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Electrical stimulation after cortical injury enhances cell proliferation in the adult rat cortex

by

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Abstract

Across all types of brain injury, 22% of individuals will manifest posttraumtic seizures (PTS). PTS are typically regarded as a negative side effect of the brain damage and attempts are made to inhibit PTS as a means of preventing further brain damage. However, it has been experimentally observed that anticonvulsant medications may retard functional recovery. Thus, PTS may be misunderstood and may elicit adaptive mechanisms, stimulating brain recovery. Furthermore, seizures are known to induce axonal sprouting and increase the release of neuronal growth factors. It is possible that PTS activate factors that promote cell proliferation and survival. The present study was conducted to determine whether seizures and stimulation can facilitate cell proliferation following injury in the mature cerebral cortex. In order to test this hypothesis, adult Long-Evans rats received bilateral aspiration lesions in the motor cortex followed by the administration of electroconvulsive shock to induce seizures. Treatment groups received either 0, 4 or 8 seizures every other day during the first 16 days post-lesion, modeling the criteria for Early and Late Seizures. Control animals underwent surgery but did not receive aspiration lesions. All animals were administered a cell proliferation marker, 5-bromo-2'-deoxyuridine (BrDU; 60 mg/kg), twice daily on post-lesion days 1-16. BrDU becomes permanently incorporated into the nuclei of dividing cells and can be visualized using immunohistochemistry. Our results show that electrical stimulation following a cortical lesion results in an increase in the number of proliferating cells in the cortex (proximal and distal to teh lesion), corpus callosum and SVZ. Seizures during a post-injury period may facilitate structural reorganization by activating compensatory cell proliferation.

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LIST OF ABBREVIATIONS

Anatomy Terms

CC	corpus callosum
DG	dentate gyrus
DGC	dentate gyrus cells
dl SVZ	dorsal lateral subventricular zone

Measurements Terms

D	day
Hz	hertz
mm	millimeters
Nla	net lesion area

Other Terms

ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factors
BrDU	5-bromo-2'-deoxyuridine
EEG	electroencephalograph
ECS	electroconvulsive shock or electroconvulsive stimulation
EGF	epidermal growth factor
FFT	fast fourier transformation
FGF2	fibroblast growth factor 2
FITC	fluorescein iso thio cyanate
GAP	growth-associated protein
GFAP	glial fibrillary acidic protein
IgG	Immunoglobulin G
LES	low-intensity (minimal) electroshock
LTP	long-term potentiation
LOC	loss of consciousness
LRTC	Lissamine
MES	maximal electroshock
NGF	nerve growth factor
NMDA	N-Methyl-D-Aspartate
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
P	post-natal day
PBS	phosphate buffered saline
PSA-NCAM	polysialylated-neural cell-adhesion molecule
PTS	posttraumtic seizures

1. General Introduction

1.1 Historical foundation

Throughout life, the brain retains the capacity to re-model its' circuitry through modification of synaptic connections. This capacity of the brain to change in response to environmental stimuli and damage is referred to as plasticity. Some factors that modify cellular organization are hormones, experience, injury, and learning. These environmental factors effect organization differentially depending upon age, sex, and prior plastic experience.

S. Ramon Y Cajal in 1928 postulated that the process of learning might produce changes in the efficiency of connections between neurons due to morphological changes and he also suggested that the synapse is the site of plastic alteration. D. Hebb in 1949 postulated that when one axon is near enough to excite or repeatedly/ persistently take part in firing another neuron, some change takes place in one or both cells such that the efficacy between these neurons is altered. This hypothesis revolutionized the thinking about the neural mechanisms of plasticity. In the years following Hebb's postulate, a body of evidence emerged to support the notion that experience modifies functional circuitry and that synaptic connections between neurons are the basis for modification. A major finding supporting Hebb's idea was the discovery that an enduring increase in synaptic efficacy occurred following electrical stimulation in the rabbit hippocampus which became known as long-term potentiation (LTP) (Bliss and Lomo, 1972). LTP is harder to obtain in the neocortex than hippocampus (Racine et al, 1995), supporting the notion that plastic response to stimuli varies depending on the brain site. The type of environmental change also influence the degree of plasticity. Historically, modification of an existing neurons' synaptic efficacy was considered the only mechanism of adult plasticity. We

now know that neuroplasticity encompasses different cellular modifications occurring on different time scales; potentiation can occur in milliseconds, synapses added or pruned in hours, and new cells added or eliminated in days. These plastic changes, occurring simultaneously in networks of excitatory and inhibitory connections, ultimately determine behavioural change. Whether behaviour is modified by the generation of new cells in adulthood is an important question and one that remains to be determined.

Neurogenesis is the generation of new neurons, and gliogenesis is the generation of new glial cells. In the adult brain, a population of proliferating cells was first observed in rodents (Altman, 1962 ; Altman and Das, 1965), and later described in other mammals, including the tree shrew (Gould et al, 1997), non-human primates (Gould et al, 1998; Gould et al., 1999), and humans (Eriksson et al, 1998). Advancements in cell labeling and imaging techniques has allowed for a better understanding of these new cells' origin, anatomical locations, and their behaviour.

1.2 Stem cells in the adult brain

New cells in the brain may originate from stem cells. The definition of stem cells used for other body systems, such as the circulatory system, considers the following criteria: 1. multipotency (ability to generate multiple cell types), 2. proliferative ability, 3. capacity for self-renewal, 4. ability to generate a large number of progeny, and 5. ability to generate new cells in response to injury or disease (Weiss et al, 1996). It has been debated whether neural stem cells are true stem cell populations, suggesting that this criteria for neural stem cells was too restrictive, since multipotency was unclear. However, recently, it was found that neural stem

cells grafted into irradiated hosts generate new blood cells (Bjornson et al, 1999), demonstrating that neural stem cells are capable of generating progeny of different cell types, depending on their environment. Under the right conditions in the nervous system, stem cell populations produce both neurons and glia. Cells with stem cell-like properties were first cultured in the adult mouse striatum (Reynolds & Weiss, 1992: Richards et al, 1992), the origin of these cells was later determined to be the lining of the lateral ventricles (Morshead et al, 1994), called the subventricular zone (SVZ). Another active germinal zone in the adult is the subgranular zone of the dentate gyrus (DG). Stem cells have also been described to exist in the olfactory epithelia (Barber, 1982) spinal cord (Richards et al, 1992), and the septal & striatal parenchyma (Palmer et al, 1995). The stem cell progeny often migrate to distant locations before they differentiate.

1.3 Proliferation and differentiation of new cells

Progeny of SVZ stem cells travel through the lateral wall of the lateral ventricles (Doetsch and Alvarez-Buylla, 1996) where they converge on a site containing a high concentration of migratory molecules (such as polysialylated-neural cell-adhesion molecule (PSA-NCAM). The cells tunnel along this molecularly distinct path, the rostral migratory stream (RMS), migrating 6-8 mm to the olfactory bulb, where most differentiate into the granule and periglomerular neurons (Lois & Alvarez-Buylla, 1993). Few cells deviate from the RMS, those that do, differentiate into glia (Luskin, 1993). Following olfactory bulbectomy, SVZ proliferation and migration persists (Kirschenbaum et al, 1999), indicating that migration is not solely based on positive feedback signaling from the olfactory bulb. Research directed towards understanding the mechanisms that regulate SVZ cell proliferation and migration is currently receiving a lot of attention because of potential use of this cell population for neurodegenerative disorder treatments.

Another brain site were proliferation exists in the adult is the dentate gyrus. Dentate gyrus neurogenesis occurs in a site located between the granular cell layer and the hilus, the subgranular zone (SGZ). Their progeny migrate to the dentate granual cell (DGC) layer and differentiate into neurons (Cameron et al, 1993; Kuhn et al, 1996), glial cells (Cameron et al, 1993), and one third remain undifferentiated (Kempermann et al, 1997), or possibly not yet identifiable. The rate of SGZ proliferation continually increases until midlife, at which time, an equilibrium forms between neuronal production and loss. After mid life there is a net loss of cells (Gage, 1998). New DGCs become structurally indistinguishable from existing DGCs, forming normal axon projections within the hippocampal formation (Stanfield and Trice, 1988; Parent et al, 1997). Emerging evidence suggests that new DGCs are also functionally integrated in the hippocampal formation, since increased DGC neurogenesis is correlated with improved learning (Kempermann et al, 1998; Gould et al, 1999).

1.4 Functional significance of adult neurogenesis

The hippocampal formation's functional association with learning and memory, makes it appealing to suggest that adult neurogenesis is linked to these processes. Dentate neurogenesis was first observed in birds (Goldman & Nottenbohm, 1983) and the speculated relationship between neurogenesis and learning and memory took flight. In chickadees, neurogenesis is seasonally modulated; neurogenesis is enhanced in seasons that correspond to seed storage and song modification, providing a possible association between neurogenesis and learning requirements. Seasonal fluctuation of neurogenesis has not been observed in bird species that do not modify songs seasonally. Further, in captive birds, not capable of navigating novel environments, neurogenesis is reduced to half the rate of the free-range birds (Barnea &

Nottebohm 1996). Access to novel environments may provide evidence for the suggested relationship between neurogenesis and learning. This relationship between neurogenesis and environmental complexity has been confirmed in the mammalian brain. Mice reared in enriched environments, comprised of social interaction, a variety of food and a large cage with toys, running wheels, and tunnels, had increased levels of neurogenesis (Gage 1998). However, this association between neurogenesis and learning in complex environments may be confounded by motor activity. Running, possibly mediated through neurotrophin activation, increases neurogenesis to levels not found in learning conditions such as the water maze (Praag et al, 1999). By responding to a number of environmental changes, including alterations in motor activity, neurogenesis may function as a preparatory mechanism for learning requirements. Dentate neurogenesis is also responsive to other environmental manipulations including a decline following stress (Gould et al 1997) and hormonal changes (Gould et al, 1992), while facilitated by ischemia (Lui et al, 1998), toxin-induced lesions (Gould and Tanapat, 1997) and seizures (Bengzon et al, 1997; Parent et al,1997, 1998; Gray and Sundstrom, 1998; Scott et al, 1998). Understanding the environmental factors that regulate dentate neurogenesis may indicate its' functional significance.

Neurogenesis can be regulated by the local environment that the precursor cells find themselves in, which includes neurotrophic factors. Neurotrophic factors are polypeptides that promote the growth, differentiation and survival of neurons. These molecules bind to tyrosine kinase receptors, whose activation causes a cascade of biochemical events leading to morphological and functional cell modification (for review see Yuen et al, 1996). The dentate and ventricular stem cell populations may respond differently to the same growth factors. *In*

vitro, infusing neurotrophic factors such as basic fibroblast growth factor (bFGF) or epidermal growth factor (EGF) into the ventricle and dentate has a differential response. Specifically, bFGF and EGF both enhance SVZ proliferation, but not dentate neurogenesis (Kuhn et al, 1997). This differential response possibly indicates unique functions of these two different stem cell populations in mammals.

Hypotheses of SVZ stem cells' functional significance has primarily focused on the adult, avian brain. In canaries, some cells can migrate to the song control center, differentiate into neurons, and respond to auditory stimuli (Paton & Nottenbohm, 1984). This association with song learning is substantiated by the finding that motor pathway neurons for song control are replenished at higher rates during song modification seasons (Alvarez-Buylia et al,1990; Goldman et al, 1993). Thus, in birds, dentate and SVZ neurogenesis can be linked to learning.

In mammals, SVZ proliferation is responsive to damage. Sensorimotor cortex lesions in adult rats result in an increase in the number of BrDU-labeled cells found at the SVZ (Willes et al, 1976; Szele & Chesselet 1996), although the cells were not found around the lesion cavity. Szele & Chesselet suggest that the lack of migration and differentiation of the SVZ cells may be due to the fact that the adult brain lacks necessary growth factors for these processes to occur. Specifically, they found that bFGF and EGF levels (thought to be necessary for differentiation) were lower in the lesion brains compared to non-operated control brains. While, in the neonate rat, Kolb et al (1998) determined that a sensory motor cortex lesion performed between post-natal day 7 -12 (P7-12), resulted in spontaneous filling of the lesion cavity, where BrDU-labeled

cells were found in and around the original lesion site. P7-12 may be unique because of developmental growth factors. Interestingly, other cortical regions do not spontaneously regenerate, suggesting to the authors that the new cells originate from the SVZ, where the RMS is in close proximity to the damaged frontal cortex. However, these cortical lesion studies indicate the necessity of growth factors for cells to proliferate and migrate to a damaged area. In the adult CNS, the required growth factors may be mediated through post-injury gliosis.

1.5 Reactive gliosis

The massive production of astroglia following injury is referred to as reactive gliosis. Reactive gliosis encompasses the hypertrophy of astrocytes and the proliferation of microglia and astrocytes. The origin of these cells is undetermined. They may arise from glial precursor cells or existing astrocytes. Reactive gliosis had long been considered inhibitory to recovery from injury. However, this process may protect surrounding tissue from further injury and provide a substraight for growth factors and axonal projection. Further, neuronal markers (nestin, tau, MAP-2) are expressed in reactive astrocytes after injury. Gliosis found at the immediate lesion site is referred to as proximal reactive gliosis. The cytoarchitecture proximal to the lesion is distorted and a glial scar forms. Reactive gliosis also occurs distal to the lesion, but a glial scar does not form. Reactive gliosis is highly plastic; the quantity and quality of gliosis depends on the type of injury and the specific environments' combination of cytokines, growth factors, adhesion molecules and other 'damage signals' from the local cells. Reactive astrocytes express an increases in recognition molecules including PSA-NCAM, enzymes, proto-oncogenes and many growth factors including: NGF, FGF and IGF. Growth factor receptors are also upregulated (for review see Ridet et al, 1997). Thus, astrocytes may have an active role in

neuronal repair and possibly regeneration. On a side note, astrocytes may play an important role in the regulation SVZ cell proliferation. *In vivo*, the direct contact of astrocytes with SVZ neuronal precursors appears to be necessary for the colonization of migratory neuroblasts (Lim and Alvarez-Buylla, 1999). Growth factors, bound to the extracellular matrix or membrane molecules on astrocytes, may assist in cell proliferation. Endogenous activation of growth factors may result in an increase in mitotic activity following cortical injury. Interestingly, growth factor production can be activated by the experimental application of seizures (Ernfors et al, 1991; Isackson et al, 1991; Lindvall et al, 1994; Yuen et al, 1996).

1.6 Seizures

Seizures can be induced in normal brains by treatments such as electroconvulsive shock, drugs, temperature increases, chemicals, or traumatic brain injury. Experimentally inducing seizures can cause a number of structural and biochemical changes in the brain, and these changes are postulated to contribute to epilepsy. The terms seizures and epilepsy are not synonymous. Seizures are the synchronous firing of a large population of neurons resulting in a transient behavioural change. Epilepsy refers to a neurological dysfunction associated with spontaneous, reoccurring seizures from excessive discharge of neurons (McNamara, 1994). Inducing seizures in the an untreated (non-seizure prone) brain causes structural changes in the hippocampal formation, including abnormal axonal projections of DG cells, referred to mossy fibre sprouting and facilitation of dentate cell proliferation (Parent et al, 1997). The majority of new dentate cells can be labeled with an antibody against TOAD-64, a neuronal marker (Parent et al, 1997; Scott et al, 1998). Seizure-induced neurogenesis is thought to contradict neurogenesis's proposed function: learning. However, the mossy fibre sprouting persists when

proliferation is temporarily ceased by whole brain radiation (Parent et al, 1999), indicating that it is not primarily the new cells that are involved in forming aberrant connections. Thus, applying seizures to a non-seizure prone brain may create a pathological condition (ie. the mossy fibre sprouting) in existing cells, then in response to this brain pathology, neurogenesis is facilitated. This postulate coincides with the evolutionary learning paradigm. Historically, those brains that were more responsive to environmental change (including damage) may have been more prepared for learning in that new environment, which resulted in a selective advantage over less plastic brains.

Seizures also result in changes that may assist in the survival of new cells. Seizures induce a cascade of proteins and neurotrophic factors including: growth-associated protein (GAP-43), glial fibrillary acidic protein (GFAP), (Dalby et al, 1995) nerve growth factor (NGF), and basic fibroblast growth factor (bFGF) (Follesa et al, 1994). Seizures cause an amplification in glial fibrillary acidic protein (GFAP) expression. One day after kindled seizures mRNA for GFAP is elevated, and remains elevated throughout the course of kindling. Within a few days of kindling termination, the levels decline to stimulation control levels (Oswald et al, 1991). Specifically, ECS-induced seizures, applied to intact rat brains, have been found to increase NGF and bFGF in all sites analyzed, including the striatum, entrorhinal cortex, hippocampus, and cerebellum (Follesa et al, 1994). NGF is produced in the cortex and hippocampus, and is thought to enhance gene expression and cell differentiation (Yuen et al, 1996). In the forebrain, bFGF is thought to increase SVZ proliferation and facilitates cell migration in the RMS (Kuhn et al, 1997). Thus, a possible function of some seizures could be the promotion of cell proliferation and survival through activation of neurotrophic factors. Neurotrophic factors are also expressed

following neuronal damage (Finklestein, 1988). Thus, the interaction of seizures after injury may enhance the availability of the appropriate combination of neurotrophic factors to promote mitotic activity, migration and differentiation.

1.7 Posttraumatic seizures

Seizures spontaneously manifested following brain injury are referred to as posttraumatic seizures (PTS). In the human brain injury literature, seizures that occur between post-injury D1 - D7 are referred to as Early Seizures. Generally, the term Late Seizure encompasses all seizures occurring beyond D7 post-injury. However, the lack of standard end points for Early and Late posttraumatic seizures influences the reported incidence rates (Kuhl et al, 1990). Other confounding variables include; lack of standardization across studies for associated complications (ie. hematoma, posttraumatic amnesia, depressed skull fractures), lack of EEG monitoring for non-convulsive seizures, and prophylactic anticonvulsant treatment.

Variables that determine the probability of posttraumatic seizures are: 1. the severity of injury, and 2. the time elapsed since the injury. For mild, closed head injuries, 2-5% of individuals will experience convulsive seizures within the first week, of which, 43% of seizures occur within the first 24 hours. Seizure severity also changes within the early period; in the first 24 hours post injury, 91 % of seizures are generalized tonic-clonic and 9% are considered partial with motor symptoms. Through Day 2 to 7 the statistics change to 79% and 21%, respectively (Lee et al, 1992). Other research suggests conservative percentages over the first year, 1.5 for mild, 2.9 for moderate injuries (loss of consciousness (LOC) for at 30 minutes to 24 hours or a skull fracture) and 17.0 for severe (LOC or amnesia for over 24 hours) (Annegers et al, 1998).

All of the described seizure incidence rates may be confounded by the lack of EEG monitoring, because only behavioural seizures are reported. A recent study assessed, over the first 14 days post-injury, the incidence of convulsive and nonconvulsive seizures in intensive care unit patients admitted with moderate and severe head injuries. Of the 92 patients, 22% had seizures, of which 52% where diagnosed based upon EEG alone because the seizures were nonconvulsive (Vespa et al, 1999).

PTS are regarded as a negative side effect resulting from a damaged brain; attempts are made to inhibit the seizures as a means of preventing further brain injury. However, one month post-injury, there is no distinct difference on various prognostic tests between head injury patients with or with out seizures (Vespa et al, (1999). The long-term prognosis of patients with PTS is assumed to be worse than those without PTS, although this discrepancy is not confirmed in the literature. Clinicians are in agreement that status epilepticus should be aggressively treated, although anticonvulsant treatment of patients with one or more seizures is controversial (Segatore & Jacobs, 1993).

Schallert et al (1986) determined experimentally in rats that inhibiting seizures through administration of anticonvulsants following brain injury may retard functional recovery, indicating that PTS may have been misunderstood. Further, experimental evidence suggests that induced seizures within the first 8 days following a motor cortex lesion improves motor function, although administering seizures beyond day 8 may retard functional recovery (Hernandez, 1997). Thus, some Early PTS may elicit adaptive mechanisms, stimulating the brain's recovery. Epilepsy may be considered as an adaptive mechanism gone awry. It is not yet understood how

seizures may act as a brain recovery mechanism, they may induce a cascade of events, like growth factor production and release, which could facilitate cell proliferation and functional recovery. However, before addressing a mechanism, it is necessary to determine if electrical simulation and seizures have an effect on structural recovery following injury in the adult.

1.8 Hypothesis and logic

One way in which seizures may promote brain recovery is by activating the proliferation of new cells. Fully generalized seizures increase levels of dentate neurogenesis (Scott et al, 1998; Parent et al 1997; 1998). To date, the role of seizure facilitated cell proliferation is un-investigated outside the hippocampal formation. Yet, in the adult brain SVZ neurogenesis exists, and the potential for these cells to deviate from the RMS and converge on the cortex is viable. In the adult primate a few new neurons have recently been isolated in the association cortex. The new cells are thought to have deviated from RMS, migrating through the corpus callosum to enter the cortex (Gould et al, 1999).

The aim of this exploratory work was to determine if mitotic activity could be increased in the forebrain by inducing stimulation (in the absence of behavioural seizures) and behavioural seizures following injury. The presence of the cell proliferation marker, 5-bromo-2'-deoxyuridine (BrDU) will indicate whether any changes in the lesion size are due to the generation of new cells. BrDU, a thymidine analogue, incorporates into the genome of actively dividing cells. This signal can be amplified and visualized by immunohistochemistry. In this experiment we induced frontal cortical injury followed by stimulation or seizures. We chose the frontal cortex because of it's proximity to the RMS, and the plastic response to injury observed at P7-10 in another forebrain

site (Kolb et al, 1998). ECS was chosen as the means to induce seizures because: 1. it reliably produced seizures, 2. we could control the level of stimulation applied, and 3. ECS is less confounding to the injury than electrode implantation or drug administration. Stimulation was applied either above or below behavioural seizure threshold to determine if the behavioural seizure itself, or the stimulation, was required to induce structural changes following injury. We administered seizures over three different periods post-lesion because we had no indication of when stimulation would be most effective, as there was no previous research testing this hypothesis. Therefore our stimulation groups corresponded with the designated Early and Late posttraumatic seizures in the human brain injury literature. We added a group, combining both Early and Late Seizures, which we referred to as the Prolonged Group. We measured the lesion area to determine if the gross differences in relative tissue amount at the lesion site was dependent on the type of stimulation. Nissl staining was conducted to determine the appropriate comparative sites across groups for the cell count analysis. We analyzed the number of new cells in the cortex proximal and distal to the lesion site to determine if there was an increase mitotic activity from stimulation or seizures, in the injured and non-injured cortex. New cells were counted in the cingulum, the body of the corpus callosum, and the SVZ to attempt to address the path and origin of any new cortical cells. We also counted new cells in the dentate gyrus to determine if: 1. ECS stimulation had similar effects on mitotic activity previously found by kindling (Parent et al, 1997), 2. stimulation and cortical injury combined changed the expression of new cells, and 3. there was positive staining for BrDU. A separate group of adult rats had electrodes implanted in the neocortex and EEG recorded to determine whether: 1. convulsive behaviour was correlated with EEG, 2. the below seizure threshold groups had electrographic seizures, and 3. the power of the EEG frequencies changed following lesions, stimulation, and/or seizures.

2. Methods and Materials

2.1 Subjects

Seventy male rats weighing 270-320 grams at the time of surgery were used in this study. All rats were of the Long-Evans hooded strain and were obtained from either Charles River Breeding Colonies (Quebec, Canada) or the University of Calgary Breeding Colony. Rats were maintained on Lab Diet #5001 (P MI Feeds Inc, St Louis, MO) and water ad libitum. Animals were housed individually in clear plastic cages in a colony room maintained on a 12 h on/ 12 h off light cycle. Animals were handled and maintained according the Canadian Council of Animal Care guidelines.

2.2 Treatment groups

The treatment groups included animals that received bilateral frontal neocortex aspiration lesions and those that received sham operation. Sham operated animals received the same treatment as lesion animals, except for bone removal and cortex removal. Each lesion animal was paired with a weight- matched sham-operated animal, and then the pair was randomly assigned to a stimulation treatment group. The three stimulation groups were: 1. No ECS (ear clips applied with no current passed), 2. Stimulation below behavioural seizure threshold, or 3. Stimulation above behavioural seizure threshold. The animals were further sub-divided into groups according to the time period that the stimulation was administered following surgery, and were designated as Early, Late and Prolonged Groups. In the early group, stimulation treatment commenced 6 hours post surgery, and they received a total of 4 stimulations at 48 hour intervals. In the late group, stimulation treatment did not commence until 8 days post surgery and the rats received a total of 4 stimulations at 48 hour intervals. The prolonged group also received stimulation

treatment commencing 6 hours post surgery, 8 stimulations were administered, at 48 hour intervals. The assignment of rats to different treatment groups is illustrated in Figure 1. In future discussion, the use of the term "untreated group" refers to animals receiving sham surgery and no stimulation. While, "intact" brains refers to any group that does not receive a surgical lesion.

2.3 Surgical procedure

Animals were anaesthetized with ketamine:xylazine (85:15), 0.8 ml/kg, injected intramuscularly. Lidocaine 2% (Austin, Joliette, QA), a local anesthetic, was administered subcutaneously at the incision site. The incision site extended approximately 10 mm on top of the head, centered between the eyes and ears. Animals were placed in a stereotaxic apparatus to maintain stable positioning of the skull and a horizontal plane between bregma and lambda. A dissecting microscope (Zeiss,Stemi 2000) was mounted above the stereotaxic apparatus. The microscope was utilized during marking of the skull surface coordinates, drilling, and cortical aspiration. A ruler and waterproof marker pen were used to mark the perimeter coordinates of skull area to be removed. Bone was removed by scoring with a hand drill (Sears, Craftman) with a 1 mm bit. A 2 mm by 5mm region of the bone was removed bilaterally. Bone removal included the area within +1.0 mm and +3.0 mm lateral to bregma, beginning at the level of bregma and extending to 5.0 mm anterior of bregma (Figure 2). The area of bone removed defined the boundary of the cortical lesion. The cortical surface was irrigated with .1 M phosphate buffered saline (PBS) to remove bone particles. The dura was cut with a #11 scalpel and peeled back with #5 forceps. The underlying cortex was aspirated down to the white matter using an 18-gauge cannula attached to a vacuum pump.

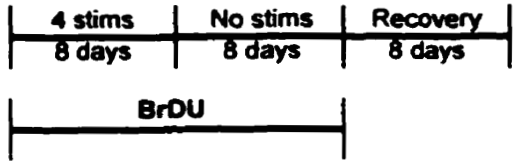
Figure 1. Treatment groups and experimental design.

There are three seizure treatment groups: Early, Prolonged and Late seizure. Within these groups animals received one of two surgery conditions and one of three stimulation conditions: Sham Surgery - No ECS, Bilateral Lesion - No ECS, Sham Surgery - ECS below seizure threshold, Bilateral Lesion - ECS below seizure threshold, Sham Surgery - ECS above seizure threshold, or Bilateral Lesion - ECS above seizure threshold. The box displays the number of animals per group (n=5). * the same animals served as controls across Early, Prolonged and Late treatments (A). (Not shown is n=12 for the electrophysiological control study.) The experimental design of seizure and BrDU administration (B) for each of the three seizure treatment groups. In the early group, stimulation was administered D1 to D8. In the prolonged group, stimulation was administered D1 to D16. In the late groups, stimulation was administered D8-D16. All animals in all groups were perfused on D 23. BrDU was administered from D1-D16 in all groups.

A **B**

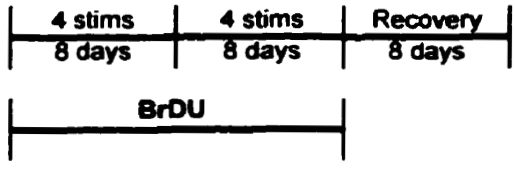
Early Stimulation (4 stimulations, Day 1-Day 8)

	Sham Surgery	Bilateral Lesion
No ECS	5*	5*
ECS below seizure threshold	5	5
ECS above seizure threshold	5	5



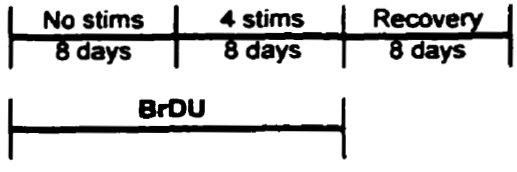
Prolonged Stimulation (8 stimulations, Day 1-Day 16)

	Sham Surgery	Bilateral Lesion
No ECS	5*	5*
ECS below seizure threshold	5	5
ECS above seizure threshold	5	5



Late Stimulation (4 stimulations, Day 8-Day 16)

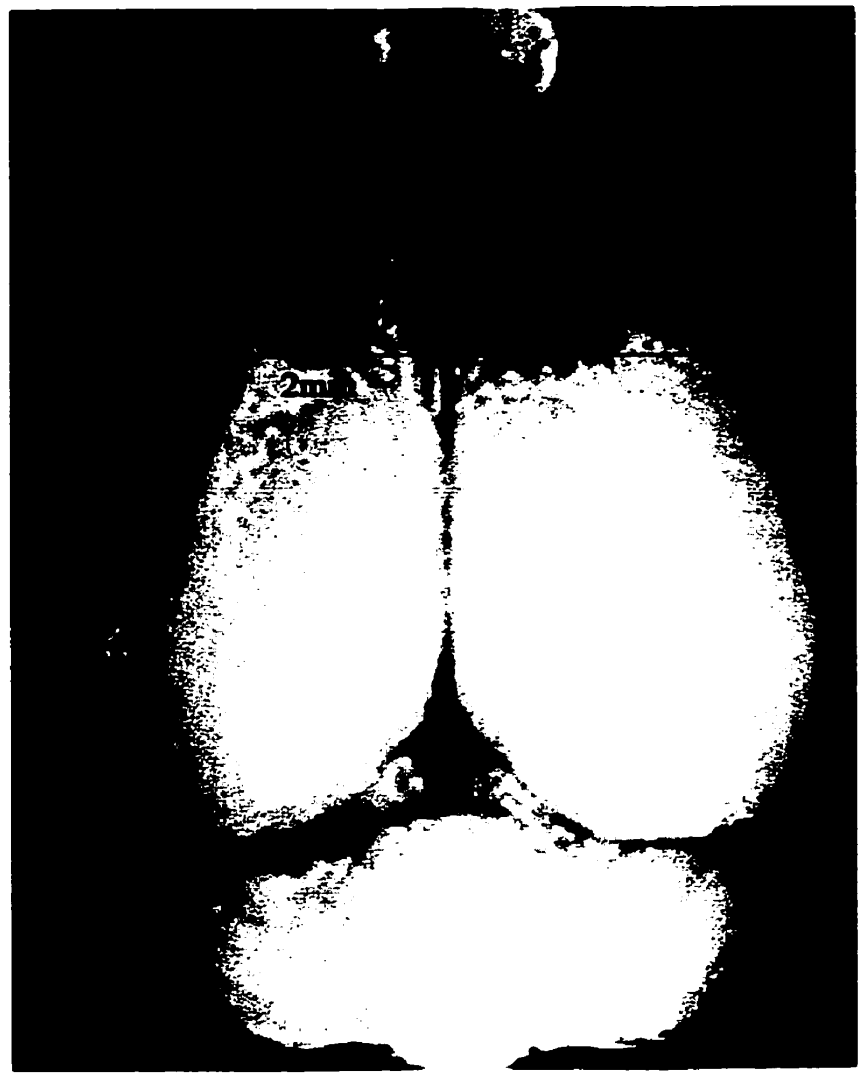
	Sham Surgery	Bilateral Lesion
No ECS	5*	5*
ECS below seizure threshold	5	5
ECS above seizure threshold	5	5



* The same animals served as No ECS controls for Early, Prolonged and Late Stimulation.

Figure 2. Dorsal view of the whole rat brain.

The position of the cortical lesion immediately after the bilateral aspiration lesion. A 2 mm x 5 mm area of the skull was removed bilaterally and the cortical area within this boundary aspirated. The horizontal dotted line denotes bregma. Actual width of the image is 17.5 mm.


2mm

The incision was closed with 3.0 silk. The animals were administered, subcutaneously, 1cc of Lactated Ringers (Cutter Laboratories, CA) to facilitate recovery from anesthetic, and allowed to recover 6-8 hours from anesthetic before the first ECS treatment.

2.4 Labeling of mitotically-active cells

Animals were injected i.p. with 60 mg/kg of 5-bromo-2'-deoxyuridine (BrDU) (Sigma B-5002) in 0 .0035 M NaOH. Fresh BrDU solution was mixed prior to each injection session. BrDU readily mixed into solution at 12 mg/ml following three minutes of stirring and was warmed to room temperature before injecting. Every animal received BrDU injections twice daily for 16 days. The two daily injections were administered between 0900-1100 and 2000-2200 hours. Animal body weights were recorded every second day and the BrDU doses were adjusted accordingly for the new weights. Animals were sacrificed eight days following the last BrDU injection.

