Examination of a Model of Thromboembolic Stroke To Understand Neuronal Plasticity and Recovery from Damage

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A Thesis Submitted to the Faculty of Graduate Studies

In Partial Fulfillment of the Requirements for the Degree of

Master of Arts

Department of Psychology

University of Manitoba

Winnipeg, Manitoba

July 2004

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

Of

MASTER OF ARTS

Acknowledgments

First and foremost I would like to thank Dr. Tammy Ivanco, my research advisor, for her support and guidance throughout the many phases of this thesis project. Dr. Ivanco has taught me to be a confident, independent and skilled researcher, and has graciously provided me with amazing opportunities to broaden my knowledge and skills. I would also like to thank the other members of my advisory committee, Dr. Todd Mondor and Dr. Marc Del Bigio, for their valuable feedback on this manuscript. Finally, I would like to thank my parents for their unwavering support and interest in everything I do. Without them I would not be where I am today.

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Abstract

The brain is capable of undergoing structural changes in response to experiences such as learning a new skill and brain damage. Damage produces structural changes similar to those resulting from learning, but on a much larger scale, allowing for a thorough investigation of brain reorganization. Rats underwent a photochemically-induced stroke within the right motor cortex. For 20 minutes, a high wattage, cold light source was used to illuminate the brain through an intact skull. The light was left off for control animals. Analysis on the cell drawings from neurons in both hemispheres showed that there was no significant difference in dendritic length between experimental and control animals. These results may indicate why small strokes do not cause behavioural deficits.

Chapter 1

General Introduction

BRAIN PLASTICITY

Plasticity is the term used to describe the ability of the brain to change its structure in response to experience or development (for review see Kolb & Whishaw, 1998). Research into plasticity falls into three main categories: anatomical/physiological, neurochemical, and metabolic (Kolb & Whishaw, 1989). Anatomical/physiological studies focus on the alteration of synaptic activity and the increased growth of dendrites. Neurochemical studies examine increases in neurotransmitter synthesis and release presynaptically, as well as the postsynaptic sensitivity to the transmitter. Finally, metabolic studies look at shifts in cortical and subcortical energy use.

A discussion of anatomical studies is relevant here, as these methods will be used to examine cortical reorganization in response to experience. The main goal of an anatomical study is to examine the morphological changes that the brain undergoes as a result of experience, or damage, and relate these physiological changes to behavioral changes. In response to experience there may be increases in the number of synapses (e.g. Kleim, Lussnig, Schwarz, Comery, & Greenough, 1996) or a change in synaptic structure within the activated area (e.g. Geinisman, Berry, Disterhoft, Power, & Van der Zee, 2001). Another response in cells produced by experience is dendritic growth (e.g. Withers & Greenough, 1989). Dendrites are branchlike structures attached to the cell body of the neuron and receive information transmitted from other neurons. There may also be an increase in astrocytes, which are a type of glial cell (e.g. Sirevaag &

Greenough, 1987). One of the functions of glial cells is to provide nourishment for the neuron; as the neuron becomes larger, more glial cells are required to support it.

There are several methods that can be used to induce plastic changes. The first involves providing experimental animals with a complex (also called enriched) environment usually consisting of a cage filled with different toys that are changed on a daily basis, as well as several littermates for social interaction. Investigators using this paradigm have found that there were more synapses per neuron in the occipital cortex of enriched animals compared to control animals housed in standard cages (Greenough & Volkmar, 1973; Volkmar & Greenough, 1972). Beaulieu & Colonnier (1987) found similar results in cats raised in either standard cages or complex environments. An additional finding in cats was that the experience of a complex environment seemed to modify the excitatory-inhibitory equilibrium by producing an increase in the number of excitatory synapses per neuron and decreasing the number of inhibitory synapses in the visual cortex. It seems that experience may serve to facilitate the formation of excitatory connections or possibly strengthen existing ones.

Using a complex (EC) environment, Green, Greenough, & Schlumpf (1983) showed an increase in dendritic material of occipital pyramidal neurons compared to animals housed individually in standard cages without any toys or other stimulating material inside the cage. Diamond (1988) conducted a series of experiments examining the anatomical changes occurring within the brain of complex housed (EC) versus standard housed (IC) animals. In these studies, rat littermates were randomly assigned either to a complex environment and maze training group, or to an individually housed condition. After 80 days samples were taken from the occipital and somatosensory

cortex. It was found that animals exposed to the complex (EC) environment had a significantly thicker occipital cortex compared to their IC counterparts. Counts of neurons and glial cells revealed a decrease in neurons for each microscopic field in the occipital cortex of enriched animals. This finding suggests that the plastic response to the complexity was not to increase the number of neurons, but rather to increase the size of existing neurons via an increase in dendritic branching (Diamond, 1988).

A second method used to study plastic changes involves training animals to do specific types of tasks. This method is more refined than the EC environment paradigm because it can demonstrate changes in specific brain areas believed to be involved in the execution of the task that is being performed. Withers & Greenough (1989) trained rats to reach into a tube for bits of cookie, either with the preferred paw or the nonpreferred paw. Dendrites of input layer II/III of the sensorimotor cortex were examined using concentric sphere intersection analysis and analysis of branching pattern. It was shown that the hemisphere contralateral to the paw used for reaching had significantly longer dendrites as well as an increase in branching compared to animals that used alternating paws or control animals.

Chang & Greenough (1982) occluded one eye of rats and then trained the animals on a visual maze. After training was completed, the neurons of the two hemispheres were compared to evaluate of the sizes of the dendritic fields. The authors hypothesized that only the cortex contralateral to the unobstructed eye would be able to process and store incoming information about the task. Results from the anatomical data revealed that neurons in the trained hemisphere had larger dendritic fields than neurons from the

untrained hemisphere, indicating the training influenced the rate of dendritic growth in the experimental hemisphere (Chang & Greenough, 1982).

A third method used to examine plasticity is to create damage. Response by the brain to damage employs mechanisms similar to those seen in learning, but on a much larger scale. Injury is a useful tool for studying the reorganizational abilities of the brain by allowing the researcher to observe the brain's innate ability to reorganize without any type of therapeutic intervention (Stroemer, Kent & Hulsebusch, 1995). Alternatively, implementing a therapeutic strategy such as an enriched environment, or skill training may be undertaken in order to enhance the brain's ability to reorganize (Chu, & Jones, 2000; Jones, Chu, Grande, & Gregory, 1999; Witte, 1998). Although damage is an important model in which to study plasticity, it also raises many controversial issues. One of the most actively debated problems revolves around a definition of what constitutes behavioral recovery and what is merely compensation. Kolb and Whishaw (1998) provide a criterion for evaluating "recovered" or "spared" behavior. One of the points they list is that the recovered behavior must be the same behavior that was observed before the damage. Many studies examining recovery simply use a quantitative improvement in performance as evidence that the behavior has been restored. The rationale being that if the time taken postoperatively for an animal to successfully complete a task has returned to preoperative level, then recovery has taken place. The problem here is that the *quality* of the "recovered" behavior is not being considered (Kolb and Whishaw, 1998). If we are to accept the hypothesis that true recovery involves the return of the *same* behavior, then the execution of the behavior becomes important (Kolb & Whishaw, 1998). One of the best paradigms for studying recovery of function

involves training rats on reaching tasks (e.g. Kolb, Cioe, & Whishaw, 2000). In these types of experiments, rats are placed in a Plexiglas box, and trained to reach through a slot to obtain a food pellet (e.g. Kolb, Cioe, & Whishaw, 2000). The sessions are videotaped so that the sequence of movements required to execute the reach can be determined. After damage, these animals are again videotaped reaching for the food pellet. Although the animal may still be able to reach for and grasp the pellet, the sequence of movements has noticeably changed. Thus, work utilizing a reaching task illustrates the importance of considering that "sparing" (behaviors apparently not affected by damage) may actually be a strategy to compensate for the abilities that are lost, rather than a reinstatement of the original behavior (Kolb & Whishaw, 1998).

ORGANIZATION OF THE CORTEX

Layers and Cells

The cortex is comprised of six layers. Each layer has a specific function: the outer four layers are input layers, receiving axons from other brain areas; the inner two layers are output layers, sending axons to other brain areas (Kolb & Whishaw, 1985). Not all cortical areas have the same distribution of cell layers. For example, the visual area of the cortex has a large number of layer IV cells receiving information from the eyes via the LGN, but a very small number of layer V cells. On the other hand, primary motor cortex has a large layer V containing cells that send axons to subcortical motor systems, but has very few layer IV cells (Kolb & Whishaw, 1985).

There are two main types of cells that make up the six cortical layers (Kolb & Whishaw, 1985). The first type is pyramidal cells, named after the shape of their soma, which is the general shape of a pyramid. These cells constitute the major efferents of the

cerebral cortex, and are found in layers II, III, and V. Layer V contains the largest pyramidal cells, which project to the brainstem and the spinal cord. The pyramidal cells of the input layers II and III are smaller and project to other cortical regions. The second type of cortical cells is stellate (star-shaped) cells. Stellate cells are interneurons and include various different types of cells each with its own unique configuration of axons and dendrites. Stellate cells can be found in every layer, but they most densely populate layer IV, particularly in the sensory cortex. This type of cell receives afferents from subcortical structures and also provides connections between cortical afferents and efferents (Kolb & Whishaw, 1985). Cortical cells in layer IV receive their most important input from the thalamus; they in turn project back to the thalamus. Cells in layer V project to other subcortical structures, while cells in layers II and III produce cortical projections (Bolz, Gotz, Hubener, & Novak, 1993).

Motor Cortex

The primary motor cortex (M1) is defined as the region from which movements can be elicited using the lowest intensity of electrical stimulation (Donoghue & Wise, 1982). Using microstimulation and Nissl stained frontal sections the cytoarchitecture of the motor cortex was determined. It was found that there are two distinct subdivisions within the frontal cortex (Donoghue & Wise, 1982). The first was termed the *medial agranular* field, and was characterized by pale-staining layer III and dense layer II. The second subdivision was termed the *lateral agranular* field, and had more uniform and superficial layers. Within this region, layer V was broad and contained large, darkly stained cells. In the rat, the M1 corresponds to the lateral agranular region. This region

was found to overlap with the adjacent granular layer of the primary somatosensory cortex (S1).

