O; Allied Chemicals), followed by washing in PBS for 5 minutes. Sections were then temporarily wet-mounted in PBS under coverslip and photographed.

2.5 Cell Counting

Sections were examined with a Nikon Optiphot-2 fluorescence microscope using a 20X objective and a Nikon B-2A filter cube (excitation 450-490 nm, dichroic 510 nm, emission 520 nm). Positively labelled nuclear profiles were counted separately in the molecular layer, granule cell layer and the hilus. The subgranular zone was considered part of the granule cell layer during cell counting. Labelled nuclei less than approximately 5 μm in diameter were not included in the counts. Some cell counts were confirmed by an independent observer (F. Dorri.).

2.6 Area Measurements

Digital images of hippocampal sections were obtained with a CCD camera system (Cohu 4915) attached to a Nikon Optiphot-2 fluorescence microscope, and connected to an IBM compatible computer with a Image Lightening Digidata 2000 image acquisition processor card (Axon Instruments Inc.), and running Axon Image Workbench version 1.0.5 for Windows® (Axon Instruments Inc.). The cross-sectional areas of counted regions of the dentate gyrus were measured using *ImageTool* for Windows® version 1.25 (developed at the University of Texas Health Science Center at San Antonio, Texas and available from the Internet by anonymous FTP from ftp://maxrad6.uthscsa.edu) running on an IBM compatible computer. Area measurements were combined with absolute cell counts to obtain cell density estimates for each hippocampal section.

2.7 Nuclear Area and Diameter Measurements

Images of BrdU positive nuclei were scanned under fluorescence illumination with a 40X objective, using the equipment described above for cross-sectional area measurements. The maximum diameter and areas of BrdU labelled nuclei in the GCL of late BrdU injected and control animals were measured with *ImageTool* for Windows® version 1.25. Nuclear profiles ranging from 3 μm to 15 μm in diameter were included in the analysis.

2.8 Single Cell Lucifer Yellow Injections

Whole-cell patch clamp recording and filling of cells with Lucifer Yellow was performed by Sabrina Wang (See Figures 1.3 & 4.1). Animals ranging in age from 40 to 60 days were killed by decapitation under Halothane anesthesia and brains were removed with hippocampi dissected out. Live hippocampal slices (400 µm thick) were incubated in an oxygenated chamber in artificial CSF. Whole-cell patch clamp recordings were made from granule cells located on the inner (hilar) and outer (molecular) edges of the GCL. Cells were simultaneously filled with Lucifer Yellow during recording and slices subsequently fixed in formaldehyde overnight. Slices were then dehydrated in graded ethanol solutions and cleared in methyl salicylate and mounted on slides under coversiip. Lucifer Yellow filled cells were reconstructed using a confocal microscope and artificial colour added using the CFOCAL software package.

Chapter 3

Results

3.1 Stimulation and Seizures

Four of seven animals in the Early BrdU injection group started experiencing stage 5 seizures before they were killed, with one animal reaching stage 5 seizure on the last day of BrdU injections (day 4 of stimulations) while the average number of stimulations before first stage 5 seizure was 7.5±1.5 (mean±SE). The average number of stimulations before first stage 5 seizure for Late injection animals was 9.8±1.4. This difference between the Early and Late groups was due to two animals in the Late injection group reaching stage 5 seizure later than the rest and is not statistically significant (t-test; $p<0.05$). The average number of stage 5 seizures experienced by Early BrdU injection animals before they were killed was 2.33±1.1. The average number of stage 5 seizures experienced by Late BrdU injection animals before they were killed was 8.3±0.8. Thus the Late BrdU group experienced more stimulations as well as more stage 5 seizures than the Early BrdU group.

3.2 BrdU Labelling Control Animals

Figure 3.1 shows examples of BrdU+ labelling within the dentate gyrus of control animals in dorsal, middle and ventral hippocampus. In control animals, BrdU+ nuclei in the DG were predominantly (~60%) found within the GCL with almost all there located at the inner edge of the GCL bordering the hilus. BrdU+ nuclei on the inner edge of the GCL displayed a size and shape typical of granule cells (Figure 3.2). The large (~8 - 12 μm) round nucleus of dentate granule cells occupies all but a thin ring of cytoplasm within the cell, making the nuclear diameter comparable to that of the entire cell diameter. BrdU+ nuclei located within the hilus (~30%) did not appear evenly distributed throughout the hilus but were instead mostly located directly beneath the GCL and appeared similar in size and shape to those within the GCL. Nuclei within the molecular layer were very often smaller than those in the hilus or GCL, and irregularly shaped or elongated (Figure 3.3). BrdU+ nuclei within the molecular layer were occasionally found to be associated with blood vessels while this was not seen within the hilus or the GCL. BrdU+ nuclei were sometimes found in closely associated pairs in all three regions of the dentate gyrus, which may indicate recent cell division (Figure 3.4).

Figure 3.1: BrdU Labelling in Dentate Gyrus of Control Animals

Transverse slices of dentate gyrus immunolabelled for BrdU from control animals. Arrow heads show examples of BrdU+ nuclei (green dots). A. Dorsal hippocampus. B. Middle hippocampus. C. Ventral hippocampus. g, granule cell layer; m, molecular layer; h, hilus. Scale bar applies to all three photos. (ctlbrdu.cdr)

Figure 3.2: BrdU+ Nuclei From Control Animals

A. BrdU+ nuclei on the inner edge of the granule cell layer. Arrow points to cell shown in B. B. A single BrdU+ nucleus shown under fluorescent light (left) and both fluorescent and white light (right), displaying the large round shape typical of dentate granule cells. g, granule cell layer; m, molecular layer; h, hilus. (ctlclose.cdr)

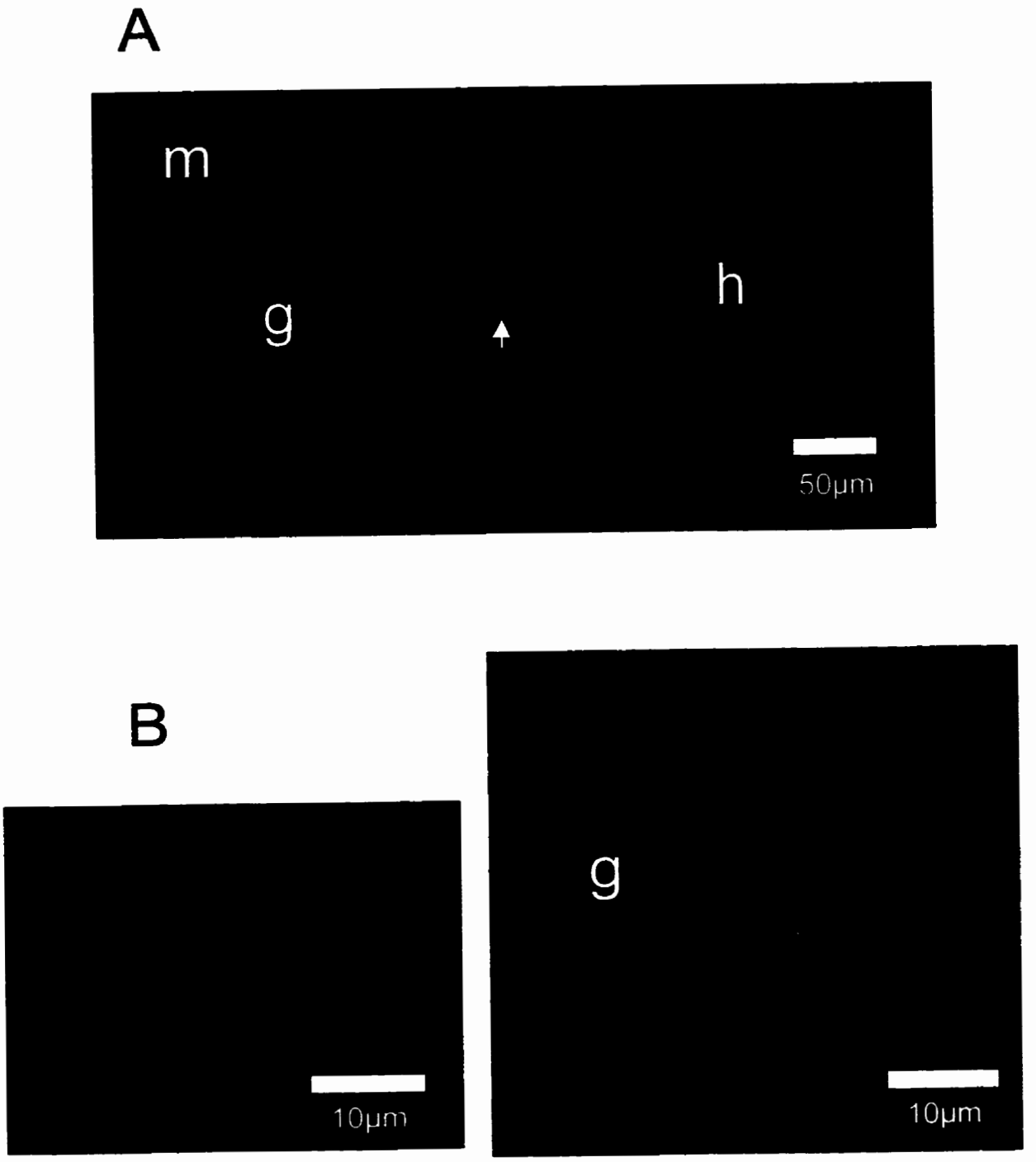
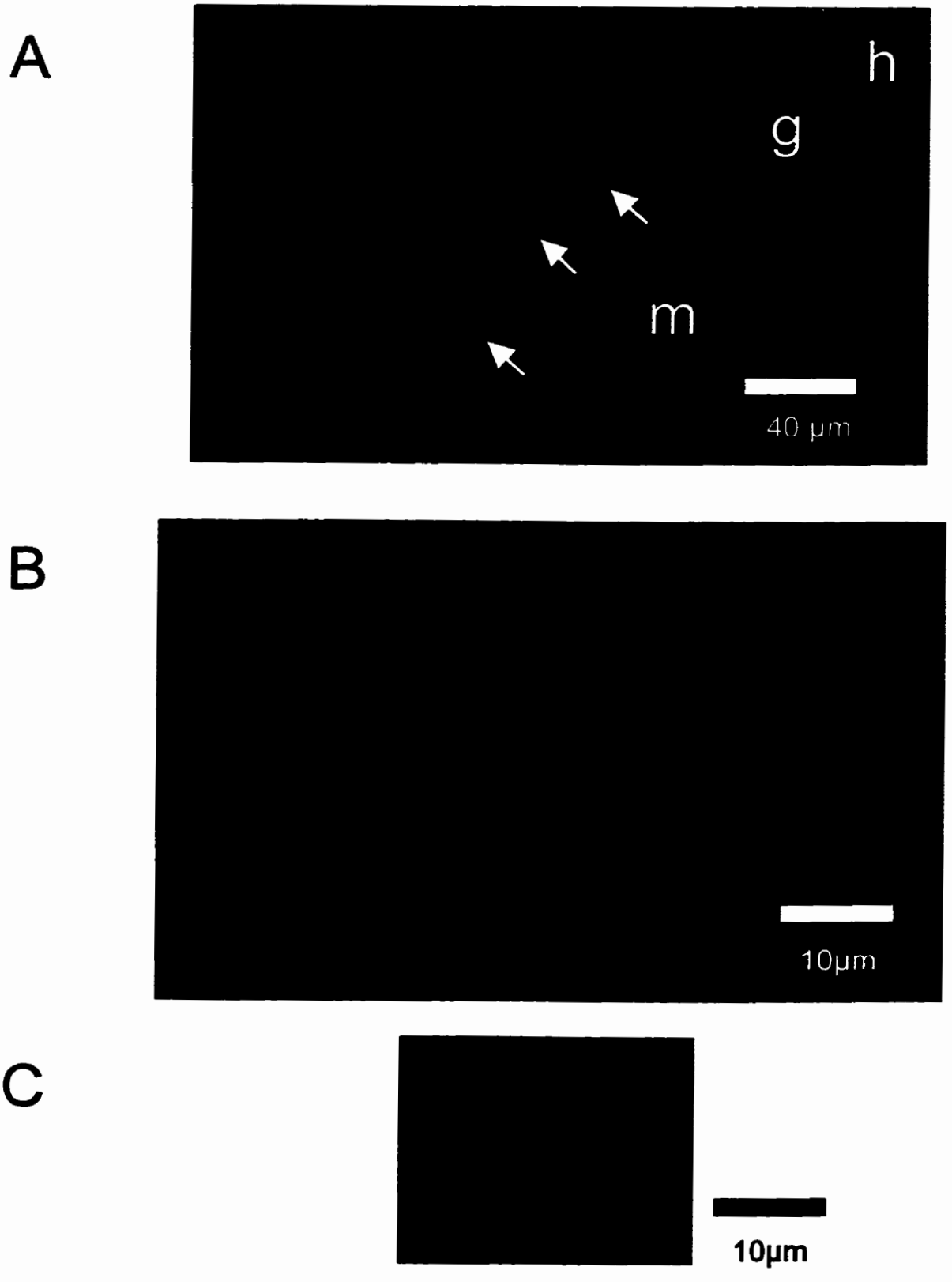