2.5 Stimulation

Electroconvulsive shock (ECS) was administered through padded alligator clip electrodes attached to the ears. Alligator clips were wrapped in gauze dressing and soaked in saturated NaCl solution. The electrical stimulation was generated by a DC power supply (Grason-Stadler,model E1100DA) and delivered through a shock generator (Grason-Stadler, model 700). Every animal was connected to the ear clips at 48 hour intervals during the 16-day experimental period, although stimulation was only administered on days indicated by the type of experimental treatment (early, late or prolonged stimulation). Animals in the Above Seizure Threshold group

were stimulated at an intensity of 40 mA for 0.2 seconds. If the animals in the Above Seizure Threshold group did not maintain a behavioural seizure for 4 seconds, the animals were re-stimulated at 40 mA for 0.3 seconds. Animals in the Below Seizure Threshold group were stimulated at 0.4 mA for 0.2 seconds. The No Stimulation group had the alligator clips applied to the ears without passage of current. Stimulation treatment took place at the same time each day, 30 minutes following the morning BrDU injection.

2.6 Electrophysiology

Neocortical activity was monitored in twelve additional rats to determine whether: 1. convulsive behaviour was correlated with EEG, 2. the below seizure threshold groups had electrographic seizures, and 3. the power of the EEG frequencies changed following stimulation, seizures and/or lesions. Twelve rats, additional to the original seventy animals, age-weight-and-sex matched to the BrDU prolonged stimulation group described above were used in this experiment. Additional animals were used for this part of the experiment because electrodes were implanted to record neocortical activity. The electrode was implanted immediately after the aspiration lesion. All animals in the electrophysiology study were handled and maintained as described above.

For the electrophysiology groups, a lesion surgery or sham surgery was made as described above. While the animal was still under anesthetic from the lesion surgery, a twisted wire bipolar electrode, prepared from Teflon-coated stainless steel wire (A-M Systems) was chronically implanted into the neocortex. The electrode tips were separated by 1.0 mm to span the cortical layers. The electrode was positioned 1 mm posterior to the lesion site (1 mm posterior to

bregma), on midline. A ground lead was soldered to a jeweler's screw and secured to the skull. The electrode and ground lead were connected to gold-plated male amphenol pins and inserted into a 9-pin McIntyre connector plug that was mounted to the skull with dental cement, anchored by three additional screws. Electrical recordings commenced 6 hours post-behavioural recovery from surgery. EEG was recorded before, immediately after, and an hour after ECS administration.

All recordings were conducted in a Faraday cage, 88 x 66 x 48 cm in diameter. A cable connected the electrode to the recording equipment. The EEG was analog filtered at half amplitude below 0.3 HZ and above 100 Hz and amplified using a Grass Neurodata Acquisition System (Model 12). The analog signal was recorded on paper (Grass polygraph-model 7) and VHS tape. The VCR recording digitized (Datawave 12-bit converter) and sampled at a rate of 512 Hz.

The EEG activity was screened for movement artifact and submitted to a frequency analysis. To determine the relative strength of all frequencies present in the EEG signal, a Fast Fourier Transformation (FFT) was performed. The FFT was performed using Datawave software, on 4 seconds of artifact-free EEG, recorded immediately before each stimulation. The Day (D) 1 and D 16 EEG data were analysis for each animal. The sampling parameters were chosen to provide 0.25 Hz resolution up to 30 Hz in the frequency domain.

2.7 Perfusion

Eight days after BrDU injections ended the animals were deeply anaesthetized with 3.0 ml/kg Sodium Pentobarbitol (Somnotol) and transcardially perfused with 40 ml of phosphate buffered saline (PBS) followed by 100 ml 4% paraformaldehyde in 0.1 M PBS. The fixative was administered over approximately 2 minutes using a perfusion pump (Vera Varistaltic Pump Plus, Manustat), attached to tubing and an 18 gauge needle which entered the left ventricle. The brains were removed from the skull, weighed and the dorsal surface photographed. The brains were post fixed in 4 % paraformaldehyde in 0.1 M PBS for 24 hours. They were then cryoprotected in 30% sucrose in PBS for at least 24 hours before sectioning.

2.8 Histology and BrDU immunohistochemistry

The brains were frozen using dry ice and cut in 50 μm sections in the coronal plane on a sliding microtome. For each brain, every section was collected and placed in a tray with twelve wells containing PBS. The sections were serially placed in the twelve wells. Each well of tissue was transferred into 1 ml of cryoprotectant solution (Appendix 1) and stored in a freezer at -20°C. The BrDU immunohistochemistry methods from Kempermann, Kuhn and Gage, (1997) were used, with modification to the pre-antibody treatment and primary antibody incubation periods. The BrDU processing was conducted in batches of 4 brains. Sections processed for BrDU were incubated in 50 % formamide/ 50 % 2 X SSC buffer at 65 degrees Celsius for 2 hours, rinsed in 2 X SSC and incubated in 2 N HCl for 30 minutes at 37 degrees Celsius, to denature the DNA, then rinsed in PBS. The sections were treated with 30 % H_2O_2 diluted 1:20 in PBS for 15 minutes to reduce background staining and washed (2 X 10 minutes) prior to the antibody incubation. The sections were incubated at 4^0 C for 48-72 hours in BrDU antibody (Boehringer

Mannheim, mouse monoclonal) diluted 1:400 in PBS containing 0.1% Triton-X-100 (PBS-plus) and 3% sheep serum. Following primary incubation, the sections were washed (3 x 10 minutes in PBS-plus before treatment with the secondary antibody. The secondary antibody used was peroxidase-conjugated AffiniPure donkey anti-mouse IgG (Jackson, PA) diluted to 1:500 in PBS for 24 hours at 4^0 C. Sections were washed in 0.05 M Tris buffer solution before the chromagen reaction step containing Ni-DAB in Tris-buffer (Appendix 1). In negative control sections the primary antibody was omitted. Sections were mounted on gelatin subbed slides, dehydrated in ascending ethanols followed by xylene and then cover slipped with Permount.

2.9 Double labeling immunohistochemistry

Prior to antibody incubation, the sections were pre-treated as above, for BrDU immunohistochemistry. The sections were incubated for 48 h at 4^0 C in a solution containing: rat monoclonal antibody to BrDU (Boehringer Mannheim, 1:1000) and mouse monoclonal antibody to NeuN (Chemicon,1:500), diluted in 0.1M PBS + 0.3% triton + 3 % normal, goat serum. The sections were then rinsed (3 X 10 minutes) and incubated in anti-rat IgG fluorescein iso thio cyanate (FITC) (Jackson, 1:500) and anti-mouse lissamine (LRTC) (Jackson, 1:500) for 72 hours at 4^0 C. Sections were rinsed in PBS, mounted on gelatin-coated slides and cover slipped using aqueous mounting medium (Appendix 1).

2.10 Nissl Stain

One brain per group was blindly chosen for nissl staining, of which one set of sections in 600 μm intervals, was stained. Mounted sections were immersed in the following solutions: water (1 minute), 70% EtOH (1 minute), 95% EtOH (5 minute), 100% EtOH (10 minute), Xylene (20 minute), 100% EtOH (5 minute), 95% EtOH (1 minute), 50% EtOH (1 minute), 1% Cresyl Violet (pH with acetic acid) (15 minute), water (dip), 100% EtOH (until differentiation of white matter and grey matter), xylene (10 minute). The slides were then coverslipped with permount.

2.11 Lesion Area Quantification

The lesion area was measured in the coronal plane located mid-lesion, 2.2 mm anterior to bregma. Using Scion NIH Image software, the lesion cavity was outlined and the area calculated in μm^2, and designated as negative area. For each section, any area of tissue extrusion was outlined and the positive area calculated (Figure 3). The net lesion area (Nla) measure is equal to the cavity region (negative) plus the protruding region (positive). Nla = Negative Area + Positive Area. For each brain the net area was calculated and submitted to ANOVA for statistical analysis.

2.12 BrDU expression by site and Quantification

A priori, cell count sites were selected from a rat brain atlas (Paxnos and Watson, 1986). The cell count sites were visualized using a light microscope (Zeiss, axioscope 2) with a camera (COHU high performance CCP) attached. The image was projected to a computer monitor. Using Scion NIH Image software, cells were counted and sized in each site discussed below. The

thresholding tool was used to select all stained areas. This thresholding could be manually adjusted to control for differences in back ground staining. However, to further eliminate background noise, a minimum particle size was set at 20 pixels. Brains were omitted from the count if they did not have labeled cells in the dentate gyrus and olfactory bulb (sites designated a priori as positive staining controls). For each brain, a count site was omitted if a tear in the tissue interfered with the cell count or there was excessive background staining that would interfere with the software generated count. Minor background staining deemed to not be BrDU-labeled cells (based on shape at higher magnification), was omitted from the analysis by using the eraser tool prior to the count. Using thresholding, each discrete object, which may have included 1 or more cells, was selected then assigned a number and area measurement (Figure 4).

Figure 3. Coronal section demonstrating the methods used to calculate the size of the lesion area.

For each brain the cavity area was outlined. This area calculated and referred to as negative. The area of protruding tissue was outlined. The area calculated and referred to as positive. The negative and positive regions were summed. In this photograph the positive region exceeded the negative, therefore the total amount of tissue at the lesion was positive for this brain.

Scale bar= 250 μm

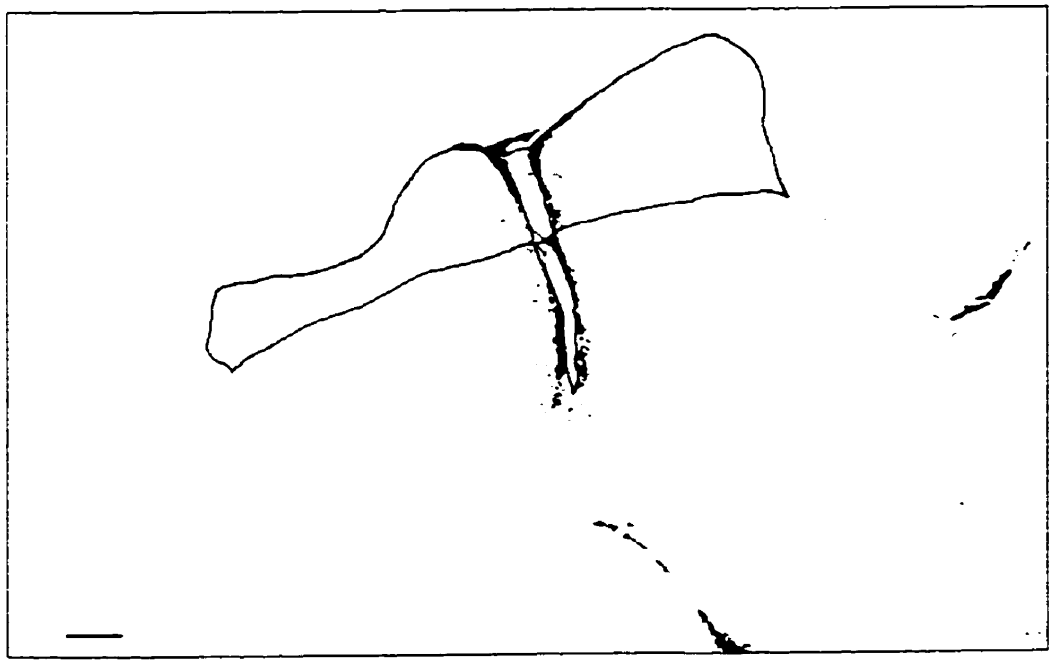
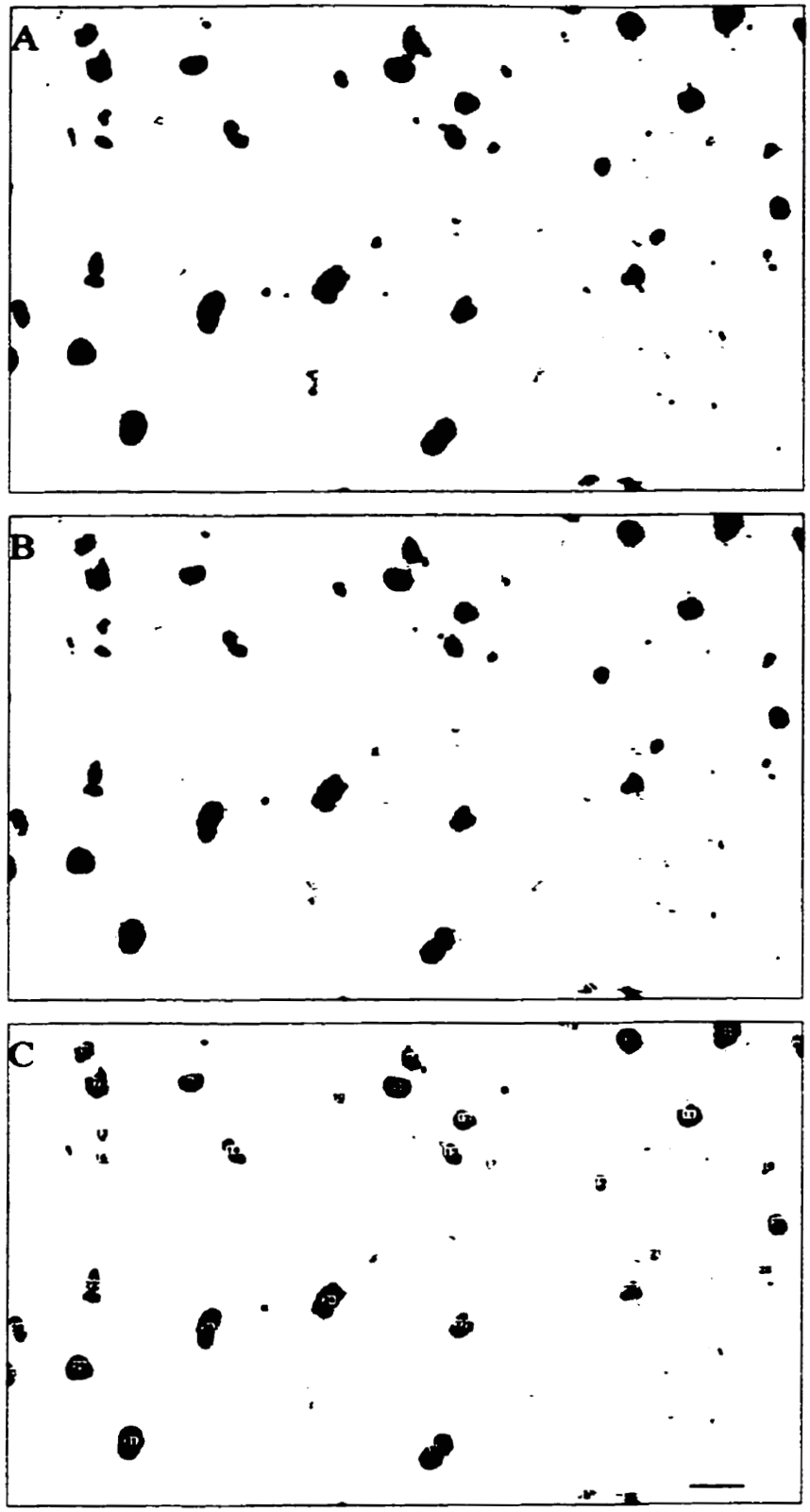


Figure 4. Methodology for estimating the cell number.

A cell count site was digitally captured (A). The thresholding tool was used to select all stained areas (B). This thresholding could be manually adjusted to control for differences in back ground staining. Any particles that did not appear to be cells were eliminated from the count. Stained areas smaller than 20 pixels were also not included, by setting a minimum particle size. Each discrete object, which may have included 1 or more cells, was assigned a number (C) and area measurement (not shown). The area values were summed, to yield the total stained area. The total stained area was divided by the average cell size to generate a cell count estimate for each site.


A
B
C

The area values for each site were saved to a computer hard drive (Macintosh, G3). The area values were later summed to yield the total stained area for each site within a coronal section (Appendix 2). The total stained area was divided by the average cell size to generate an estimate of the total number of cells for each site. The average cell size was selected by randomly sampling 5 cells per site in each brain. The cell size of 20 square μm was consistent across brains. To validate the software generated count, 3 brains were manually scored. The automated analysis was slightly more conservative possibly because the image was captured in one focal plane and/or a conservative cell size was used. This analysis was done blind to group membership.

2.12.1 Cortex

To ensure that the cortical count sites were selected from the same cell layers between brains with cortical protrusion and those without, one brain per group was nissl stained and the cell layers determined. A priori, it was decided that only coronal sections through the extent of the lesion would be analyzed. The coronal planes counted were from 3.2mm, 2.8 mm, 2.2mm, 1.4mm, 0.8 mm and .2mm anterior to bregma (Paxnos and Watson, 1986). The 3.2mm and .2mm counts were omitted from the analysis (prior to determining group membership) because not all brains had a lesion scar in the extreme coronal planes. Only coronal planes ranging from 2.8mm to 0.8mm were included in the analysis. In each cortical section, 8 sites were analyzed per hemisphere. Each count site was a total rectangular area of 65,100 μm^2 (310 μm long X 210 μm wide). The camera was positioned so that the areas were captured parallel to the lesion scar. The eight cortical sites included the following locations in each section and brain: 1. Four sites immediately adjacent to the lesion scar (Proximal Sites) in cortical layers II/III medial of the

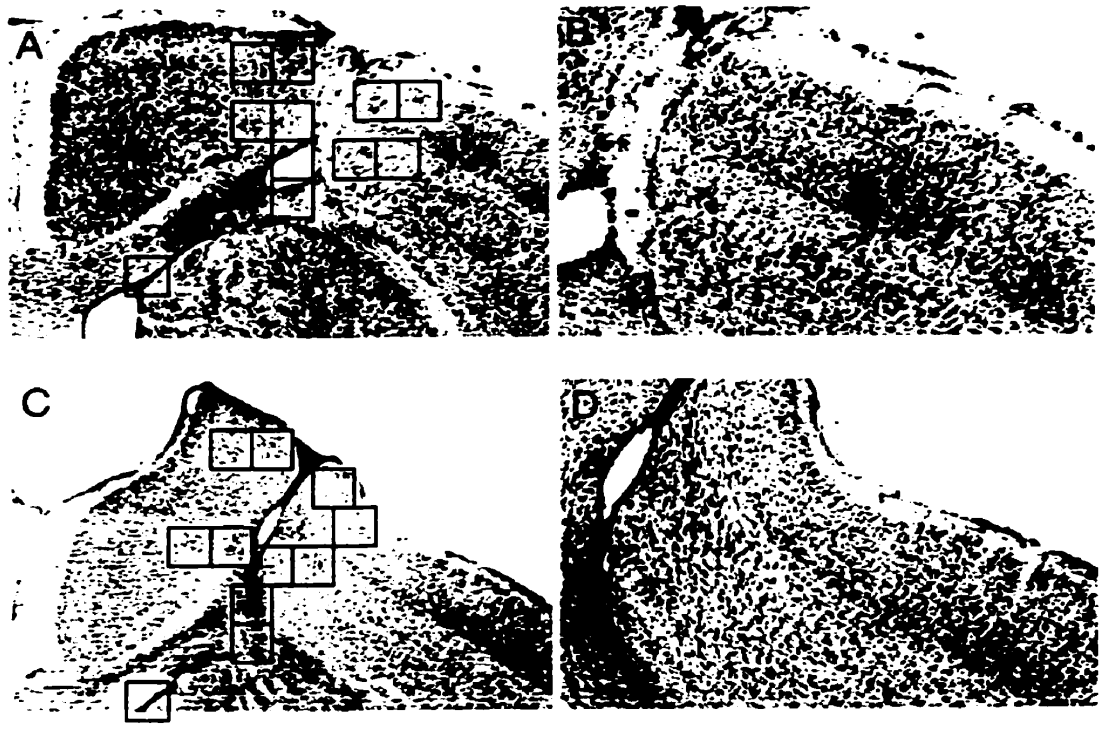
lesion scar, b. cortical layers II/III lateral to the lesion scar, c. mid-way between the corpus callosum and cortical surface medial to the lesion scar, d. mid-way between the corpus callosum and cortical surface lateral of the lesion scar, and 2. Four sites distal to the lesion scar. All four distal sites were positioned immediately beside a proximal site, so that the distal sites were all 310 μm away from the lesion scar (Figure 5). The camera was rotated, so that the lesion scar was always perpendicular to the bottom edge the screen. This rotation was made to ensure that all images were captured relative to the edge of the lesion scar. Measures for right and left hemispheres were averaged within a brain before being included in the statistical analysis, to maintain n=1 for each brain.

2.12.2 Corpus Callosum

Images were also captured of the cingulum and the body of the corpus callosum in all coronal planes listed above. The total area captured was the same as above. The cingulum placement was immediately below the lesion scar and included some Layer VI cortex. The placement within the corpus callosum was dorsal and ventrally centered within the structure, and medial-lateral position was directly below the cingulum site. Estimates of cell number were generated as described above. Group membership was revealed for the statistical analysis. The mean number of cells was determined per group, and a 1-Way ANOVA was conducted across all groups for each site. Upon a significant F-test, a follow-up ANOVA was conducted across Early, Prolonged and Late groups, if there was no difference in the number of cells for that site they were combined for further follow-up t tests. An alpha level of 0.05, bonferroni adjusted (0.05/ the number of follow-up tests), was used for each site. And, the more conservative, 2-tailed t-test was used for every follow-up t-test.

Figure 5. Nissl stained sections displaying the cell count sites.

Nissl stained coronal sections from a lesion and non-stimulated brain in (A). Lesion alone did not distort the cortical cell layer surrounding the lesion. A lesion and above seizure threshold stimulation (C-D) reliably resulted in the cortex expanding into the area above the brain. Comparative areas for the cell count were chosen based on the Nissl staining. The boxes indicate the BrDU-labeled cell measured areas. The boxes are not precisely to scale, each box represents a 310 x 210 μm area. The dl SVZ area analyzed was 150 x 100 μm. The boxes are also not precisely oriented in the figure, because during analysis the photographs were rotated to capture all areas parallel to the lesion scar. The coronal sections (A,C) are expanded (B,D) to better see the cell layers. Scale bars in C (250 μm) refers to A as well and scale bar in D (250μm) refers to B as well.


A
B
C
D

2.12.3 SVZ

For the dorsal lateral subventricular zone (dl SVZ), every 600 μm from the most anterior extent of the lateral ventricles (2.2 mm) to the most posterior extent of the lesion scar (0.2mm anterior to bregma), was included in the analysis. The area captured at the dl SVZ was 15,000 μm^2 (150 μm long X 100 μm wide). The area captured at the dl SVZ was smaller than the cortex and corpus callosum, so as to include less surrounding cortex and corpus callosum and there was more background staining in the dl SVZ than cortex, so the count was obtained under a higher magnification, to increase count accuracy for this site. The camera was rotated to orientate the dl SVZ the same across sections. The image was positioned so that the ventricular space was just visible at the ventral edge of the image and the dl SVZ was vertical in the middle of the image.

2.12.4 Dentate Gyrus

Three sections, spaced 200 μm apart, starting where the dentate had a complete dorsal and ventral blade, were counted per animal, blind to group membership. Cells were manually counted, under the light microscope, at 40 X magnification for the objective lens and an additional 2.5X on the eye piece. The following counting rules applied: 1. Amorphous shapes were counted as one cell, and 2. Labeled nuclei greater one cross-hair distance away from the dentate cell layer were excluded from the analysis. Group membership was revealed for the statistical analysis. The mean number of cells was determined per group, and a 1-Way ANOVA was conducted across all groups. Upon a significant F-test, follow-up tests were conduced, using an alpha level, bonferroni adjusted for the number of follow-up tests (0.05/ the number of follow-up tests).

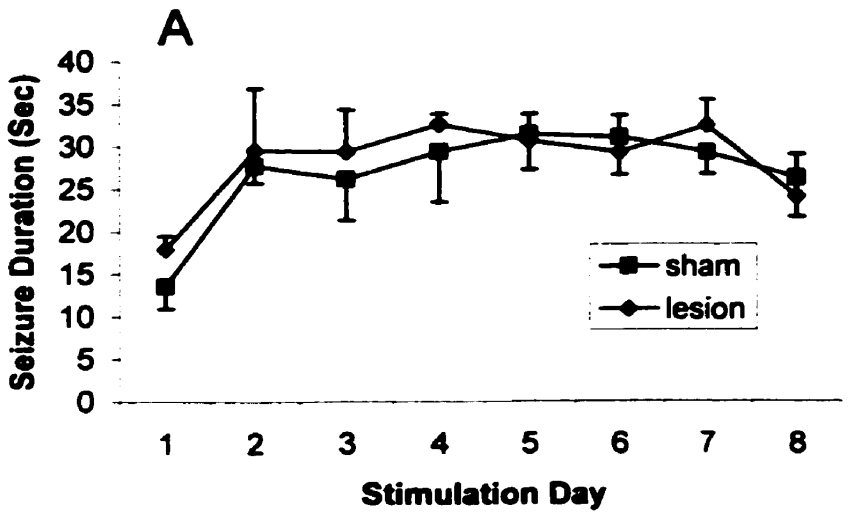
3. Results

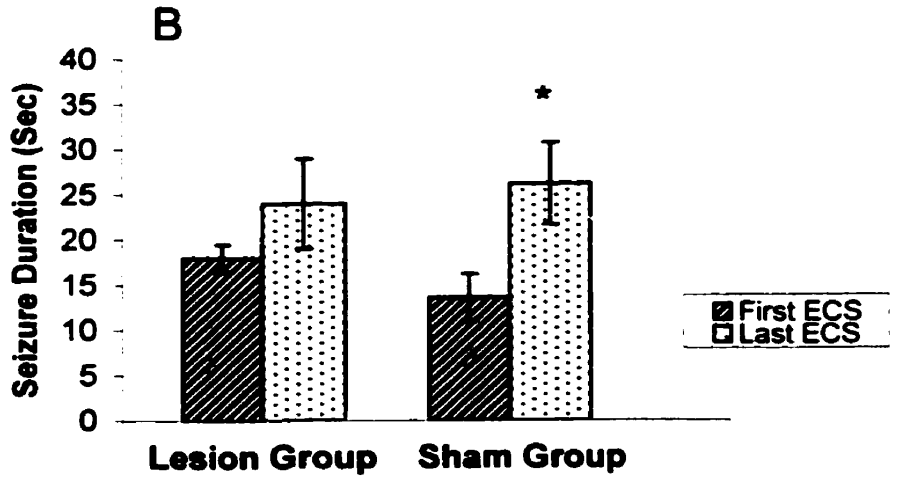
3.1 ECS seizures

All animals receiving above threshold stimulation had behavioural seizures that were stereotypical and similar regardless of the (sham or lesion) surgery condition. All animals receiving above seizure threshold stimulation achieved fully generalized seizures in every stimulation session. Upon stimulation the animal lost postural control and landed in a semi-pronate position. Immediately thereafter the animal would curl into a rigid ball, with limbs retracted. This behaviour lasted 1-2 seconds and was accompanied by pronounced muscle rigidity. Full tonic limb extension, head and spinal arching, tail erection and circling, and clonic twitching of the limbs occurred for approximately 10 seconds. The clonic limb twitching rapidly progressed into bilateral hind limb clonus, and occasionally, but not consistently across all animals and days, bilateral forelimb clonus was observed. The bilateral hind limb clonus was maintained for 5-10 seconds in duration. Following the behavioural activity, the animals would remain immobile for 1-4 minutes, except for regular, deep breathing and occasional teeth chattering in some animals. In the prolonged seizure groups the total duration of the behavioural seizures ranged between 10 -20 seconds for the first stimulation and 20-33 seconds for the last (8th) simulation. The duration of the behavioural seizure progressed over time in the sham surgery condition ($t(8)=2.398, p=.022$, 2-tailed) but there was no difference between the duration of the first and last seizure treatment for the lesion surgery condition (Figure 6).

Figure 6. Change in behavioural seizure duration.

The behavioural seizure duration for lesion and sham treatment groups, in the prolonged stimulation group. There is no difference in the mean (± SEM) seizure duration between the two surgery condition across all days (A). The mean (± SEM) seizure duration from Day 1 and Day 16 (B) progressed in the sham surgery condition. *indicates significance ($p<0.05$) from Day 1 to Day 16 for the sham surgery group.


A
Seizure Duration (Sec)
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
Stimulation Day
sham
lesion


B
Seizure Duration (Sec)
40
35
30
25
20
15
10
5
0
*
Lesion Group
Sham Group
First ECS
Last ECS

The animals receiving stimulation below seizure threshold had no observable behavioural seizures. The absence of behavioural seizures remained the case even with repeated stimulation. Immediately upon stimulation, all animals receiving below threshold stimulation would momentarily freeze and vocalize (a squeak), for approximately 1 second. Following ear clip removal this group appeared to be slightly more active than non-stimulated control rats, where ear clips were applied without passing of current.

3.2 EEG Record & FFT Analysis

Seizure afterdischarge (AD) activity was only seen in the above threshold groups and correlated with the behavioural seizure. However, the end point of the AD continued for 1-2 seconds beyond completion of the behavioural seizure. The EEG activity recorded during the post-seizure immobility period was characterized by high amplitude and high frequency, as well as the presence of post-seizure interictal spikes. This post-seizure EEG activity was observed for hours after stimulation treatment (Figure 7).

3.2.1 No stimulation groups

The EEG for the non-stimulated, sham surgery group was consistent between D1 and D16. The FFT revealed maximum power between 5-8 Hz range (Figure 8A-D). In the lesion brains, the EGG changed over time. The FFT frequency range and power changed between D1 and D16. The 5-8 Hz range had 4 times more power on D 16, and the 1-2 Hz and 4-10 Hz had more power (Figure 8E-H).

Figure 7. EEG recorded before, during and after ECS.

EEG was recorded before ECS stimulation (A), during the afterdischarge activity (B), one minute after stimulation (C) during post-seizure immobility, and one hour after stimulation (D). The abbreviations above the after discharge activity correspond to the commencement of that behaviour: ART. = Stimulation Artifact, BL. = Balling, EX.=Extension, BHC= Bilateral hindlimb clonus, IM=Immobility. Total duration=76 sec.

Maximum pen deflection=approximately 3 mV.

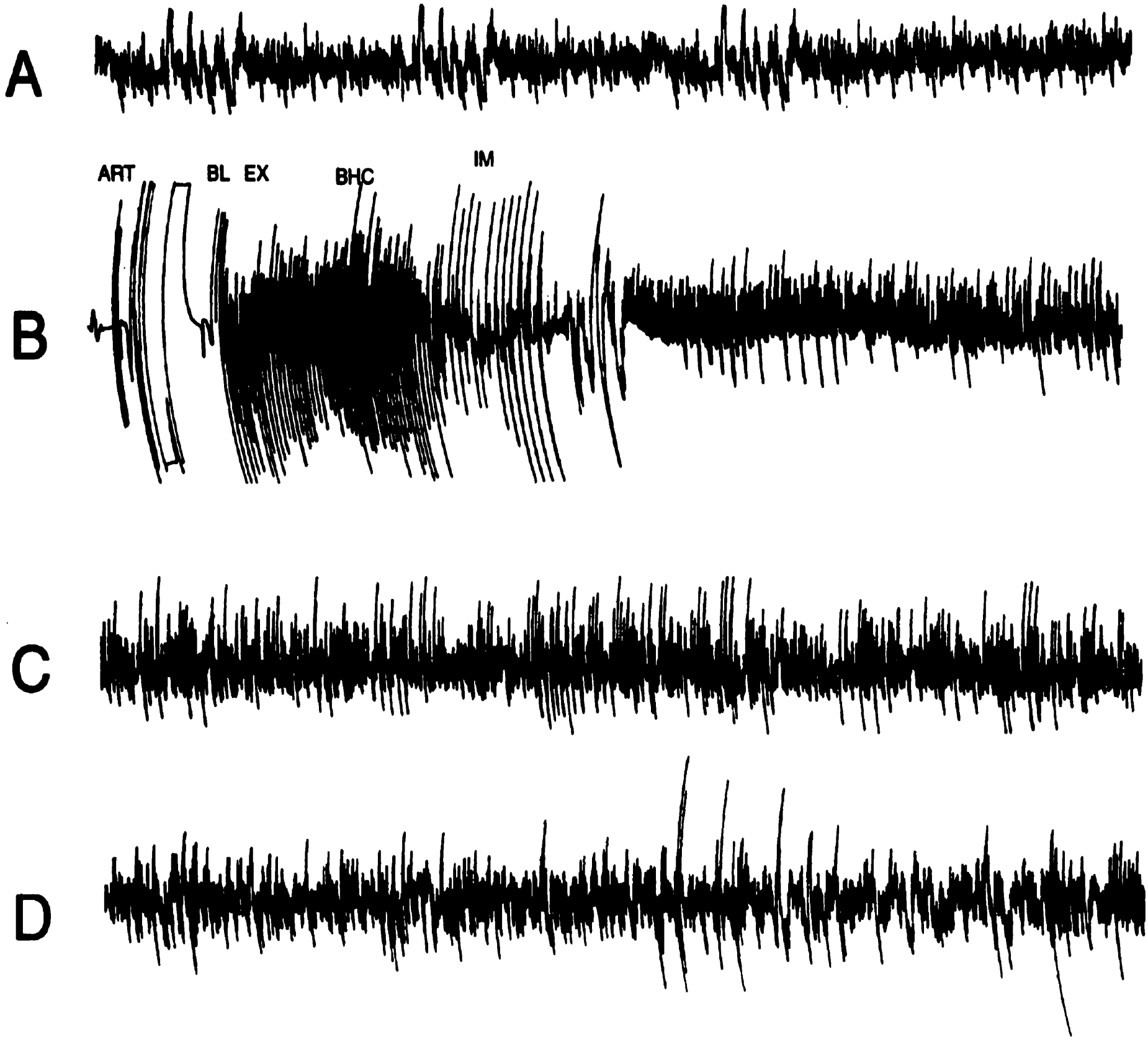

A
B
ART
BL EX
BHC
IM
C
D

Figure 8. EEGs and FFTs from the sham and lesion groups receiving no ECS.