One of the reasons the motor cortex is of interest is its ability to reorganize in response to experience. Studies have shown that a series of horizontal connections within the motor cortex may be partly responsible for the region's plasticity. Evidence of these horizontal connections has been provided mainly through electrical stimulation. Jacobs & Donoghue, (1991) pharmacologically blocked GABA induced cortical inhibition in one area of M1 representation. Upon stimulating the adjacent M1 areas, movements of nearby representations were elicited. The authors speculated that local adjustments in GABA activity, like those occurring after the type of stimulation used in the experiment may produce the recovery of function that is seen after ischemic injuries such as stroke. Damage may disrupt the inhibitory connections providing temporary communication between adjacent groups of cells, facilitating reorganization (Jacobs & Donoghue, 1991).

Hess and Donoghue (1994) used long-term potentiation (LTP) to reduce the inhibition imposed on the horizontal pathways and observed changes in the interactions of cortical neurons. The authors suggested that lowering the inhibition on horizontal connections, increased the reorganizational capabilities of the motor cortex, and that this heightened plastic ability may be useful in creating new sensorimotor associations and in preparing the motor regions for learning new movements (Hess & Donoghue, 1994).

The human brain is capable of changing in response to experience. These experiences include exposure to novel items or situations, learning a new skill, or damage to the brain. The motor cortex has been found to have an impressive ability to reorganize in response to experience. Early research focused on learning-induced changes, however,

with the discovery that damage can create the same kinds of changes, but on a larger scale, more recent work has looked at the response of the motor cortex to injury.

Chapter 2

Ischemia and Excitotoxicity

Defining Ischemia

Ischemia is precipitated by a disruption of blood flow to the brain. There are two types of ischemia. *Global ischemia* is usually the result of a prolonged stoppage of the heart, which halts entire blood flow to the brain. It often follows the systematic hypotension that results from cardiac arrest. The ischemic event is usually intense and brief in duration. If blood flow is restored within four minutes, the individual may only suffer temporarily from symptoms such as confusion and will have no permanent damage. Prolonged cardiac arrest causes the brain to sustain a global ischemic injury. The pathology to the brain depends on the duration of the ischemia, and the severity and length of patient survival. The cerebral tissues supplied by the most distal branches of the cerebral arteries experience the most severe ischemia during global cerebral hypoperfusion. This produces widespread ischemic damage with associated cerebral edema. If the intracranial pressure increases and becomes greater than systemic blood pressure, there will be no blood flow and the brain will undergo a process termed "autolysis *in vivo*." *Focal ischemia* is a regionally specific loss of blood flow due to a blockage, such as a blood clot. It results in an infarction (or lesion) in a circumscribed region in the distribution of an arterial territory. This type of ischemia is usually long-lasting and can be permanent, but is less severe than global, particularly in penumbral regions. The lesion typically only affects the area supplied by the occluded artery, later invading the penumbral region.

Since the underlying mechanisms of ischemia cannot fully or comprehensively be studied in humans, researchers have turned to animal models.

Animal Models of Ischemia

There are various different methods researchers employ to create ischemia in animals. One of the most prevalent methods involves middle cerebral artery occlusion. Blockage of the artery is most often accomplished by tying it off using very fine surgical thread (e.g. Ohlsson & Johansson, 1995; Johansson, 1996, Johansson & Ohlsson, 1996; Johansson & Belichenko, 2002). Another method involves inserting a surgical thread through the artery (e.g. Ding, Zhou, Lai, Li, Park & Diaz, 2002). This second procedure is useful because it allows for reperfusion. The artery can be blocked for certain amounts of time, then blood flow can be reinstituted and behavioral and anatomical deficits assessed.

A novel method of inducing ischemia has been developed that is of interest because of its characteristics. This method involves creating a thrombosis or blood clot within the vessels of the brain. The procedure for creating the clot involves injecting a photoreactive dye, then irradiating through an intact cranium (e.g. Watson, Dietrich, Busto, Wachtel, & Ginsberg, 1985; Gajkowska, Frontczak-Baniewicz, Gadamski, & Barskov, 1997). The theory is that free radicals may be released during the irradiation of the dye, leading to an increased adhesion of platelets to the inner walls of blood vessels. This model is useful because more than half of all strokes in humans are thromboembolic in nature (Bergeron, 2003), so it better mimics what is actually occurring in a human being. Another advantage is that the procedure is not invasive. It does not require removal of skull or dura, thus eliminating potential bleeding, edema, that would impact

the measures of interest and potentially decreasing the mortality rate of experimental animals.

Histopathological Consequences of Ischemia

An ischemic event can have devastating effects on brain tissue. Disruption of blood and oxygen to the affected region sets into motion the mechanisms of cell death. There are two major types of cell death. Necrosis is the result of serious toxic or traumatic events, causing cell swelling, injury to cytoplasmic organelles such as mitochondria, and the breakdown of internal homeostasis (Bonfoco, Krainc, Ankarcrona, Nicotera, & Lipton, 1995). In comparison, apoptosis is an active process of cell damage and death. The specific morphological and molecular features are still under investigation, but it is known that apoptosis is characterized by cell shrinkage, and extensive damage to DNA material (Bonfoco et al, 1995). Another major difference between these two types of cell death is the widespread involvement of neighboring cells in the necrotic process (Bonfoco et al, 1995). Inflammatory processes are also triggered after vessel occlusion. These cascades begin in the parachyma and serve to further increase the tissue damage. Reactive microglia, macrophages and leukocytes are recruited into the ischemic area, while at the same time inflammatory mediators are generated by these cells or by neurons and astrocytes. (Lo, Dalkara and Moskowizt, 2003).

These pathological changes have been observed in the brains of both animals and humans who have suffered a stroke. Pevesner, Eichenbaum, Miller, Pivawer, Eichenbaum, Stern, Zakian, and Koutcher (2001) compared the brains of rats that had undergone a photochemically-induced stroke with autopsy specimens from human stroke

patients. Examination of the infarcts in human and animal tissue revealed many common features between the two such as vacuolated edematous neuropil, shrunken nuclei and evidence of thrombi in the small vessels over the infarct. These finding indicate that animal models of stroke can produce the pathological changes seen in humans.

Physiological Consequences of Ischemia

At the onset of an ischemic episode, there is an abrupt rise in the extracellular level of potassium due to a disruption of the sodium-potassium pump (Feldman, Meyer, & Quezner, 1997). There is then a concomitant fall in the extracellular sodium concentration. The rise in potassium leads to a rapid depolarization termed *anoxic depolarization* due to the lack of oxygen present. There is speculation that the changes in ionic and voltage gradients across the membrane causes a reversal of the glutamate transporter. The disruption of the transporter leads to massive glutamate release. Under normal conditions, the glutamate transporter takes up glutamate along with two sodium ions. Due to the interruption of oxygen produced by ischemia, the uptake reverses pushing glutamate out of the transporter causing a build up of the transmitter in the synaptic cleft ultimately leading to the excessive stimulation of glutamate receptors located on the postsynaptic terminal (Feldman, Meyer & Quezner, 1997). Since glutamate plays such a vital role in the nervous system as well as excitotoxicity, the next several sections will be dedicated to a discussion of this important neurotransmitter.

Importance of Glutamate in Central Nervous System

Glutamate is the main excitatory neurotransmitter in the central nervous system. Glutamatergic neurons separate glutamate into two "compartments" (Feldman, Meyer & Quezner, 1997). Within "small compartments", glutamate synthesis is high and is

thought to correspond to glutamate metabolism taking place in astrocytes. In "large compartments", the glutamate synthesis rate is low and is thought to correspond to metabolic activity occurring in neurons. This compartment is rapidly replenished by glucose breakdown.

Several ionotropic glutamate receptors have been identified. The first type is the non-NMDA (AMPA and kainate) receptors. These receptors are permeable to both sodium and potassium and contribute to the early peak of the excitatory postsynaptic potential (EPSP) (Kandel, Schwartz & Jessell, 2000).

The second type of glutamate receptor is the N-methyl-D-aspartate (NMDA) receptor. This receptor is permeable to calcium as well as sodium and potassium. A unique feature of the NMDA receptor is that it is both ionotropic and voltage-dependent. The channel is blocked by extracellular magnesium while in the resting state. Upon depolarization, the magnesium is removed. Thus, both glutamate and depolarization are required to open the NMDA channel (Carlson, 1994). A binding site for phencyclidine (PCP) is also believed to be located inside the channel, making PCP a very potent NMDA antagonist. Since the NMDA channel has a fairly slow activation, it contributes to the latter part of the EPSP (Kandel, Schwartz & Jessell, 2000). The calcium that flows through the NMDA receptor channels is of particular importance. Normal amounts of calcium are believed to play a significant role in stimulating pathways involved in memory; excessive amounts of calcium are thought to result in brain damage. The final type of glutamate receptor is a metabotropic receptor that uses the second messengers insositol triphosphate (IP_3), diacylglycerol (DAG), and cyclic adenosine momophosphate (cAMP) to act directly on the channel.

Glutamate is used in many descending pathways originating from neocortical pyramidal cells. Examples of pathways include fibres from the neocortex, caudate nucleus, putamen, fornix, thalamus and hypothalamus (Carlson, 1994). Glutamate is also used by excitatory interneurons in the spinal cord. Despite the importance of glutamate within the central nervous system, excessive amounts are devastating to the brain.

Negative Effects of Glutamate

The destructive effects of glutamate were serendipitously discovered by Lucas & Newhouse in 1957. The authors found while studying retinal dystrophy that applying the preservative monosodium glutamate (MSG) to the mouse retina produced lesions. Twelve years later, Olney (1969) found that MSG also causes lesions in the brain, especially the hypothalamus. It was also found that the damaged areas either lacked a blood-brain barrier or were near the ventricular system, leading Olney (1969) to speculate that it was the location of the brain region that allowed the access to the glutamate. The MSG model also showed that the pathological changes were contained to the postsynaptic sites (dendrites, soma). Presynaptic sites remained unharmed.