A
m
g
h
50µm
B
10µm
g
10µm

Figure 3.3: BrdU Labelling In Molecular Layer

Examples of irregular and elongated shaped nuclei in the molecular layer of control animals. A. BrdU+ labelling associated with a blood vessel. B. Higher magnification of two BrdU+ nuclei associated with a blood vessel. C. High magnification of an elongated BrdU+ nucleus. g, granule cell layer; h, hilus; m, molecular layer. (vessel.cdr)


A
h
g
m
40 µm
B
10µm
C
10µm

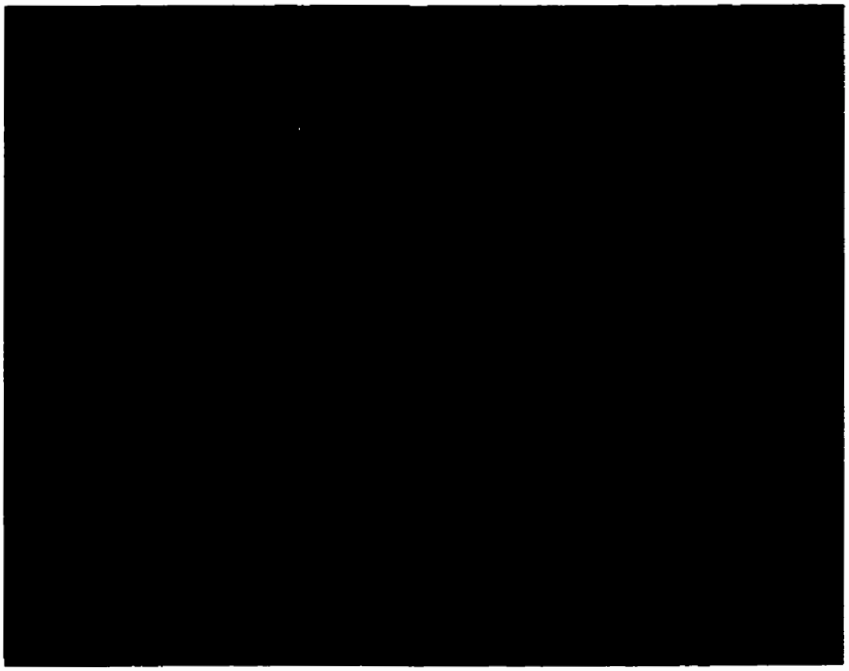


Figure 3.4: Paired Nuclei in Hilus

Example of BrdU+ nuclei found in closely associated pairs. This may be evidence of recent cell division as cells have not had a chance to migrate apart. (pair.cdr)

3.3 Regional Differences

BrdU+ densities in Early and Late injected control animals did not differ significantly from each other and are therefore shown combined in Figure 3.5. The GCL of dorsal and middle hippocampus were found to have BrdU+ nuclei at a density of approximately 80/mm^2 while ventral hippocampus had a significantly lower density (46±5.4/mm^2; 1-way ANOVA, $p<0.05$). No difference between the regions of the hippocampus was found for BrdU+ densities in hilus or molecular layer. Densities in this study are generally similar to those of a previous report using BrdU (Kuhn *et al.*, 1996) although density differences can be expected due to differences in methodology such as number BrdU injections given, time delay between injections and tissue processing as well as thickness of tissue slices.

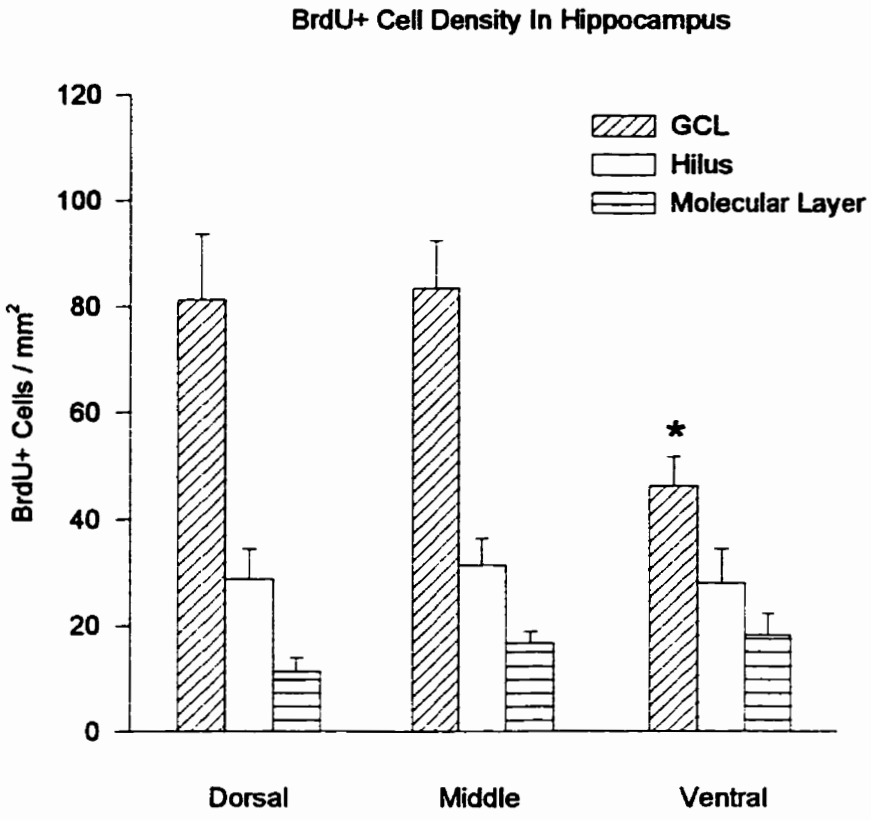


Figure 3.5: BrdU+ Cell Densities In Control Animals

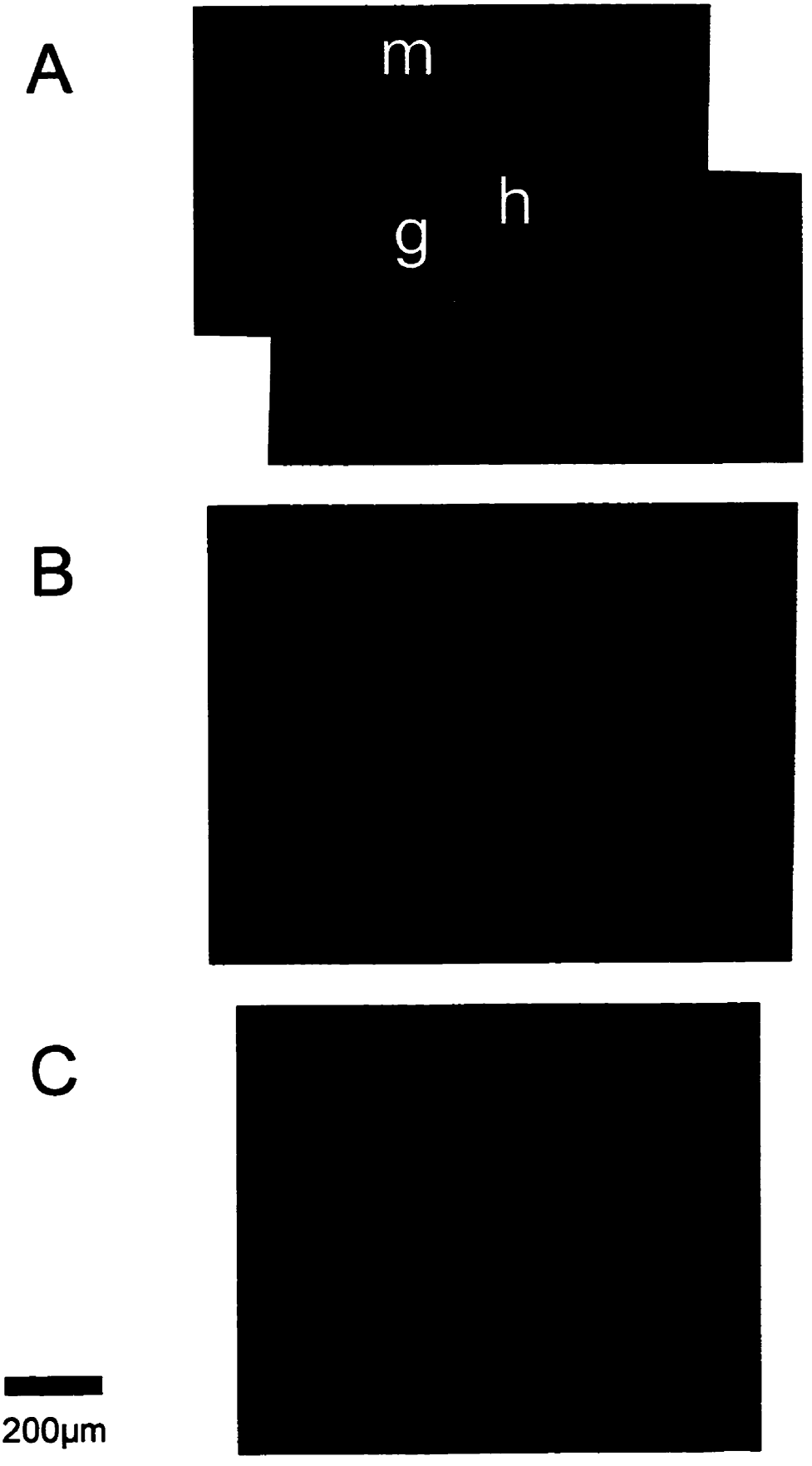
BrdU+ densities in granule cell layer (GCL), hilus and molecular layer of dorsal, middle and ventral dentate gyrus of Early and Late BrdU injected control animals combined. BrdU+ density of ventral GCL differed significantly (*) from dorsal and middle regions (1-way ANOVA, $F=4.88$, $p=0.0128$, $\alpha=0.05$; Student-Newman-Keuls all pairwise multiple comparison procedure). BrdU+ densities in molecular layer did not differ between regions (1-way ANOVA, $F=1.35$, $p=0.2711$, $\alpha=0.05$). BrdU+ densities in hilus did not differ between regions (1-way ANOVA, $F=0.10$, $p=0.91$, $\alpha=0.05$). Data shown as Mean±SE. n=14 animals in each group. (contsum.spw)