Sham surgery condition (A-D).The EEG for the non-stimulated, sham surgery group was consistent between day1 (A) and day16 (C) post-lesion. The FFT revealed maximum power between 5-8 Hz range (B, D). **Lesion surgery condition** (E-H). In the lesion brains (E-H), the EGG changed over time (E,G). The FFT frequency range and power changed between day1 and day16. The 5-8 Hz range had up to 4 times more power on D 16, and the 1-2 Hz and 4-10 Hz had more power (F,H).

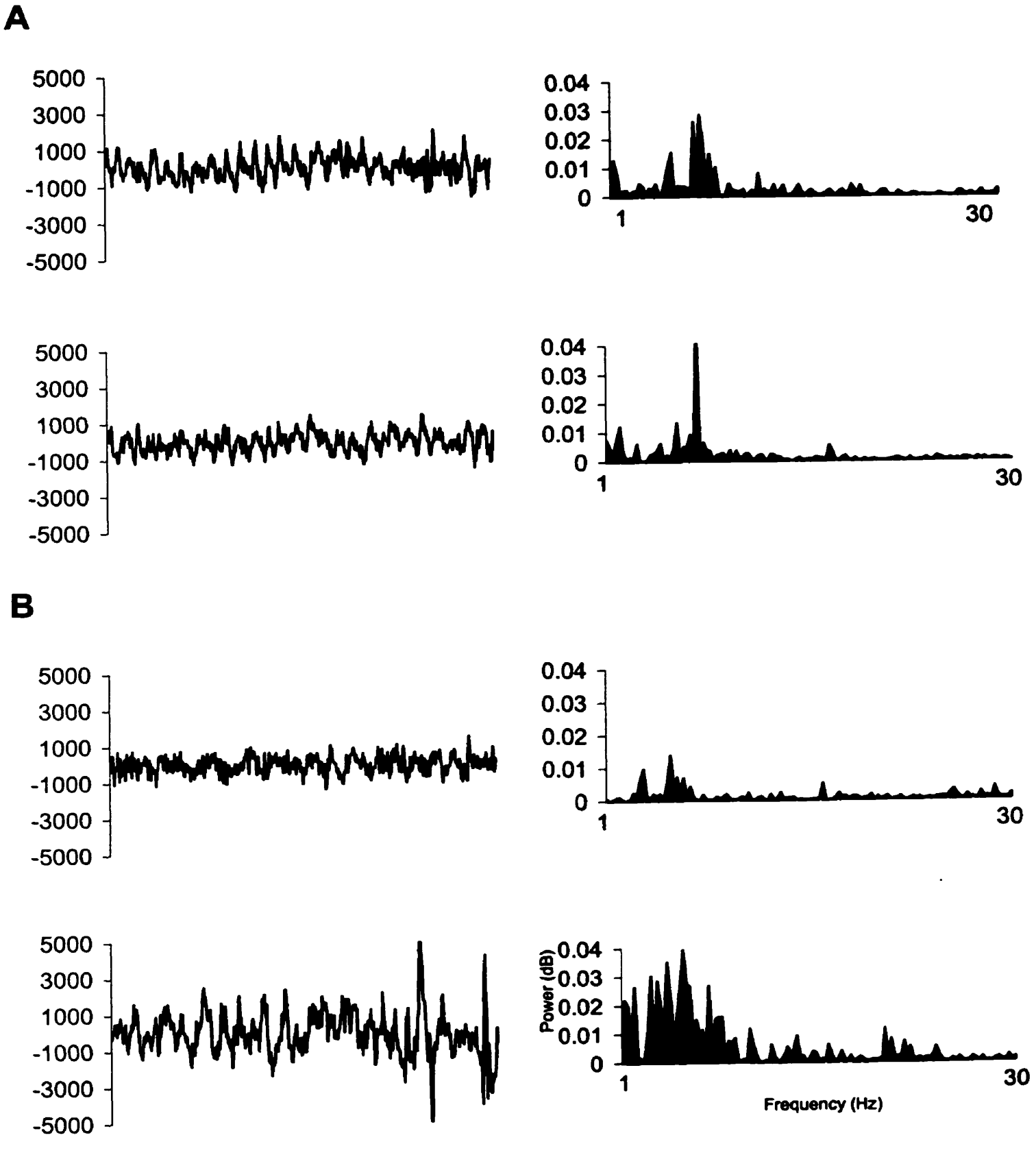

A
5000
3000
1000
-1000
-3000
-5000
0.04
0.03
0.02
0.01
0
1
30
B
Power (dB)
Frequency (Hz)

Figure 9. EEGs and FFTs from the sham and lesion conditions receiving ECS below behavioural seizure threshold.

Sham surgery condition (A-D) The EEG day 1 (A) and day 16 (C) for the corresponding FFTs appear similar following 8 stimulations. The FFT on day1 (B) and day16 (D) are within the same frequency range, between 2- 8 Hz, although the power was 2-3 times greater on day 16. **Lesion surgery condition (E-H)** Day 1 EEG (E) and Day 16 EEG (G) for the corresponding FFT's (F,H). In the lesion surgery condition, the FFT revealed two prominent frequency ranges at 5 Hz and 7 Hz on Day 1. On Day 16, the 5 Hz frequency lost power, but the 7 Hz frequency remained prominent. Additionally, the 2-4 HZ range of frequencies had more power on Day 16 (H).

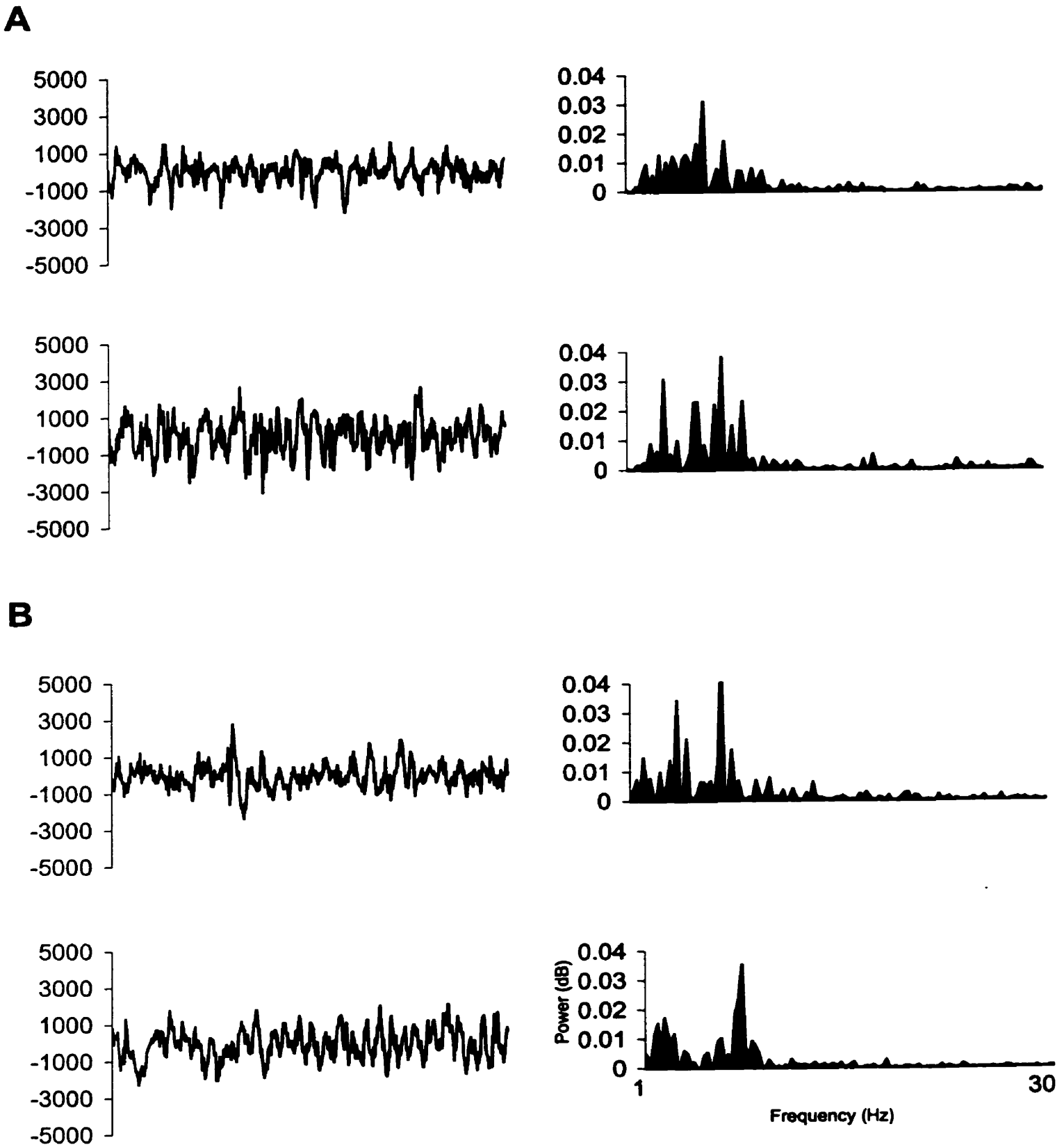
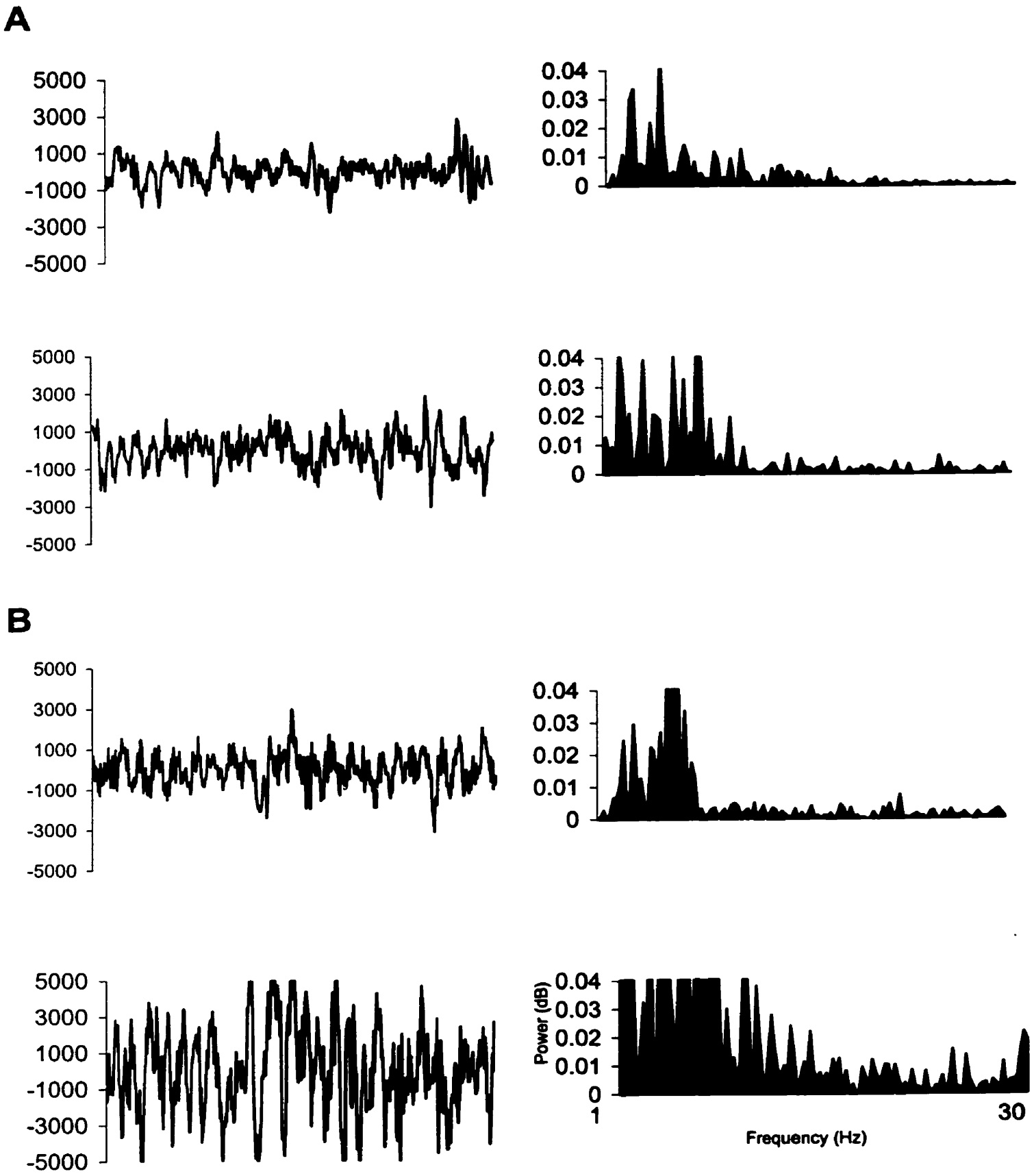

A
5000
3000
1000
-1000
-3000
-5000
0.04
0.03
0.02
0.01
0
B
Power (dB)
1
30
Frequency (Hz)

Figure 10. EEGs and FFTs from the sham and lesion conditions receiving above behavioural seizure threshold stimulation.

Sham surgery condition (A-D) The Day 1 FFT (B) and Day 16 FFT (D) for the corresponding EEG (A, C) revealed power concentrated between 3-8 Hz. On D 16 this same range of frequencies had four times more power. **Lesion surgery condition (E-H)** EEG and FFT Day1, before simulation (E,F) EEG and FFT Day 16, after 8 stimulations (G,H). On day 1 (E,F) the power was concentrated between 3 -8 Hz. On day 16 (G,H) there was additional power in the frequency ranges 1-2 Hz and 8-15 Hz. **Note: There is a scale change in Figure 10 (B, D, F, H) compared to the figures 8-9.**


A
5000
3000
1000
-1000
-3000
-5000
0.04
0.03
0.02
0.01
0
B
Power (dB)
1
30
Frequency (Hz)

3.2.2 Below threshold stimulation groups

In the sham surgery condition, the FFT on D1 and D16 are within the same frequency range, between 2- 8 Hz, although the power was 2-3 times greater (Figure 9A-D). In the lesion surgery condition, the FFT revealed two prominent frequency ranges at 5 Hz and 7 Hz on D1. On D 16, the 5 Hz frequency lost power, but the 7 Hz frequency remained prominent. Additionally, the 2-4 Hz range of frequencies had more power on D16 (Figure 9E-H).

3.2.3 Above threshold stimulation groups

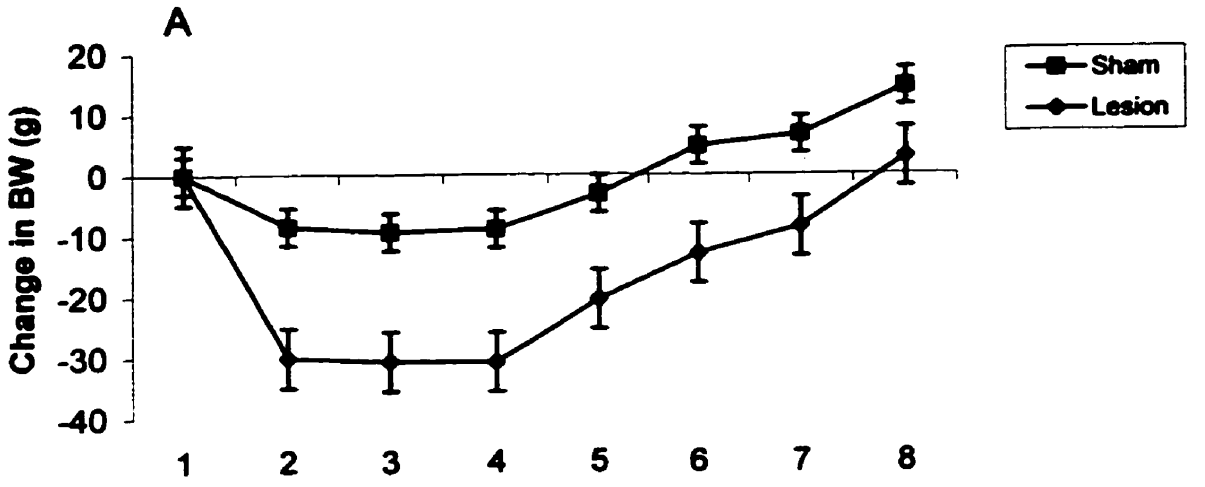
In the sham surgery condition power was concentrated between 3-8 Hz. On D 16 this same range of frequencies had four times more power (figure 10A-D). In the lesion surgery condition, the power was concentrated between 3 -8 Hz. On D16 there was additional power in the frequency ranges 1-2 Hz and 8-15 Hz (Figure10E-H).

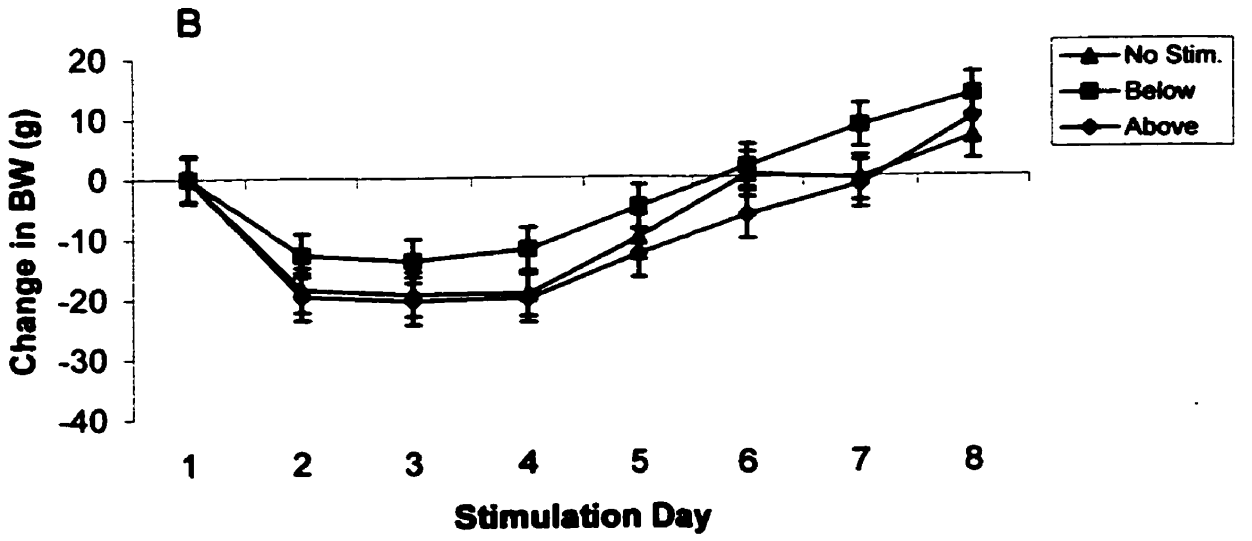
3.3 Body and Brain Weight

For all sham and lesion groups, the general trend for body weight as recorded every 48 hours, was to drop in the first 48 hours. The animals weights stabilized at this lower weight level for 6 days, and then slowly rose across the rest of the days. Fourteen days post surgery, the body weight had recovered to or slightly exceeded the day 1 weights for most groups (Figure 11). There was a significant effect of surgery condition; Lesion groups weighed less than sham groups between the second and eighth measurement ($t(1,12)=2.179$, $p=.0115$, 2-tailed) at the .05 level (Figure 11A). There were no differences in body weight between all of the stimulation groups (Figure 11B). The brain weights were equivalent in all groups.

Figure 11. Effect of surgery and stimulation on body weight.

Body weight was measured prior to each of the 8 stimulation sessions, every 48 hours. The general trend for all groups was to drop between session 2-4, followed by a slow, steady rise. There was a significant effect of surgery on mean (± SEM) body weight (A). The lesion groups lost significantly more weight than the sham surgery animals, across all days ($p<.05$). In all the stimulated and non-stimulated groups (B) the mean (±SEM) body weight dropped equivalently over the first 2-4 days, after which body weight steadily rose at the same rate in all stimulation condition. BW=Body Weight


A
Change in BW (g)
20
10
0
-10
-20
-30
-40
1
2
3
4
5
6
7
8
Sham
Lesion


B
Change in BW (g)
20
10
0
-10
-20
-30
-40
1
2
3
4
5
6
7
8
Stimulation Day
No Stim.
Below
Above

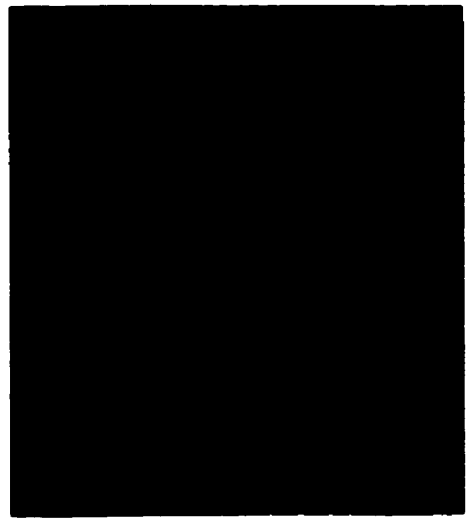
3.4 Lesion Site - net amount of tissue at the lesion site

Different treatment conditions resulted in differences in the amount of tissue at the lesion site. Some brains had tissue protruding into the space above the brain, where the skull had been removed (Figure 12). Brains that received a lesion and no stimulation had a defined cavity and the cortical tissue at the perimeter of lesion did not extrude in to the space above the brain (Figure 12A). Thus, this control group's net lesion area, with minimal variability between animals, was negative. In the group that received lesions and early above seizure threshold stimulation, a small lesion cavity (negative area) was present. However, some tissue surrounding the lesion cavity, unrestrained by skull, protruded beyond the normal cortical boundaries (referred to as positive area). In this group, the summation of an equivalent area for lesion cavity (negative area) and protruding issue (positive area) resulted in a near zero net area. The total amount of tissue was not significantly greater than the non-stimulated group after bonferroni adjustments. In the late and prolonged above seizure threshold groups net amount of tissue at the lesion site was greater than the non-stimulated brains, ($t(11)=-4.237$, $p=0.00139$) and ($t(11)=-3.716$, $p=0.00207$), respectively. In these late and prolonged groups the positive extruding tissue area was far greater than the remaining cavity region, yielding a net positive area, with little variability (Figure 13A).

There was a higher degree of variability seen in the three below threshold stimulation groups than all other groups. The variability was not only between brains of the same group, but between hemispheres of an individual brain (Figure 13B). The overall trend for the hemispheres with protruding tissue was for the lesion scar to extend into the corpus callosum, conversely, when the lesion scars had a net negative area, the scar did not breach the corpus callosum, and this was the case in 72% of the below seizure threshold brains. The net amount of tissue in these groups was not significantly different than the non-stimulated group.

Figure 12. Dorsal View of whole rat brains on day 24 post lesion.

The no stimulation brains (A) had a lesion cavity remaining. The above seizure threshold stimulation brains (B) had a narrower cavity and tissue protruded beyond the normal cortical surface. Actual width of the image is 17.5 mm.



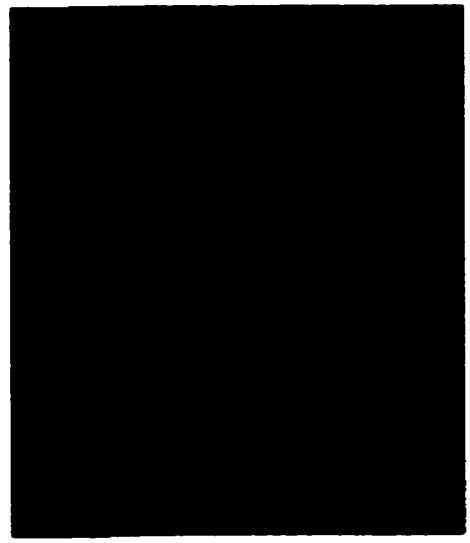
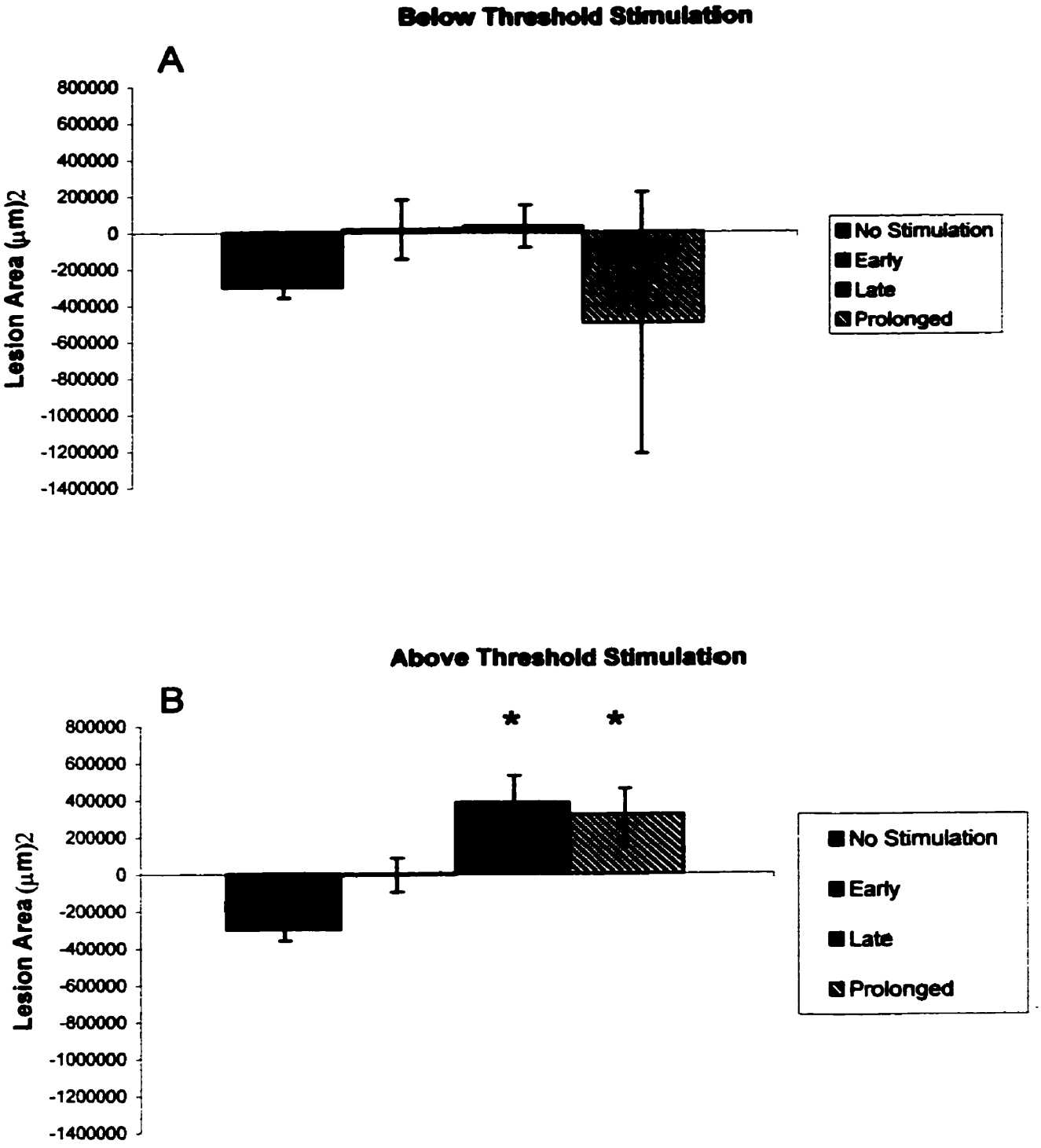


Figure 13. The effect of seizures and stimulation on the amount of tissue at the lesion site.

The amount lesion cavity and cortical protrusion beyond the normal brain surface, varied across stimulation groups. Comparing the mean (+SEM) of the non-stimulated group with below threshold stimulation (A), the means (+SEM) of the below threshold stimulation was highly variable, and not different than no stimulation. Comparing the mean (±SEM) of the non-stimulated group to the above threshold stimulation treatments (B), Prolonged, and Late seizures had an effect on the amount of tissue at the lesion site. Less than zero total tissue area shows that the group mean (+SEM) net amount of tissue is comprised of more cavity than protruding tissue, while a group mean greater than zero displays the reverse. * indicates significantly different, ($p<.01667$) than the non-stimulated control group.


Below Threshold Stimulation
A
800000
600000
400000
200000
0
-200000
-400000
-600000
-800000
-1000000
-1200000
-1400000
Lesion Area (μm)2
No Stimulation
Early
Late
Prolonged
Above Threshold Stimulation
B
*
*
800000
600000
400000
200000
0
-200000
-400000
-600000
-800000
-1000000
-1200000
-1400000
Lesion Area (μm)2
No Stimulation
Early
Late
Prolonged

3.5 BrDU expression by site

For each brain site I addressed the effect of lesion, stimulation and seizure treatments on the number of BrDU-labeled cells in the following order: 1. *The effect of lesion.* The non-stimulated, lesion group was compared to the untreated brains (no lesion and no stimulation group). 2. *The effect of stimulation.* The non-lesioned, below seizure threshold stimulation groups were compared to the untreated brains. 3. *The effect of seizures.* The non-lesioned, above seizure threshold stimulation groups were compared to the untreated brains. 4. *The interaction of lesion and stimulation.* The lesion & below seizure threshold stimulation groups were compared to the lesion & no stimulation group, as well as no lesion & below threshold stimulation. 5. *The interaction of lesion and seizures.* The lesion & above seizure threshold stimulation groups were compared to the lesion & no stimulation group as well as no lesion & above threshold stimulation.

3.5.1 Cortex

Stimulation that either did or did not result in a seizure had an effect on the number of BrDU-labeled cells in the cortex. There was no effect of coronal position; data obtained from all coronal sections (2.8mm, 2.2mm, 1.4mm and .8mm relative to bregma) within each treatment condition and site were not significantly different, therefore they were combined for the analysis. However, to maintain each brain as n=1, the grand mean was calculated across the sections. For both proximal and distal sites, there was no difference in the number of BrDU-labeled cells between medial and lateral aspects of the lesion scar, therefore a total cell count of all four distal sites and all four proximal sites was obtained, thus the total area for both of these sites was (6,5100 μm^2 x 4) 260,400 μm^2. Thus, the total area from which cells were counted in the cortex was exactly four times greater in the cortex than the corpus callosum areas.

Cortical Sites distal to the lesion

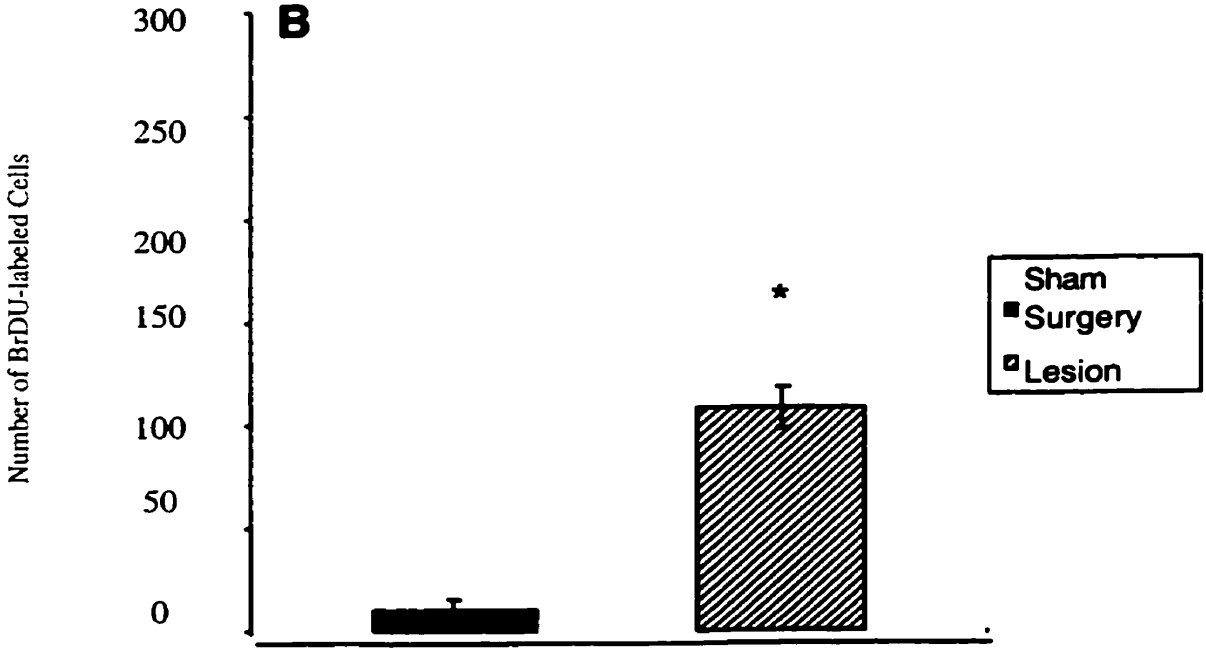
1. *The effect of lesion.* The brains that received a lesion had several times more labeled cells in the cortex (distal to the lesion) than the untreated group ($t(4)=-12.912, p=0.000207$) (Figure 14B) 2. *The effect of stimulation.* Early, prolonged, and late below threshold stimulation in the non-lesioned brain had no effect on the number of BrDU-labeled cells found in the cortical sites distal to the lesion, relative to the normal brain (Figure 14C). 3. *The effect of seizures.* There was also no effect of above threshold stimulation on the non-lesioned brains Figure 14D). 4. *The interaction of lesions and stimulation.* There was a significant effect of below threshold stimulation and lesion ($F(3,12)=5.274$, $p=0.0145$). Therefore follow-up tests were conducted to see which groups differed. There was no difference between the stimulation groups (early, prolonged and late) therefore they were combined for further follow-up tests. There was a significant difference between all the combined below threshold stimulation groups and the non stimulated lesion group ($t(14)=6.018, p<0.0001$) (Figure 14E). A second control group (for the effect of below threshold stimulation on the injured brain) was the below threshold stimulation in the intact brain. There was a significant effect of stimulation on lesion compared to non-lesioned brains, ($t(22)=10.886$, $p<0.0001$). Data not shown. 5. *The effect of lesion and seizures.* Seizures following injury increased the number of BrDU-labeled cells in the distal count sites. There was a significant difference between no stimulation, early, prolonged and late stimulation groups, ($F(3,16)=14.158, p<0.0001$). Early, Prolonged, and Late stimulation groups differed significantly.