These early findings led to the *excitotoxicity hypothesis* which states that: the effects produced by glutamate and other excitatory amino acids are caused by a prolonged depolarization of receptive neurons that eventually leads to their death.

Mechanisms of Excitotoxicity

Rothman (1985) was among some of the early researchers examining the underlying mechanisms of excitotoxicity. He conducted a series of experiments in an attempt to elucidate possible mechanisms. First, cultured neurons were exposed either to glutamate, NMDA, or kainic acid. The cultures were kept in a balanced salt solution

containing tetrotoxin (TTX blocks regenerative sodium conductance) and magnesium. After 30 minutes, neurons exhibited swelling of the soma and processes. The second experiment examined the effects of excessive calcium. Cultures were exposed to 30μM of A23187 a calcium ionophore (Rothman, 1985). Neurons in a solution containing 0.5mM of calcium did not noticeably change after 30 minutes of exposure. The third experiment involved chloride substitution. The hypothesis for this experiment was that the steady depolarization produces a chloride influx because of the altered electrochemical gradient for chloride resulting in the cell swelling and eventually bursting. The extracellular chloride in the culture medium was replaced with the sulfate anion. Neurons exposed to glutamate, NMDA or kainic acid under these conditions remained unchanged. Based on these results, Rothman (1985) concluded that excitotoxicity is simply due to an influx of chloride that is due to prolonged depolarization. Although Rothman (1985) was quick to discount the role of calcium in excitotoxicity, experiments conducted later revealed that calcium might indeed have played a part in cell death.

Role of Calcium in Excitotoxicity

Choi (1988) provided evidence that calcium was involved in excitotoxicity. It was found that heptaocytes were destroyed by calcium. If the extracellular calcium was removed before exposure to an excitatory amino acid, injury to the cell was reduced. Similar results were found with neurons. When the extracellular calcium was removed and the culture exposed to an excitatory amino acid, cells showed a temporary swelling, but were able to recover. Choi (1988) speculated that the NMDA receptor was involved.

Current Excitotoxicity Hypothesis

The studies conducted by Rothman (1985) and Choi (1988) provided conflicting information. Rothman's experiments led him to conclude that chloride influx, not calcium was responsible for excitotoxicity. Choi's research showed that calcium might in fact be involved in excitotoxicity. In light of these findings, the excitotoxicity hypothesis was revised.

The current hypothesis states that there are two phases to the excitotoxic process. The first phase involves the influx of chloride into the cell due to prolonged depolarization. The accumulation of ions in the interior of the cell draws water across the membrane in an attempt to restore osmotic equilibrium. The large volume of water entering the cell causes the cell to swell. The second phase is defined as a delayed neurotoxic effect. This phase is the result of massive calcium influx through the NMDA receptors, leading to the disintegration of the neuron (Olney, Samson, & Labruyere, 1986).

EVIDENCE FOR NMDA ACTIVITY IN ISCHEMIC INJURY

Rothman (1983) exposed cultured hippocampal cells to cyanide to mimic the anoxic environment that occurs during ischemia. The experiment was carried out in cultures that were less than two hours old and in cultures that were two days old. It was found that exposure to cyanide only destroyed the older cultures, leaving the younger cultures relatively unharmed. Upon closer examination, it was discovered that only the older cultures displayed swelling resembling synaptic boutons leading to speculation that synaptic activity may be involved in excitotoxicity. To test this theory, magnesium chloride was added to the bath to block synaptic activity. Cells treated with magnesium

chloride showed almost no cell death. Several theories concerning the protective effects of magnesium have been put forth. The first was that magnesium blocks neurotransmitter release. A second, more modern theory is that magnesium elevates the threshold for action potential generation by exerting a postsynaptic effect by gating NMDA receptor channels.

In vitro studies have shown that pretreating CA1 hippocampal cells with an NMDA antagonist almost completely prevents the irreversible loss of evoked synaptic potential that otherwise occurs in this region after 40 minutes in an anoxic environment. NMDA antagonists have also been shown to repolarize neurons that have begun to depolarize in hypoxic hippocampal slices, thus reversing the process before it has a chance to get started (Kemp, Foster, & Wong, 1987).

In vivo studies have also provided evidence for NMDA involvement in ischemia. Direct intrahippocampal injection of the competitive NMDA antagonist APH reduced the loss of CA1 pyramidal neurons produced by transient carotid ligation in rats. Lesioning glutamate inputs to the hippocampus result in dramatically preserved hippocampi in animals that have been subjected to carotid ligation. The CA1 region of the hippocampus is of particular interest to researchers studying ischemia because it is rich with NMDA receptors and is the most susceptible to damage and death during ischemia (Kemp, Foster, & Wong, 1987).

PROTECTION AGAINST ISCHEMIC DAMAGE

NMDA Antagonists

There are two types of antagonists. A competitive antagonist prevents activation of the receptor by competing with the agonist for the binding site. This type of antagonist

can be displaced by excessive amounts of the agonist (Carlson, 1994). An example of an NMDA competitive antagonist is D-AP5. The second type is the non-competitive antagonist, which acts at a site different from that of the agonist. Non-competitive antagonists are not displaced by excessive amounts of the agonist, making them more effective (Carlson, 1994). Examples of NMDA noncompetitive antagonists include magnesium, which physically blocks the channel preventing ionic conduction. MK-801 and PCP bind to sites within the channel that are separate from (but interact with) sites for divalent cations when the channel is in its open state, preventing the passage of ions.

Extracellular Acidity

During ischemia, the pH level falls, becoming more acidic. Most studies examining ischemia use a normal physiological pH of 7.2 to 7.4. Kaku, Gifford & Choi (1993) examined the efficacy of glutamate antagonists in in-vitro preparations under conditions of extracellular acidity relevant to hypoxic-ischemia. Cultures subjected to hypoxic-ischemia at a pH of 7.4 were destroyed. When the pH was lowered to 6.4 there was markedly attenuated neuronal damage after 40-80 minutes of oxygen deprivation. NMDA antagonists were shown to prevent neuronal damage at low pH, proving that their efficacy was not compromised. Although it was also found that combining two antagonists provided more protection than one antagonist by itself (Kaku et al, 1993).

Elevated Extracellular Potassium

Despite the fact that the extracellular concentration of potassium is elevated during ischemia, studies attempting to explain the mechanisms of excitotoxicity are generally conducted under low levels of extracellular potassium. Kiedrowski (1999) tested the effects of elevating the potassium concentration from 5.6 mM to 60 mM. It

was found that a high potassium concentration curtailed NMDA induced calcium and sodium influx, preventing cell death. The theory put forth by Kiedrowski was that the high extracellular concentration of potassium leads to an increased suppression of cytoplasmic sodium. As the membrane depolarizes, the sodium/calcium exchanger reverses operation, preventing calcium influx and ultimately any damage to the neuron.

ZINC AND EXCITOTOXICITY

Although calcium has received the most attention in terms of its role in excitotoxicity, researchers have discovered that zinc may also play an important part in neuronal damage after ischemia. Zinc is the second most abundant mineral in the body after iron (Fredrickson, 1989). In the central nervous system, the amount of zinc (dry weight) in the mammalian brain is generally lower in the cortical white matter, higher in cortical grey matter, and the highest in hippocampal mossy fibers (Choi & Koh, 1998).

Zinc-Induced Neuronal Damage

The first evidence that prolonged zinc exposure can be harmful came from work by Gaskin, Kress, Brosnan, and Bornstein in 1978. The study focused on the effects of zinc on microtubules in cultures of dorsal root ganglion cells. Electron microscopy revealed that exposure to 1mM of zinc for 24 hours induced degenerative changes in the cultured cells.

Cultured mouse cortical neurons exposed to 300-600 μM of zinc for 15 minutes were found to undergo rapid structural changes (Yokoyama, Koh, & Choi, 1986). Specifically, somata would become swollen and agranular, and processes would begin to look beaded in appearance. The following day most of the neurons were reduced to debris. Choi, Yokoyama & Koh (1988) found that if the zinc concentration was raised to

1mM, neuronal destruction would begin after an exposure time as brief as 5 minutes. Injection of zinc chloride into in vivo cultures of rat hippocampus was shown damage neurons as well as glia (Lees, Lehmann, Sandberg, & Hamberger, 1990). Neuronal death could be prevented by introducing the zinc chelator tetrakis- (2-pyridylmethyl) ethylenediamide (TPEN) to the cultures.

Entry Routes for Toxic Zinc

There are several possible routes of zinc entry. First, depolarization may serve to facilitate toxic zinc entry via voltage-dependent calcium channels. When calcium levels were raised, zinc-induced neuronal death was reduced (Weiss, Hartley, Koh, Choi, 1993; Koh & Choi, 1994). Researchers have speculated that these findings lend support to the theory that zinc competes with calcium for entry into the neuron (Choi & Koh, 1998).

Secondly, zinc may enter into neurons via glutamate-activated calcium channels. Evidence for this as a possible entry route lies in zinc's ability to cause a "flicker" block of NMDA channels (Christine & Choi, 1990). This may indicate that zinc may not only act at a sight outside the receptor, but also one inside the channel. The zinc block may be faster than the magnesium block, so upon depolarization zinc is able to pass through the channel before the block is replaced. Koh & Choi (1994) provided further support by showing that zinc-induced neuronal death was sensitive to NMDA antagonists, suggesting a possible interaction with NMDA channels. The competitive NMDA antagonists D-2-amino-5-phosphonovaleric acid (D-APV) or CGS-19755 reduced zinc-induced neuronal damage and death in a more permanent way when the extracellular concentration of zinc was increased compared to the protection produced by the noncompetitive antagonist MK-801.