3.4 BrdU Labelling in Stimulated Animals

BrdU+ labelling in the DG appeared greater in stimulated animals compared to controls (Figure 3.6). Individual labelled nuclei appeared similar to that of controls. Figure 3.7 shows examples of BrdU+ nuclei in the GCL of a Late BrdU injected animal, displaying the large round morphology typical of granule cells. Stimulated animals which received BrdU injections early during the kindling procedure were found to have BrdU+ densities which did not differ significantly from those of control animals, and yet were consistently lower in all regions of the dentate examined (Figures 3.8 to 3.10). In contrast, stimulated animals which received BrdU injections after experiencing a level 5 seizure had BrdU+ densities greater than controls in all regions of the dentate examined. This increase was significant within the GCL and molecular layer but not in the hilus (2-way ANOVA, $p<0.05$), and was found in all three regions of the hippocampus. Increases in BrdU+ density which occurred within the GCL ranged from 75 to 137.8 %, with ventral hippocampus showing the greatest, while increases in the molecular layer were generally larger except in ventral hippocampus (Table 3.1). The regional presentation of BrdU+ densities in GCL of Late BrdU injection animals was similar to controls in that the ventral hippocampus had the lowest density compared to dorsal and middle, but this difference was not significant (1-way ANOVA, $p<0.05$; Figure 3.11).

Figure 3.6: BrdU Labelling In Dentate Gyrus of Stimulated Animals

An increase in BrdU+ labelling in Late BrdU injected animals from dorsal (A), middle (B) and ventral (C) dentate gyrus. Notice what appears to be more prominent blood vessels compared to controls. g, granule cell layer; h, hilus; m, molecular layer. Scale bar applies to all photos. (stbrdu.cdr)


A
m
h
g
B
C
200µm

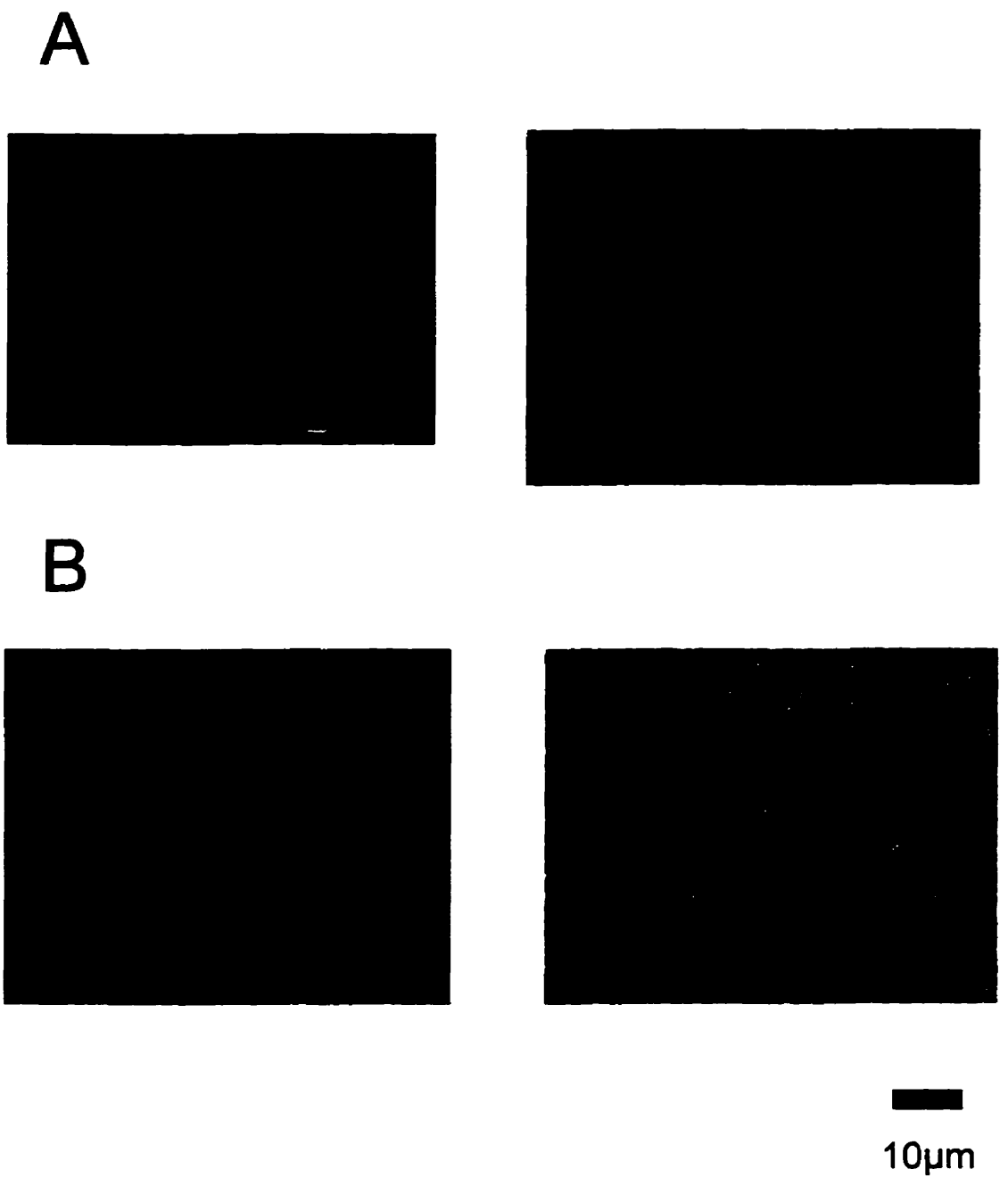


Figure 3.7: Higher Magnification of BrdU+ Nuclei from Stimulated Animals

A. Three BrdU+ nuclei on the inner edge of the granule cell layer under fluorescent illumination (left panel) and both fluorescent and white light (right panel). B. A single BrdU+ nucleus deeper within the granule cells layer. Notice the correspondence in shape and size to surrounding granule cells. Scale bar applies to all photos. (stclose.cdr)

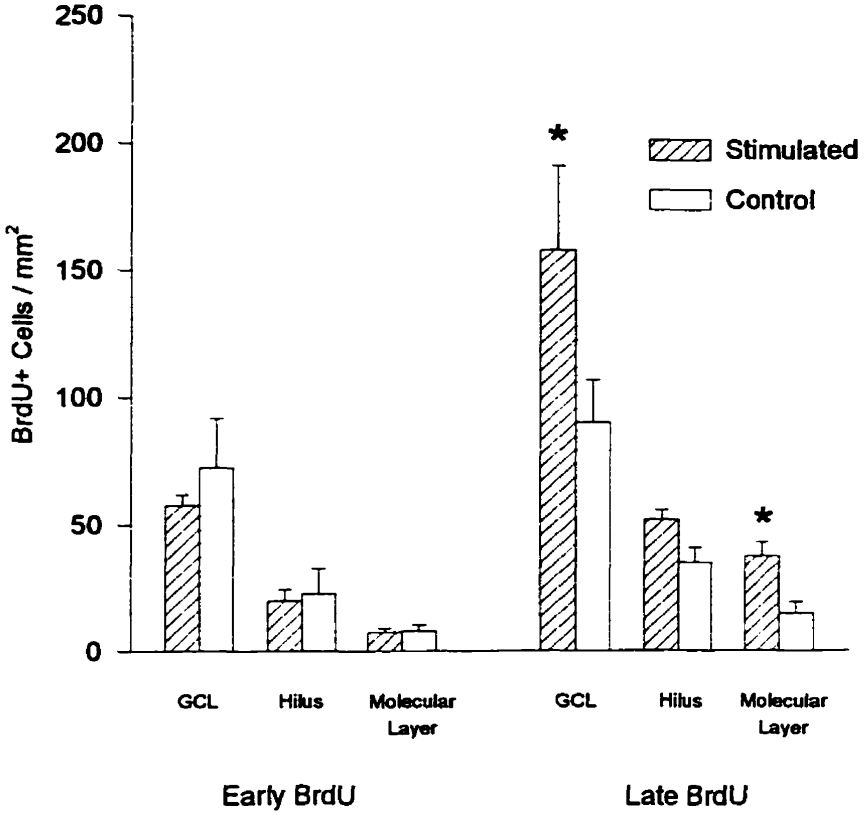


Figure 3.8: BrdU+ Densities in Dorsal Dentate Gyrus

BrdU+ densities of Early BrdU injected stimulated animals did not differ from controls, while densities of Late BrdU injected stimulated animals were significantly (*) different from controls in the granule cell layer ($F=4.34$, $p=0.0486$) and molecular layer ($F=9.12$, $p=0.0061$) but not the hilus ($F=2.3$, $p=0.1431$). 2-way ANOVAs were performed on each region with $\alpha=0.05$, using Student-Newman-Keuls all pairwise multiple comparison procedure. Data shown as mean±SE. n=7 animals in each group except Late BrdU stimulated, in which n=6 animals.