Figure 14. The effect of lesions, stimulation and seizures on cortical BrDU-labeled cell counts distal to the lesion.

The distal cortex sites were not different from each other across all treatment, therefore the total number of cells from all four sites in (A) were compared between groups. Boxes denote the areas captured for cell count analysis.

There was a significant effect of lesion (B) compared to sham surgery conditions. For sham surgery conditions (C-D) there was no effect of below (C) or above (D) threshold stimulation In the lesion surgery conditions (E-F), the below threshold stimulation groups (early, prolonged, and late) combined had more BrDU-labeled cells than the non-stimulated control (E). The early and late above threshold treatment also significantly increased the number of new cells (F) compared to the non-stimulated control. * indicates significance ($p<.0167$) when all stimulation (early, prolonged and late) groups were combined. * indicates significance ($p<.0125$) when the stimulation group were not combined. Scale bar in A (250 μm).


A


B
300
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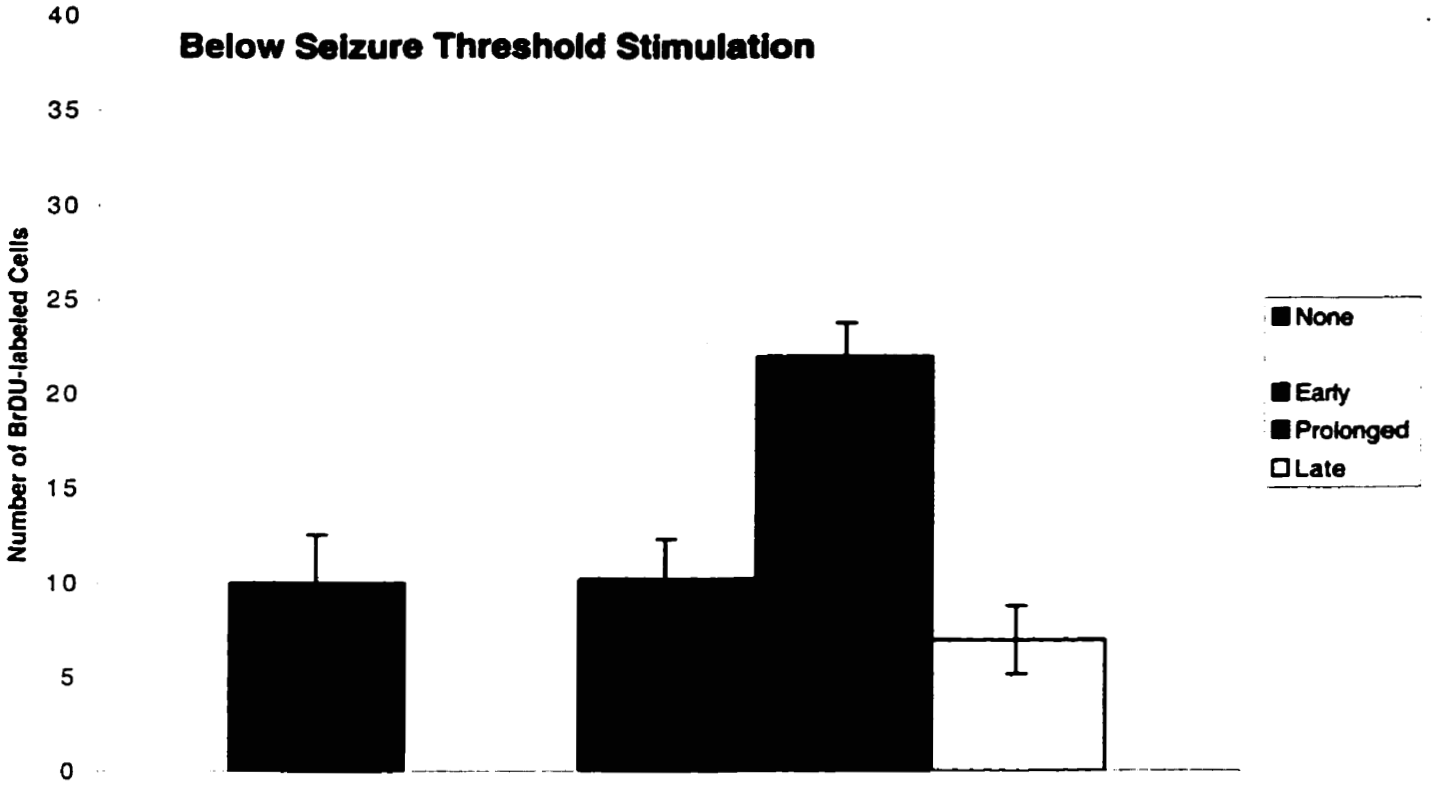
Number of BrDU-labeled Cells
*
Sham
Surgery
Lesion

C

SHAM SURGERY GROUPS



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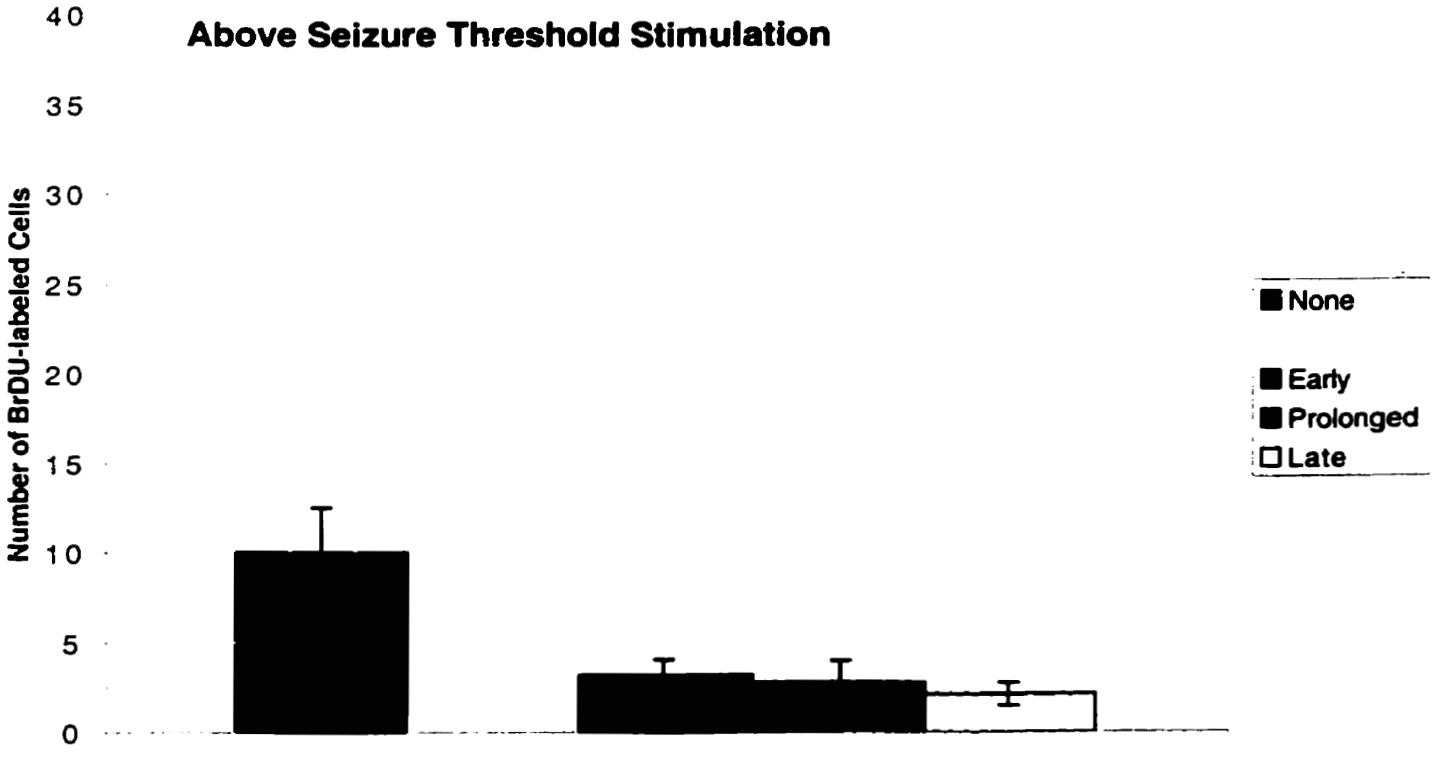
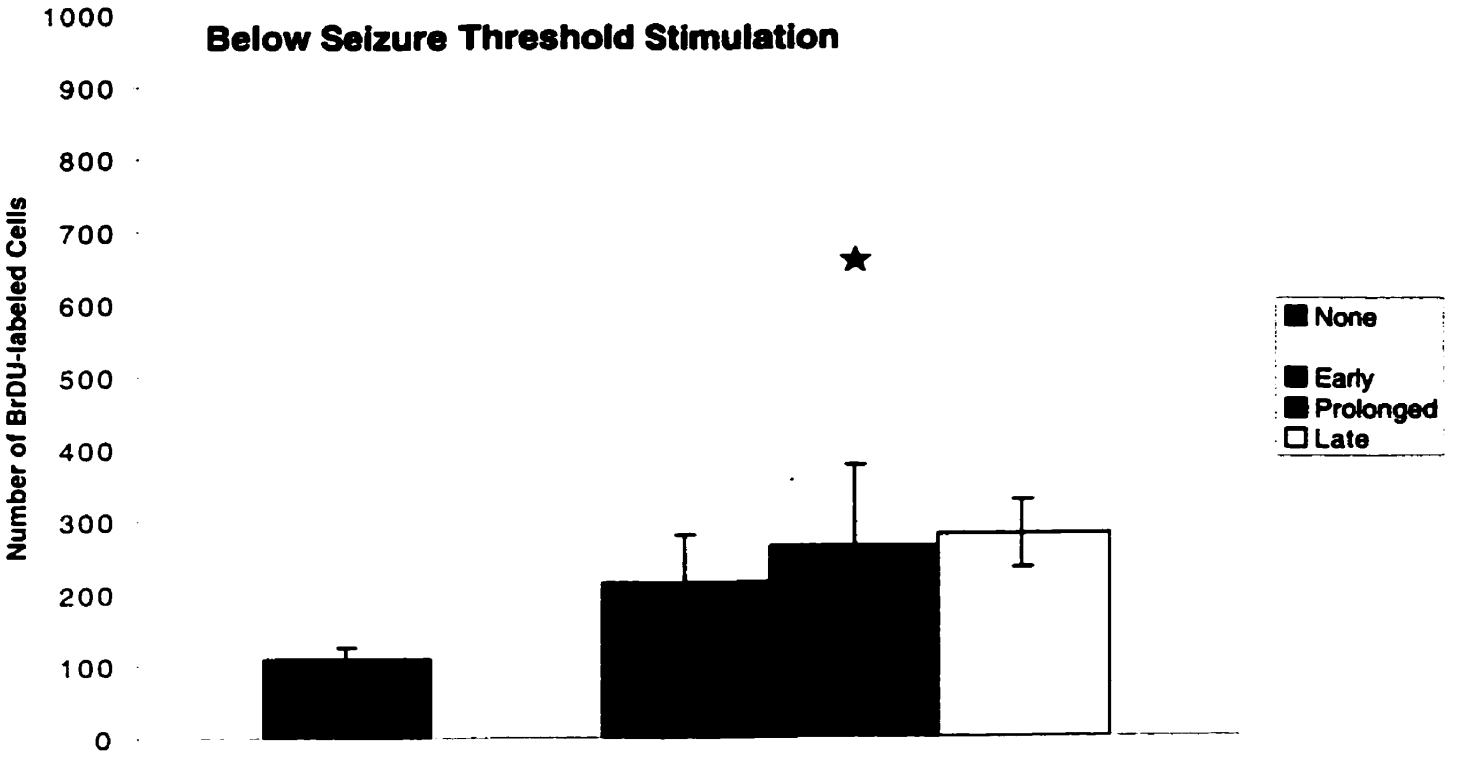
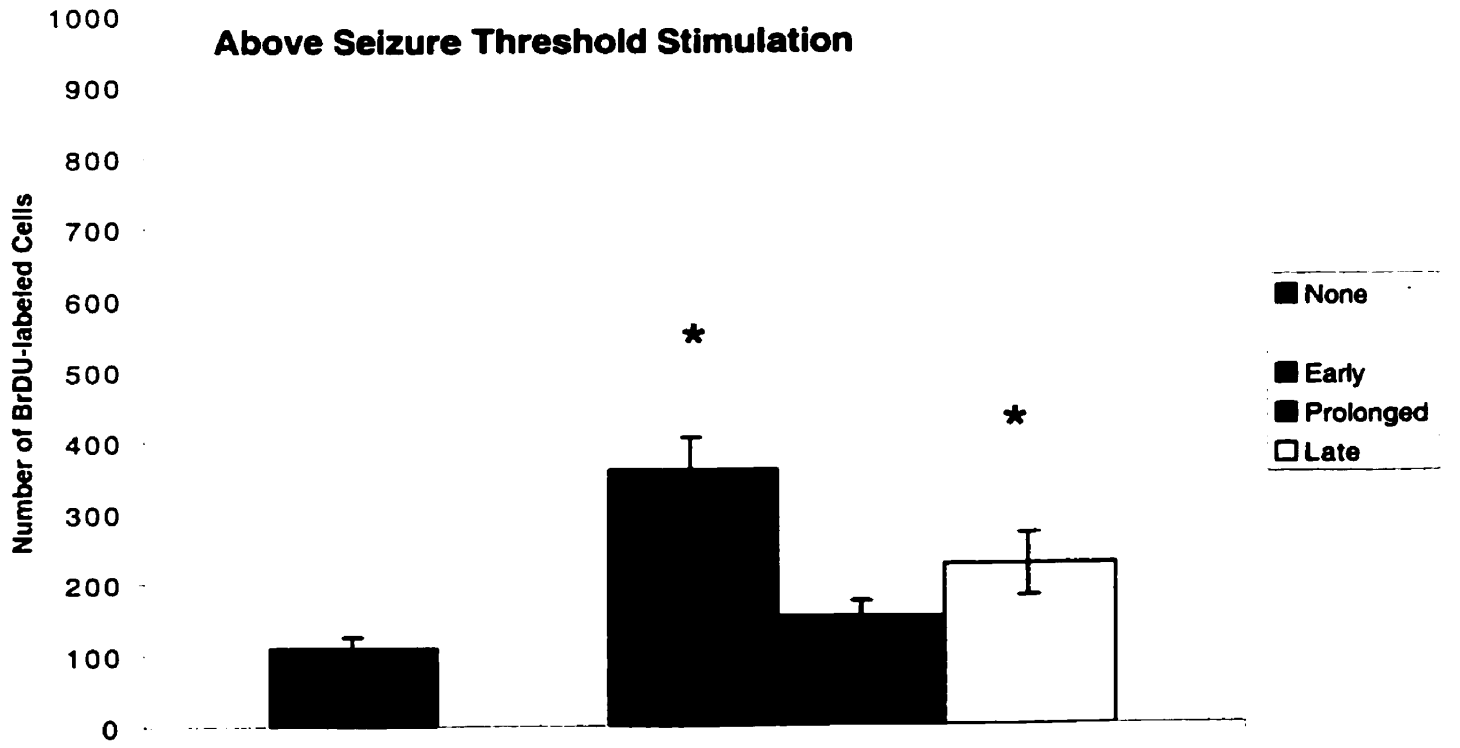


E

LESION GROUPS



F



(F(2,12)=9.945,p=.00284). These groups were compared separately to the no stimulation group. After making the bonferroni adjustment, the alpha level was 0.0125. The Early and Late stimulation groups had significantly more labeled cells than the non-stimulated control, (t(6)=3.700,p=0.0100) and (t(6)=3.717,p=0.00988), respectively (Figure 14F). A second control group for the effect of above threshold stimulation and lesions was above threshold stimulation in the intact brain. Seizures applied to the injured brains had more BrDU-labeled cells than seizures applied to the intact brains (t(22)=7.040, p<.0001) data not shown. Across all lesion groups, there were far more cells immediately adjacent to the lesion (proximal) than in the sites that were over 310 μm away from the lesion scar (distal).

Cortical sites proximal to the lesion

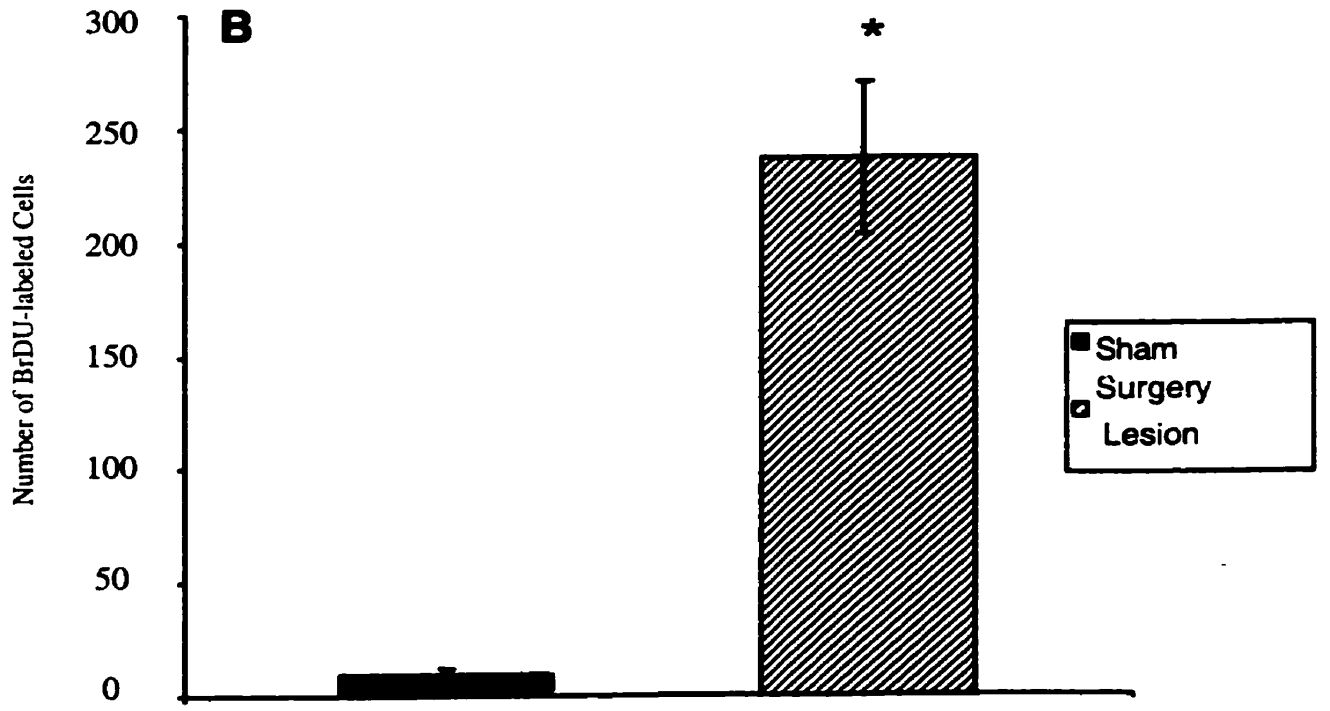
1. *The effect of lesion.* The brains that received a lesion had several times more labeled cells in the cortex (proximal to the lesion) than the untreated brains (t(6)=36.882, p<0.0001) (Figure 15B) 2. *The effect of stimulation.* There was an effect of below threshold stimulation on the number of BrDU-labeled cells in the intact brains (F(3,12)=114, p<0.0001). There was a significant difference between Early, Prolonged and Late treatments (F(2,9)=124, p<0.0001), therefore these groups were separately compared to no stimulation in the intact brain. Prolonged and late stimulation had more cells than the non-stimulated control, (t(6)=11.361, p<0.0001) and (t(6)=13.659, p<0.0001), respectively (Figure 15C). 3. *The effect of seizures.* There was an effect of above threshold stimulation on the number of BrDU-labeled cells in the intact brains (F(3,12)=6.274, p=0.00832). There was no difference in the number of cells in the proximal site between early, prolonged and no stimulation groups in the intact brains. However, with late above threshold stimulation there was a significant reduction in the number of labeled cells in the intact brains, (t(6)=-4.276, p=.00522) (Figure 15D). 4. *The interaction of lesions*

and stimulation. There was a significant effect of below threshold stimulation and lesion ($F(3,12)=5.161$, $p=0.0161$) (Figure 16). Therefore follow-up tests were conducted to see which groups differed. There was no difference between the stimulation groups (early, prolonged and late) therefore they were combined for further follow-up tests. The combined below threshold stimulation groups had more BrDU-labeled cells than the non-stimulated lesion group ($t(14)=6.000$, $p<0.0001$) (Figure 15E). A second control group for the effect of below threshold stimulation on the lesioned brains was below threshold stimulation in the intact brain. There was a significant effect of stimulation and lesion compared to the stimulated and intact brains, ($t(11)=5.439$, $p<0.0001$), data not shown. 5. *The effect of lesion and seizures*. Seizures following injury increased the number of BrDU-labeled cells in the proximal count sites. There was a significant difference between no stimulation, early, prolonged and late stimulation groups, ($F(3,12)=45.709$,$p<0.0001$). Early, Prolonged, and Late stimulation groups differed significantly ($F(2, 9)=9.945$,$p<0.0001$). These groups were compared separately to the no stimulation group. After making the bonferroni adjustment the alpha level was 0.0125. The Early and Late stimulation groups had significantly more labeled cells than the lesion & non-stimulated control group, ($t(6)=12.097$, $p<0.0001$) and ($t(6)=3.918$, $p=0.00782$), respectively (Figure 15F). A second control group for the effect of above threshold stimulation & lesions was above threshold stimulation in the intact brain. Seizures & lesions compared to seizures applied to the intact brains had more BrDU-labeled cells ($t(11)=5.439$, $p=0.000200$), data not shown.

Figure 15. The effect of lesions, stimulation and seizures on cortical BrDU-labeled cell counts proximal to the lesion.

The total number of cells from all four proximal sites in (A) were compared between groups. Boxes denote the areas captured for cell count analysis. There was a significant effect of lesion (B) compared to sham surgery condition. For sham surgery condition (C-D) prolonged and late below seizure threshold stimulation (C) increased the number of labeled cells compared to the normal brain. Late above threshold stimulated (D) decreased the number of BrDU-labeled cells compared to the untreated brains. In the lesion surgery condition (E-F), the below threshold stimulation groups (early, prolonged, and late) combined had more BrDU-labeled cells than the non-stimulated control (E). The early and late above threshold treatment also significantly increased the number of new cells (F) compared to the non-stimulated control. Scale bar in A = 250 mm. * Indicates significant ($p<.0167$) when all stimulation (early, prolonged, late) groups were combined. *Indicates significance ($p<.0125$) when the stimulation group were not combined.


A


B
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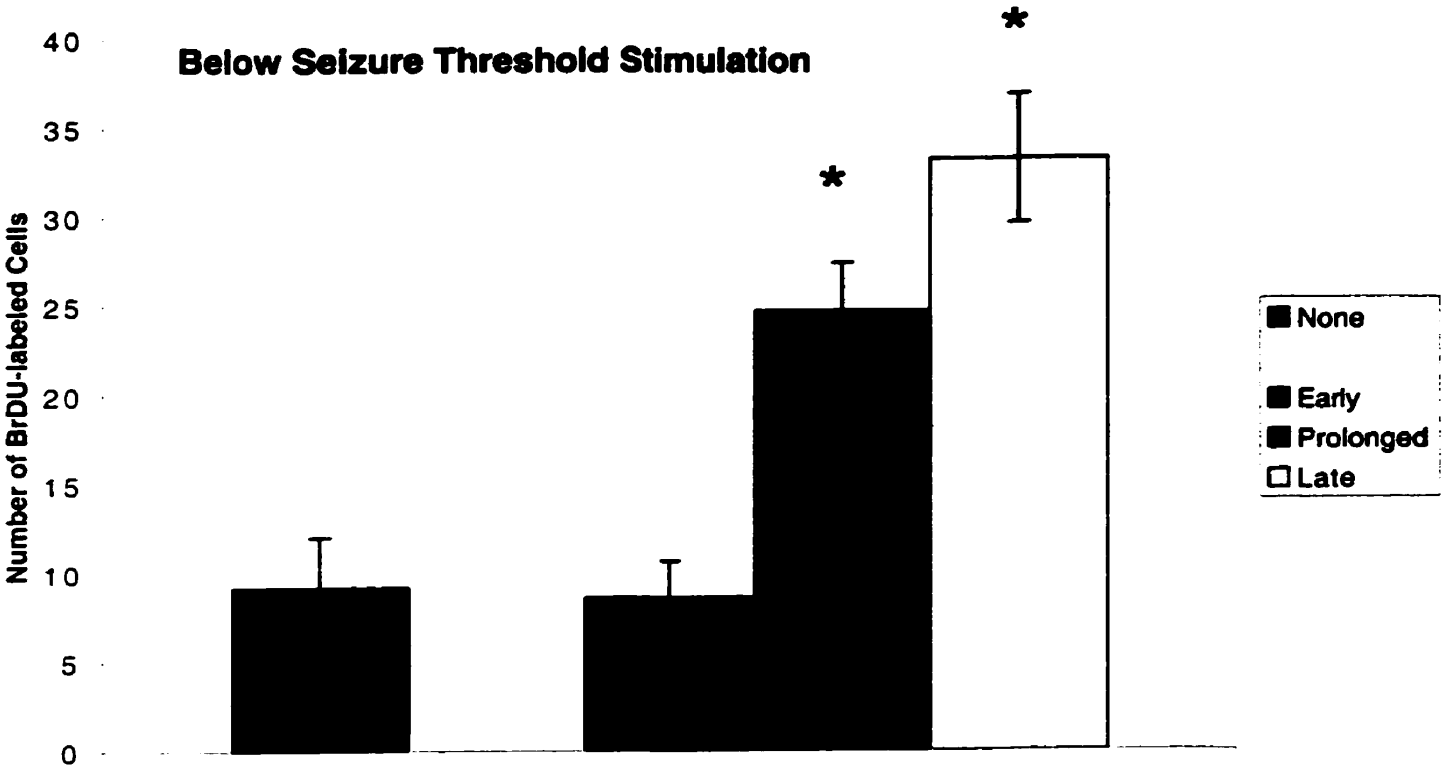
Number of BrDU-labeled Cells
Sham Surgery
Lesion

C

SHAM SURGERY GROUPS



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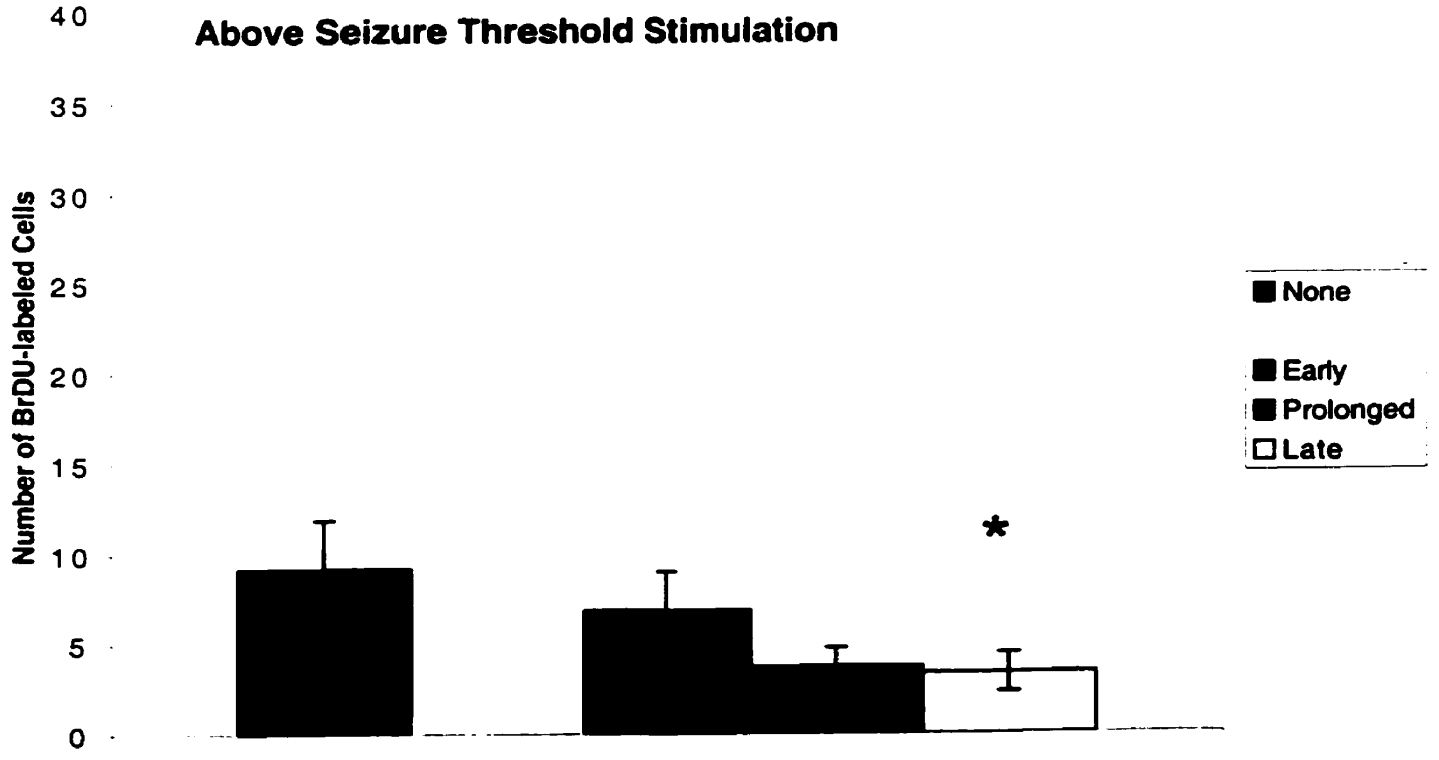
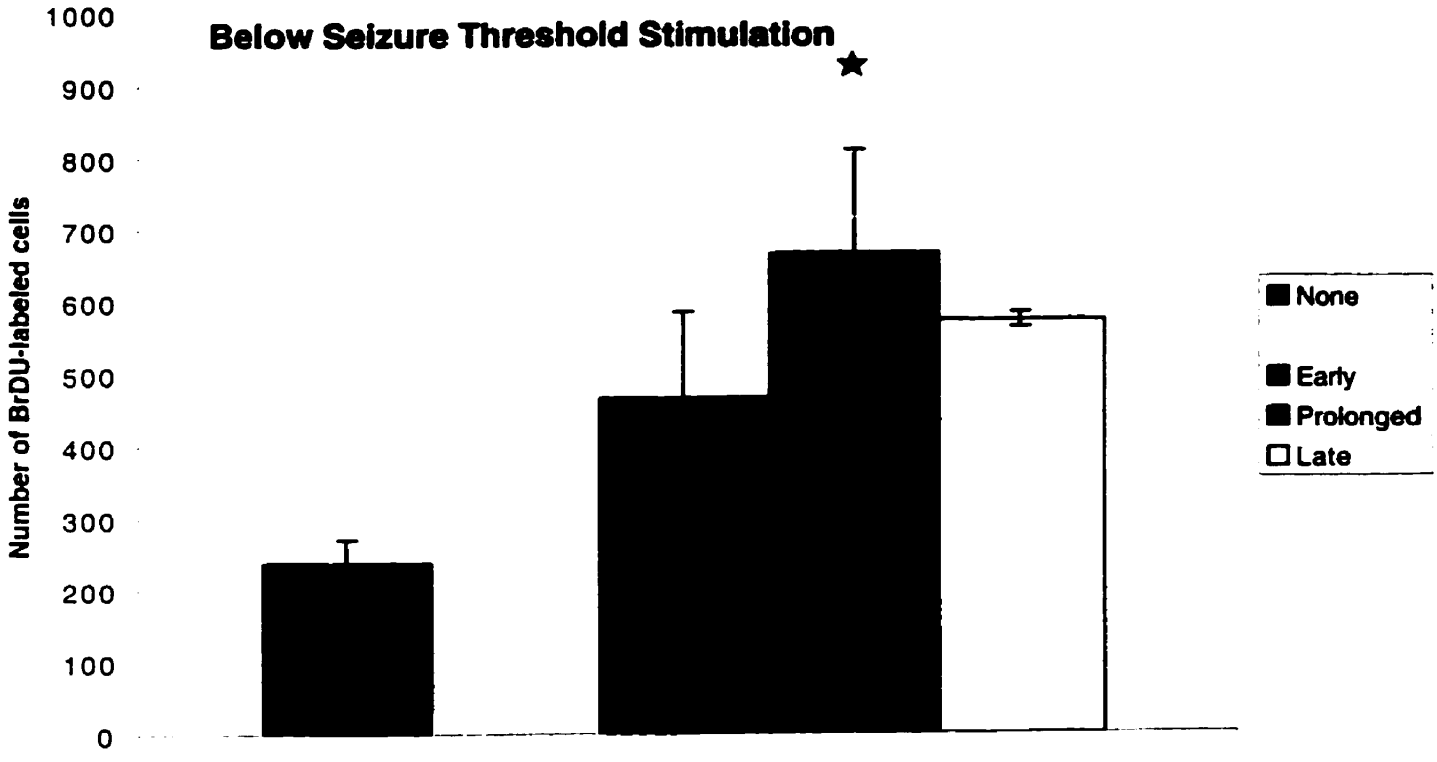