Thirdly, zinc may be able to pass through a small group of AMPA receptors that show a high permeability to calcium. These receptors were found either to lack or have a different version of the GluR-2/GluR-B subunits (e.g. Verdoorn, Burnashev, Monyer, Seeburg, & Sakmann, 1991). Testing of various combinations of KA-AMPA receptor subunits revealed that the calcium permeability varied as a function of subunit composition. GluR1 plus GluR2 and GluR2 plus GluR3 seemed to form channels impermeable to calcium. GluR1, GluR3 and GluR1 plus GluR3 seemed to create channels that were permeable to calcium (Hollmann, Hartley, & Heinemann, 1991). It stands to reason that if zinc and calcium do compete for entry, a channel permeable to calcium may also be permeable to other divalent cations such as zinc. It has been speculated that this may account for why blocking NMDA channels does not always prevent neuronal damage. It seems as though zinc influx through certain KA-AMPA channels may be responsible for some of the damage produced in excitotoxicity (Choi & Koh, 1998).

Finally, Simons (1991) showed that zinc efflux in human erythrocytes is, in part mediated by a calcium/zinc exchanger. Thus, it may be that just as depolarization disrupts the glutamate transporter, or the sodium/calcium exchanger, it may also affect the calcium/zinc exchanger. Reversal of the exchanger would result in massive zinc influx into the cell.

Zinc and Ischemia

The damage produced by high levels of extracellular zinc is the result of the same disturbances initiated by excessive calcium influx, including the activation of various lipases, proteases and endonucleases (Choi, 1992).

Tonder, Johansen, Frederickson, Zimmer, and Diemer (1990) provided evidence that zinc may have a role in neuronal damage resulting from ischemia. Rats were subjected to 20 minutes of cerebral ischemia followed by 2-24 hours of reperfusion. Brain sections were stained for chelatable zinc using N- (6-methoxy-8-quinolyl)-para-toluenesulfonamine (TSQ) to examine the distribution of zinc in the hippocampus. In normal brains, TSQ stained the neuropil, specifically the mossy fiber layers of the dentate hilus. Within 2 hours of ischemia, TSQ stained cells were observed in the dentate hilus. Longer survival times revealed that cells stained with TSQ tested positive for degeneration. Hippocampal tissue taken from normal and ischemic animals showed two main differences in TSQ staining. First, intact brains almost never contained TSQ stained neurons, ischemic brains contained TSQ stained neurons particularly the soma and proximal dendrites (Tonder et al, 1990). Secondly, a decrease in normally densely stained TSQ cells of the mossy fiber region was observed in the dentate hilus and CA3. This change was not observed in all rats. Of particular interest was that the neurons were the first to show degeneration were somatostatin-immunoractive (Ssi) neurons in the dentate hilus. These cells are located in zinc-rich areas. Ssi cells located in less zinc-rich areas were spared. The authors suggest that a translocation of zinc from the mossy fiber terminals may be one possible explanation for the presence of zinc in degenerating neurons. Koh, Suh, Gwag, He, Hsu and Choi (1996) have found evidence that translocation of zinc from presynaptic to postsynaptic terminals was not restricted to just the mossy fiber region of the hippocampus, but that the phenomenon occurs early throughout the brain, subsequent to degeneration.

In summary, there are two types of ischemia. Global ischemia results from a prolonged stoppage of the heart (i.e. during a heart attack), which halts blood flow to the entire brain. Focal ischemia occurs in a specific region of the brain, where blood flow is compromised by a blockage such as a blood clot. Research has focused on discovering the underlying mechanisms. It has been found that glutamate, calcium and zinc all play crucial roles in neuronal damage and destruction after ischemia. Animal models have also been developed in order to better understand what occurs during the ischemia process. Middle cerebral artery occlusion is the most popular method, however, a novel method involving the reaction between illumination of the skull and a photoreactive dye to create a blood clot is becoming more widely used. Another important area of research involves examining possible rehabilitative strategies to try to improve function after ischemia.

Chapter 3

Rehabilitation After Ischemic Damage

Patients who suffer from a stroke often experience some functional recovery (Ward & Frackowiak, 2004). The extent to which rehabilitative procedures benefit recovery have been the subject of debate (Johansson & Ohlsson, 1996). Recent studies have examined the effects of enriched environments, and motor skill training as possible therapeutic interventions. The role that the contralateral hemisphere may have in recovery of function has also been an issue under investigation.

Rehabilitative Effects of Environmental Enrichment

Ohlsson and Johansson (1995) set out to determine if preoperative and postoperative exposure to an enriched environment would have any effect on functional recovery after ischemia. Nine- week old male rats placed into one of three groups. In group A rats were kept in individual cages before and after surgery. In group B rats were kept in individual cages before the surgery, but transferred to an enriched environment afterwards. Finally, animals in Group C were kept in an enriched environment at all times. The enriched environment consisted of elevated horizontal boards placed at various heights, a chain, a swing and wooden blocks. Functional recovery was examined for 12 weeks after surgery. Behavioral tests were performed postoperatively to assess postural reflex, limb placement and coordination.

Results showed that whereas all rats demonstrated improved performance over time, there were considerable differences between the groups. Rats housed individually showed more disturbances in postural reflex than animals in the other two groups. These deficits were most pronounced three weeks after surgery, but at no other time point. Rats

housed individually also had significantly more difficulty in the limb placement tasks. Animals housed in enriched environments before and after surgery performed significantly better than those exposed to the enriched environment only after the surgery. This difference between the two groups presented itself at five weeks after surgery only.

Two tests were used to examine coordination: a beam and a rotating pole (eg. Hilber & Caston, 2001). On the beam, animals in groups C and B performed better than animals in group A. On the rotating pole, rats in group C were only slightly better than rats in group B. Interestingly, when time to commence climbing and time spent climbing were measured, in both cases group C had shorter times. Based on these findings it was concluded that exposure to an enriched environment both pre and postoperatively provided the best opportunity for recovery. Animals in group C were found to recover sooner and to a somewhat higher degree than those in group B.

In an extension of the abovementioned study, Johansson and Ohlsson (1996) examined not only environment, but also social interaction and physical activity as possibly influencing functional recovery. Once again animals were postoperatively separated into three groups. In group A, animals were housed together in an enriched environment; in group B, animals were housed together with no stimulating objects in the cage; in group C, animals were housed individually in cages containing a running wheel. Behavioral tests were conducted to assess limb placement, coordination and climbing ability and ability to traverse an inclined plane.

Results showed that over the entire postoperative behavioral assessment period, group A performed better than group C on all of the tasks and better than group B on the rotating pole (Johansson & Ohlsson, 1996). As time passed, group A also performed

better than group B on the limb placement test, the climbing task and the inclined plane. Group B consistently performed better than group C on the climbing task and on the inclined plane. After thirteen weeks group B also began to outperform group C on the limb placement and beam task. It appears as though while social interaction was superior to wheel running, exposure to an enriched environment in combination with social interaction provided the best outcome in terms of performance.

In both studies, animals that were assigned to an enriched environment were placed in the condition soon after recovery from surgery. Johansson (1995) conducted a study to see whether delaying exposure to an enriched environment would affect outcome on behavioral tasks. Performance on the rotating pole, limb placement, and postural reflexes were assessed in 15 male rats for two weeks after middle cerebral artery ligation. Seven of the rats were transferred to an enriched environment, and the two groups were tested at 1, 3, and 5 weeks. Performance on all tasks was significantly better for animals that had been moved to an enriched environment compared to animals that had not. These findings prove that even though enrichment was delayed for 15 days after inducing ischemia, performance still significantly improved.

Rehabilitative Effects of Motor Skill Training

Whereas exposure to an enriched environment either before or after ischemic damage has been shown to improve some motor behaviors, it has not been able to improve performance on tasks requiring forelimb or digit use. Biernaskie and Corbett (2001) combined enriched environment with daily reach training to try to stimulate the use of the affected limb, which would in turn activate the corresponding sensorimotor area of the cortex. Two weeks after being subjected to either a focal ischemia or a sham

surgery, animals were assigned to an enriched-reach training or standard-housing condition. Assessment of forelimb function was conducted at four and nine weeks after treatment. Animals in the enriched-reach training condition showed an increased improvement on a staircase-reaching task and performance on traversing a beam was comparable to that of sham animals.

Examination of the dendritic material of layer V pyramidal cells of the intact hemisphere revealed an increased complexity and length in experimental animals compared to controls. The authors suggest that this is an indication that combining environmental enrichment with specific skill training serves to enhance the brain's natural plastic ability within the undamaged hemisphere, and increases functional recovery.

The effects of motor skill training on lesion-induced plasticity were also studied by Jones, Chu, Grande, and Gregory (1999). After undergoing unilateral forelimb sensorimotor cortex lesions or sham surgery, rats received 28 days of training on an acrobatic task. Control animals received only simple repetitive exercise. Behavioral results showed that lesioned animals had improved forelimb function as evaluated using a footfault test. Interestingly, the most improved forelimb performance was observed in the ipsilateral (intact) forelimb rather than the contralateral (damaged) forelimb. The authors suggest that strengthening the abilities of the intact limb may serve as a compensatory strategy. Anatomical analysis revealed that acrobat training increased the number of synapses per neuron of layer V of the undamaged cortex compared to motor controls. It appears the intact hemisphere may play a crucial role in the outcome of functional recovery.

Role of Intact Hemisphere in Improved Performance

Chu and Jones (2000) set out to determine if motor skill training following lesions of the forelimb sensorimotor cortex impacted structural changes in the hindlimb representation of either hemisphere. The hindlimb region was chosen for two reasons. First, the hindlimb region is situated in close proximity to the forelimb region, thus allowing the analysis of the same region both ipsilateral and contralateral to the lesion. Secondly, the hindlimbs may be involved in building a compensatory strategy (Chu & Jones, 2000). Animals exposed to motor skill training showed a significant improvement in performance on the footfault test compared to sham-operated animals. Anatomical analysis revealed that acrobat training produced increases in cortical and dendritic volume in the hindlimb representation opposite the lesion site. Animals exposed to the acrobatic task showed an increase in cortical volume, the volume of neuropil per neuron in layer II/III, and in the amount of dendritic material compared to sham operated animals that experienced the acrobat task. It appears as though the structural changes occurring in the intact hemisphere may have contributed to the improved performance of lesioned animals that were given skilled motor training.