(d-sumall.spw)

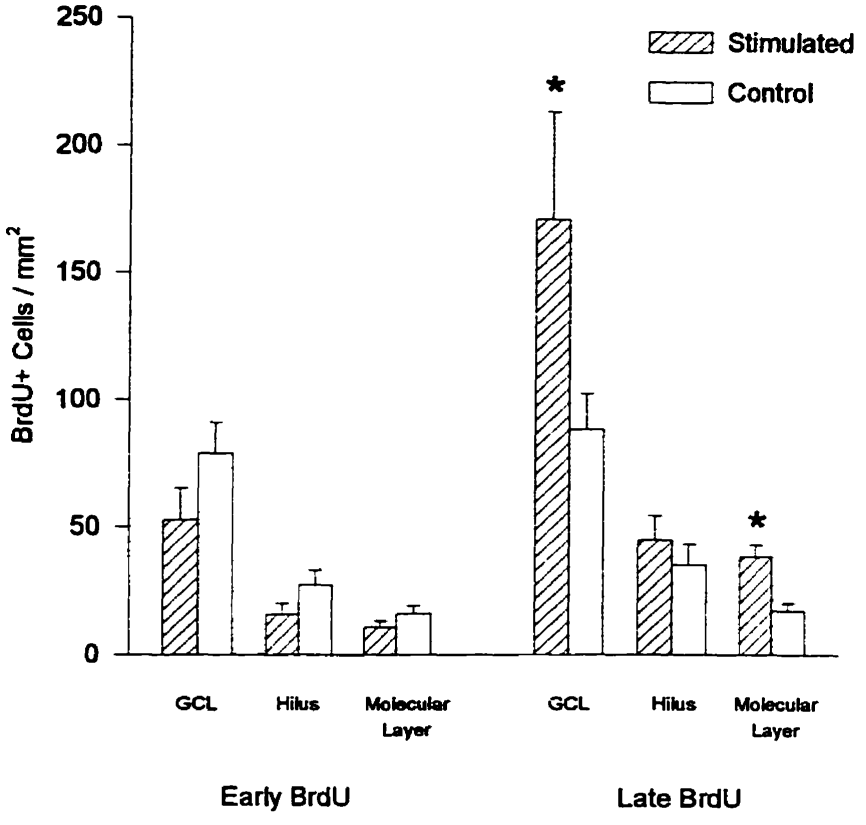


Figure 3.9: BrdU+ Densities in Middle Dentate Gyrus

BrdU+ densities of Early BrdU injected stimulated animals did not differ from controls, while densities of Late BrdU injected stimulated animals were significantly (*) different from controls in the granule cell layer ($F=6.20$, $p=0.0204$) and molecular layer ($F=14.88$, $p=0.0008$) but not the hilus ($F=2.29$, $p=0.1443$). 2-way ANOVAs were performed on each region with $\alpha=0.05$, using Student-Newman-Keuls all pairwise multiple comparison procedure. Data shown as mean±SE. n=7 animals in each group except Late BrdU stimulated, in which n=6 animals.
(m-sumall.spw)

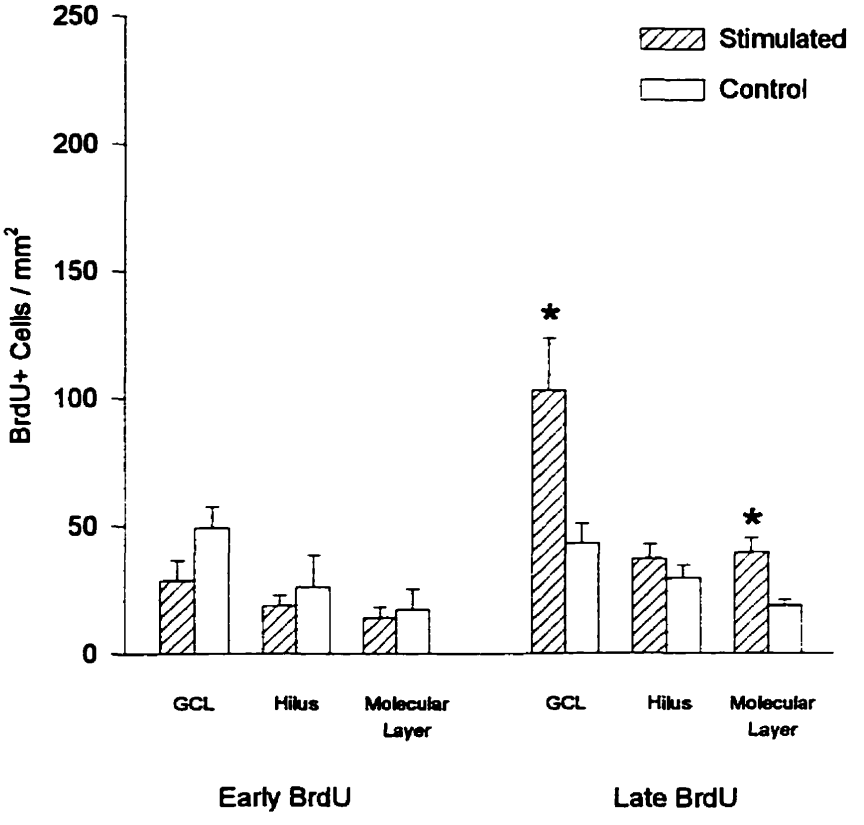


Figure 3.10: BrdU+ Densities in Ventral Dentate Gyrus

BrdU+ densities of Early BrdU injected stimulated animals did not differ from controls, while densities of Late BrdU injected stimulated animals were significantly (*) different from controls in the granule cell layer ($F=12.12$, $p=0.002$) and molecular layer ($F=4.47$, $p=0.0455$) but not the hilus ($F=0.963$, $p=0.337$). 2-way ANOVAs were performed on each region with $\alpha=0.05$, using Student-Newman-Keuls all pairwise multiple comparison procedure. BrdU+ densities were generally lower than in dorsal and middle regions.Data shown as mean±SE. n=7 animals in each group except Late BrdU stimulated, in which n=6 animals.
(v-sumall.spw)

Hippocampal Region	GCL (%Δ)	Hilus (%Δ)	Molecular Layer (%Δ)
Dorsal	75.3 *	49.2	153.1 *
Middle	94.2 *	27.2	129.1 *
Ventral	137.8 *	25.9	109.3 *

Table 3.1: Percent Change in BrdU+ Density in Late BrdU Group

Changes in BrdU+ density in dorsal, middle and ventral regions of the hippocampus expressed as % change relative to non-kindled controls. Significant changes are indicated with an * (from 2-way ANOVAs performed as in Figs. 3.8 to 3.10, $p<0.05$). n=7 in each group except Late BrdU stimulated, in which n=6. (table1.doc)

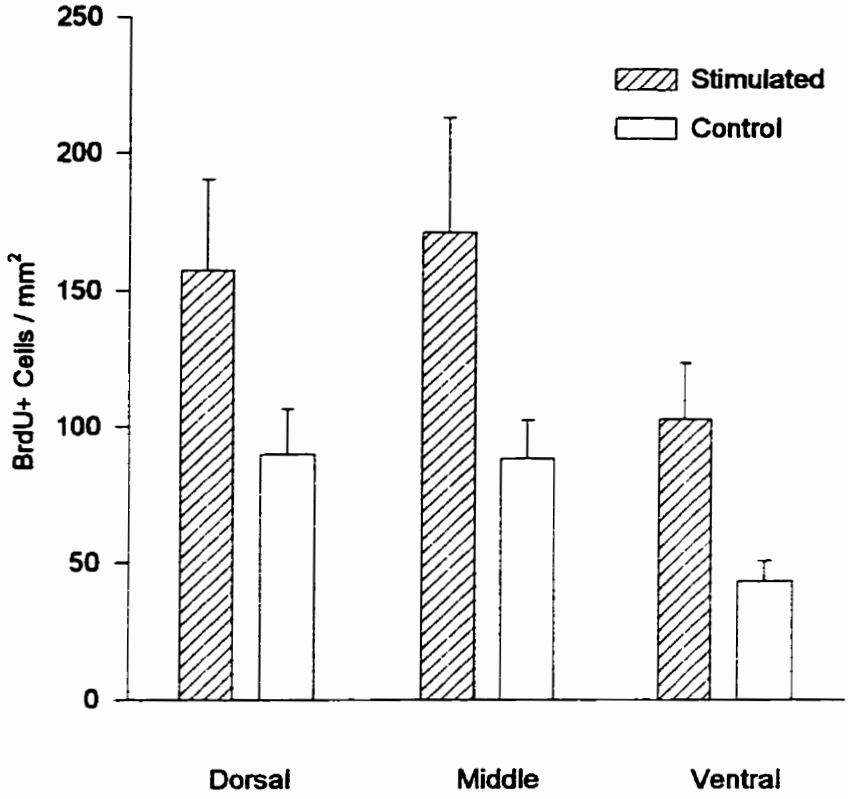


Figure 3.11: Regional Profile of BrdU+ Densities in Granule Cell Layer

Data from Figures 3.8 to 3.10 compiled for clarity. BrdU+ densities in the granule cell layer of Late BrdU injected stimulated animals were significantly greater (see Figs. 3.8 to 3.10, $P<0.05$) than controls in all three regions of the hippocampus. The controls were slightly lower in ventral hippocampus when compared to dorsal and middle hippocampus, but not significantly. Data shown as mean±SE. (gclp.spw)

Blood vessels within the molecular layer often appeared larger and more numerous in kindled animals compared to controls (compare Figures 3.1 and 3.6). The association of BrdU+ nuclei and blood vessels within the molecular layer was also noticeably increased in Late BrdU animals, as was the incidence of irregularly shaped and elongated nuclei.

No significant correlation (Pearson r) was found between BrdU+ densities and the total number of kindling stimulations an animal received or the total amount of time an animal experienced afterdischarges following stimulation (Figures 3.12 and 3.13).

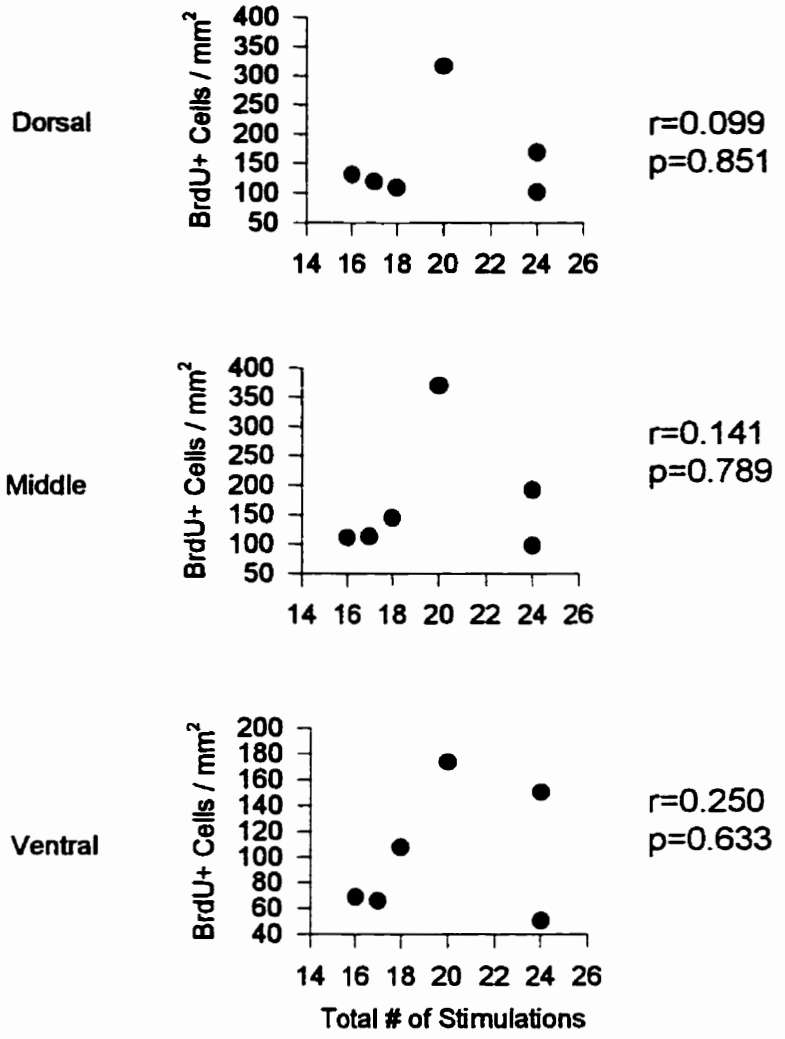


Figure 3.12: BrdU+ Densities Vs. Total # of Stimulations

No significant correlation (Pearson r and p value shown for each region, α=0.05) was found between the number of kindling stimulations and the density of BrdU+ labelling in any region of the hippocampus.