E

LESION GROUPS



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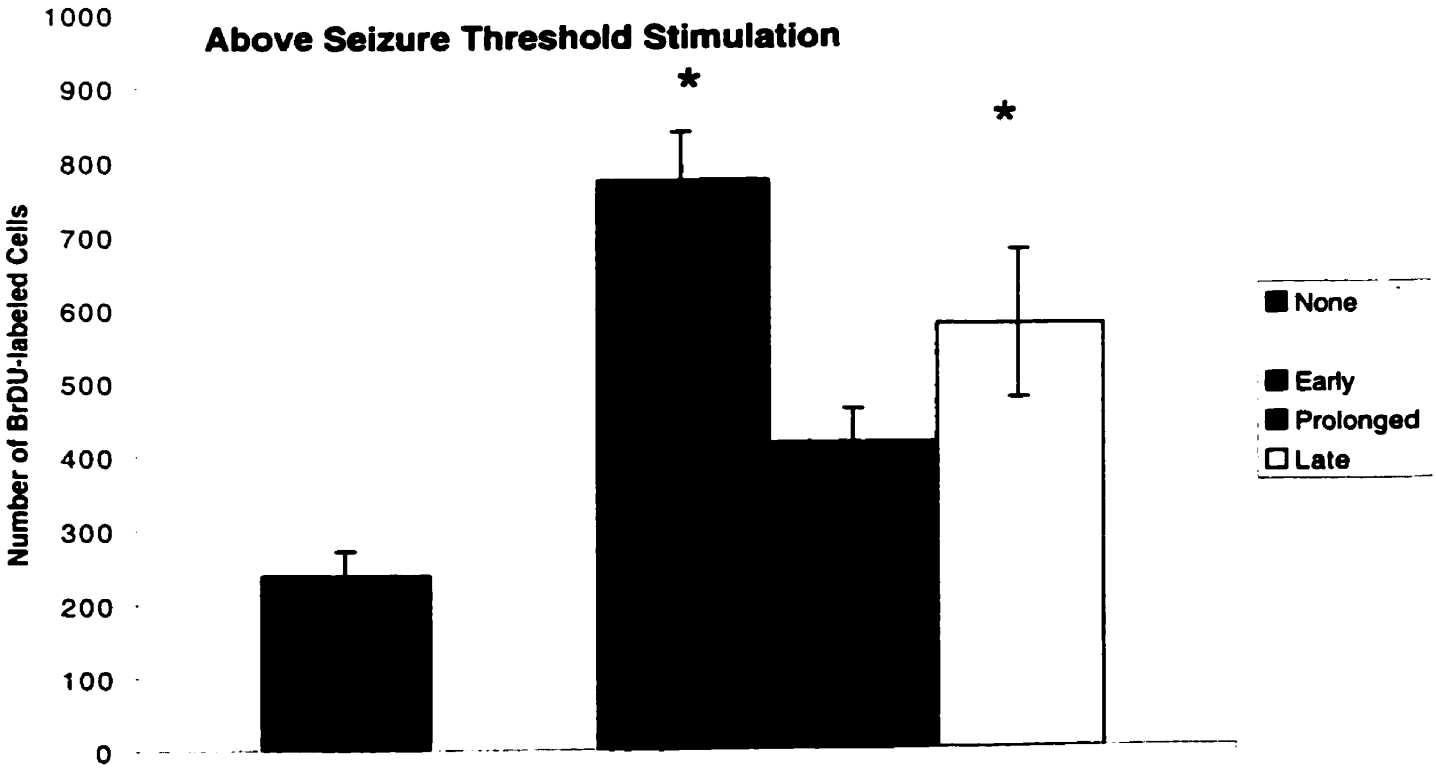
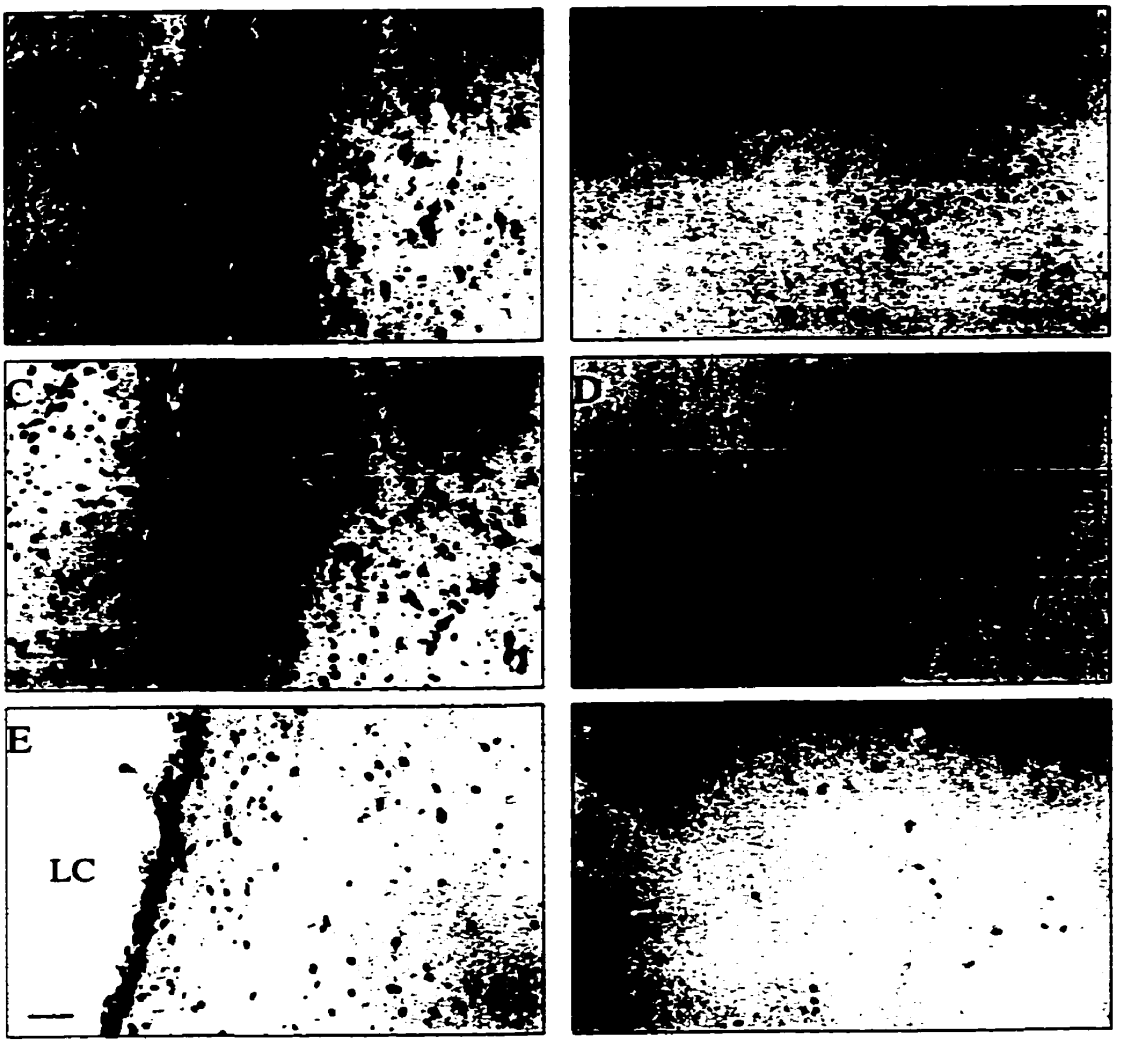


Figure 16. Photomicrographs of the cortical lesion site.

BrDU-labeled cells in the cortex are predominantly found at the lesion scar (A, C, E) Following above (A) and below (B) seizure threshold stimulation in the lesion cavity is non existent or small relative to the non-stimulated-lesion brains in (E). And the number of BrDU-labeled cells is greater in the stimulated brains. All sham surgery controls for above (B), below (D) and no stimulation (F) have few labeled cells in the cortex. Scale bar in E (20 μm) refers to A-F as well. LC=lesion cavity.


C
D
E
LC

3.5.2 Corpus Callosum

Across all groups, there was a much greater proportion of cells in the corpus callosum than the cortex. *Note- The unit of area measured was exactly four times greater for the proximal and distal cortex counts than the corpus callosum area because the four areas were totaled for the proximal and distal measures.

Cingulum

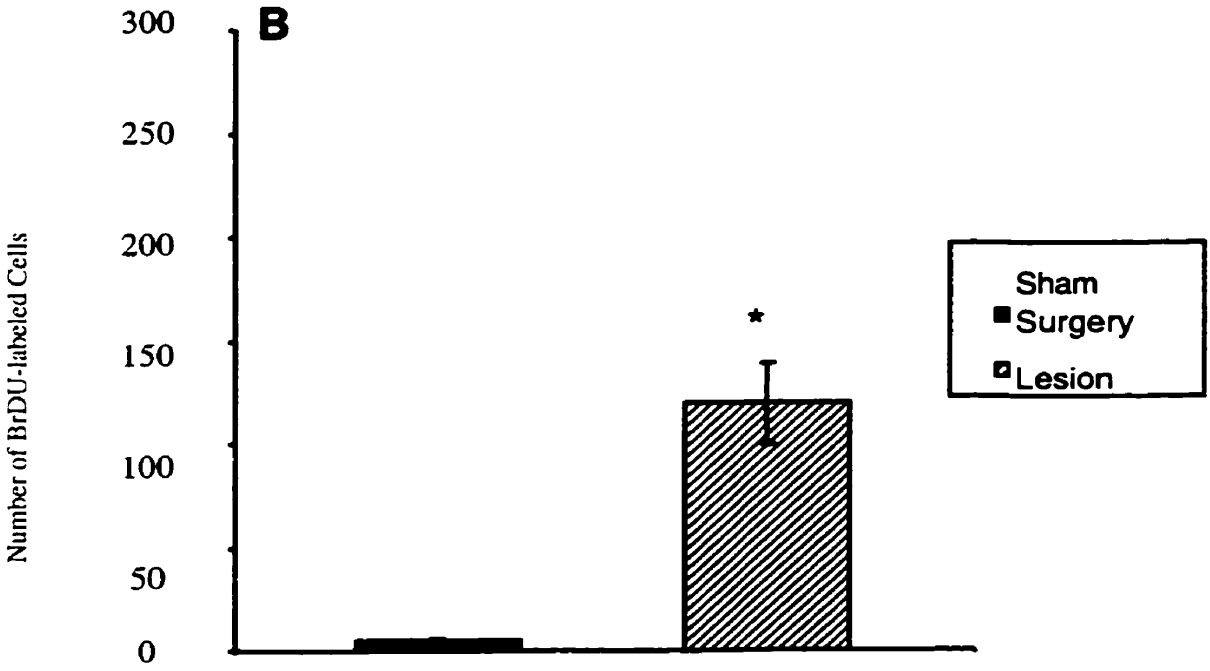
1. *The effect of lesion.* The brains that received a lesion had more labeled cells in the cingulum than the untreated brains ($t(6)=4.347$, $p<0.0001$) (Figure 17B). 2. *The effect of stimulation.* There was an effect of below threshold stimulation on the number of BrdU-labeled cells in the intact brains ($F(3,12)=11.641$, $p=0.000724$). Only prolonged stimulation had more cells than the non-stimulated control, ($t(6)=3.358$, $p=0.0152$), using an alpha level of 0.01667 (Figure 17C). 3. *The effect of seizures.* There was an effect of above threshold stimulation on the number of BrdU-labeled cells in the intact brains ($F(3,12)=6.424$, $p<0.00767$). Late stimulation reduced the number of labeled cells in the cingulum compared to non-stimulated, intact brains, ($t(6)=3.354$, $p=0.0153$), using an alpha level of 0.01667 (Figure 17D). 4. *The interaction of lesions and stimulation.* There was a significant effect of below threshold stimulation and lesion ($F(3,12)=7.851$, $p=0.00365$), therefore follow-up tests were conducted to see which groups differed. There was no difference between the stimulation groups (early, prolonged and late) therefore they were combined for further follow-up tests. The combined below threshold stimulation groups had more BrDU-labeled cells than the non stimulated lesion group ($t(14)=3.097$,$p=.00788$) (Figure 17E). A second control group for the effect of below threshold stimulation on the injured brains was below threshold stimulation in the intact brain. There was a significant effect of stimulation and lesion compared to stimulating the intact brains, ($t(11)=7.066$, $p<0.0001$), data not shown. 5. *The effect of lesion and seizures.* Seizures following injury increased

the number of BrDU-labeled cells in the cingulum site. There was a significant difference between no stimulation, early, prolonged and late stimulation groups, ($F(3,12)=14.111$, $p=0.000306$). The Early, Prolonged, and Late stimulation groups did not differ significantly, therefore, these groups were combined and then compared to the lesion & non-stimulated group. There was more BrDU-labeled cells in the seizure/lesion groups than the lesion/no stimulation group ($t(13)=9.017$,$p<0.0001$) (Figure 17F). A second control group for the effect of above threshold stimulation and lesions was above threshold stimulation in the intact brain. There was a significant effect of seizures and lesions compared to seizures applied to the intact brains ($t(11)=16.651$, $p<0.0001$), data not shown. The expression of new cells was lower in the body of the corpus callosum than cingulum across all lesion groups.

Figure 17. The effect of lesions, stimulation and seizures on BrDU-labeled cell counts in the cingulum.

The cingulum count in (A) was at the base of the lesion. Boxes denote the area captured for the cingulum cell count. There was a significant effect of lesion (B) compared to sham surgery conditions. For sham surgery conditions (C-D) prolonged below seizure threshold stimulation (C) increased the number of labeled cells compared to the normal brain. Late above threshold stimulation (D) decreased the number of BrDU-labeled cells compared to the normal brain. In the lesion surgery conditions (E-F), the below threshold stimulation groups (early, prolonged, and late) combined had more BrDU-labeled cells than the non-stimulated control (E). In the lesion surgery conditions (E-F), the above threshold stimulation groups (early, prolonged, and late) combined had more BrDU-labeled cells than the non-stimulated control (F). Scale bar in A (250 μm). * indicates significance ($p<.0167$) when all stimulation (early, prolonged and late) groups were combined. * indicates significance ($p<.0125$) when the stimulation group were not combined.


A


B
300
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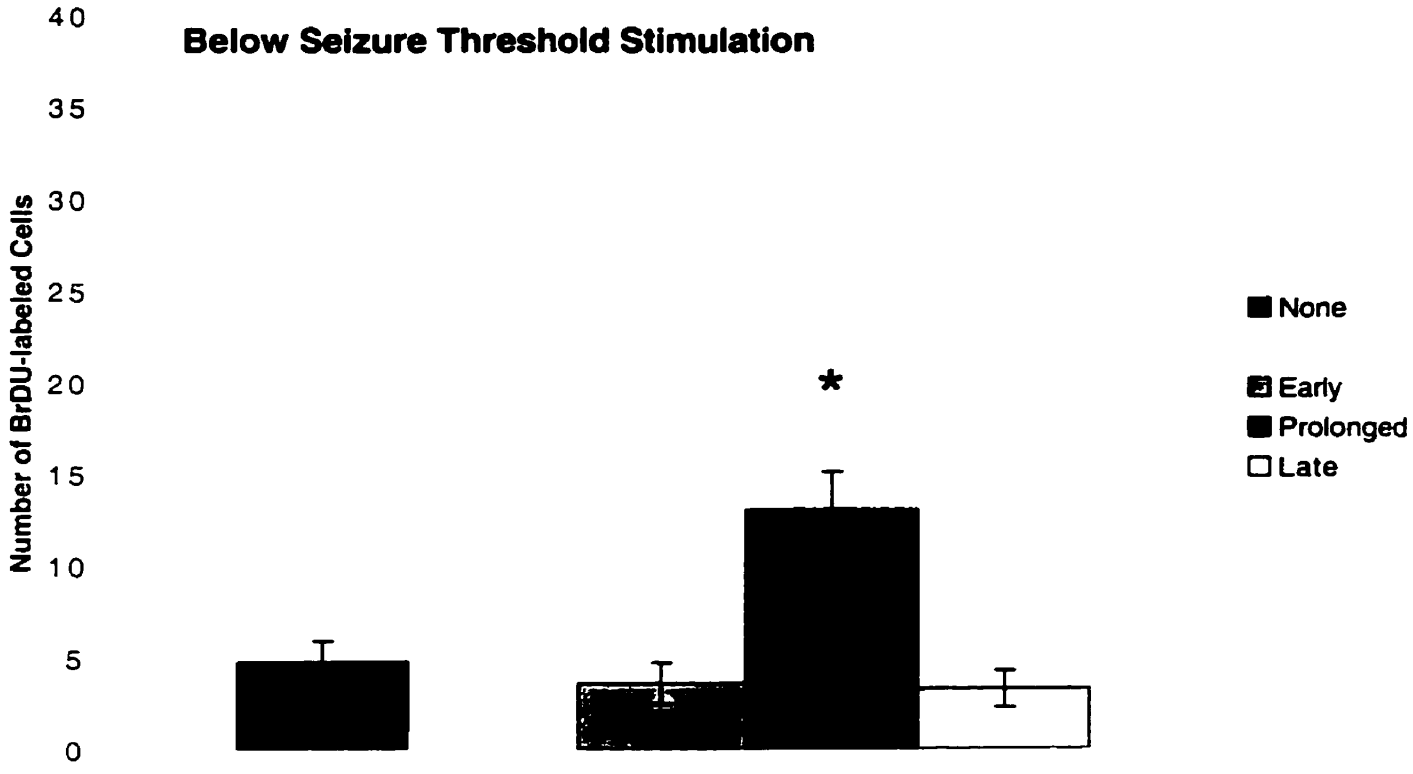
50
0
Number of BrDU-labeled Cells
*
Sham
Surgery
Lesion

C

SHAM SURGERY GROUPS



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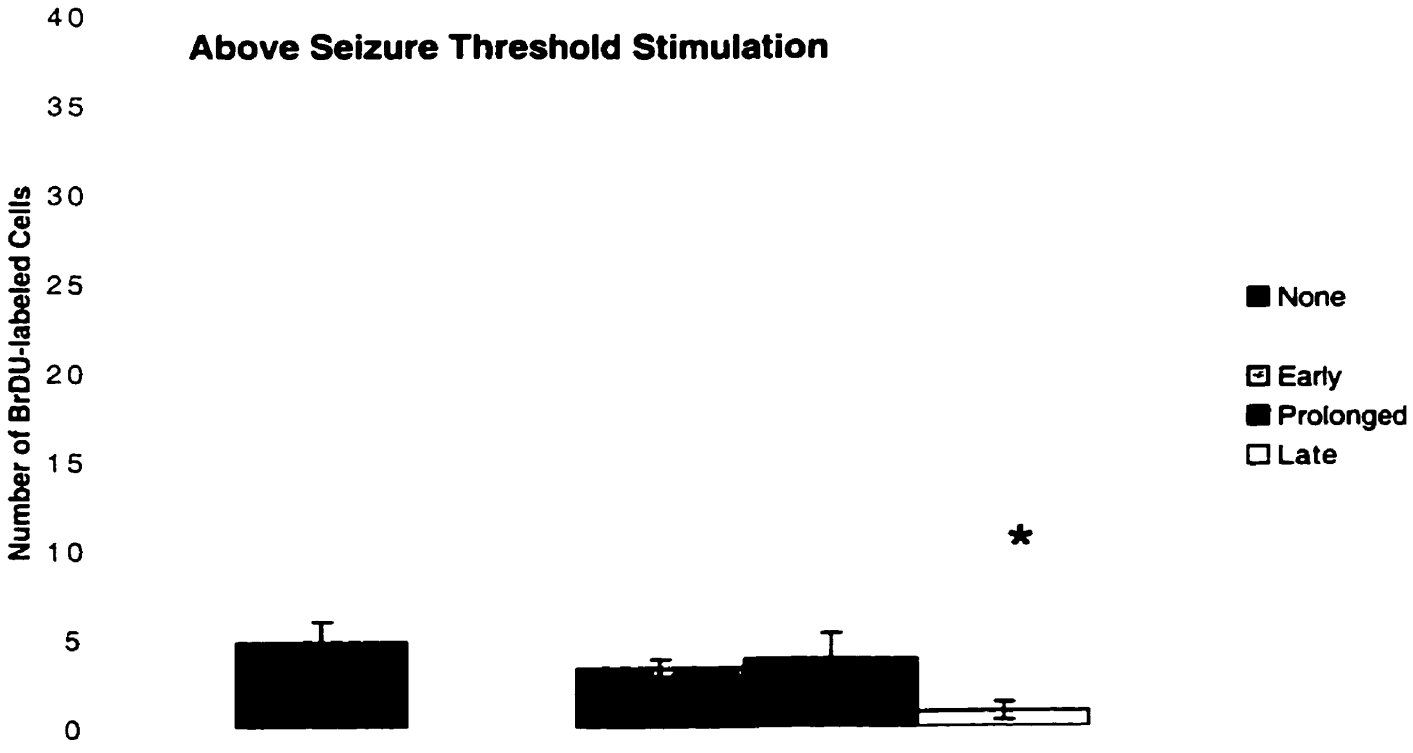
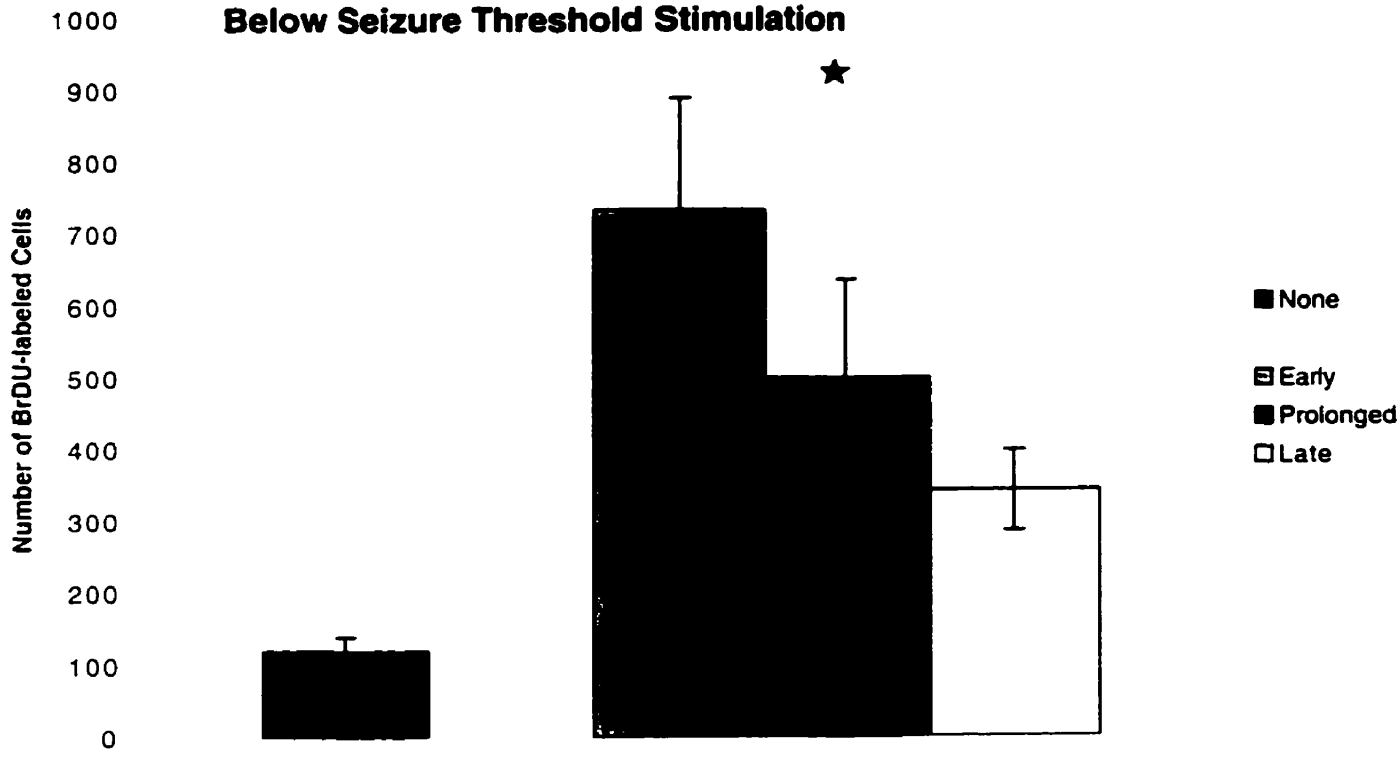
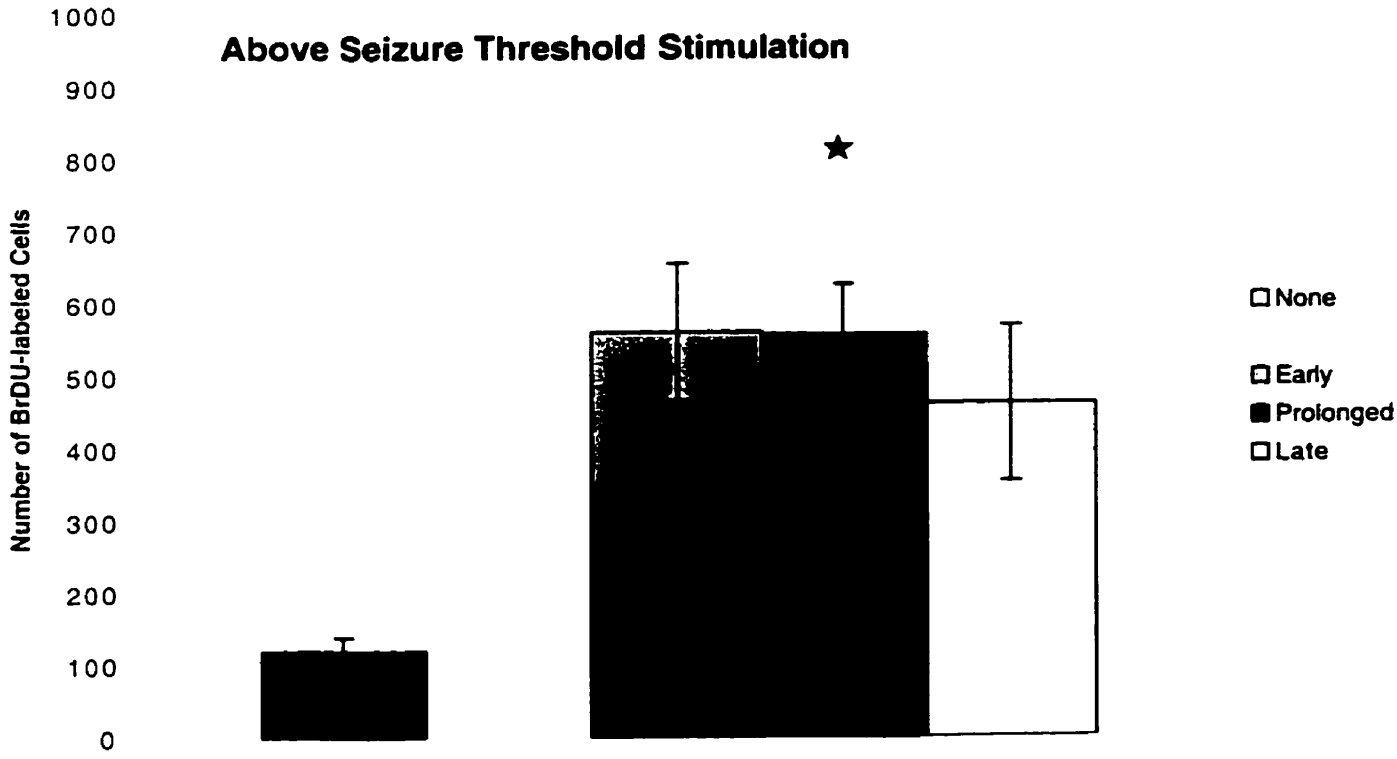


E

LESION GROUPS



F



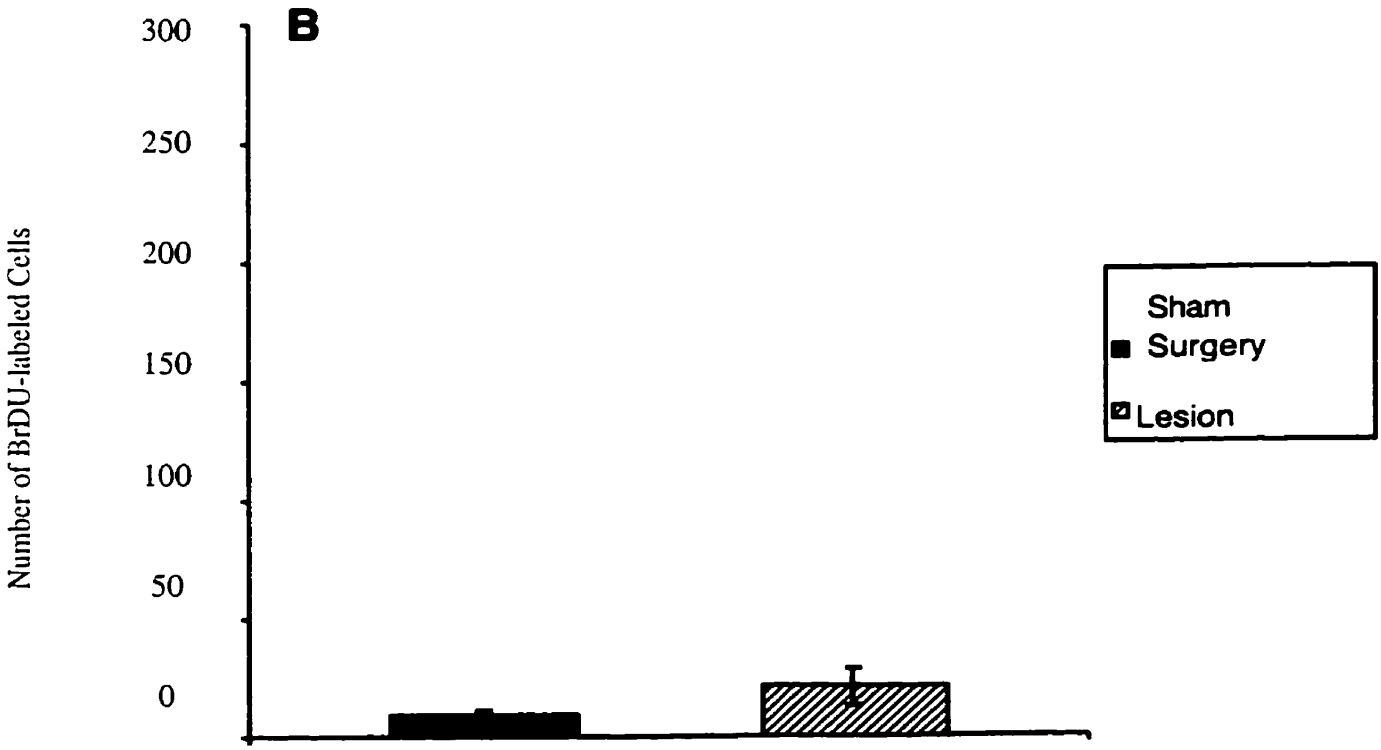
Body of the corpus callosum

1. *The effect of lesion.* Unlike all other more dorsal sites, the brains that received a lesion did not have significantly more labeled cells in the body of the corpus callosum than the untreated brains (Figure 18B). 2. *The effect of stimulation.* There was an effect of below threshold stimulation on the number of BrDU-labeled cells in the intact brains ($F(3,12)=4.214$, $p=0.0298$). There was no difference between the individual stimulation groups and no stimulation (Figure 18C). 3. *The effect of seizures.* There was an effect of above threshold stimulation on the number of BrdU-labeled cells in the intact brains ($F(3,12)=9.663$, $p<0.00160$). Early, Prolonged and Late groups differed, ($F(2,9)=12.715$, $p=0.00239$), therefore they were separately compared to the no stimulation group. Prolonged and late stimulation reduced the number of labeled cells in the corpus callosum compared to untreated brains, ($t(6)=3.852$, $p=0.00421$) and ($t(6)=4.066$, $p=0.00661$), respectively. After making bonferonni adjustments the alpha level was 0.01667 (Figure 18D). 4. *The interaction of lesions and stimulation.* There was no effect of below threshold stimulation and lesion compared to the non-stimulated, lesion group (Figure 18E). A second control group for the effect of below threshold stimulation and lesions was below threshold stimulation in the intact brain. There was a significant effect of stimulation and lesion compared to the intact brains, ($t(22)=2.454$, $p=0.0225$), data not shown. 5. *The effect of lesion and seizures.* Seizures following injury increased the number of BrDU-labeled cells in the corpus callosum site. There was a significant difference between no stimulation, early, prolonged and late stimulation groups, ($F(3,12)=3.958$, $p=0.0356$). Early, Prolonged, and Late stimulation groups did not differ significantly, therefore, these groups were combined and then compared to the lesion & no stimulation group. Seizure and lesions had more BrDU-labeled cells than lesions alone ($t(12)=3.369$, $p=0.00558$) (Figure 18f). A second control group for the effect of seizures and lesions was seizures applied to the intact brain. There was a significant effect of seizures and lesions compared to seizures applied to the intact brains ($t(21)=3.950$, $p=0.000732$), data not shown.