Jones, Kleim and Greenough (1996) used electron microscopy to determine whether structural changes in the sensorimotor cortex opposite the lesion site included synaptogenesis, specifically in layer V. Unilateral lesions of the forelimb area were found to produce an increase in the number of synapses, and the number of dendritic processes within layer V of the contralateral hemisphere compared to sham-operated controls. The changes in dendritic material and synapses were found to follow a specific time course. Increases in number of synapses were found after 30 days, following

increases in dendritic volume. The authors make the interesting point that the changes seen in synapses may not simply reflect an increased usage of the unimpaired forelimb, but also the way in which it is used. Lesioned animals may develop a compensatory strategy that requires the limb to be used differently than a normal animal would use it. The new method of using the limb would require growth and reorganization of the forelimb representation of the cortex.

Research into developing therapeutic interventions has found that environmental enrichment lead to improved performance on tests of motor ability. Similar results were found when motor skill training was provided after ischemic damage. Investigators conducting studies involving motor skill training also discovered the impact the training was having on the intact hemisphere in terms of increased dendritic growth and synaptic connections, bringing to light the importance of the intact hemisphere in the formation of compensatory strategies.

It is important before evaluating possible rehabilitative strategies that an investigation into anatomical correlates is made. In order to be able to determine which interventions would be most beneficial, an understanding of the effect the injury is having on the brain tissue is crucial as a first step. After stroke is induced in the somatomotor cortex, it is expected that cells immediately surrounding the area of damage will show dendritic growth in response to the injury. It is also expected that cells from the intact hemisphere may also exhibit dendritic growth. Neuronal changes in the hemisphere contralateral to the damage are believed to play an important role in recovery of function, or in development of compensatory strategies.

Chapter 4

Induction of Thromboembolic Stroke

METHODS

Subjects. Male (n = 16) and female (n = 17) Long Evans rats approximately 2 months of age at the start of the experiment were used. Rats were housed in pairs, permitted food and water *ad libitum*, and were maintained on a 12 hr light/dark cycle. Animals were handled once a day for 14 days to allow them to become accustomed to being held and moved around by an experimenter prior to surgery.

Surgical Procedures. All animals were anesthetized with sodium pentobarbital (65mg/kg for males; 40mg/kg for females). Animals were then placed in a stereotaxic frame where the skull was exposed and a fiber optic bundle with an aperture of 1.5mm positioned over the primary motor cortex in the right hemisphere. Stereotaxic coordinates of the irradiated site with respect to bregma were as follows: anteroposterior, +3mm, mediolateral, +0.5mm. Tails were rubbed with Emla cream once the animals were anesthetized to provide extra analgesic protection. The topical also reduced pain when the animal was recovering. The tail was then placed into warm water (<50 degrees Celsius) to cause the lateral tail vein to dilate and become more visible. Pressure was also applied to the tail to make the vein more visible. Animals were injected intravenously via a 25 gauge butterfly needle inserted into the lateral tail vein with Rose Bengal in physiological saline over 1-2 minutes (Watson, Dietrich, Busto, Wachtel & Ginsberg, 1985). The tip of the needle was used to inject the dye had lidocaine dripped onto it prior to injection; a single drop will cause dilation of vein and increase analgesia. Rose Bengal is a dye that is reactive to light of a wavelength of 520nm and appears to cause no

adverse effects in the central or peripheral nervous system after injection. Once the injection of the dye had begun, the cold light source was turned on. The brain was illuminated through the skull for 20 minutes. Control animals underwent the same procedure except the light was not turned on. The lesion site was chosen because it would include not only motor cortex, but sensorimotor cortex as well (see figure 1). Ideally, the thrombosis would appear a large lesion encompassing the motor cortex and the forelimb region of the sensorimotor cortex.

Quantitative Morphology

Twenty days after surgery animals were given a lethal dose of sodium pentobarbital. The animals were then perfused through the heart with saline. Once the perfusion was complete, brains were extracted and placed in Golgi – Cox solution. Brain tissue becomes impregnated with heavy metal as it sits immersed in the Golgi – Cox solution. After 21 days the tissue was transferred to a sucrose solution for 7 days. The tissue was then sectioned into thick slices (200μm) with a vibratome (Vibratome 1000 plus sectioning system) and placed on slides. After forty-eight hours, sections were processed using ammonium hydroxide, Kodak fix and alcohol baths (as per Gibb & Kolb 1998). Processing with ammonium hydroxide produces a reaction that causes a precipitate to form inside the cell. In a small percentage (1-4%) of cells the precipitate can completely fill the cell, allowing it to be easily visualized under a light microscope. Under the microscope, the cell body, dendrites, and dendritic spines are clearly visible. Layer II and layer V pyramidal cells from the right and left motor cortex were drawn from each animal. Neurons from these layers were chosen because research has shown that they exhibit the most change in response to damage (eg. Kolb, Cioe, & Whishaw,

2000). Sections from an animal with a visible lesion were used as a guide to determine stereotaxic coordinates for cell selection. The distance from the midpoint of the lesion to the edge was determined using the X and Y-axis located on the left side of the microscope stage. This measurement was used to determine how far to move each slide to ensure that cells were being drawn from the correct area in both hemispheres for all animals. Cells selected were those immediately surrounding the lesion site. These cells were traced using an Olympus CX40 microscope equipped with a drawing tube at 960X magnification. Sholl analysis was used to estimate dendritic length. Sholl analysis involves using a series of equidistant rings centered on the cell body of a drawing (Sholl, 1967). The space between each ring was 10μm. Intersections with dendrites at each ring were counted and a total dendritic length (in μm) was estimated by multiplying the number of intersections by 10μm (see tables 1-3). Analysis for layer II cells was conducted on basilar and apical dendrites. Only basilar dendrites were analyzed for layer V cells, as apicals are often cut during sectioning, making growth estimation difficult.

Statistical Analysis

A power analysis was conducted post hoc to determine how many animals would be needed for each group to ensure significance. A similar study by Kolb, Cioe and Whishaw (2000) found a significant group x ring intersection effect with an F-value of 15.6. We used this F-value along with the parameters $\alpha = 0.5$ and a confidence level of 90%, to calculate power. The analysis revealed that an effect would be seen with 7 or greater animals per group if it were of the magnitude seen previously.

Changes in dendritic morphology were assessed with a repeated measures 4-way ANOVA using group (3) x side (2) x cell (10) x ring (15). Due to the size of the data file, intersections were collapsed such that statistics were done on 20μ ring (200μ) totals.

Chapter 5

Results and Discussion

Results

One animal died as a result of anesthesia, no animals died as a result of the surgical procedure. Animals were observed in their homecage and were handled daily after surgery, but no obvious deficits were observed after damage. Due to the differences in lesion size (see Fig 2), animals were divided into three groups: control (n = 15), mild lesion (n = 12), moderate/severe lesion (n = 6). Experimental animals were divided into two groups according to lesion size. Mild lesions were defined as involving only layer I and the terminal fields of layer II. Moderate and severe lesions were defined as anything that reached beyond the terminal fields of layer II. Under a microscope moderate and mild lesions both had obvious discoloration of the tissue at the lesion site. This discoloration was so prominent, that a blind examiner evaluating each slide from every animal could consistently distinguish between experimental and control animals regardless of the size of lesion the animal had.

The ANOVA conducted on basilar dendrites showed there was no main effect of group for cells in layer II, F (2, 9) = .473, p = 0.63 (right hemisphere) and F (2, 9) = .367, p = 0.69 (left hemisphere). Analysis on apical dendrites of layer II cells also revealed no main effect of group (see figure 3), F (2, 9) = .156, p = 0.86 (right hemisphere) and F (2, 9) = .322, p = 0.73 (left hemisphere). There was also no significant group x ring interaction for either basilar dendrites (see figure 3), F (28, 126) = .552, p = 0.97 (right hemisphere), F (28, 126) = .414, p = 0.99 (left hemisphere) or apical dendrites (see figure

4), F (28, 126) = .639, p = 0.92 (right hemisphere) and F (28, 126) = .637, p = 0.93 (left hemisphere).

Analysis conducted on basilar dendrites of layer V cells revealed a similar pattern to that found for layer II cells. The ANOVA showed no main effect of group, F (2, 9) = 1.10, p = 0.34 (right hemisphere) and F (2, 9) = .874, p = 0.43. There was also no significant group x ring interaction for the left hemisphere (see figure 5), F (28, 126) = .987, p = 0.49. The results for the group x ring interaction for the right hemisphere, F (28, 126) = 1.38, p = 0.09 approached significance only in animals with small lesions, indicating that the dendrites of layer V cells in the damaged hemisphere may be somewhat smaller than those in the intact hemisphere.

Discussion

The purpose of this study was to examine the morphological changes of layer II and V pyramidal cells of the motor cortex in response to damage. Results showed there were no differences in dendritic length in layer II cells between experimental and control animals. There was a trend approaching statistical significance indicating that layer V cells had a less complex dendritic arborization in experimental animals.

Despite the fact that the study produced non-significant results, these results are still of interest. Many people experience small strokes that they never know about or seek medical treatment for. This study has shown that one small stroke does not produce any anatomical changes, but the occurrence of one stroke makes one vulnerable for more. The question then becomes, what are the consequences of multiple small strokes on the brain and consequently on behaviour. In terms of the present study, what might be some

possible explanations for why there were no differences found between experimental and control animals.

Importance of Lesion Size

The damage created in this experiment was not as large as some other methods (eg. Kolb, Cioe, & Whishaw, 2000). It may be more comparable in scope to that produced by mild traumatic brain injury (TBI), which is caused by a blow to the head, subjecting the brain to a variety of mechanical forces. Mild trauma can also lead to a decrease in blood flow to the brain, causing an increase in intracranial pressure resulting in neuronal damage (Kolb & Whishaw, 1990). Bruises and strains caused by the impact may produce bleeding, known as a hemorrhage. The blood trapped within the skull acts as a growing mass exerting pressure on surrounding structures. Blows to the brain, as with blows to other parts of the body, may produce edema. Edema is a collection of fluid in and around the damaged tissue, producing another source of pressure on the brain tissue.