(g-stim1.spw)

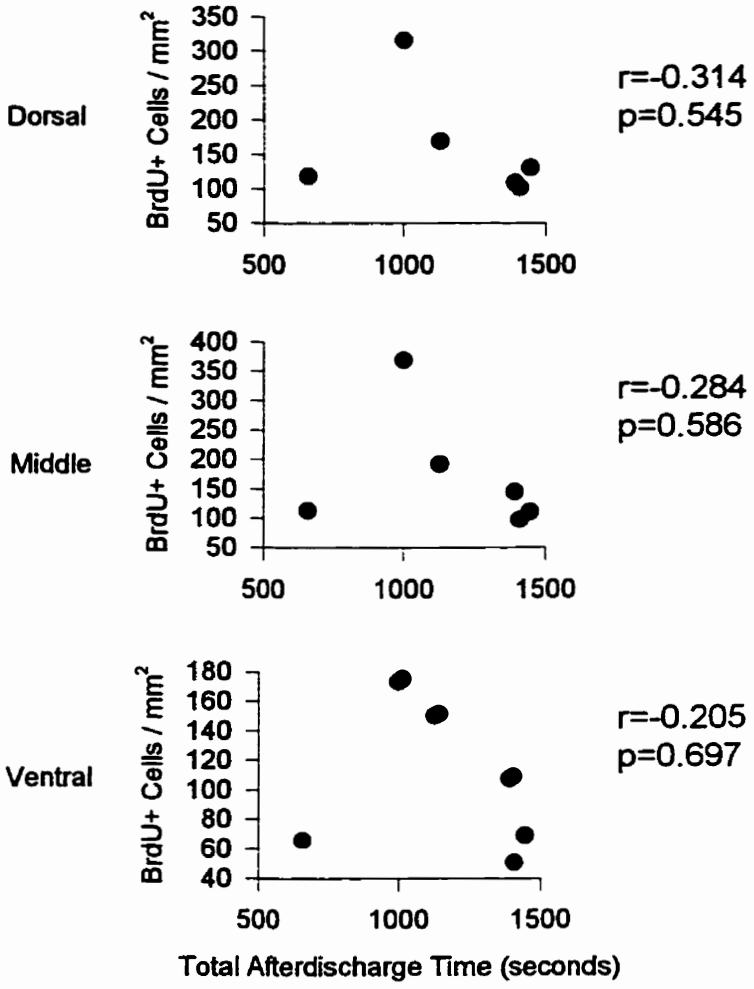


Figure 3.13: BrdU+ Densities Vs. Total Afterdischarge Time

No significant correlation (Pearson r and p value shown for each region, α=0.05) was found between the amount of time afterdischarges continued in the amygdala and the density of BrdU+ labelling in any region of the hippocampus.
(g-stim2.spw)

3.5 Area

Cross-sectional areas of the GCL, hilus and molecular layer of Early BrdU stimulated animals did not differ from controls, and neither did that of GCL and molecular layer in Late BrdU stimulated animals. In contrast, the cross-sectional area of the hilus in dorsal and middle hippocampus of Late BrdU stimulated animals increased significantly by 25.0% and 35.3% respectively, compared to control animals (2-way ANOVA, $p<0.05$; Table 3.2), while that of GCL and molecular layer did not.

Hippocampal Region	GCL Area (%Δ)	Molecular Layer Area (%Δ)	Hilus Area (%Δ)
Dorsal	15.8	15.2	25.0 *
Middle	17.7	6.4	35.3 *
Ventral	7.0	14.6	10.0

Table 3.2: Percent Change in Cross-sectional Area in Late BrdU Group

Changes in cross-sectional area in dorsal, middle and ventral regions of the hippocampus expressed as % change relative to non-kindled controls. Significant increases (*) were found in dorsal ($F=10.54$, $p=0.0036$) and middle hilus ($F=11.26$, $p=0.0027$). 2-way ANOVAs were performed on each region with $\alpha=0.05$, using Student-Newman-Keuls all pairwise multiple comparison procedure, but only Late BrdU group is shown here. n=7 animals in each group except Late BrdU stimulated, in which n=6 animals. (table2.doc)

Chapter 4

Discussion

In this study the kindling model of epilepsy was used to examine the production of cells within the dentate gyrus in response to the increased activity during epileptiform afterdischarge. BrdU immunolabelling revealed regional differences among the dorsal, middle and ventral regions of the hippocampus in basal level of production, with ventral showing fewer BrdU+ cells within the GCL. Increased densities of BrdU+ cells were found within the GCL and molecular layer of animals which experienced severe (stage 5) kindling evoked seizures. This suggests that cell production - especially that of new dentate granule cells - may be enhanced by the increased activity associated with kindled seizures.

The possibility that more severe seizures results in a greater increase in BrdU labelling cannot be definitely proven with this experimental design as a number of other factors may be at work. Although the Early BrdU group did experience fewer numbers of stage 5 seizures than the Late BrdU group, they also received fewer stimulations in general. Also, the Early BrdU group was killed before the animals in the Late BrdU group, and therefore survival time after the beginning of the stimulations may also be a contributing factor. A longer survival time will allow cells to proliferate more and thus produce more descendants. Therefore it cannot be precisely determined whether the total number of stimulations, severe

seizures or time after stimulations began are most important in influencing the increase in cell production found here. Using low-level electrical stimulation of the perforant path as opposed to the high-intensity kindling stimulations may reveal whether stimulation alone is capable of increasing cell production. Killing animals soon after BrdU injections as opposed to waiting one week, could help explain whether proliferation itself is affected (see below). Regardless of the precise mechanism though, the kindling procedure in general can be said to alter the amount of new cells produced within the dentate gyrus.

4.1 Use of Nuclear Profile Counts

In this study uncorrected counts of BrdU labelled nuclear profiles were taken in transverse hippocampal slices. A problem with this technique is that profile counts do not accurately estimate true object numbers but instead estimate profile numbers. Profile counts are therefore biased in that as more profiles are counted, the estimate approaches the total number of profiles and not the true number of actual objects in the sample space. A number of other counting procedures have been developed in order to minimize biasing of object counts (Coggeshall & Lekan, 1996). The most accurate of these is complete serial reconstruction of the sample space such that all objects in the space are identified, though this is time-consuming and impractical with large sample spaces. In order to estimate object numbers in larger sample spaces, the

application of correction formulas has also been used. These formulas are usually applied to profile counts to generate corrected estimates of true object numbers. These formulas are usually based on a number of assumptions such as a consistency of object shape, size and section thickness which might not be true. To the extent that these assumptions differ from reality, corrected profile counts can differ from true object numbers.

More recently, unbiased stereological methods have been introduced to estimate true object numbers in a large sample space. In these methods each counted object is only counted once and as sample size increases estimated counts approach true object numbers (Coggeshall & Lekan, 1996). A number of techniques have been developed to ensure that this is the case, with the two most common of these techniques being the physical disector and the optical disector methods. The physical disector method uses two serial sections such that one is a "reference" section and the other is a "look-up" section. Objects are counted in the reference section only if they do not appear in the look-up section. In this way, objects are never counted twice. The optical disector method is a variation of the physical disector except two optical planes are used.

Although these stereological methods are becoming more accepted by the general scientific community, they are not yet considered to be the standard techniques for object number estimation. A number of recent published articles

in this field have used uncorrected profile counts as were used in this study (Kuhn *et al.*, 1996; Gould *et al.*, 1997; Parent *et al.*, 1997). The Journal of Comparative Neurology has now made the stereological methods the default techniques to be used in order to be considered for publication in their journal (Saper, 1996). However, they do point out one case in which they will accept biased object counts - estimates of labelled nuclear profile numbers leading to a ratio of number of cells. This case is exactly what was done in this study. If object size (relative to slice thickness) is the same in control and experimental groups then changes in nuclear profile counts reflect changes in true object number as nuclei are roughly spherical. A recent study in this field has justified their use of nuclear profile counts in this way after comparing measurements of nuclear area between experimental groups and finding no difference (Kuhn *et al.*, 1996).

Nuclear area and nuclear diameter were therefore measured in control and late BrdU injected stimulated animals. No significant difference was found between the two groups and therefore the use of nuclear profile counts remains valid (Tables 4.1 & 4.2). In accepting this, one must still make an assumption regarding nuclear shape being constant as well. Nuclear shape in the transverse sections did not appear different between the two groups but whether nuclei became elongated or shortened in the longitudinal plane of the hippocampus is not known and cannot be determined without serial sectioning of

the hippocampus. It does seem highly unlikely though that BrdU labelled nuclei would change their diameter only in one plane of the hippocampus, which itself curves in a "C" shape from dorsal to ventral thus drastically changing its orientation relative to the rest of the brain.

	Animal Means				Individual Diameters			
Group	**N**	**Mean (μm)**	**Std. Dev.**	**Std. Err.**	**N**	**Mean (μm)**	**Std. Dev.**	**Std. Err.**
Late Stimulated	6	8.76	0.796	0.325	36	8.46	1.42	0.236
Late Control	6	8.12	1.441	0.588	36	7.91	1.8	0.3

Table 4.1: Nuclear Diameter

Measurements of nuclear diameter made in Late BrdU injected stimulated and unstimulated control animals and presented as animals means (left) and individual diameters (right). No significant difference (t-test, $p<0.05$) was found between these groups.

	Animal Means				Individual Areas			
Group	**N**	**Mean (μm^2)**	**Std. Dev.**	**Std. Err.**	**N**	**Mean (μm^2)**	**Std. Dev.**	**Std. Err.**
Late Stimulated	6	46.9	6.93	2.83	36	45.4	16.6	2.77
Late Control	6	40.0	11.91	4.86	36	38.8	16.0	2.67

Table 4.2: Nuclear Area

Measurements of nuclear area made in Late BrdU injected stimulated and unstimulated control animals and presented as animals means (left) and individual areas (right). No significant difference (t-test, $p<0.05$) was found between these groups.

4.2 Cell Proliferation Vs. Cell Production

The data from this study might not only represent the effects of cell proliferation but could also include those of cell survival. An increase in the number of BrdU+ cells can result from an increase in cell proliferation or a decrease in the number of dying BrdU+ cells. It is therefore possible that seizures might not alter the rate of cell division but the rate of death of newly generated cells. Such a phenomenon was observed by Kempermann *et al.* (1996), who found that raising mice in an enriched environment did not result in a change in cell proliferation in the dentate as evidenced by the number of BrdU+ cells immediately after injections, but did affect BrdU+ cell numbers at 4 weeks post-injections. This is possible in my study, as animals were killed one week after the last BrdU injection after which the existence of BrdU+ nuclei was examined, and not immediately after a BrdU injection which would show the number of cells undergoing replication at that time. Regardless of the cause, an increased number of BrdU+ cells is interpreted to mean a greater net rate of cell production.

It is also possible that precursor cells incorporating BrdU during the cell division process could dilute the BrdU label with further cell divisions during the 1 week waiting period. Dilution of BrdU label might result in precursor cells being missed during cell counting. This would mean that those cells which are counted are more likely to be cells which have left the cell cycle and are in the

process of terminal differentiation as opposed to proliferating precursors. Therefore, a delay between BrdU injection and subsequent tissue processing could allow us to examine the effects of seizure on the production of actual granule cells for example, and not just a precursor population which is merely dividing.