Figure 18. The effect of lesions, stimulation and seizures on BrDU-labeled cell counts in the corpus callosum.

The body of the corpus callosum count in (A) was positioned immediately below the cingulum site. Boxes denote the areas captured for the corpus callosum cell count. There was no effect of lesion (B) compared to sham surgery conditions for this site. In the sham surgery conditions (C-D) no below seizure threshold stimulation (C) increased the number of labeled cells compared to the normal brain. Late and prolonged above threshold stimulation (D) decreased the number of BrDU-labeled cells compared to the normal brain. In the lesion surgery conditions (E-F), the below threshold stimulation groups (early, prolonged, and late)did not differ from the the non-stimulated control (E). The above threshold stimulation groups (early, prolonged, and late) combined had more BrDU-labeled cells than the non-stimulated control (F).Scale bar in A (250 μm). * indicates significance ($p<.0167$) when all stimulation (early, prolonged and late) groups were combined. * indicates significance ($p<.0125$) when the stimulation group were not combined.


A


B
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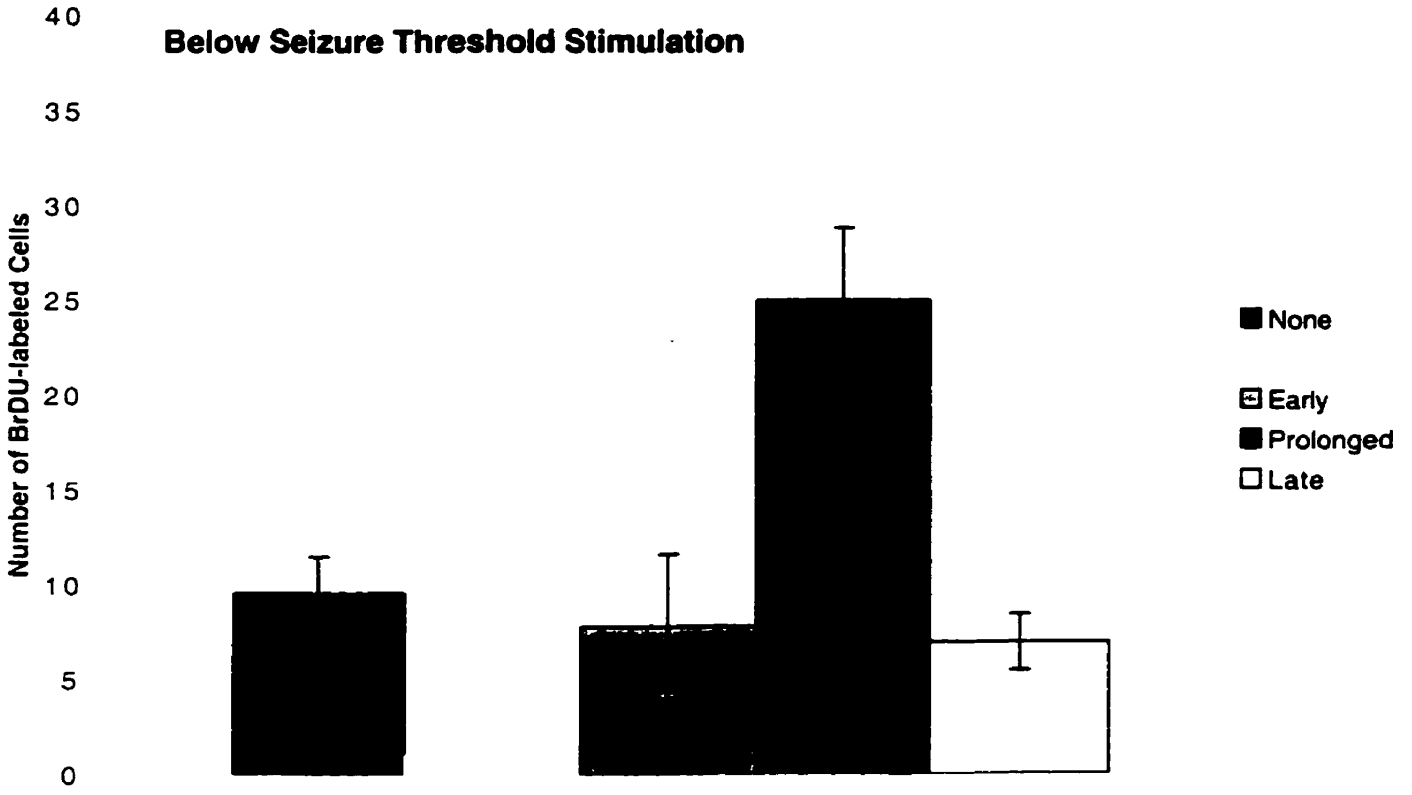
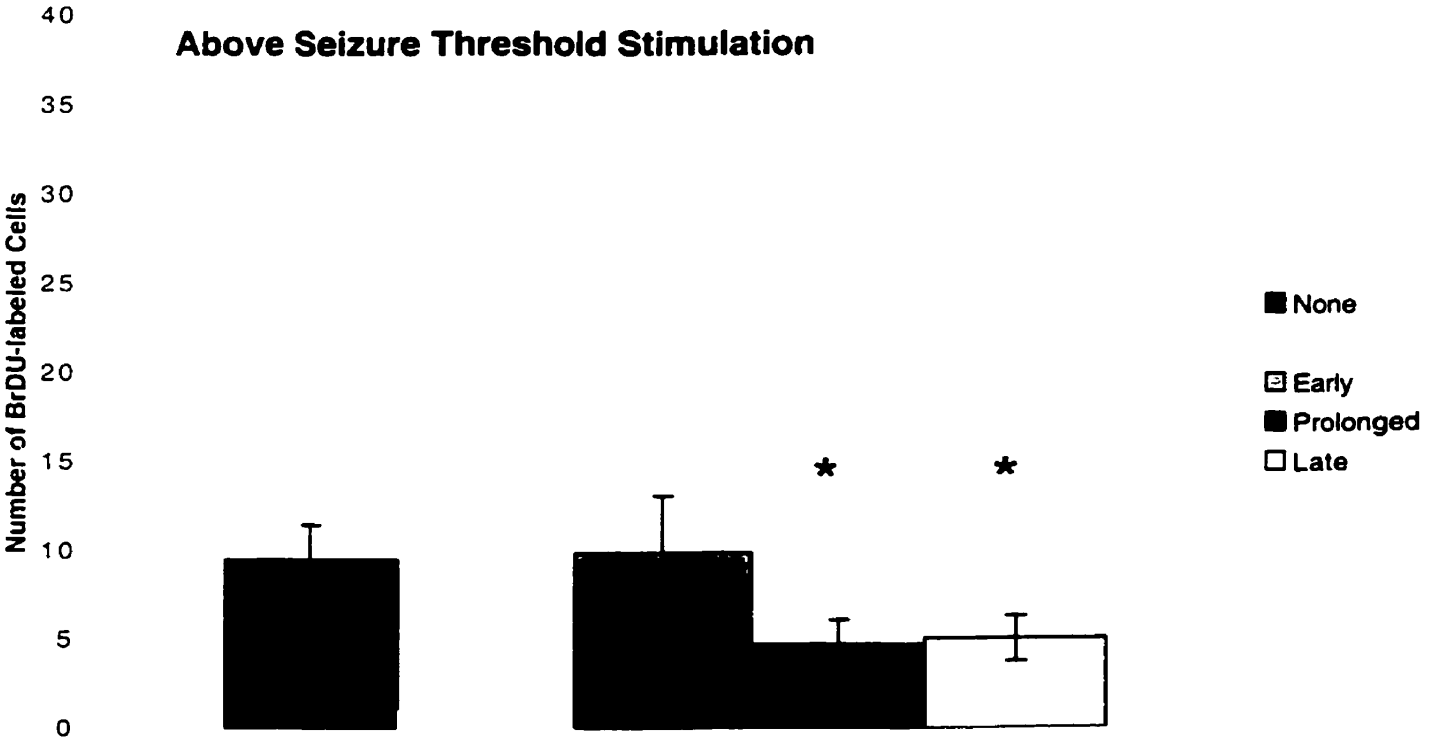
50
0
Number of BrDU-labeled Cells
Sham
Surgery
Lesion

C

SHAM SURGERY GROUPS

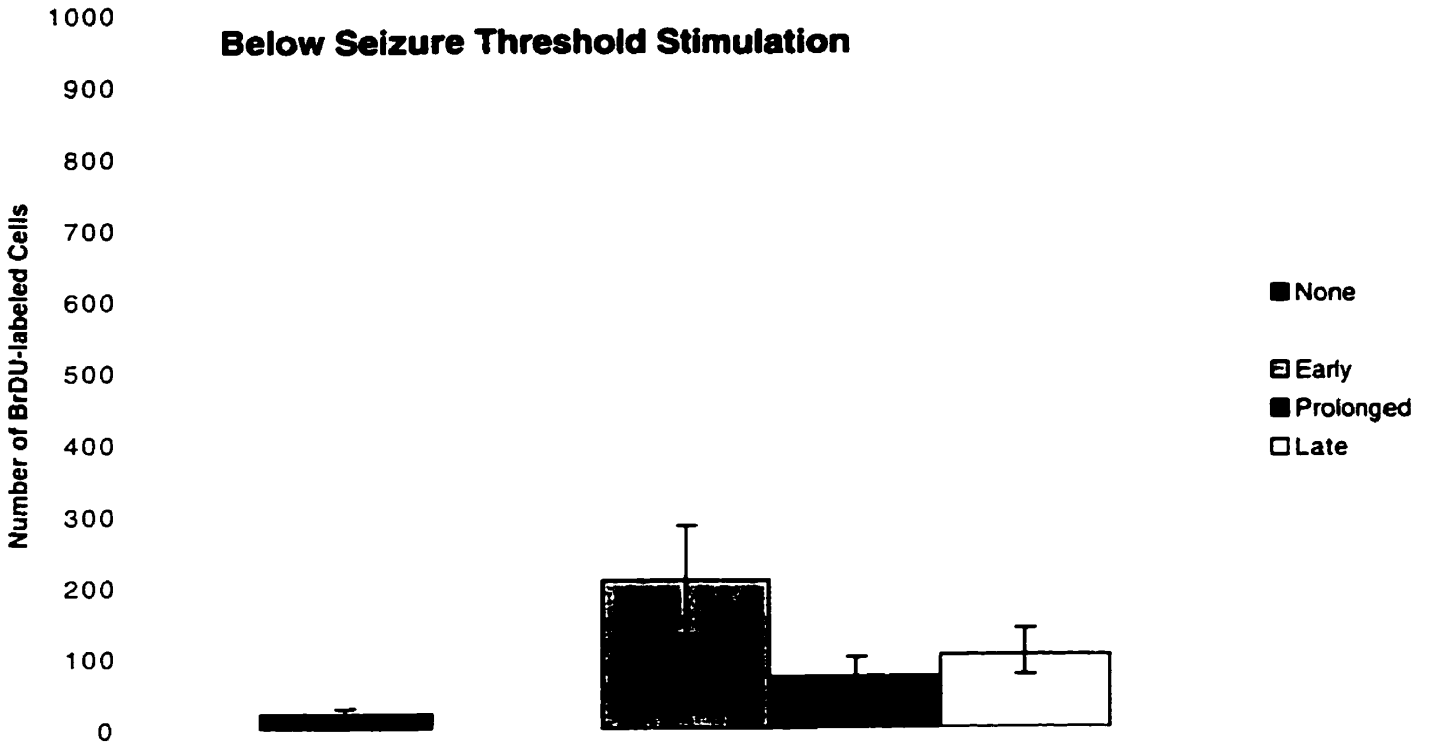


D

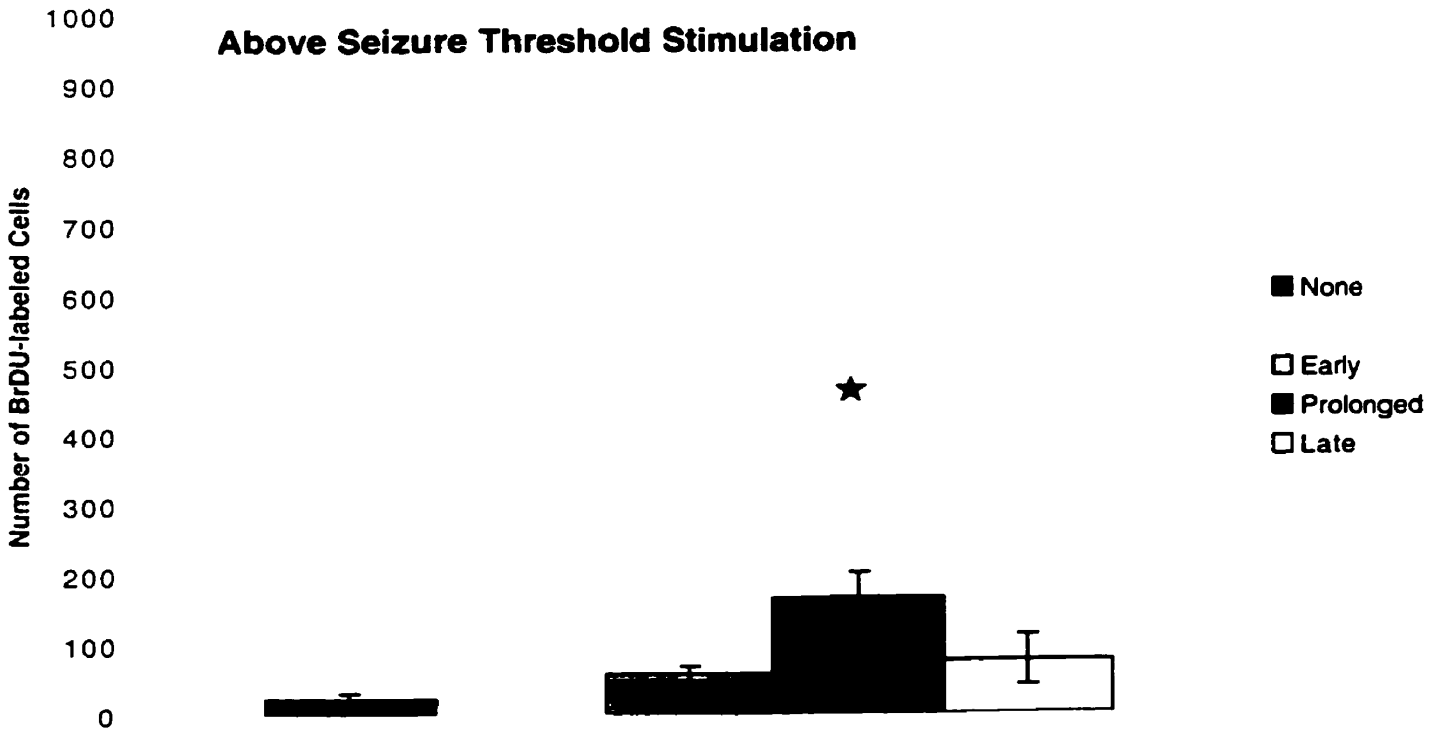


E

LESION GROUPS



F



3.5.3 SVZ

Like the cortex and corpus callosum, ECS increased the number of BrDU-labeled cells in the dl SVZ of the injured brains (Figure 19). Prolonged stimulation of both above and below seizure threshold stimulation intensities resulted in profound increases the number of new cells at the dl SVZ (Figure 20). 1. *The effect of lesion*. Similar to the corpus callosum, lesions alone did not produce an increase in the number of labeled cell observed at the dl SVZ compared the untreated group. In fact, there was a significant decrease in cell number at the dl SVZ in the lesion group compred to the untreated brains ($t(6)=3.756$, $p=0.00944$) (Figure 21B). 2. *The effect of stimulation*. There was an effect of below threshold stimulation on the number of BrDU-labeled cells in the intact brains ($F(3,12)=3.490$, $p=0.000787$). Early below threshold stimulation applied to the intact brains slightly reduced the number of new cells in the dl SVZ ($t(6)=6.202$, $p=0.000810$), relative to the untreated brains (Figure 21C). 3. *The effect of seizures*. There was an effect of above threshold stimulation on the number of BrDU-labeled cells in the intact brains ($F(3,12)=7.393$, $p<0.004$). Only late seizures were statistically different than no seizures following injury, ($t(6)=4.809$, $p=0.00297$) (Figure 21D). 4. *The interaction of lesions and stimulation*. There was a significant effect of stimulation on the injured brains ($F(3,12)=17.275$, $p=0.000119$), relative to the lesions alone group (Figure 21E). Early and prolonged below threshold stimulation in the injured brains had more BrDU-labeled cells in the dl SVZ than the lesion/ non-stimulated group ($t(6)=4.635$, $p=0.00178$) and ($t(6)=6.953$, $p=0.000439$), respectively. A second control group for the effect of below threshold stimulation and lesions was below threshold stimulation in the intact brain. There were more labeled cells in the stimulation and lesion group compared to stimulation in the intact brains, ($t(22)=4.136$, $p=0.000431$), data not shown. 5. *The effect of lesion and seizures*. Seizures following injury increased the number of BrDU-labeled cells in the dl SVZ site.

Figure 19. Photomicrographs of the cortex, cingulum and SVZ.

The number of BrDU labeled cells varied between the cortex (C, D), cingulum (E, F), and dl SVZ (G, H) and between late above threshold simulation brains in (A, C, E, G) and the non-stimulated, lesion control (B, D, F, H). Scale in (A) 250 μm also refers to (B) Scale in (G) 20 μm also refers to (C-H).

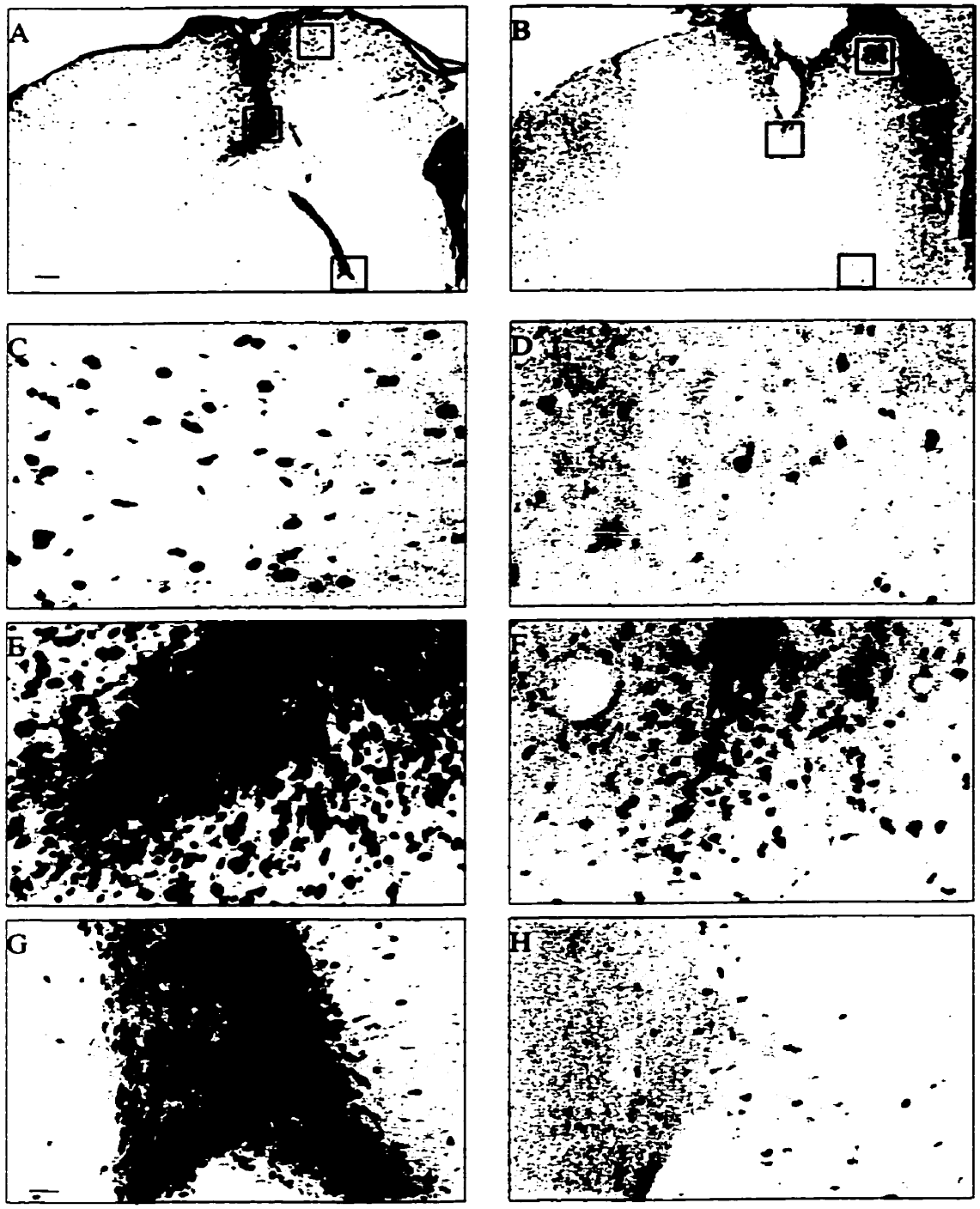

A
B
C
D
E
F
G
H

Figure 20. Photomicrographs of BrDU-labeled cells in the dl SVZ.

The number and expression of BrDU-labeled cell in the dl SVZ was different between groups. Lesions and above threshold stimulation (A) as well as below threshold stimulation (C) increased the number of labeled cells compared to the lesion and non-stimulated control group (E). There was very few new cells in the dl SVZ for above (B), below (D) and non-stimulated (F) sham surgery conditions.

Bottom right corner is the lateral ventricle. Scale bar in (E)= 20 μm also refers to (A-F).

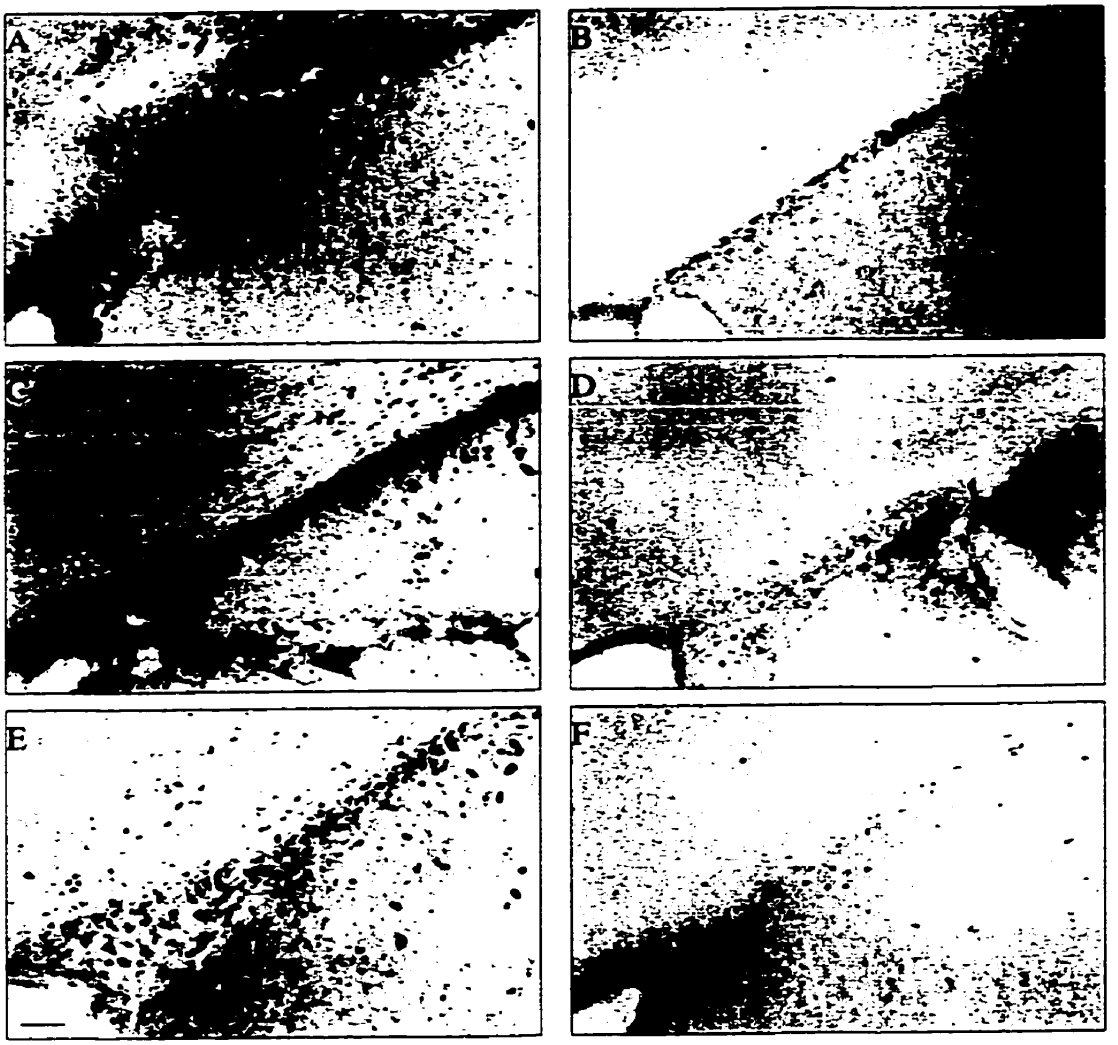
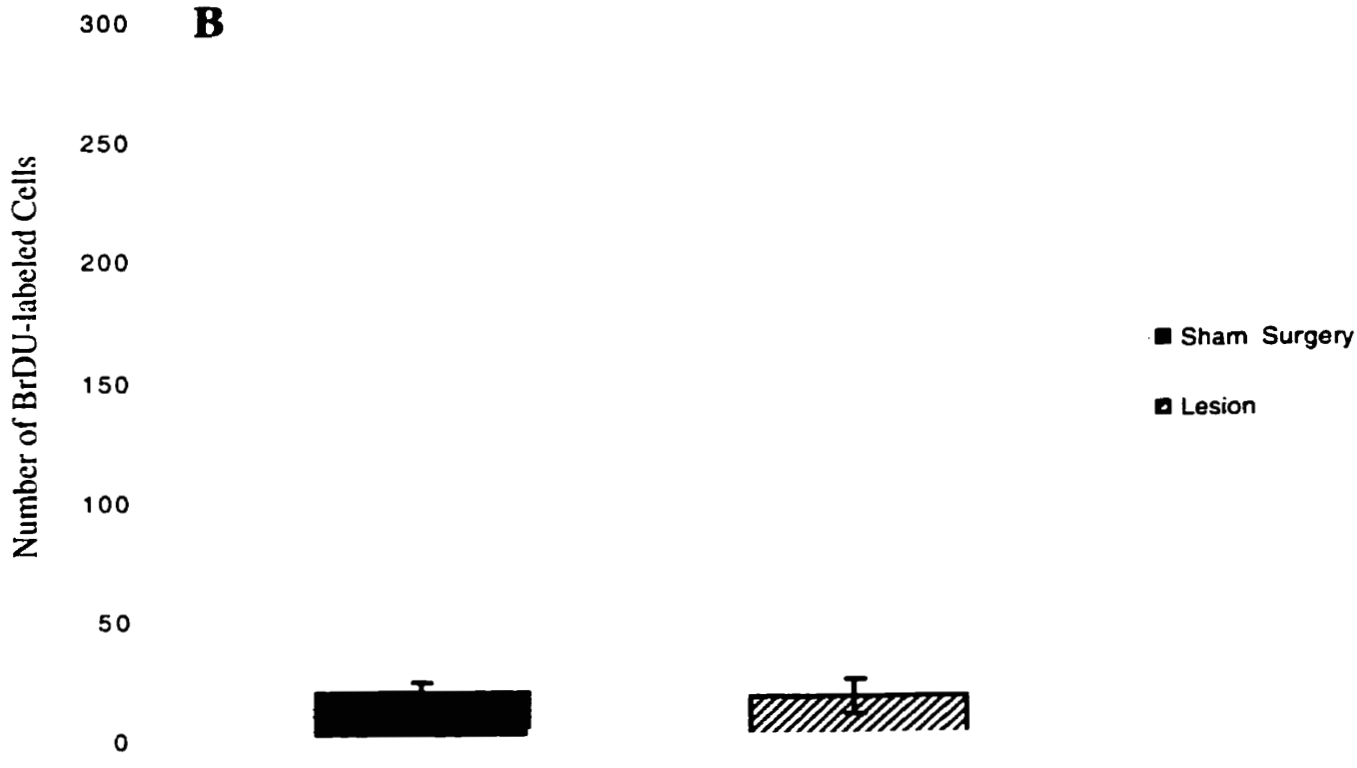

A
B
C
D
E
F

Figure 21. The effect of lesions, stimulation and seizures on BrDU-labeled cell counts in the dl SVZ

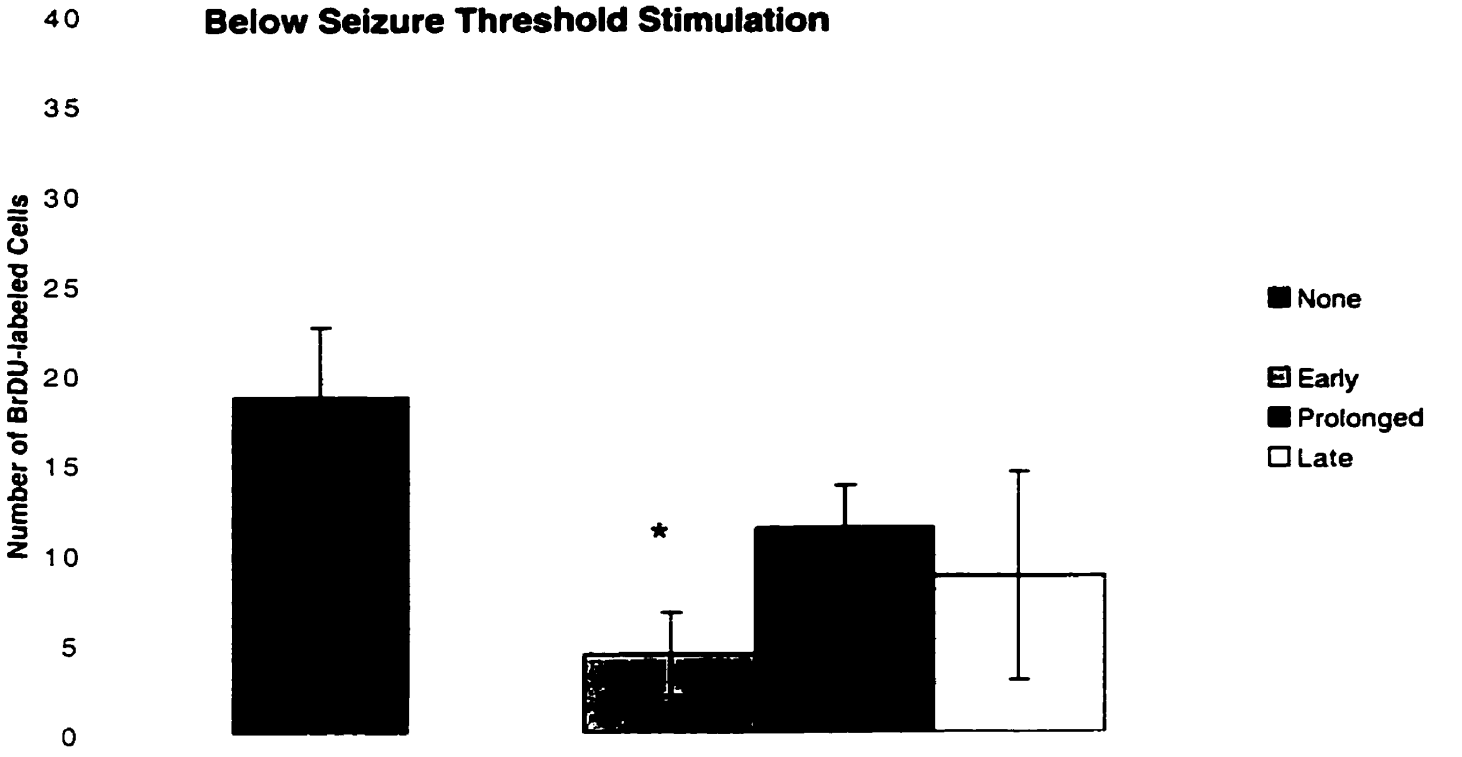
The dl SVZ count site (A). Lesions decreased the number of BrDU-labeled cells compared to sham surgery (B). In the sham surgery conditions (C-D) early stimulation (C) decreased the number of labeled cells compared to the normal brain. All seizures (early, prolonged and late) (D) decreased the number of BrDU-labeled cells compared to the normal brain. In the lesion surgery conditions (E-F), the below threshold stimulation groups (early, prolonged, and late) combined had more BrDU-labeled cells than the non-stimulated control (E). Only late seizures had more BrDU-labeled cells than the non-stimulated control (F). Scale bar in A (250 μm)* indicates significance ($p<.0167$) when all stimulation (early, prolonged and late) groups were combined. * indicates significance ($p<.0125$) when the stimulation group were not combined.