In order to study the effect of TBI, in particular closed-head injury, several animal models have been developed. One of the most popular, the fluid percussion injury has been shown to reproduce the behavioral and physiological effects of TBI observed in humans (Sato, Chang, Igarashi, & Noble, 2001). Briefly, the fluid percussion method involves exposing the skull, and drilling a 3-4mm hole above the area of interest. A polyethylene tube filled with saline is inserted through the hole and held in place against the intact dura using dental cement. The tube is connected to a fluid percussive device, which produces a pulse of increased intracranial pressure through the rapid injection of saline into the closed cavity, resulting in a brief displacement and deformation of neural

tissue (Hicks, Smith, Lowenstein, Saint Marie, & McIntosh, 1993). Use of this procedure has enabled researchers to control the severity and location of the damage created as well as to examine the pathological and behavioral consequences following traumatic brain injury. Pathological changes after traumatic brain injury include tiny lesions and lacerations throughout the brain. These lesions can in turn result in behavioral problems such as an inability to concentrate, loss of complex cognitive functions, and reduction of mental speed.

Existence of Ischemic Penumbra

Many studies examining stroke rely on the penumbra as an indicator of where viable tissue begins. This method was not used for the present study as the majority of histological stains (including golgi) do not allow for the visualization of the penumbra as a distinctive band of tissue. The existence of a penumbra has long been a topic of debate. There is currently no universal definition of the ischemic penumbra (Warach, 2003).

The concept of the ischemic penumbra was introduced by Astrup, Siesjo & Symon (1981) based on their work using a stroke model in baboons. They described the penumbra as a moderately hypoperfused cerebral region in which electrical failure had occurred, but in which membrane homeostasis was preserved because extracellular potassium concentration was normal or slightly elevated. This tissue was thought to form a ring around a severely hypoperfused center, in which energy and ion pump failure had developed, causing this tissue to become rapidly irreversible. This concept has been refined in more recent literature. It is now believed that that the penumbra is a volume of brain tissue that suffers from ischemia, but in which the damage is at least partly reversible (Touzani, Roussel & MacKenzie, 2001).

The fact that there is no solid definition for the penumbra has lead to the debate that it may not always be present in every circumstance. For example, the type of stroke experienced, or the methodology used to create the stroke may determine whether or not there is the formation of a penumbral region (Bergeron, 2003). Specifically, the photochemical cortical lesion method employed in the present study may compromise the development of a perceptible penumbra due to the generation of massive vasogenic edema (Dietrich, Ginsberg, Busto, & Watson, 1986; Dietrich, Busto, Watson, Scheinberg, & Ginsberg, 1987). Similarly, there is controversy about whether a penumbral region develops during perihemorrhagic ischemic stroke (Warach, 2003). The hypothesis concerning this type of stroke is that there is a penumbra surrounding the hematoma partially the result of pressure exerted by the mass on surrounding vasculature. Warach (2003) points out that this hypothesis is very difficult to study in humans who have suffered an intracerebral hemorrhage, due to the fact that if the penumbra does exist, then it would be found in the sickest patients who would then be too unstable to undergo imaging, or who would be immediately sent for surgical intervention before the presence of a penumbra could be determined. Warach also argues that when trying to determine if a penumbra exists, one must consider the fact that the various types of strokes have different imaging features; that the cellular and molecular mechanisms involved differ among stroke types, and that the biology of one type of stroke may be different than another. For example, a perihemorrhagic stroke differs from a purely ischemic stroke as the brain is exposed to blood cells and other molecules from which it is usually protected. A study by Schellinger, Fiebach, Hoffman, Becker, Orakcioglu, & Kollmar (2003) supported the hypothesis that the existence of a penumbra may be dependent on the type

of stroke suffered. Their study set out to determine the presence or a perihemorrhagic penumbra in a perspective study of 32 patients that had suffered a primary intracerebral hemorrhage and received a stroke MRI within 6 hours after symptom onset. The results showed that despite the presence of a mild diffuse as well as focal perihemorrhagic hypoperfusion in some of the patients, this was neither predictive of outcome or with definite ischemia. More convincing findings that support the findings of Schellinger et al (2003) that there is no perihemorrhagic penumbra can be found in a recent PET study conducted by Zazulia, Diringer, Videen, Adams, Yundt, Aiyagari, Grubb, & Powers (2001). Cerebral blood flow, cerebral metabolic rate of oxygen and oxygen extraction fraction were measured in 19 patients 5 to 22 hours after the onset of intracerebral hemorrhage. It was found that both periclot cerebral blood flow and cerebral metabolic rate of oxygen were significantly reduced compared to the values for the contralateral hemisphere. The values for the rate of oxygen, however, were found to be reduced to a larger extent than those for cerebral blood flow, and consequently the oxygen extraction fraction was reduced, rather than increased as would be seen in ischemia.

Schellinger et al. (2003) posit that diaschisis, not ischemia may be responsible for the hemodynamic changes seen in intracerebral hemorrhage, and this may be the reason that no penumbra is found. Diaschisis can be likened to a kind of shock the brain undergoes after damage during which areas connected to the region damaged show a temporary arrest of function (Kolb & Whishaw, 1985). Brain function lost as a result of diaschisis is often restored through rehabilitation or restoration of blood flow. It is therefore possible that the considerable edema produced by a photochemically- induced lesion may also prevent the formation of a penumbra. It seems that the type of stroke, or

the methodology used to induce stroke must be taken under consideration when determining whether to use the penumbra as a parameter for cell selection.

. Another factor that may affect the presence of a penumbra was suggested by Jovin, Yonas, Gebel, Kanal, Chang, Grahovac, Goldstein, & Wechsler (2003) who pointed out the high degree of correlation between the presence of penumbra and the incidence of large-vessel occlusion. The presence of penumbral tissue estimated on the basis of perfusion/diffusion imaging mismatch on brain MRI was found to be highly correlated with large-vessel occlusion (Staroselskaya, Chaves, Silver, Linfante, Edelman, & Caplan, 2001).

The target of stroke in the present study was not a large vessel such as the middle cerebral artery, but rather the smaller vessels lying on the surface of the motor cortex. The cup-like shape of the lesion produced would also suggest small vessel occlusion. If a larger vessel had been occluded, the lesion would have been much larger and more widespread. It stands to reason then, that focusing the damage on smaller vessels may have also contributed to the failure in the development of a penumbra.

Contributions of Apoptosis and Necrosis in Ischemic Cell Death

The type of cell death occurring after damage may also play a role in the time course for the development of the infarct. While this issue was not examined in this study, it still might provide a possible explanation for the small size of the lesion. Necrosis and apoptosis are distinct mechanisms of cell death with very unique characteristics and time courses. Studies using traumatic brain injury research have been conducted to determine a temporal profile for apoptotic and necrotic changes. It was once assumed that cell death resulting from insults such as traumatic brain injury and

ischemia primarily involved necrosis. Recent research has shown that these types of injuries also involve apoptosis (Newcomb, Zhao, Pike, & Hayes, 1999) and that the severity of the injury may influence the extent of the role apoptosis and necrosis play in cell death. This point is of the greatest interest, as the lesion produced in my study can be considered mild. Raghupathi, Conti, Graham, Krajewski, Reed, Grady, Trojanowski, & McIntosh (2002) examined the temporal pattern of apoptosis in the cortex following mild and moderate traumatic brain injury. Results showed that after mild injury, apoptotic cells were restricted to the region of maximal injury. These cells were present between 12 hours and 1 week post-injury, and were maximal at 24 hours. After moderate injury, apoptotic cells were also restricted to the site of maximal injury. In contrast to mild trauma, these apoptotic cells were present between 12 hours and 1 month post-injury, and were maximal at 24 hours and 1 week. The difference in the temporal progression of apoptosis in mild versus moderate traumatic brain injury suggests that the mechanisms underlying this process may be dependent on the severity of the insult (Raghupathi et al, 2002), which would, in turn, affect the progression of the resulting lesion.

Research has also been conducted to determine the contributions of apoptosis and necrosis after ischemia. Bonfoco, Krainc, Ankarcrona, Nicotera, & Lipton (1995) exposed 15 day old embryonic cerebrocortical cultures to varying concentrations of NMDA or peroxynitrite ($OONO^-$) to mimic mild and severe insults. It was found that necrotic damage was most prevalent in response to an acute and intense excitotoxic (2mM NMDA) or free radical induced injury (100μM $OONO^-$). Conversely, cultures with milder insults created with 300μM NMDA or 10μM $OONO^-$ showed mostly features of apoptotic damage that developed over many hours after the initial damage. Lee, Kim,

Kim, Kim, Chi, Roh, & Yoon (2002) using MCA occlusion in rats, created mild (20 minute occlusion) and severe (2 hour occlusion) damage to observe the cell death mechanisms involved. It was found that apoptosis played a role in delayed infarction following focal cerebral ischemia whether mild or severe in nature (Lee et al, 2002). Interestingly, quantitative analysis of the delayed infarction present in the frontoparietal cortex after 2 hours of MCA occlusion showed that it was only in part generated by apoptosis. This suggests that the severity of the ischemia may affect the distribution of the delayed infarction.

The findings of the previous studies may provide a possible explanation for the anatomical results found in this thesis. The mechanisms of cell death (mainly apoptosis) that occur after mild damage progress slowly. It may be that the damage produced in the present experiment was so mild that a large enough apoptotic response was not produced, delaying the development of the infarct further.

The age of the animals used may also have had some influence here. Adult rats were used as subjects in my study, and it has been shown that ischemic insult generally progresses more quickly in the immature brain compared to its adult counterpart (Rice, Vannucci, & Brierley, 1981).

Summary

The study here puts forth the possibility that not all damage leads to cortical reorganization. The fact that no significant changes in neuronal morphology were found between experimental and control animals may be due to the very mild nature of the lesion created. Insults that are mild, potentially those with without penumbras, may not pose as much of a threat to normal brain functioning, so there may be less reorganization

in an attempt to compensate for what was lost. There is, however, a possibility that there were changes in the injured cortex that were not examined in the present study, such as spine and/or synaptic changes. Future research should examine possible synaptic changes at the electron and light microscopy levels. LTP may also be used to examine the functional efficacy of the surviving cells in the immediate vicinity of the lesion site. The findings presented here may provide some evidence as to why small strokes do not lead to a behavioural deficit.