4.3 Identity of BrdU Labelled Nuclei in Control Animals

4.3.1 Granule Cell Layer

BrdU+ nuclei within the GCL showed characteristics typical of dentate granule cells in size and morphology and were located almost entirely within a 2 cell body wide strip bordering the GCL and hilus which is the known location of a proliferative zone from which new dentate granule cells are normally produced and therefore most likely represent newly generated granule cells. A low level of glial proliferation is known to occur within the dentate gyrus, primarily within the hilus and molecular layer (Cameron *et al.*, 1993; Kuhn *et al.*, 1997). Those glial cells which do exist within the GCL are found at the border of the GCL and hilus and have the morphology of radial glia which are much smaller than granule cells in the same location (Cameron *et al.*, 1993, Gould *et al.*, 1997). Figure 4.1 shows a typical granule cell and glial cell from the inner edge of the GCL, both filled with Lucifer Yellow during patch clamp recording. Smaller nuclei were not

included in the counting, thus reducing the likelihood of including glial cells in the data analysis.

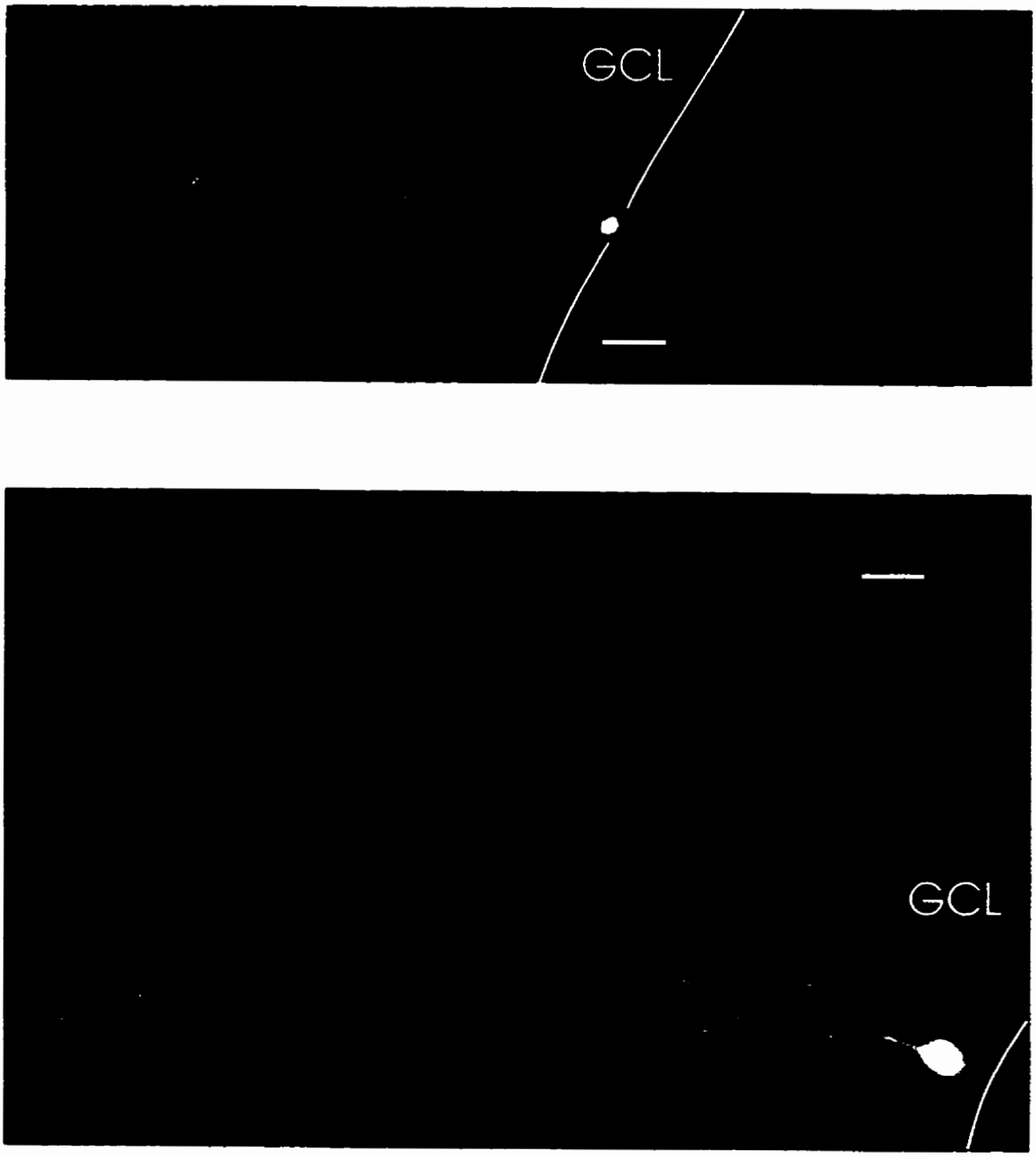


Figure 4.1: Glia and Granule Cell

A glial cell (upper panel) and a granule cell (lower panel), both on the inner edge of the granule cell layer (GCL) and filled with Lucifer Yellow. Note the much smaller size of the glial cell. Scale bar = 10 μm in both panels. Experiments and photos by Sabrina Wang.

4.3.2 Hilus

The identity of BrdU+ cells within the hilus is less certain. Proliferation of astrocytes is known to occur within this region (Cameron *et al.*, 1993) and it is possible that this accounts for a proportion of the BrdU+ nuclei found there. A number of BrdU+ nuclei in the hilus may also be that of neurons. BrdU+ cells in the hilus were more often found just below the GCL as opposed to deep within the hilus. Some precursors might not have fully migrated from the hilus to subgranule zone during development and thus may rest slightly below the subgranule zone and were therefore included in hilar cell counts.

4.3.3 Molecular Layer

Neuronal proliferation has not previously been found within the molecular layer and very few neuronal cell types are known to exist there. It is therfore more likely that some BrdU+ nuclei may be that of glial cells, as glial proliferation has in fact been found within the molecular layer (Cameron *et al.*, 1993). The relatively frequent association of BrdU+ nuclei in the molecular layer with blood vessels, along with the irregular or elongated nuclear morphology suggests that many of the proliferating cells found there may be either within the blood or perhaps part of the endothelial lining of the blood vessels.

4.4 Identity of BrdU Labelled Nuclei in Stimulated Animals

4.4.1 Granule Cell Layer

As in control animals, the BrdU+ nuclei within the GCL were located on the inner edge of the GCL in a known neuronal proliferative zone, and also appeared similar in size and morphology to that of granule cells. BrdU+ labelling in the GCL of stimulated animals is therefore also likely to be of neuronal origin, although this is not certain. Even though this increase is occurring within a known neuronal proliferative zone, it is possible that the ratio of newly produced glia to neuron may be altered with kindling stimulations. For example, Kuhn *et al.* (1997) found that in control animals the number of BrdU labelled cells in the dentate gyrus which became neurons was 92% while those which became glial cells was less than 1%. With infusion of epidermal growth factor, this ratio was changed to 52% becoming neurons and 39% becoming glial cells, while the total number of BrdU labelled cells did not change. Thus the glial fate of newly produced cells increased while the neuronal fate was decreased, without changing the total number of newly generated cells. It is possible that kindling stimulations could also alter the ratio of newly produced glia to neurons within the dentate. Double labelling with BrdU and glial or neuronal specific markers would allow the ratio of neuron to glia in kindled and non-kindled groups to be determined (see below).

The cross-sectional area of the GCL of stimulated animals was found to be slightly larger than that of control animals (7.0% to 17.0%; Table 3.2). This trend may be the result of the addition of granule cells to the GCL. On the other hand, Parent *et al.* (1997) found ectopically located BrdU labelled cells with granule cell-like morphology which presumably migrated from the proliferative zone. This migration of new-born granule cells might result in a dispersion of the GCL which could be causing the increase in cross-sectional area found here.

4.4.2 Hilus

A proportion of BrdU+ cells within the hilus may be glia as glial proliferation has been found there. On the other hand, neurons of granule cell morphology have been found in ectopic locations within the hilus and molecular layer after seizure (Parent *et al.*, 1997) and it is possible that newly generated granule cells might migrate to these locations and therefore be included in the counts. An increase in BrdU+ cell density was not found in the hilus, although an increase in BrdU+ cell number in the hilus may have been masked by the simultaneous increase in the cross-sectional area which was found. This might then result in a BrdU+ cell density lower than would be expected if no increase in area had occurred.

4.4.3 Molecular Layer

An increase in BrdU+ cell density was found in the molecular layer and may be due to the ectopic migration of new granule cells as mentioned above.

It is also possible that angiogenesis may be occurring in the molecular layer in response to seizures and/or seizure-induced damage, which may result in the proliferation of endothelial cells there. In a seizure model using suxamethonium to induce status epilepticus, blood vessels in the substantia nigra appeared larger and more numerous in seized animals compared to controls (Eriksdotter-Nilsson *et al.*, 1987). Laminin immunolabelling was used to assess the changes in the blood vessel network within the substantia nigra, as laminin is a component of the basement membrane of blood vessels and has been used as a marker for brain blood vessels (Eriksdotter-Nilsson *et al.*, 1986). Laminin immunolabelling was found to be increased in seized animals, suggesting that angiogenesis may be occurring. Angiogenesis may therefore be occurring within the molecular layer of Late BrdU injected kindled animals resulting in an increase in BrdU labelling there as well as the trend towards increased cross-sectional area.

4.5 Confirmation Of Cell Identities

The identification of cell types labelled with BrdU in this study cannot be determined with certainty and is largely based on assumptions regarding nuclear morphology, size and location within the dentate, as well as extrapolations from previous reports using double labelling techniques. To firmly establish the identities of the BrdU labelled cells in this study, double labelling with BrdU and cell-specific markers such as glial fibrillary acidic protein (GFAP) or vimentin to label glia, and neuron specific enolase (NSE) or neuronal nucleus (NeuN) to label neurons, is necessary. Many cell-specific marker proteins are not expressed until cells are fully differentiated (e.g. NSE) and thus the delay of one week between BrdU injections and tissue processing may not allow for the use of these markers as the cells might not be developed enough. In this case, the neuron-specific marker TOAD-64 might be more appropriate as it is turned on shortly after cell division (Minturn *et al.*, 1995; Parent *et al.*, 1997).

4.6 DNA Repair

It is possible that BrdU can be incorporated into the DNA of cells undergoing DNA repair as a response to DNA damage and not the proliferation of new cells as believed. A number of points suggest that this is not the case. BrdU+ nuclei were occasionally found in pairs which would be consistent with recent cell division. Paired nuclei were not often seen though and this may be due to

migration of cells away from the original site of division during the one week delay between BrdU injection and tissue processing. Within the GCL, BrdU+ nuclei were found almost entirely on the border of the hilus and GCL, and primarily below the GCL in the hilus. It is not likely that DNA damage would be occurring in such a restricted area, and not throughout the GCL.

In the pilocarpine model of epileptiform seizure (Parent *et al.*, 1997) the number of BrdU+ cells was also found to increase at the border of the GCL and hilus, suggesting that although seizure-induced damage may be occurring throughout the dentate gyrus in this model, BrdU is only being taken up in a restricted area and not by those cells which are particularly susceptible to damage (i.e. hilus). The Parent *et al.* study also found double labelled cells for BrdU as well as another independent mitotic marker, proliferating cell nuclear antigen, which supports the view that BrdU is indeed incorporated into the DNA of dividing cells after pilocarpine induced seizures. TOAD-64 was also found to be turned on in BrdU+ cells, again suggesting cells being newly generated and not incorporating BrdU for DNA repair.