A

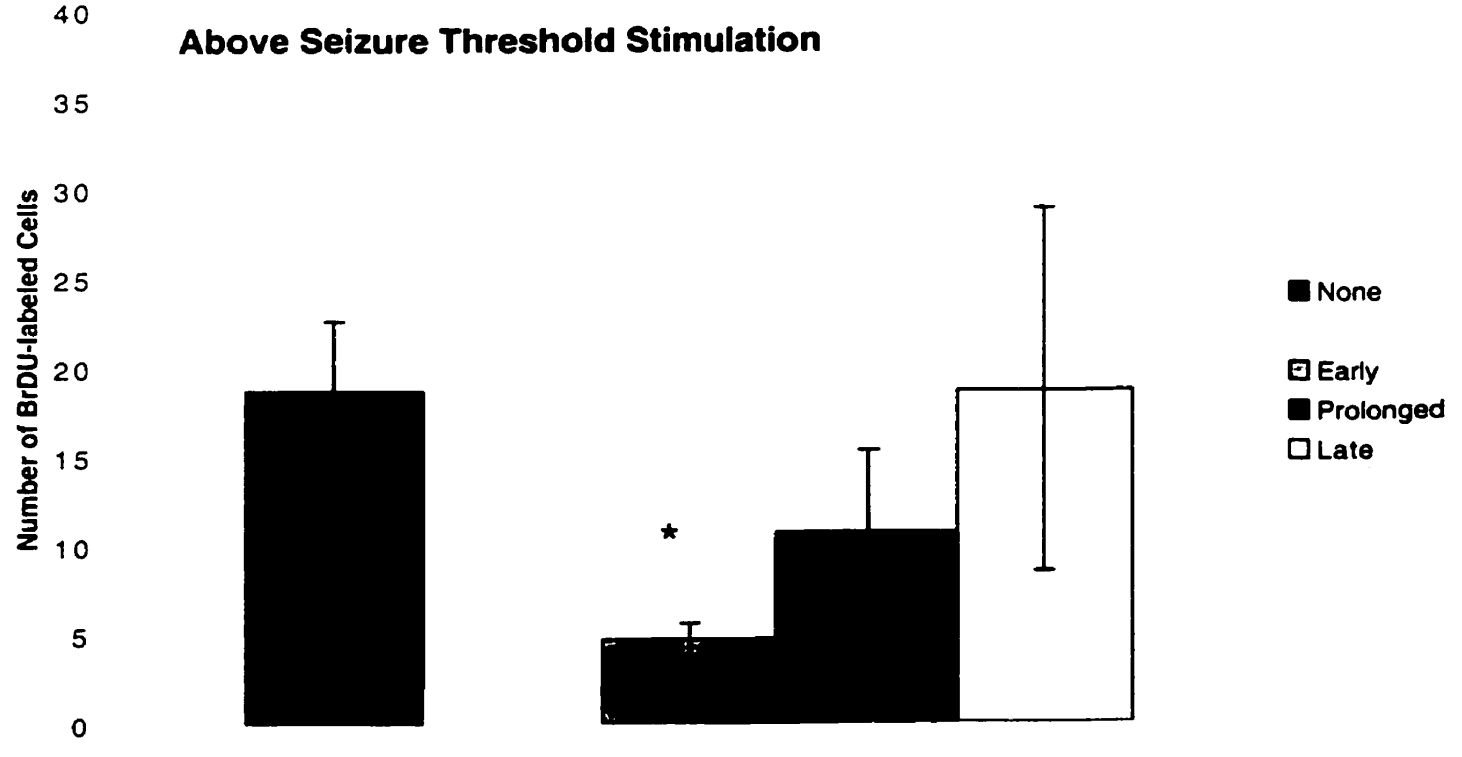

B
300
250
200
150
100
50
0
Number of BrDU-labeled Cells
Sham Surgery
Lesion

C

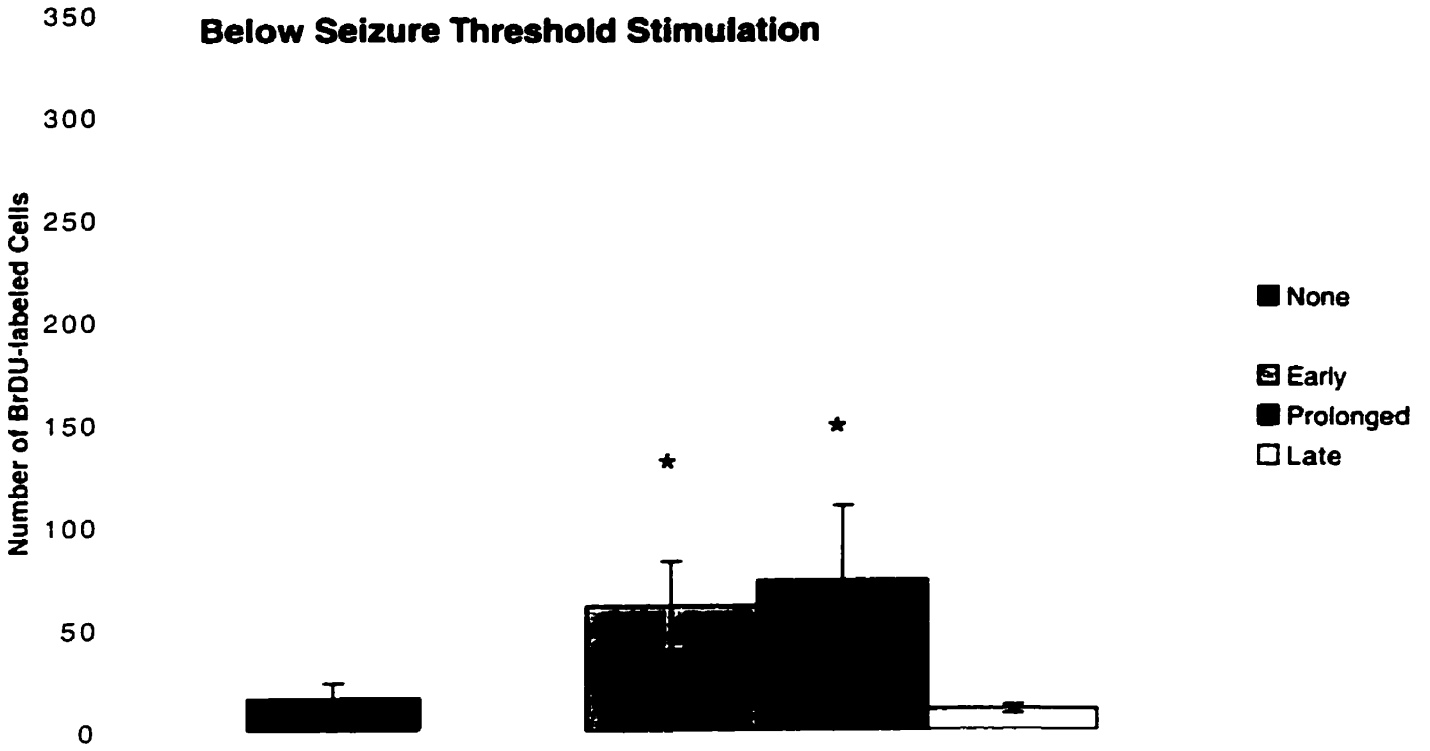
SHAM GROUPS



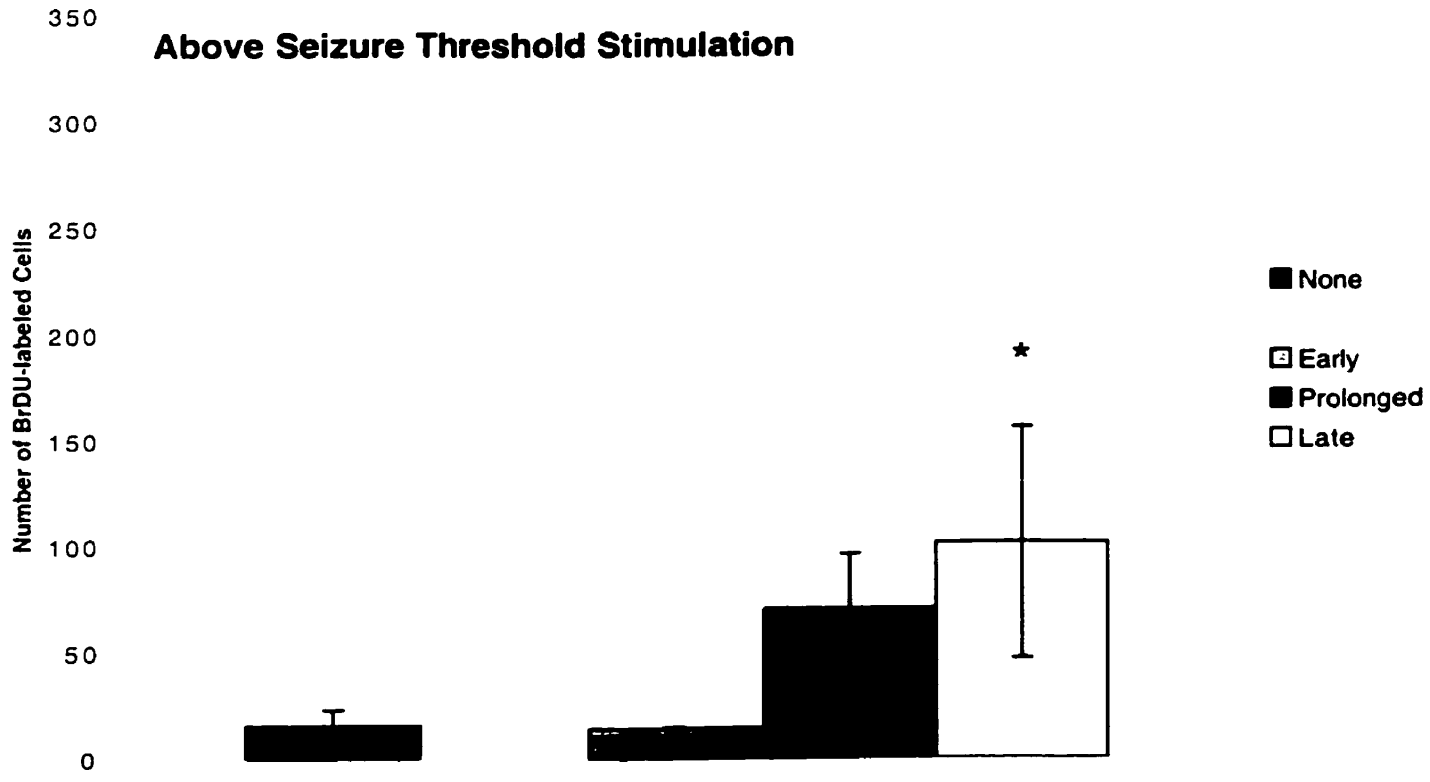
D



E


LESION GROUPS
Below Seizure Threshold Stimulation
Number of BrDU-labeled Cells
350
300
250
200
150
100
50
0
None
Early
Prolonged
Late

F

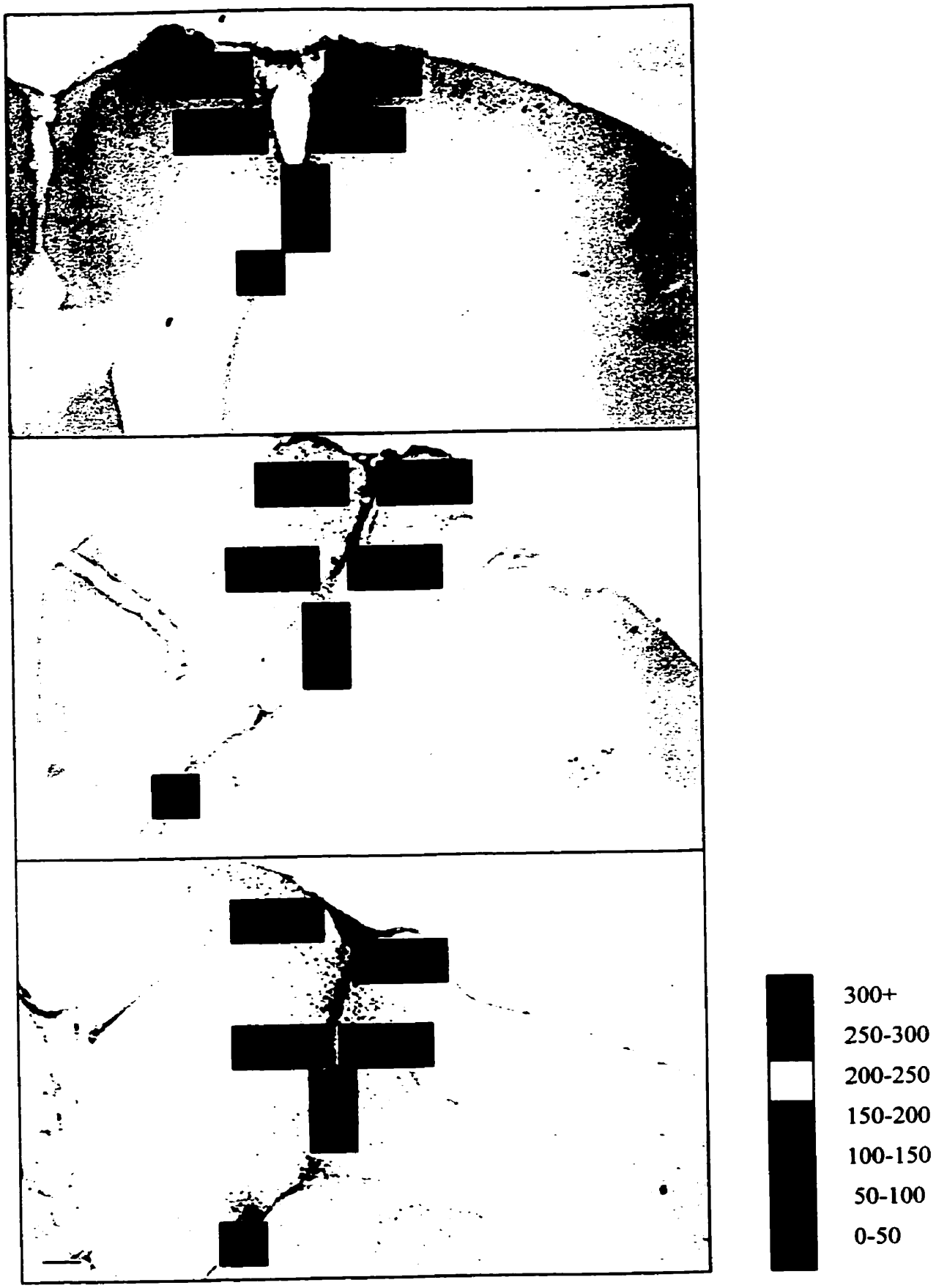

Above Seizure Threshold Stimulation
Number of BrDU-labeled Cells
350
300
250
200
150
100
50
0
None
Early
Prolonged
Late

There was a significant difference between no stimulation, early, prolonged and late stimulation groups, ($F(3,12)=9.751$, $p=0.00154$). After adjusting the alpha level to 0.01667, only seizures applied late had significantly more BrDU-labeled cells than non seizures in the injured brain, ($t(6)=4.809$, $p=0.00297$ (Figure 21F). A second control group for the effect of seizures following lesions was seizures in the intact brain. There was a significant effect of seizures and lesions compared to seizures applied to the intact brains ($t(22)=3.430$, $p=0.00120$), data not shown.

The above presentation of the data, does not fully describe the vast difference in BrDU-labeled cell number between sites. Figure 22, gives an idea of the effect of no stimulation, stimulation and seizures following injury on the mean number of cells for each site.

Figure 22. Densitometry of cell count sites

Color densitometry provides a within and between group comparison of the number of cells at each site analyzed. The mean number of cells at each site in the no stimulation group (UPPER) was mostly less than 50, indicated by the purple boxes. In the late, below threshold stimulation group (MIDDLE) and above threshold stimulation group (BOTTOM), the number of cells varies depending on the site. In B and C, the highest concentration of cells is at the base of the lesion in the cingulum. And generally, there are more cells in the cortex proximal to the lesion than distally. The color bar scale on the figure corresponds to cell count ranges listed to the right of the color bar. *The SVZ cell count means were adjusted to account for the same area measured as the other sites.


300+
250-300
200-250
150-200
100-150
50-100
0-50

3.5.4 Dentate Gyrus

Seizures increased the number of BrDU-labeled cells in the dentate gyrus for both sham surgery and lesion conditions (Figure 23). There was significance between all lesion and stimulated groups ANOVA ($F(13,36)=2.384$, $p=.0199$). Thus, follow-up analyses were conducted. Stimulation (below seizure threshold) had no effect on the number of new cells in the dentate gyrus, as all below seizure threshold stimulation groups combined had slightly higher levels than no stimulation, but this effect was not significant (Figure 24A). There was an effect of seizures on the number of new cells in the dentate gyrus (Figure 24B). Combining surgery conditions, early, prolonged and late above threshold stimulation had significantly more BrDU-labeled cells than the non-stimulated brains, for Early, ($t(12)=2.667$, $p=0.0100$); Prolonged ($t(12)=3.542$, $p=0.00203$) and Late ($t(13)=2.852$, $p=0.00681$), with an alpha level of .05/4, bonferonni adjusted to .0125. For the dentate site, we predicted a priori that seizures in the normal brain would have the most proliferation; this prediction was based on previous kindling data (Parent et al. 1997), therefore the dentate statistics were based on 1-tailed t-tests. There was an effect of surgery condition on the number of dentate BrDU labeled-cells for the stimulation conditions (Figure 21C). The sham surgery groups had more BrDU-labeled cells than the lesion surgery groups ($t(39)=2.026$, $p=.0248$). This follow-up test was based on a bonferonni of 0.05/2, since all groups involved this follow-up test were only subjected to one previous follow-up test.

Figure 23. Photomicrographs of the dentate gyrus.

In the sham surgery condition, compared to the normal brain (A-B), seizures (C-D) significantly increased the number of BrDU-labeled cells in the dentate gyrus. Cortical lesions in non-stimulated brains (E, F) had fewer cells than intact controls (A, B). Seizures and lesions increased the number of labeled cells (G, H) compared to non-stimulated lesion control. Scale bar in (A) 250 μm refers to C, E G as well. B, D, F, H were taken at 20 x magnification (scale bar missing).

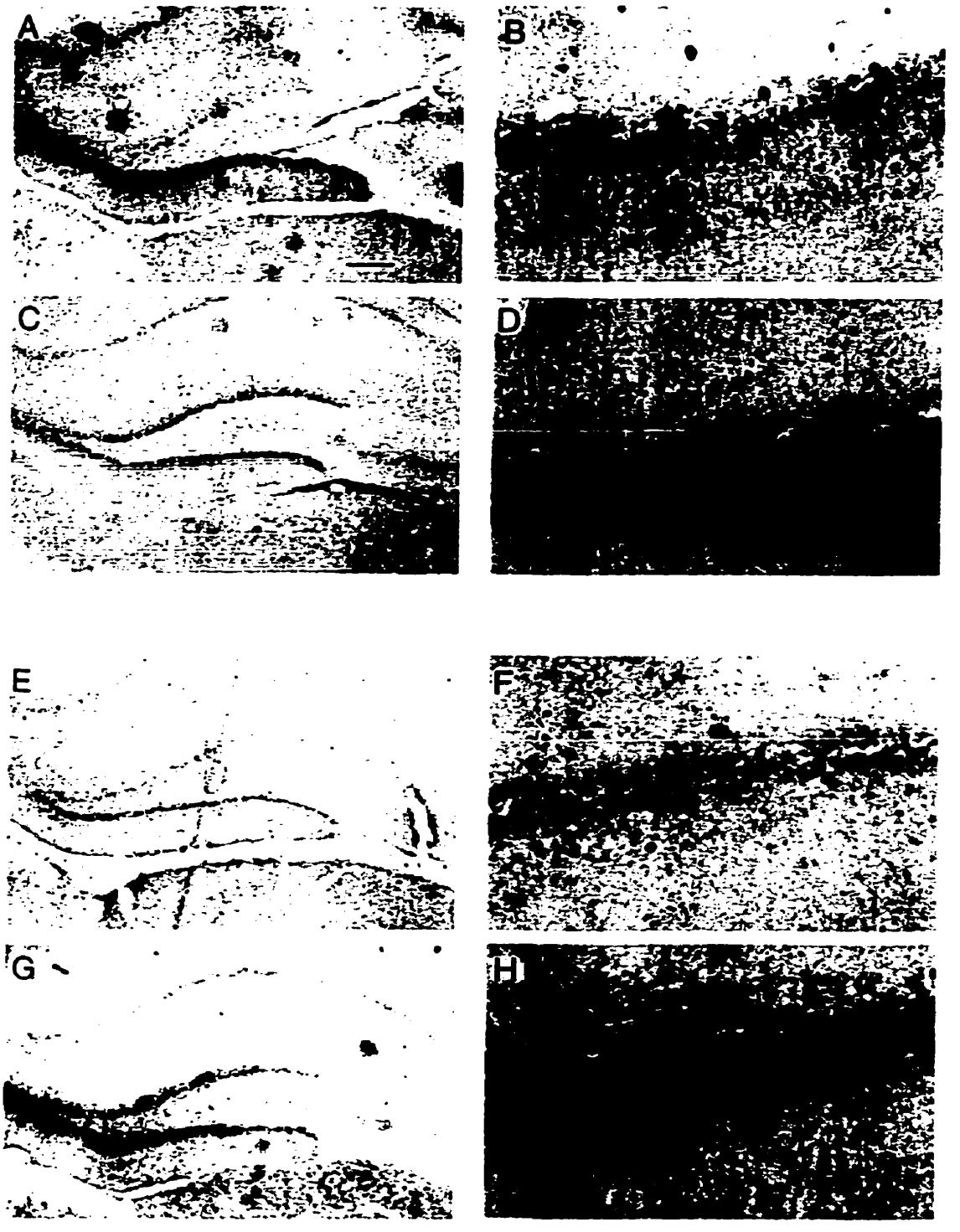
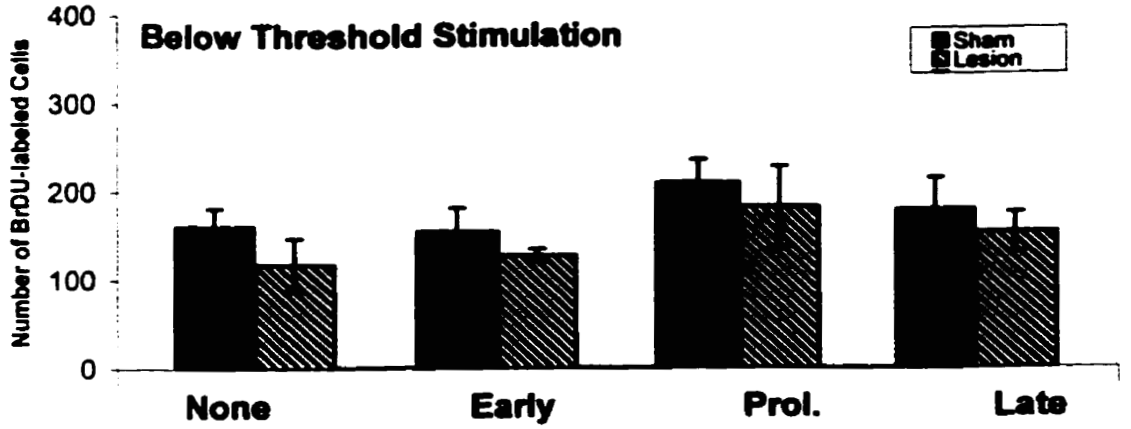

A
B
C
D
E
F
G
H

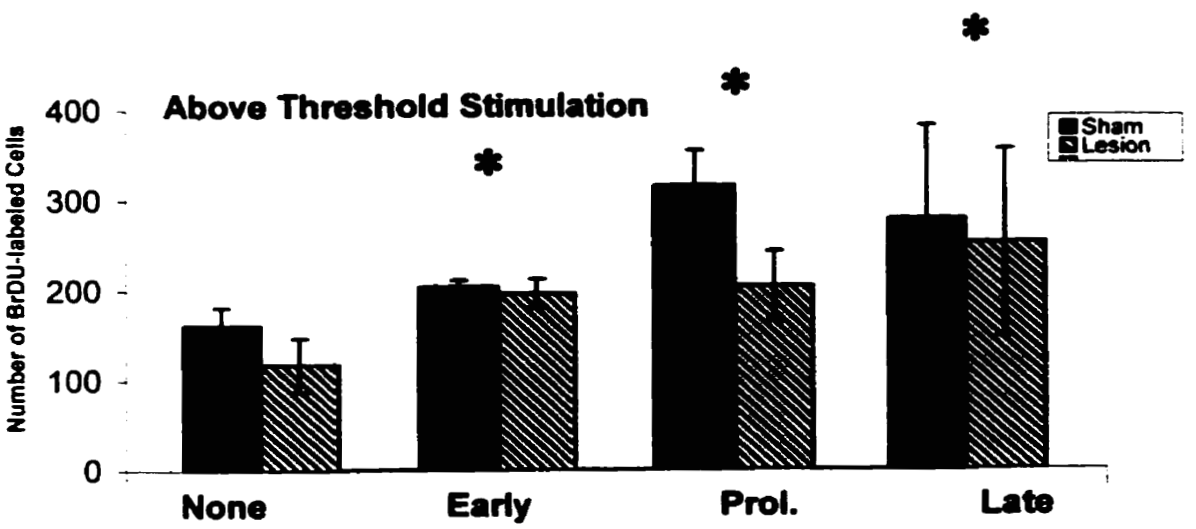
Figure 24. The effect of below and above seizure threshold stimulation on BrDU-labeled cell count in the dentate gyrus

Below threshold stimulation did not increase the number of BrDU-labeled cells in lesion or sham surgery conditions compared to the non-stimulated control groups (A). Early, prolonged and late above seizure threshold stimulation for the sham and lesion groups combined increased the number of BrDU labeled cells in the dentate compared to the non-stimulated controls (B). Across stimulation groups, sham surgery conditions had more labeled cells than the lesion surgery condition (C). * indicates statistical significance ($p<.0167$). * indicates statistically significance ($p<.025$).

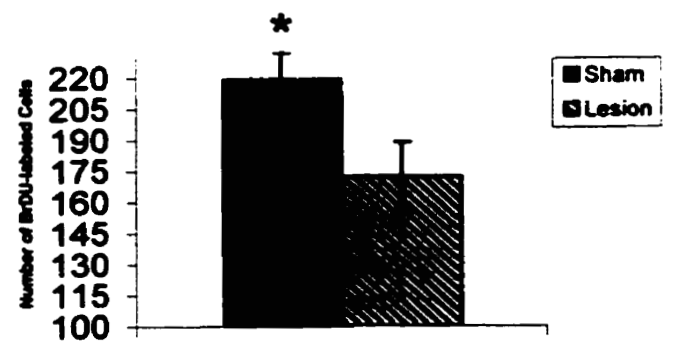
A


Below Threshold Stimulation
Sham
Lesion
Number of BrDU-labeled Cells
400
300
200
100
0
None
Early
Prol.
Late

B


Above Threshold Stimulation
Sham
Lesion
Number of BrDU-labeled Cells
400
300
200
100
0
*
*
*
None
Early
Prol.
Late

C


All Stimulation Groups
Sham
Lesion
Number of BrDU-labeled Cells
220
205
190
175
160
145
130
115
100
*

4. Discussion

This research was exploratory, as no previous research groups have assessed the effects of seizures and stimulation on cell proliferation in the injured brain. Due to the lack of previous research we did not know whether post-injury seizures would facilitate cell proliferation, nor did we know what time points following injury would be the most effective in enhancing cell proliferation. Thus, we maximized the number of groups in the experimental design, by applying seizures at different times post-lesion and applied different stimulation intensities. Further, numerous sites, within each brain, were analyzed for BrDU incorporation in order to trace the possible cell origin and path. The results show that there is reason to believe PIS do facilitate cell proliferation following injury and that these cells may have migrated from the SVZ through the corpus callosum to enter the cortex. While, in the intact brain, SVZ and dentate cell populations may respond differently to seizures.

ECS following injury had a profound effect on the brains' neural activity, gross structural appearance and the number of newly born cells occupying the cortex, corpus callosum, SVZ and dentate gyrus. Lesions alone produced a marked increase in the number of BrDU-labeled cells in the cortex, and cingulum, but not the corpus callosum, SVZ and dentate. Some seizure groups resulted in a significant amount of excess tissue at the lesion site compared to the non-stimulated group. Further, stimulation and seizures following injury produced increases in the number of labeled cells in the cortex, cingulum. The cingulum had the highest cell counts of all sites following lesions, up to four times more new cells occupied this site at the base of the lesion scar than each proximal or distal cortical area. Counter to our predictions, seizures administered to intact brains decreased the number of BrDU-labeled cells in all forebrain sites.

4.1 Effect of stimulation on the cortex

The cortical surface of some brains receiving lesion & stimulation protruded through the surgical opening made in the skull for the aspiration lesion. Structurally, this tissue was well adhered to the underlying tissue, and did not break away during sectioning or tissue processing. It was possible that this protrusion could either have been a manifestation of new, re-generated tissue or swelling. The non-stimulated brains had no protruding tissue and the mean area of the lesion cavity, measured mid-lesion in a coronal plane, was over -20,000 square μm (negative representing a lesion cavity). It appeared the tissue protrusion was an effect of stimulation. The prolonged and late above stimulated brains had extensive tissue protrusion and relatively little lesion cavity remaining, and the mean lesion area was over + 30,000 square μm (a net of excess tissue). However, in the early above stimulated brains there was a lesion cavity equivalent in size to the protruding tissue, therefore the net lesion area was approximately zero. This seizure group difference could be based on recovery time from injury. Referring to the experimental design (figure 1), in the early group, there was a 16 day period between the last ECS and the perfusion, and this recovery period was only 8 days for the prolonged and late groups. Thus, the protrusion could have been a temporary effect of the stimulation. Based on the experimental design, it is unclear whether a stimulated brain's lesion area, over more time, would revert back to having a lesion cavity equal in size to the non-stimulated brains. Nissl staining revealed a cytoarchitecture consistent with the notion that above threshold ECS increased intracranial pressure (unrestrained by the skull opening), allowing the cortex to shift.

The net amount of tissue in the below threshold stimulation groups was extremely variable. This variability may have resulted from differing lesion depths. The variability was not only

between brains of the same group, but between hemispheres of an individual brain; a few brains had with one hemisphere devoid of extruding tissue and the other hemisphere had a net positive region. As a side note, this variability between hemispheres justified the argument that each hemisphere (receiving a slightly different lesion) was independent of the other, allowing each hemisphere to serve as a separate observation. This would have increased power, although the more conservative approach was taken, and each animal served as one sample. Post hoc, it is difficult to account for the below threshold seizure group variability based on the original lesion size, although depth of the lesion scar may account for some variability. It may seem that measuring lesion area and lesion depth would be confounding, in that one would expect that a larger cavity would be accompanied by greater depth. However, this prediction was not observed. In fact, the overall trend for the hemispheres with protruding tissue was for the lesion scar to extend into the corpus callosum. When the lesion area was net negative (more cavity, than protrusion), the scar did not breach the corpus callosum. Thus, with the low intensity stimulation a deeper lesion may have been necessary to permit mechanical folding from the pressure below. However, to confirm this observation, a future study would need to incorporate superficial and deep lesions followed by below threshold stimulation. Lesion depth may not have been as crucial for cortical protrusion in the above threshold group where the stimulation intensity was 100 times greater. In summary, based on tissue composition, nissl staining, and the pattern of protrusion across the groups, the tissue protrusion possibly resulted from ECS-induced mechanical forces, rather than over zealous tissue regeneration. Regardless, new BrDU-positive cells were abundantly found in the forebrain following lesions. The expression of these new cells may illuminate a possible functional significance of PTS.