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Ring Number	Controls	Mild Lesion	Moderate/Severe Lesion
1	7.47	7.80	7.29
2	15.74	16.44	14.70
3	20.23	21.46	19.17
4	20.99	22.30	19.55
5	19.20	20.63	18.24
6	15.62	17.34	14.59
7	10.73	12.22	9.66
8	5.81	6.84	5.55
9	2.49	2.77	2.55
10	0.94	1.02	0.91

Table 1: *Mean Dendritic Intersections for Layer II Basilars*

Ring Number	Controls	Mild Lesion	Moderate/Severe Lesion
1	5.16	4.25	5.96
2	13.35	11.29	14.87
3	17.77	15.40	20.36
4	19.01	17.02	21.74
5	18.67	16.67	21.31
6	16.83	15.35	19.43
7	14.14	13.21	16.48
8	10.76	10.37	12.72
9	7.34	7.24	9.14
10	4.78	4.49	5.22

Table 2: *Mean Dendritic Intersections for Layer V Basilars*

Ring Number	Controls	Mild Lesion	Moderate/Severe Lesion
1	1.25	1.34	0.99
2	3.10	3.24	2.70
3	5.15	5.59	4.80
4	6.94	7.36	6.50
5	7.80	8.22	7.23
6	7.90	8.11	7.36
7	7.52	7.64	7.10
8	6.64	6.87	6.34
9	5.72	5.77	5.62
10	5.03	4.88	5.10
11	4.43	4.29	4.56
12	4.00	3.92	3.94
13	3.38	3.64	3.30
14	2.87	3.24	2.66
15	2.50	3.00	2.40

Table 3: *Mean Dendritic Intersections for Layer II Apicals*

Figure Captions

Figure 1. Coronal section of rat brain highlighting lesion site at primary motor cortex and sensorimotor cortex.

Figure 2.

A: Digital image of large lesion

B: Digital image of moderate lesion

C: Digital image of small lesion

All images taken at 10X magnification using an Olympus BX51 microscope with a CoolSNAP-Pro-monochrome camera

Figure 3. Graph of Sholl ring intersections for layer II basilar dendrites. Ring number along the x-axis, mean number of intersections along the y-axis. No difference in the mean number of intersections between lesion groups and controls.

Figure 4. Graph of Sholl ring intersections for layer II apical dendrites. Ring number along the x-axis, mean number of intersections along the y-axis. No difference in the mean number of intersections between lesion groups and controls.

Figure 5. Graph of Sholl ring intersections for layer V basilar dendrites. Ring number along the x-axis, mean number of intersections along the y-axis. Note that there are slightly fewer intersections in the mild lesion group compared to the severe lesion group and controls.