4.7 Causes Of Increased Proliferation In Dentate Gyrus

4.7.1 Cell Death

There is some evidence that cell death in the hippocampus may occur as a result of seizures in both human epilepsy and animal models of seizure disorders (Dam, 1982; Cavazos & Sutula, 1990; Bertram & Lothman, 1993). In human temporal lobe epilepsy, the most common lesion found is hippocampal sclerosis which features neuronal loss and gliosis in the hilus, CA1 and CA3, with the granule cell layer also affected in many cases (Babb & Brown, 1987). In the kindling model, the hilus and CA1 appear to be particularly susceptible to damage while the GCL is more robust (Cavazos *et al.*, 1994). In a number of organ systems cell loss is offset by the production of new cells to replace those which have died. For example, skin or gut epithelium is replaced as it is lost or damaged in order to maintain tissue integrity. A similar mechanism might be operating within the DG in response to kindled seizures.

4.7.2 Excitotoxic Cell Death

A potential mechanism which might be responsible for the loss of cells within the DG during kindling is glutamate excitotoxicity. Glutamate is the predominant excitatory neurotransmitter in the mammalian central nervous system. The increased excitation in hippocampus due to amygdaloid kindling has been

shown to result in a large increase in extracellular glutamate (Bradford, 1995). Overactivation of glutamate receptors can lead to excitotoxic neuronal death (Watkins & Evans, 1981) most likely resulting from excessive Ca^{2+} influx through the NMDA ionotropic glutamate receptor subtype especially, which has a very high Ca^{2+} permeability, as well as possible enhancement by Ca^{2+} release from intracellular sources (Mody & MacDonald, 1995). The resulting loss of Ca^{2+} homeostasis with high Ca^{2+} levels may lead to the generation of free radicals which can be involved in lipid membrane damage as well as mitochondrial dysfunction and other potentially toxic effects (Greene & Greenamyre, 1996). Excitotoxicity resulting from seizure activity may therefore lead to loss of neurons in the DG which may in turn act as a trigger for the increase of proliferation there.

4.7.3 Robustness of Granule Cell Layer

This study finds an increase in the production of cells in the GCL, an area believed to be resistant to seizure-induced damage, while there was no significant increase in production in the hilus, a region known to be particularly susceptible to damage. This result would not be expected if increased proliferation occurs to replace cells lost due to excitotoxic damage. An important difference between human temporal lobe epilepsy and animal seizure models has been that patients frequently show reduced or severely depleted granule

cell numbers while the GCL in animal seizure models remains relatively intact (Meldrum & Bruton, 1992; Sutula *et al.*, 1994). The belief that granule cells are less vulnerable to neuronal loss due to kindling may be based on cell density estimates as well as total cell number estimates which may not reveal a turnover of granule cells in which dying cells are replaced by new cells generated in the subgranular proliferative zone. Recent work by Sloviter *et al.* (1996) has found that a single prolonged period of afferent excitation produced a rapid death of granule cells apparently by an apoptotic mechanism followed by rapid removal of debris by macrophages, while evidence of degeneration in other regions of the hippocampus, with pyknotic cells and debris, remained for weeks. Significant loss of granule cells might therefore be occurring but is missed as no evidence of cell death remains if left too long after seizure. Therefore loss of granule cells as well as hilar neurons may be occurring in response to severe seizure, possibly due to excitotoxicity, which in turn may be acting as the trigger for increased granule cell production.

Removal of adrenal steroids by adrenalectomy has been shown to result in both an increase in cell death - also by apoptosis - as well as an increase in proliferation which may also be a response to cell loss (Sloviter *et al.*, 1993; Cameron & Gould, 1996). Sloviter *et al.* (1993) found massive cell death occurring after adrenalectomy, with degenerating cells concentrated near the molecular layer. Cameron & Gould (1996) also noted that older granule cells

labelled with ^{3}H-Thymidine were being lost while younger granule cells were not. Therefore it is possible that the death of older, perhaps less robust granule cells, combined with an upregulation of granule cell production as well as rapid removal of evidence of cell death may make granule cells appear to be more resistant to seizure-induced injury while in fact cell death is being compensated for by neurogenesis.

4.7.4 Other Neuronal and Glial Influences

The actual cue by which cell death might influence production of new granule cells is unknown but may involve the loss of a direct tonic synaptic input to granule cell precursors from dying target cells or perhaps a signal from granule cells which have lost target neurons through collateral contacts with precursor cells (Figure 4.2). The discovery of synaptic contacts on the cell bodies of presumptive granule cell precursors can support either argument as the origin of this afferent input is unknown (Kaplan & Bell, 1984). Another possible cue which might be influencing granule cell production may come from local glial cells. Glial proliferation has been reported to occur in response to kainic acid-induced seizures in regions believed susceptible to injury but not in the GCL (Niquet *et al.*, 1994). Seizures also produce astrocyte hypertrophy as evidenced by increased levels of glial fibrillary acidic protein (GFAP) as well as a reorganization of the astrocytic cytoskeleton and upregulation of the synthesis of

a number of proteins (Khurgel & Ivy, 1996). This glial reaction to seizure along with an alteration of the permeability of the blood-brain barrier (Nitsch *et al.*, 1986) may represent a kind of inflammatory response in which glial or other cell types may release mitogenic factors such as basic fibroblast growth factor (bFGF) which could affect cell proliferation or survival in the dentate. Basic fibroblast growth factor has been found to be primarily localized within glial cells in the CNS (Mocchetti & Wrathall, 1995), and is known to promote vascularization (Joseph-Silverstein & Rifkin, 1987) as well as able to maintain survival and induce proliferation *in vitro* of neuronal progenitors isolated from adult rat hippocampus (Gage *et al.*, 1995). Therefore the release of a factor such as bFGF from glial or other cell types may influence cell proliferation in the dentate gyrus as part of a general response to seizure induced damage.

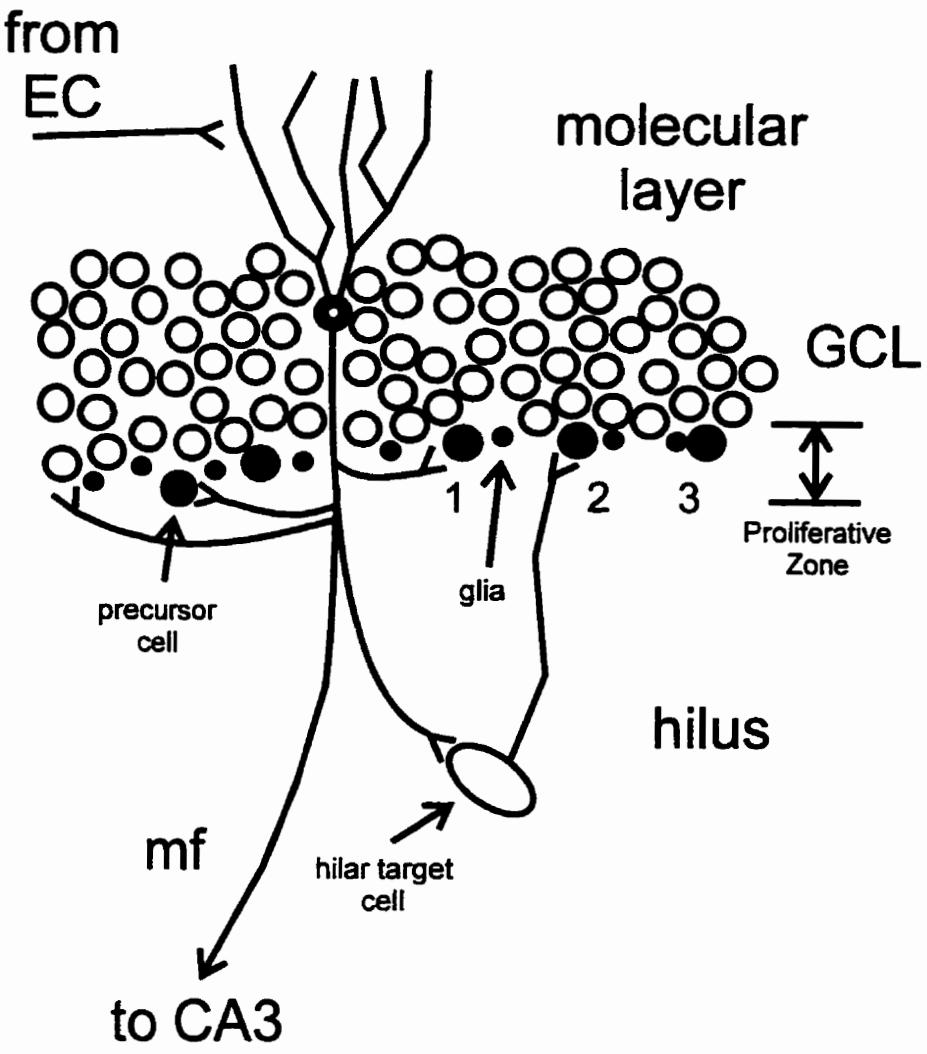


Figure 4.2: Mechanisms For Influencing Proliferation of Precursors

Schematic representation of possible mechanisms which may be able to influence the proliferation of precursor cells within the proliferative zone. 1) Collateral connections from dentate granule cells may synapse directly onto precursor cells (black filled circles) providing regulatory information which may change if target cells are lost. 2) Death of hilus target cells would remove tonic input to precursor cells leading to alteration of proliferation. 3) Local glial cells (small light filled circles) may "sense" extracellular levels of glutamate and release a mitogen which might influence proliferation of precursors. EC, entorhinal cortex; GCL, granule cell layer; mf, mossy fibre. (summary.cdr)

4.7.5 Growth factors

A number of factors have been found to have mitogenic effects or are able to stimulate differentiation of neural precursors from embryonic and adult brain (Gage *et al.*, 1995). Precursor cells isolated from the adult rat hippocampus were able to survive and proliferate for one year in medium containing bFGF (Gage *et al.*, 1995). Further, those same cells, when transplanted into the hippocampus of another host rat were found to migrate up to 3 mm away from the injection site and differentiate into neurons only within the GCL, suggesting that an environmental cue local to the GCL will promote survival and differentiation of neuronal precursor cells. Whether this cue is secreted by granule cells or glia or another cell type is unknown.

Although bFGF appears to be a potent mitogen and survival factor for adult hippocampal precursor cells *in vitro*, bFGF levels do not change in the dentate with amygdaloid kindling (Sato *et al.*, 1996) and bFGF does not appear to affect proliferation in the dentate gyrus *in vivo (*Kuhn *et al., 1997)*, questioning its role as a potential cue for increased neurogenesis in the dentate. Basic fibroblast growth factor as well as epidermal growth factor do appear to affect proliferation of precursor cells within the sub-ventricular zone. Infusion of either bFGF or epidermal growth factor into the lateral ventricle has been found to increase the proliferation of progenitor cells within the sub-ventricular zone (Weiss *et al.*, 1996) but not the dentate gyrus, and thus have site-specific effects on

proliferation (Kuhn *et al.*, 1997). Epidermal growth factor was found to alter the ratio of glial cells versus neurons produced within the dentate gyrus, by increasing the proportion of cells which become glia and reducing that which become neurons, while bFGF had no effect on cell fate. It is possible though that these differential effects of bFGF and epidermal growth factor may be due to differences in penetration of either molecule through the brain to the hippocampus, as well as degradation by enzymes.