One problem when quantifying the BrDU-labeled cell count within a specific cortical area was the massive cortical distortion in the brains with tissue protrusion. Specifically, the expansion of tissue in the brains with protrusion may have biased the counts in favor of the non-stimulated brains because of an increased distance from the SVZ and tissue density may have decreased. Ideally, every cell in the cortex would have been counted, although there were thousands of new cells per coronal section through the lesion. Thus, sampling defined areas was the alternative. Sampling sites were chosen based on structural reference point within each brain. Some of these reference points were more "fixed" than others, for instance, images captured from the cingulum and the body of the corpus callosum would be less biased by the cortical protrusion's structural and density alterations than the other sites. Reference points for cortical count sites included: the lesion scar (medial, lateral and dorsal aspects) and the cortical surface. Based on these biases, the increase in BrDU-positive cells in stimulated verses non-stimulated brains may have been conservative estimates.

4.2 BrDU-expression following injury

In the non-stimulated brains, new cells were in close proximity to the lesion scar and there were few cells. Based on the known glial response following injury, these may all be glial cells. In the stimulated brains, there were more cells, densely clustered surround the lesion scar, and in addition, there was a higher proportion of BrDU-labeled cells dispersed through out the surrounding cortex (distal) (Figure 25). Often these isolated, new cortical cells were larger in diameter, than those densely packed along the lesion scar. Specifically, lesions without stimulation produced a marked increase in the number of BrDU-labeled cells in the cortex, and cingulum, but not the corpus callosum and SVZ, relative to the intact brain (Figure 26A, column1).

This distribution of new cells suggests that immediately post-injury, cell proliferation was activated, evident by the increase in cells in the cortex and cingulum. However, by Day 24 post-lesion, the number of BrDU-labeled cells in the corpus callosum was the same as the number found in untreated brains. Further, the dl SVZ cell number following lesions alone was less than the untreated brains. This data suggests that immediately following lesion (without ECS) is a massive production of cells, although prior to Day 24, post-lesion cell proliferation declines. This observed post-lesion cell proliferation was most-likely reactive astrocytes. Reactive astrocytes (including proliferation of microglia and astrocytes) has historically been considered inhibitory to recovery. However, reactive gliosis may function to create a barrier around the lesion, inhibiting secondary lesions of intact tissue. Further, reactive astrocytes produce neurotrophic factors and increase the expression of receptors (See review: Ridet et al, 1997).

Figure 25. Cell density at the lesion site.

In the lesion and seizures group (A) there was more cells at the lesion site, indicated by the colour red, surrounding the lesion scar. In the lesion and no stimulation group, there were fewer cells at the lesion site. The colour scale ranges from Red-representing a high concentration of BrDU labeled cells to Purple/Blue-representing no cells. Scale in (B) 250 μm also refers to (A). (The sections are rotated so that the lesions are vertical-for the medial cell counts) LC=lesion cavity, Fr2=frontal cortex. The red color above the lesion and on the cortical surface is noise.


A
Fr2


B
Fr2

26. Summary of Findings

For the lesion surgery condition (A-B) and the sham sugery condition (C) the statistical results for the number of BrDU-labeled cells for each site are summarized in table format. In (A) Lesion alone, Lesion/Stimulation, and Lesion/Seizures are compared to those stimulation groups in the sham surgery condition. In (B), the changes in Lesion/ Stimulation and Lesion/ Seizures are compared to the lesions alone. In (C) Stimulation and Seizures in the intact brains are compared to the untreated group. INCREASE= a statistically significant increase for all early, prolonged and late groups compared to the sham control. DECREASE= a statistically significant decrease for all early, prolonged and late groups compared to the sham control. NO DIFF.= no statistical difference for all early, prolonged and late groups compared to the sham control. (e, p or l) after the increase or decrease refers to only the early, prolonged or late group being statistically different than the control group.

Lesion Surgery

A.

	Lesion Alone	Lesion/Stimulation	Lesion/Seizure
Distal Cortex	INCREASE	INCREASE	INCREASE
Proximal Cortex	INCREASE	INCREASE	INCREASE
Cingulum	INCREASE	INCREASE	INCREASE
Corpus Callosum	NO DIFF.	INCREASE	INCREASE
dl SVZ	DECREASE	INCREASE	INCREASE

B.

	Lesion/Stimulation	Lesion/Seizures
Distal Cortex	INCREASE	INCREASE (e,l)
Proximal Cortex	INCREASE	INCREASE (e,l)
Cingulum	INCREASE	INCREASE
Corpus Callosum	NO DIFF.	INCREASE
dl SVZ	INCREASE (e,p)	INCREASE (l)

Sham Surgery

C.

	Stimulation	Seizure
Distal Cortex	NO DIFF.	NO DIFF.
Proximal Cortex	INCREASE (p,l)	DECREASE (l)
Cingulum	INCREASE (p)	DECREASE (l)
Corpus Callosum	NO DIFF.	DECREASE (p,l)
dl SVZ	DECREASE (e)	DECREASE

The lesion and stimulation produced more BrDU-labeled cells in all sites analyzed compared to stimulation in the intact brains (Figure 26A, column2). However, this comparison was confounded by the massive proliferation of cells following lesions alone. Thus, the number of BrDU-labeled cells in the stimulation/lesion groups were also compared to the group receiving lesions alone (Figure 26B,column 1). Stimulation increased the levels of proliferation in the cortex and cingulum compared to lesions alone. Further, unlike the lesion alone group, enhanced cell proliferation may have been sustained until at least until Day 24, evident by the amount of cell proliferation at the SVZ. Similarly, seizures following injury increased proliferation compared to injury alone (Figure 26B,column2). However, not all seizure groups effected BrDU-labeled cell count equally; the prolonged administration of seizures did not statistically increase the number of new cells compared to lesions alone, reliably across all sites. This result indicates that prolonged changes in neural activity may over time may begin to attenuate proliferation.

Generally, cortical lesions alone activated proliferation, but stimulation/seizures after lesions facilitated the proliferation and may have assisted in sustaining proliferation. This observation leads to the hypothesis that there may be an interaction between lesion-induced proliferation and the biochemical effects of stimulation. Specifically, reactive gliosis may produce growth factors, and the stimulation/seizures may function to sustain the activation of these growth factors that promote cell proliferation and survival. If ECS interacts primarily with reactive gliosis, then stimulation/seizures in the intact brain may not be capable of inducing SVZ proliferation. This hypothesis could explain why seizures in the intact brains did not increase cell proliferation. In fact, seizures alone may have temporarily decreased SVZ cell proliferation (discussed below). Experimentally inhibiting reactive gliosis (See review: Ridet et al, 1997) could

test the prediction that seizures assist in it's protective function.

Most of the BrDU-positive cells may be astrocytes. However, if stimulation/seizures effects the number and expression of glial cells around the lesion, then the prediction of PIS as a functional adaptation is still viable. Glial response following injury may protect the intact surrounding tissue from further injury and promote growth factor production (Ridet et al, 1997). However, to find even one new neuron in the adult rat cortex following the application of stimulation would be important for the recognition of PTS as having a functional role in recovery. The presence of neurons would also more likely influence treatment from injury (or lack of anticonvulsive drug treatment), as well as providing an avenue of research for treatment of neurodegnerative disorders. In our study, preliminary double label findings suggest that new neurons can be rapidly located in the cortex of injured brains following seizures (Figure 27). It is now necessary to 1. confirm this finding using confocal microscopy, and 2. determine the relative proportion of neuron and glial cells.

Figure 27. Photomicrograph of BrDU and NeuN double labeled cell

A cortical cell in the lesion and stimulation group that is double labeled for BrDU (green) and NeuN (red). The existence and proportion of double labeled cells needs to be determined for all groups.

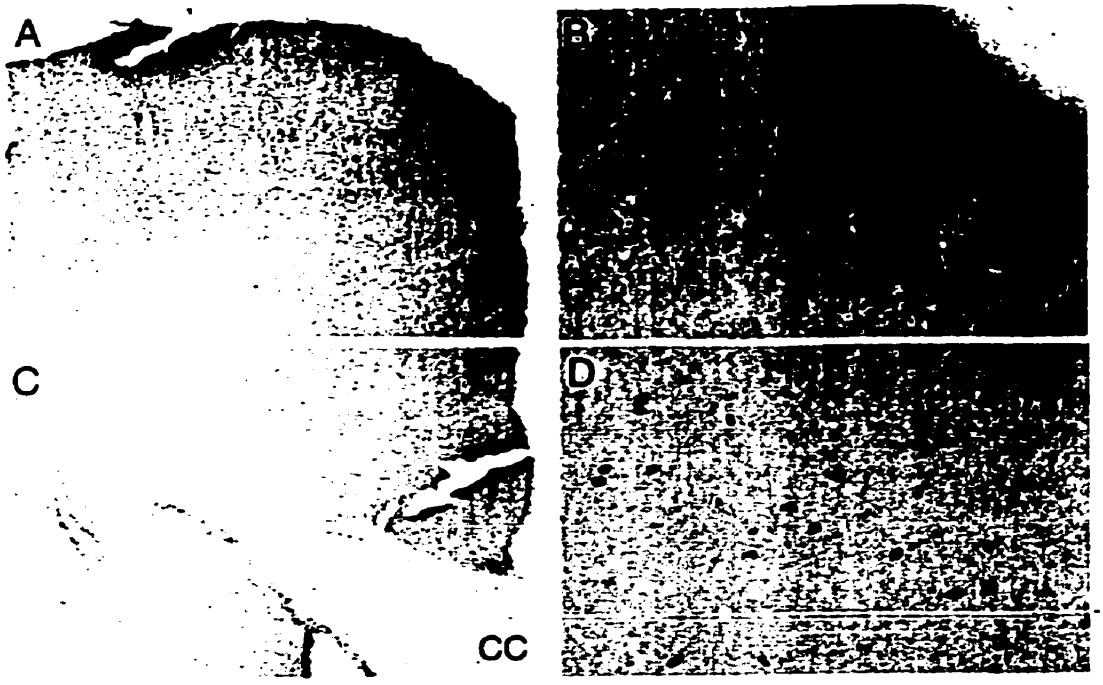
4.3 BrDU expression in the intact brain

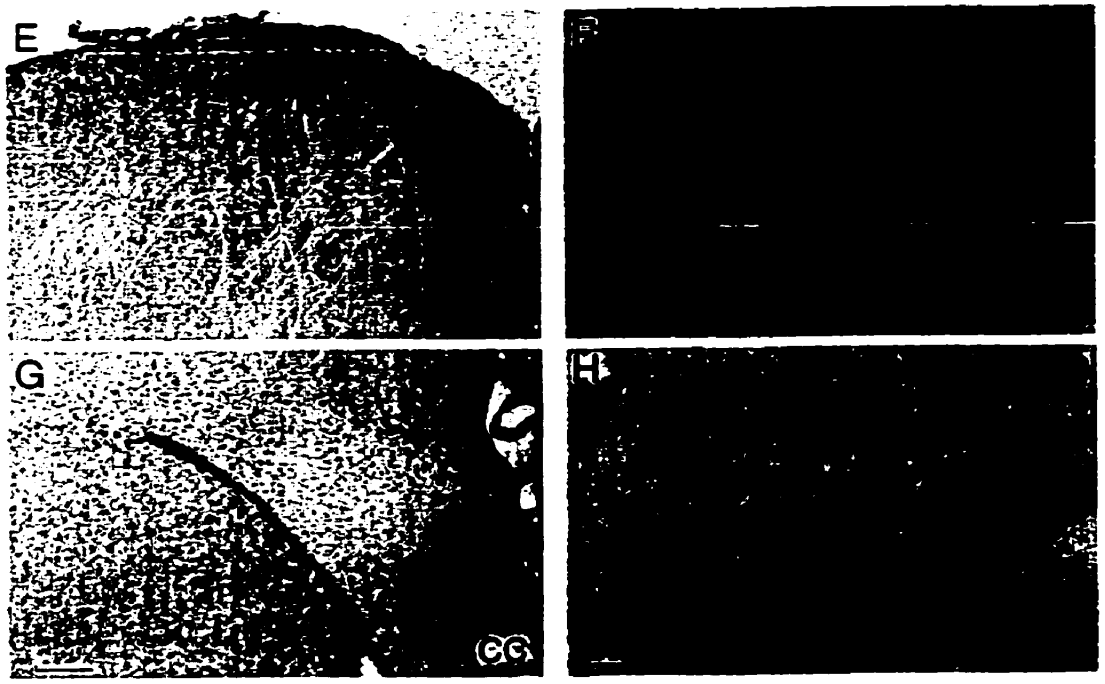
Two of five untreated brains contributed the most to cell count numbers in that group, having BrDU-labeled cells dispersed throughout cortex. New cells were also observed in the fornix, olfactory cortex-posterior of the RMS, and surrounding the anterior commisure. In light of the recent Gould et al (1999) article, which found new neurons in the adult, monkey cortex, the possibility that these new cells in the normal, rat cortex are in fact new cells and not an artifact of staining, is viable. However, the authors contend that cortical neurogenesis in mammals is an anomaly limited to primates. The phenotype of these new cells in the normal rat cortex needs to be determined. Glial cells are added to the mammalian brain through out life. Thus, it is highly likely that these cells are of glial phenotype.

Below threshold stimulation in the intact brain resulted in the expression of disperse cells through out the frontal cortex and cingulum (Figure 28). This effect was mostly observed with prolonged stimulation (Figure 26C, column 1) and less reliably late seizures. However, after stimulation is ceased, there may be a slight rebound in SVZ cell production, indicated by the decrease in dl SVZ proliferation in the early stimulation group. This rebound may not be observed in the prolonged and late group until after day 24. It appears that chronic low-level stimulation may facilitate cell proliferation of some proliferative zones. However, surprising and possibly unexplainable, late seizures applied to the intact brain decreased the number of new cells in the cortex (proximal site), cingulum and corpus callosum, and SVZ but not in the distal cortex, relative to the non-stimulated group (Figure 26 C, column 2). Thus, intense stimulation in the normal brain may temporarily interfere with SVZ cell production.

Figure 28. Photomicrograph of BrDU-labeled cells in the untreated and stimulation treated brains.

The expression of BrDU-labeled cells in the cortex and corpus callosum in the sham surgery groups were few regardless of stimulation treatment. The cortex (A, B) and corpus callosum (C, D) of the untreated brain had some BrDU labeled cells. Below threshold stimulation increased cortical cell number (E, F) and corpus callosum cell number (G, H).


A
B
C
CC
D


E
F
G
GG
H

4.4 Where do the new cells originate?

Structurally, it is feasible that the new cortical cells originated from the SVZ because of the close proximity of the frontal cortex and RMS. In the adult, mammalian brain, it takes less than six days for cells to travel from the SVZ to the olfactory bulb (Lois and Alvarez-Buylla, 1994). In our study, there would have been enough time for cells to migrate to the cortex, since injections were over a 16 day period. However, a time course study, where each animal is perfused at different time intervals following the BrDU injection (and injury) would better confirm this speculation.

Assuming that a high proportion of cells born early in the experiment migrated away from the SVZ, sampling the number of new dl SVZ cells gave an estimate of recent proliferation. The application of late seizures to the injured brain produced significantly more BrDU-labeled cells than no stimulation. As well, the Prolonged Seizures group was meaningfully different than applying no stimulation, although after the bonferroni adjustment, the number of new dl SVZ cell in this group was not statistically different than no seizures. In the Early Seizures group the number of SVZ BrDU-labeled cells was few and statistically equivalent to no stimulation (refer to Figure 21F). Based on this finding, there are two hypotheses: 1. Early seizures had no effect on facilitating cell proliferation, or 2. Seizures and Lesions had an acute effect on proliferation and this proliferative zone is no longer profoundly activated by Day 16. The second hypothesis is more likely because new cells were found extensively throughout the forebrain in this group. Seemingly counter to this alternative hypothesis, the early below seizure threshold group had significantly more new cells in the dl SVZ than the non-simulated brains. It is possible that below threshold stimulation does not facilitate migration as effectively as the higher intensity stimulation

for injured brains. This rationale is also applicable to differences found between the Late (stimulation and seizure) groups. Of the four groups receiving stimulation in the later part of the experiment (i.e. above & below threshold for prolonged & late groups), all had significant increases in dl SVZ cell number, except the late below group. This may suggest that it takes longer to "turn on" proliferation mechanisms with less intense stimulation, where as the prolonged below group was also activated throughout the early period. Based on the cell count pattern across early and late groups the two stimulation intensities (above and below) may not have identical effects on cell proliferation and migration. The effect of posttraumatic stimulation/seizures on the proliferation of new dl SVZ cells may indicate the following generalizations: 1. Post-injury stimulation and seizures facilitate cell proliferation in the dl SVZ, 2. Seizures may have an acute effect on this proliferative zone, where proliferation may decline soon after ECS is terminated, and 3. Below threshold simulation may recruit proliferation and migration mechanisms slower than more intense stimulation and the resulting seizures.

In the above rational was the generalization that all cells born at the SVZ migrated away to occupy the cortex and RMS/olfactory bulb. However, each cell may have unique, complex behaviours and environmental interaction, since there are varying cell phenotypes arising from the SVZ, including: Type A (which react positive to TuJ1 and PSA-NCAM, a neuronal marker and molecule for cell migration, respectively), Type B (GFAP positive for astrocytes), and Type C (non-reactive to neural and glial stains, but contain Nestin, found in undifferentiated stem cells) (Doetsch et al, 1997). Thus, some cells may not migrate, but continue to divide at the SVZ (indicated by lightly stained nuclei presumed to be an effect of subsequent cell divisions) and cell death, not accounted for in this experiment, is likely to be substantial (Pretel et al, 1997).

4.5 Effect of stimulation on the dentate gyrus

The anterior of the dentate gyrus was included in the study because these new cells were born from a separate pool of stem cells. ECS stimulation increased the number of BrDU-labeled cells in the dentate gyrus. All of the above threshold simulation groups (early, late and prolonged) had a significant effect on the number of new cells over the non-stimulated group, suggesting that a minimum of four ECS stimulations enhances proliferation. However, this enhancement is different than previously reported. Parent et al (1998) found that at least 9-10 fully generalized kindled seizures were required to induce proliferation. During kindling, it is possible that the non-convulsive stimulation prior to reaching generalized seizures attenuates proliferation. However, ECS seizures may have produced more intense limbic activation than kindling, resulting in more rapid recruitment of the proliferative response, as chemically induced seizures and status epileptus can produce proliferation after one session (Parent et al, 1997).

In the intact brains, seizures increased new dentate cell number for the early, prolonged and late groups. The increases in each of these stimulation conditions was matched by slightly lower levels of proliferation in the respective lesion groups. This reduction across the lesion groups may be due to the fact that animals with lesions were less mobile. Environmental enrichment (Kempermann et al, 1998) and specifically running (van Praag et al, 1999), increases dentate proliferation. Thus, the lowered cell proliferation in lesion groups may have been a result of motor activity differences between the animals in the two surgery conditions (Lawrence, unpublished observation). This activity level postulate assumes there is an interaction between motor activity and neural activity (seizures). An interaction could possibly be mediated through

growth factor response from motor activity (Kuhn et al, 1997) and known growth factor/receptor activation with seizures (Ernfors et al, 1991; Isackson et al, 1991 ; Lindvall et al, 1994 ;Yuen et al, 1996). This postulated interaction between motor activity and seizure activity can not be confirmed by this study, but warrants further investigation. However, the cortical lesions' 'injury signals' could be causing or contributing to the attenuation in the number of dentate BrDU-labeled cells following injury.

4.6 Possible Mechanisms

Cortical brain injury radically increases gene expression for neurotrophic factors. Neurotrophic factors are polypeptides that facilitate the growth, differentiation and survival of neurons. One family of neurotrophic factors is the neurotophins, including: nerve growth factor (NGF), brain-derived neurotrophic factors (BDNF) neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). These molecules function by binding to tyrosine kinase receptors. The receptor activation causes a cascade of biochemical events leading to morphological and functional cell modification (See Review: Yuen et al, 1996). Brain insult such as hypoglycemia, ischemia, traumatic brain injury and epilepsy increase neurotrophin levels (Lindvall et al, 1994). For example, seizure activity increases NGF mRNA (Gall et al, 1989) and BDNF (Ernfors et al, 1991; Isackson et al, 1991) expression in the cortex and hippocampus. Neurotrophins may function as a neuroprotective mechanism, stimulating sprouting and synaptic reorganization. There are many families of growth factors. Thus, different combinations of the growth factors may be activated following brain injury and others in response to the ECS induced post-injury seizures. Specifically, ECS induced seizures, in intact rat brains, has been found to increase NGF and bFGF in all sites analyzed, including the striatum, entrorhinal cortex, hippocampus and cerebellum

(Follesa et al, 1994). NGF is produced in the cortex and hippocampus, and is thought to enhance gene expression and cell differentiation (Yuen et al, 1996). bFGF increases the mitotic rate of SVZ stem cell proliferation and facilitates RMS migration (Kuhn et al, 1997). However, it is likely that specific combinations of neurotophins function to maintain the unique processes involved in adult neurogenesis, and these neurotrophin combinations likely vary for the different cell populations. The posttraumatic induced seizures may endogenously activate fundamental combinations of growth factors.

4.7 Are seizures necessary?

Based on the hypothesis that seizures facilitate post-injury cell proliferation, it was predicted that below seizure threshold stimulation would not increase cell proliferation compared to the non-stimulated brains. This group was added to the experiment to serve as a stimulation control. So, why did cell proliferation intensely respond in the below seizure threshold group? If the below threshold stimulation was intense enough to produce mechanical changes (i.e. the tissue protrusion), then possibly biochemical changes also occurred. However, the animals did not behaviourally manifest seizures, nor did repeated stimulation result in behavioural convulsions. Further, in the electrophysiology control animals, EEG recorded from the neocortex, did not show any afterdischarge activity. However, the EEG was not recorded continuously, therefore the below threshold stimulation may have facilitated spontaneous seizures or non-convulsive seizures in the lesion groups (already prone to spontaneous seizure activity from the brain injury). As a side note: All of the above seizure threshold stimulation animals displayed fully generalized seizures. However, the seizure duration progressed between the first and last session in the sham surgery animals, but not for the lesion surgery animals. This surgery group difference was

probably a reflection of an initially shorter seizure duration in the sham group, indicating that the cortically injured brains may have had a predisposition to seizure activity. The FFT changed dramatically following above threshold stimulation, and little following below threshold stimulation. However, the FFT analysis for the below threshold stimulation was different than non-stimulated group, indicating minor, yet detectable, changes in neural activity. Thus, an increase in neural activity and neurotrophin response may have occurred independent of the behavioural seizures. Follesa et al (1994) applied corneal maximal electroshock (MES) or low-intensity (minimal) electroshock (LES) and assessed growth factor levels. For stimulation to qualify as MES, animals had to display tonic hind limb extension, and for LES clonic movements in the face and forelimbs were required for 5 seconds. Follesa et al found that both MES and LES produced increases in bFGF mRNA and NGF mRNA in the entrorhinal cortex and hippocampus. However, the mRNA increases for the neurotrophic factors were greater in the LES group than the MES group. The fact that less intense stimulation produced greater growth factor activation may provide intriguing predictions for the mechanisms behind below threshold stimulation cell proliferation. Although our below threshold stimulation was less intense than LES, the discrepancy may be countered by the brain injury compromised state in our study, relative to their intact rats. In other words, there may be an interaction between the below threshold stimulation and the brain injury, resulting in facilitation of the growth factor response, leading to the observed cell proliferation. However, these suggestions need to be addressed empirically. A prolonged duration of low level of stimulation may better maintain the cells survival than intense, acute stimulation. Electrical stimulation does enhance bone, skin and peripheral nerve regeneration (see review: Sisken et al, 1993). Stimulation may also be a successful treatment following CNS injury, stroke and for neurodegenerative disorders.

4.8 Implications of PTS as a functional adaptation

The first decade of this century is in line to be marked by great neuroscience advances, including better treatments and possibly cures for Alzheimer's Disease, Parkinson's Disease, aging, and brain injury. The potential of these treatments to include cell replacement, or some means of activating and harnessing endogenous cell proliferation, migration and differentiation is likely.

No previous research groups have assessed the effects of seizures and stimulation on cell proliferation in the injured brain, therefore we did not have an indication of when seizures would be most effective in enhancing cell proliferation. Thus, we maximized the number of groups in the experimental design, by applying seizures at different times post lesion and applied different stimulation intensities. Further, numerous sites, within each brain, were analyzed for BrDU incorporation in order to trace a possible cell origin and path. The results show that there is reason to believe PIS do facilitate cell proliferation following injury and that these cells can migrate from the SVZ to occupy the cortex. And, cells displaying neuronal phenotype were expeditiously located in the cortex surrounding the lesion following induced seizures. It is now critical that phenotypic ratios be determined. However, in light of the notion that post injury glial proliferation may be beneficial to structural recovery and axonal sprouting, increases in either, or both, glial cells and neurons in the adult following PIS has astounding implications. Whether these new cells integrate into cortical circuitry needs to be determined. Structurally, it is important that the new cells form axonal projections, although, ultimately, facilitation of behavioural improvement following injury would fully express the neuroplastic adaptive potential of PIS.

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Appendix 1: Recipes for immunohistochemistry procedures

Paraformaldehyde

Heat 100 ml of 2X DH_2O, add
8 g paraformaldehyde, add
1M NaCL a few drops at a time, until clear, add
100 ml .2M phoshate buffer

10 X PBS: 1 L
To 600 ml of heated 2X DH_2O, add:
80 g sodium chloride
2 g potassium chloride
14.4 g sodium phosphate
2.4 g potassium phosphate
disolve solutes and fill to 1 L with 2X DH_2O
Dilute sample to 1 X PBS to measure pH, pH to 7.4.

Mounting Media (for Fluorescence)

2.5 % PVA/DABCO Solution for 50 ml:
1. weigh 12 g glycerol
2. Add 4.8 g PVA, mix until PVA coated
3. Add 12 ml DH_2O, mix overnight
4. Add 24 ml of .2 M Tris-HCl pH 8-8.5
5. Heat to 50 ^{0} in water bath 10-30 min
6. Add 1.25 g of DABCO and mix well
7. Centrifuge @ 5000 g for 15 min, remove supernatant and store @ -20 ^{0}

DABCO= 1,4 diazabicyclo[2.2.2] octaine (Sigma, #D2522)
PVA= polyvinyl alcohol (Sigma, # P 8136)

Saline Citrate Buffer (SSC)

20 X Stock
175 g NaCl
88 g Na Citrate (tri)
900 ml pH to 7.0
1 N H Cl final volume 1 L, use as 2 X

PBS + .3 % Tx100

897 ml DH_2O
3 ml of tritmx
100 ml 10 X PBS

Cryoprotectant

25%	Ethylene Glycol
25%	Glycerin
	.05M PBS

Ni-DAB

25 ml	.05 M Tris-HCl (pH 7.6)
10 mg	Nickel Chloride
5 mg	DAB
7.5 µl	30% H_2O_2

Appendix 2: Thresholding technique for estimating cell quantity

Thresholding, Smoothing and Counting Particles

Discrete objects such as particles, cells, filter pores, etc. can be sized and counted with NIH Image. Start with the original test image, or restore it with the *Revert to Saved* command. To count and size the pores in the filter, enable the bi-level thresholding mode using *Options - Density Slice* (or double-click on the LUT tool) and set the threshold limits in the LUT window with the LUT tool to 243-254. Do this by double clicking on the LUT tool. A red band will appear in the color bar, and the corresponding pixels in the image will appear red. Drag the top of the red band in the color bar with the LUT tool to the top of the LUT window. Practically all of the image will appear red. Drag the bottom of the red band with the LUT tool to value 243 - read the values in the Info window. Now the red areas are associated mostly with the pores of the filter. (Fig. 9)

Figure 9-- Filter image with pores selected using thresholding (see text).

Note that intensity levels of 0 and 255 are not accessable for thresholding. I.e., zero and 255 can't be within the density slice because those entries in the lookup table can never be colored red. 0 is always white and 255 is always black. This is intentional, to prevent loosing the mouse, tools, etc. due to thresholding. If an image shows saturation, either in the black or white, then pixels with values of 0 or 255 might likely appear within the areas of interest, but not be selectable via thresholding. If the image has values of 0 that need to be selected, for example, they may be changed to value 1 using the *Process - Arithmetic command* twice: 1) *Arithmetic - subtract* , then use 1 as the constant to subtract. NIH Image clips results of arithmetic so that they lie within the range 0-255, so this subtraction results in all pixels being lowered by one intensity unit, except for the zero valued pixels which are still zero. Note that this affects the internal pixel values - if the Invert Pixel Values box is checked, then the values to read are in the parentheses to the right in the Value: line of the Info window. 2). *Arithmetic - add* , then use 1 as the constant to add. This results in all pixel values being restored, except for the old zero valued pixels, which now have the value 1, along with the pixels which used to have the value 1. Use the reverse arithmetic sequence to lower pixels with value 255 down to 254. (Note 1. This can also be done using *Process - Fix Colors* .) Noise in the image causes lots of little red selected areas and some holes in the red dots representing the pores. Most of the noise in this case can be ignored by the *Analyze - Analyze Particles* command by setting the 'Min Particle Size' to something greater than the area of the small red dots (such as 10 pixels), and by checking the 'Include Interior Holes' box. Alternatively, smoothing the image will get rid of a lot of the dots (Fig. 10). Do this with the *Process - Smooth* command.

Figure 10-- Pores in smoothed TEM Filter image selected by thresholding.

When using the LUT tool, holding the option key down will temporarily turn the red highlights off, allowing for closer inspection of what is being selected. Double-clicking the LUT tool will toggle the red highlights on and off. The zoom tool (the magnifying glass) and the scroll tool (the hand) can also aid inspection.

To count the holes, first choose the parameters to be measured by clicking the appropriate boxes shown in the *Analyze - Options* dialog box if desired. The defaults - Area, Mean Density, X-Y Center, and Include Interior Holes were used here. Invoking the *Analyze - Analyze Particles* command and then checking all of its boxes, results in Fig. 11. The numbers by the pores correspond to the numbers in the table of results, viewed using the *Analyze - Show Results* command (Fig. 12). There are various ways to save this table, using the *File - _Export...* command (see About NIH Image), for example it can be exported as a text file for importing into other applications or incorporating into a report

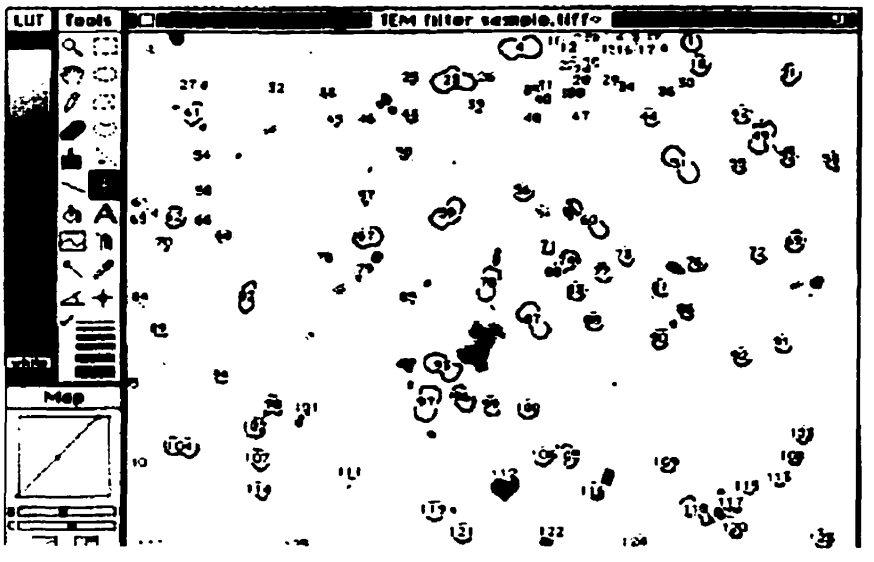

Figure 11-- Result of Analyze Particles command. Pores are outlined and numbered.

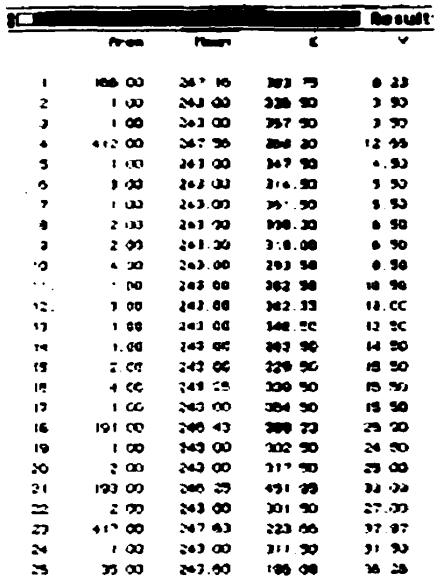

Figure 12-- Table of results corresponding to Fig. 11 (only first 25 rows shown), shown with Show Results command.

Figure 13 shows the thresholded particles rather than the pores. The threshold levels were set at 1 and 216. Fig. 14 shows the outlined particles. This time, the 'Label Particles' box was not checked. For review:

http://rsb.info.nih.gov/nih-image/more-docs/Tutorial/Particles.html