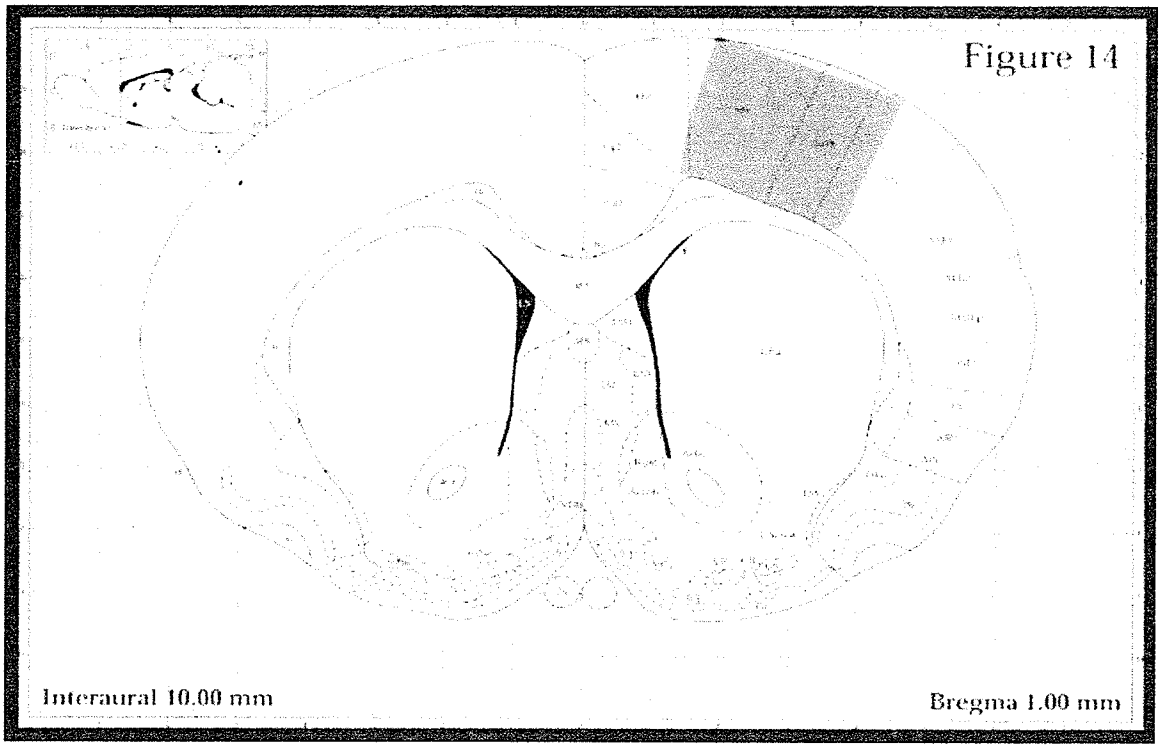

Figure 1: Coronal section of rat brain highlighting lesion site

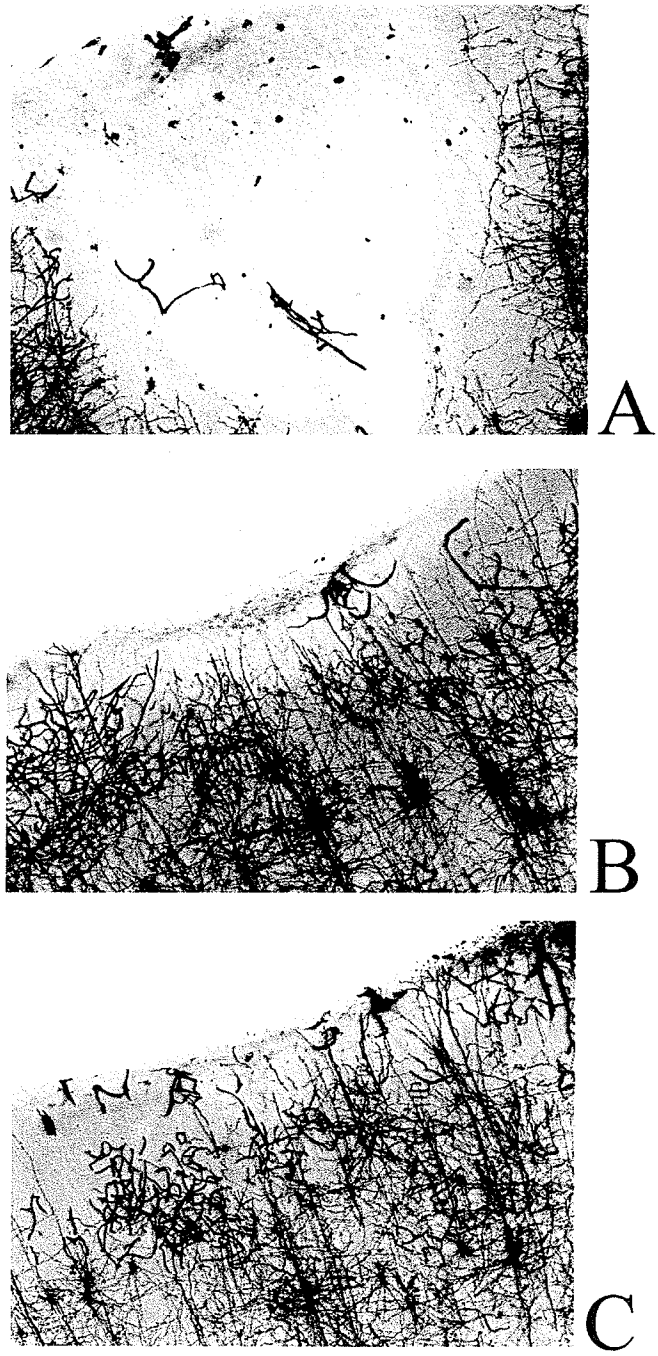

Figure 2: Digital images of different lesion sizes

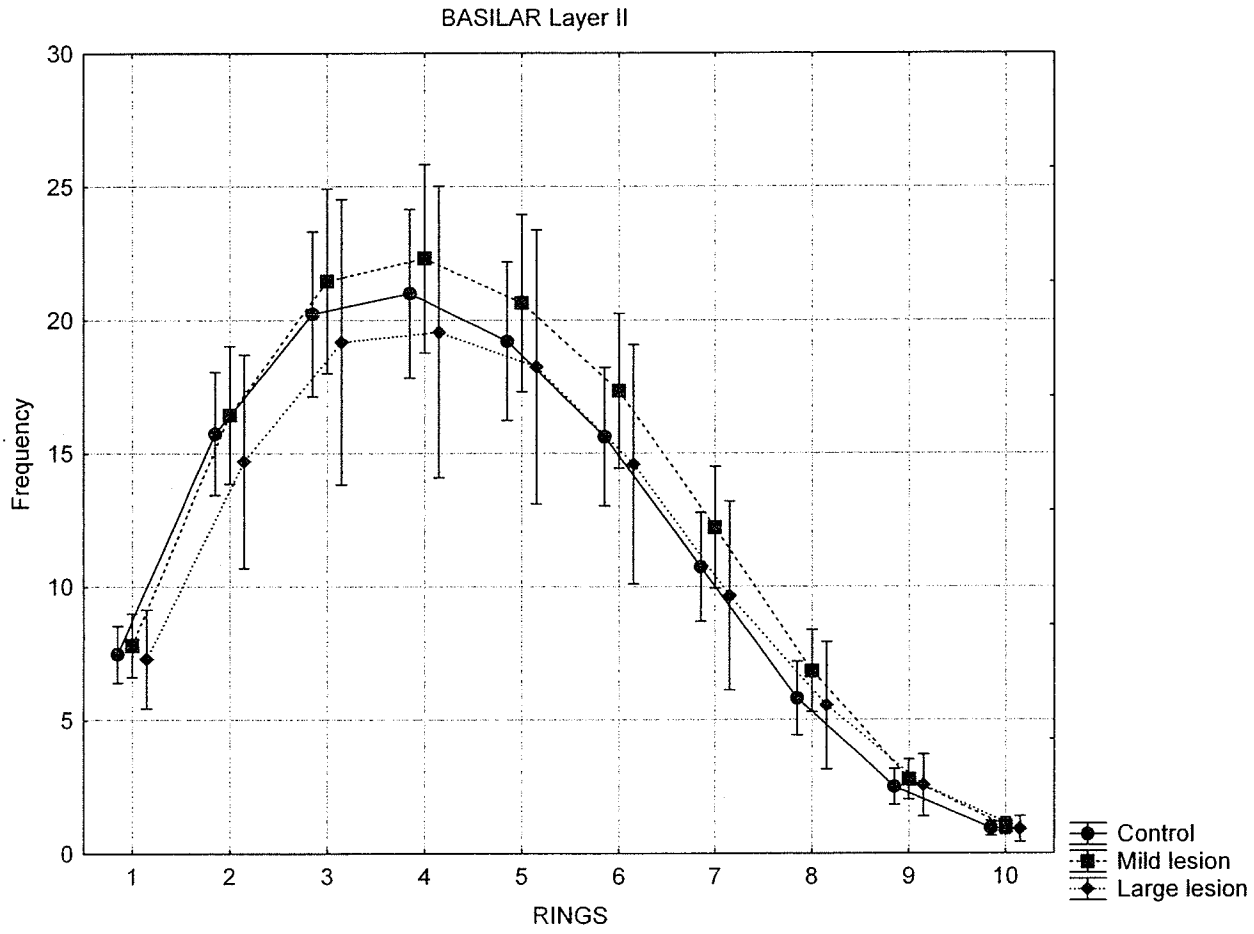

Figure 3: Mean Number of Intersections for Layer II Basilar Dendrites

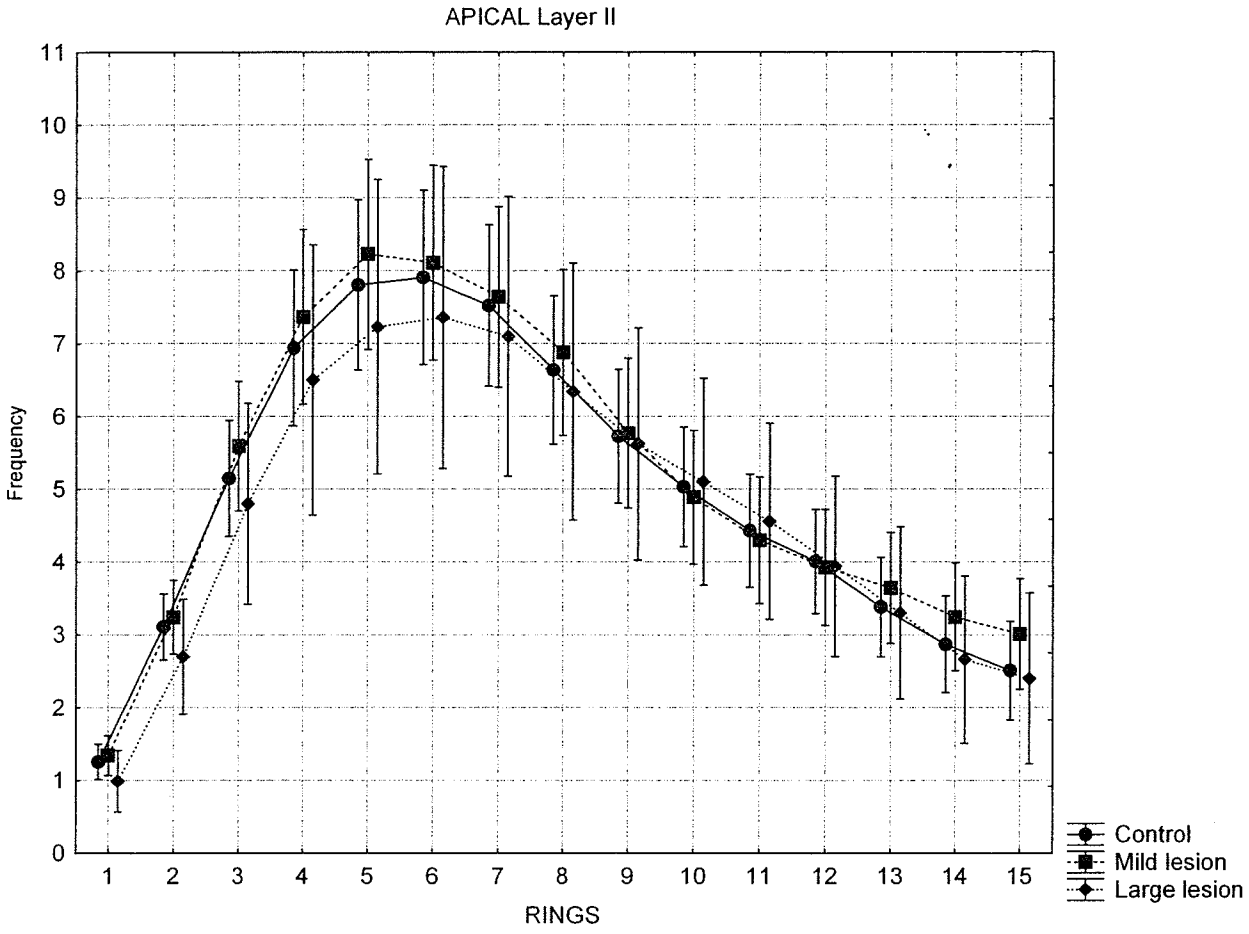

Figure 4: Mean Number of Intersections for Layer II Apical Dendrites

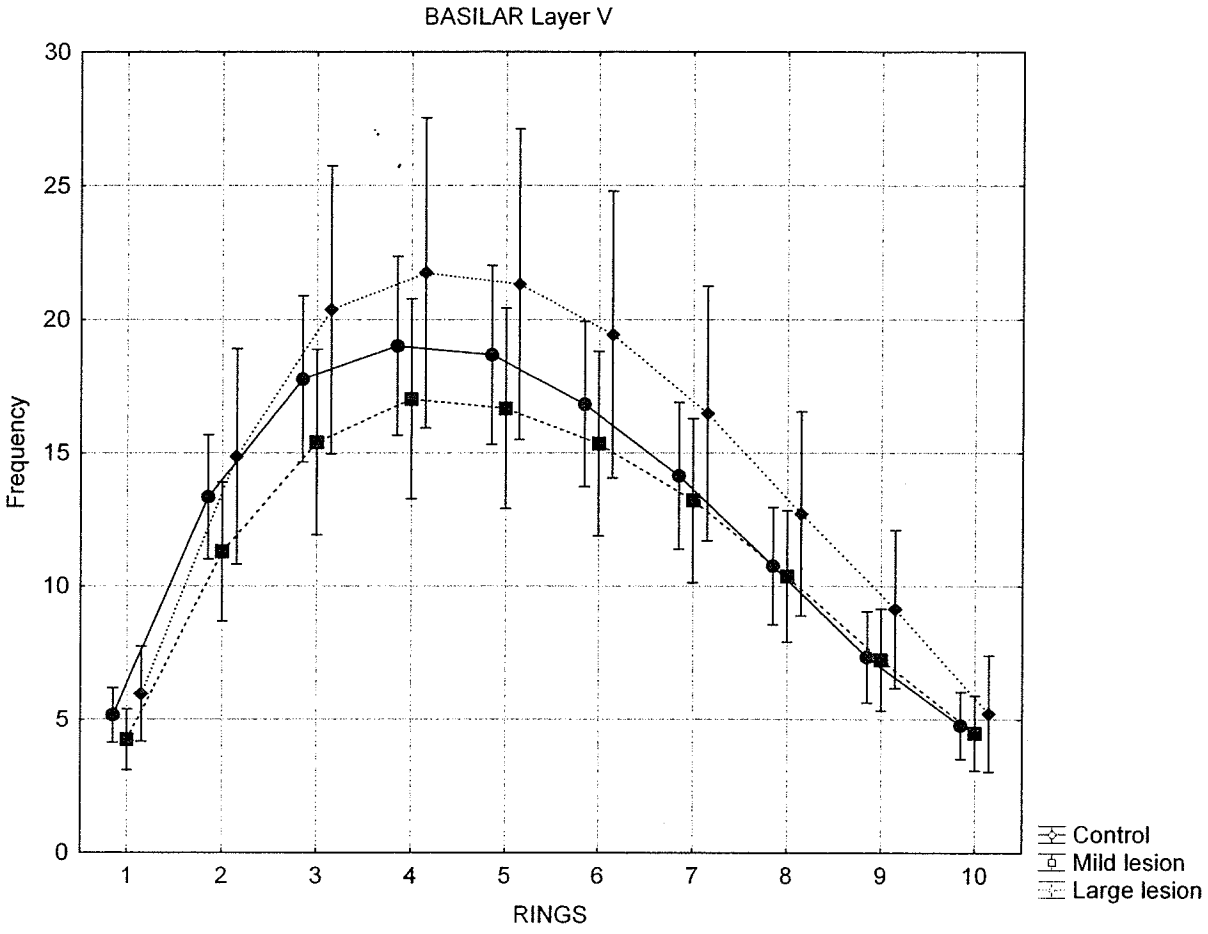

Figure 5: Mean Number of Intersections for Layer V Basilar Dendrites