The synthesis of mRNA for a number of neurotropins including brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in dentate granule cells has been found to sharply but transiently increase after chemically and electrically evoked seizures, followed by a rise in protein levels and specific receptors (Lindvall *et al.*, 1994). Both of these factors have been found to have some mitogenic abilities but seem to be better able to influence neuronal differentiation (Gage *et al.*, 1995; Ahmed *et al.*, 1995; Lachyankar *et al.*, 1997). It is believed that BDNF and NGF may be involved in protection against neural damage or even the sprouting and synaptic reorganization found to occur with seizures (Lindvall *et al.*, 1994), but their role in neurogenesis in response to seizure has yet to be explored.

4.7.6 Stress

Adrenal steroid hormones have been shown to selectively regulate both granule cell production and death in the adult rat (Gould, 1994). Adrenalectomy has been found to cause both an increase in granule cell genesis as well as massive cell death in the GCL (Gould *et al.*, 1992; Sloviter *et al.*, 1993), while treatment of intact rats with corticosterone decreases the low level of naturally occurring cell death (Gould, 1994) and will reduce the number of ^{3}H-Thymidine labelled cells in the dentate (Cameron and Gould, 1994). It is possible then that stress may influence granule cell production via changes in adrenal hormones.

The psychosocial stress associated with a subordinate position in the social hierarchy of tree shrews has been found to decrease the production of granule cells (Gould *et al.*, 1997). The possibility therefore exists that the stress associated with the daily kindling procedure and repeated seizures may influence neurogenesis in this study. If the differences in BrdU labelling between the stimulated and control groups were caused primarily by the greater stress experienced by the stimulated group, then one would expect a decline in BrdU labelling and not the substantial increases in the stimulated group seen here. The effects of seizures are therefore able to overcome any modulation of granule cell production stress may have. On the other hand, excitatory input via NMDA receptors is also thought to modulate granule cell production, but in a negative fashion whereby NMDA receptor activation decreases proliferation

while NMDA receptor blockade increases proliferation (Gould *et al.*, 1994; Cameron *et al.*, 1995). Presumably then the increased excitatory transmission via glutamate associated with seizures should result in a decrease in granule cell production, which is not seen in this study or that of Parent *et al.* (1997) using pilocarpine induced seizures. The effects of seizure on granule cell production must then be accomplished via a separate mechanism than mere NMDA receptor activation.

The myriad of changes in dentate associated with seizure activity provide a number of possible routes through which neurogenesis may be influenced, including target cell loss as a trigger, or collateral input to precursors from mature granule cells, as well as the effects of trophic factors released from neurons or glia. The modulation of granule cell neurogenesis by afferent input may be a natural regulator under normal conditions, but may be overcome in certain pathological states.

4.8 The Role of Granule Cell Neurogenesis In Kindling

Among the abnormalities found in the hippocampus of temporal lobe epilepsy patients, granule cells have been found ectopically located within the hilus and molecular layer (Houser, 1990; 1992). Granule cell mossy fibers have been shown to make aberrant projections to the inner molecular layer, forming what

are believed to be recurrent excitatory synapses on granule cell dendrites, which might contribute to hyperexcitability and seizure maintenance (Babb *et al.*, 1991; Dudek & Spitz, 1997). Animal models of epilepsy show similar changes. Using pilocarpine induced status epilepticus, Parent *et al.* (1997) found that an increasing number of BrdU+ nuclei were located in the hilus and molecular layer with increasing time after BrdU injection suggesting the possibility that they had migrated there from the GCL. Cells labelled with antibodies to TOAD-64 - which is turned on after cell division and thus serves as an early post-mitotic marker - were found to have granule cell-like morphology and were also occasionally found in similar ectopic locations as well as with axonal projections toward the molecular layer. The abnormal development of young granule cells may therefore account for the aberrant excitatory synapses which are hypothesized to be involved in seizure maintenance.

Granule cells are known to be under strong GABA-ergic inhibition under normal conditions and paradoxically, a significant increase in GABA mediated inhibition is found to occur within the dentate gyrus of rats which have experienced kindled seizures (Oliver & Miller, 1985; Titulaer *et al.*, 1995). Results from our laboratory have revealed that granule cells located closer to the hilus (younger) have greater GABA-ergic inhibition than those cells located closer to the molecular layer (older) (Scott *et al.*, 1997). This strong inhibition may be a mechanism to prevent hyperexcitable young cells from excessive firing. This

mechanism might then be acting in an attempt to prevent the excessive activity associated with invading seizures, but is ultimately overcome. It is therefore possible that these newly generated cells may account for a significant proportion of the kind of anatomical pathology and physiological alterations seen in animal models of seizure disorders and perhaps human temporal lobe epilepsy.

4.9 Summary

In order to examine the effects of kindling evoked seizures on cell production in the dentate gyrus of the rat, this study used the electrical kindling technique combined with bromodeoxyuridine immunolabelling to identify cell production under these conditions. Cell production in the granule cell layer and molecular layer of the dentate gyrus was found to increase with kindled seizures, while cell production in the hilus did not. Regional differences in the basal level of cell production in the granule cell layer were found such that ventral hippocampus had a lower rate when compared to dorsal and middle. No such regional difference was found in hilus or molecular layer. The cross-sectional area of the hilus in dorsal and middle hippocampus was found to be increased in kindled animals when compared to unstimulated controls, confirming previous reports. An increase in the prominence of putative blood vessels in the molecular layer of kindled animals was noted, although this was not quantified.

The short duration and widely spaced seizures resulting from kindling stimulations therefore increases cell production in the granule cell layer and molecular layer of the dentate gyrus. This is the first report of this kind of anatomical change resulting from kindling evoked seizures in the rat, and is similar to the increase in granule cell production found in the chemical seizure model of pilocarpine status epilepticus.

4.10 Significance

The alteration of granule cell neurogenesis by kindled and chemically induced seizures, excitatory afferent input and NMDA receptor activation, as well as adrenal steroid levels and external factors such as psychosocial stress and enriched environment shows that it is very sensitive to a wide variety of modulators. Since granule cell production also continues throughout adulthood, it is likely that the new granule cells contribute in an important way to the normal functioning of the dentate gyrus. What that contribution might be is unknown at present, but if a number of the changes occurring in the dentate gyrus in animal seizure models are in fact primarily due to the young cells produced there, then this would attest to the morphological and maybe functional plasticity of these young cells as well as their ability to withstand insult. Understanding these properties and how modulation of neurogenesis can occur in the adult brain would not only contribute to our understanding of the hippocampus with respect to its role in learning and memory, but to also to the role neuronal development might play in diseases such as epilepsy.

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IMAGE EVALUATION
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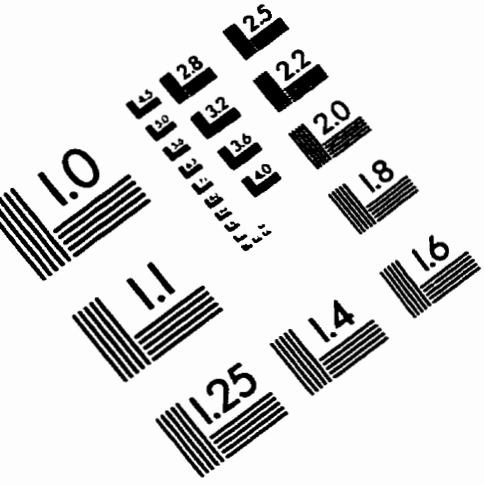

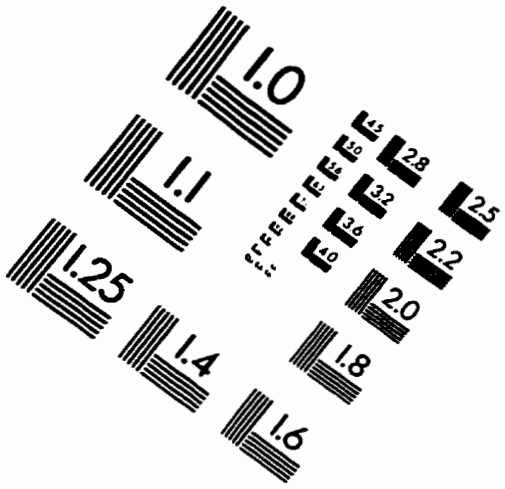

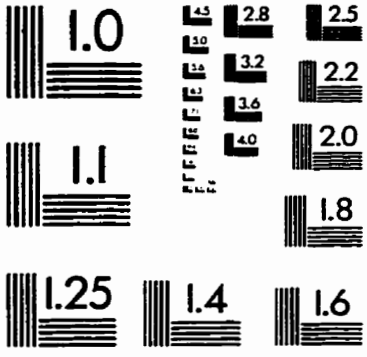

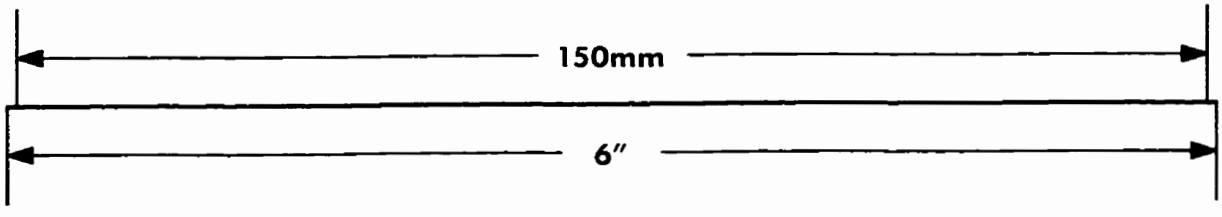

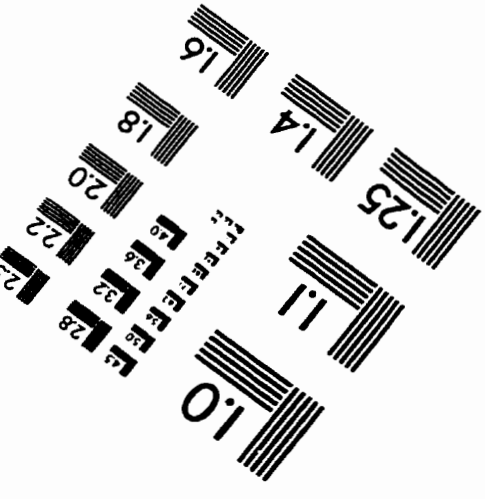

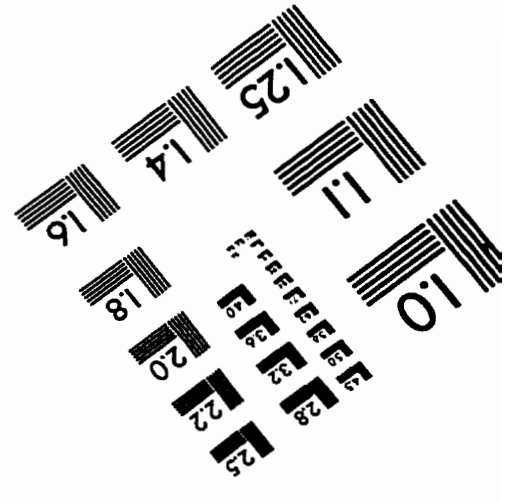

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