Does extra-hippocampal sprouting accompany epileptogenesis?

by

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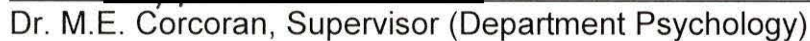

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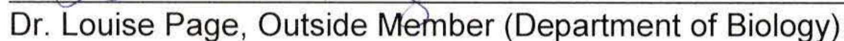

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Abstract

To determine whether extra-hippocampal axonal sprouting occurs in association with epileptiform activity I used two preparations: kainic acid-induced status epilepticus and kindling. Tissue from rats was collected five and 30 days after status epilepticus and following five stimulations or five stage five seizures in amygdaloid kindled rats. Using antibodies for synaptophysin and GAP-43, as well as Timm staining, the tissue was processed and analyzed for axonal sprouting. As previously described, rats treated with kainic acid as well as rats kindled to five stage five seizures displayed an increase in Timm granules in the inner molecular layer of the dentate gyrus. Furthermore, five days following kainic acid-induced status epilepticus, there was an increase in synaptophysin immunoreactivity in the inner molecular layer of the dentate gyrus. Nevertheless, there were no changes in immunoreactivity or Timm stain observed in the piriform cortex, the perirhinal cortex or the basolateral amygdala. As suggested by these results, extra-hippocampal axonal sprouting may not occur following epileptogenesis. However, it is also plausible that the changes are subtle and remained undetected by the methods of quantification I used. Furthermore, mechanisms other than axonal sprouting may be responsible for epileptogenesis.

Examiners

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List of Abbreviations

ILAE	International League Against Epilepsy
SE	status epilepticus
TLE	temporal lobe epilepsy
PTZ	pentylenetetrazol
KA	kainic acid
IML	inner molecular layer
MFS	mossy fiber sprouting
IR	immunoreactivity, immunoreactive
NGF	nerve growth factor
bFGF	basic fibroblast growth factor
BDNF	brain derived nerve growth factor
aFGF	acidic fibroblast growth factor
NT3	neurotrophin 3
trk	tyrosine protein kinase
p75NTR	neurotrophin receptor p75
GAP-43	growth associated phosphoprotein-43
PBS	phospho-buffered saline
PBS-T	phospho-buffered saline and 10% triton
OD	optical density
AD	after discharge
ADT	after discharge threshold
AP	anterior-posterior
ML	medial-lateral
DV	dorsal-ventral

Dedication

This work is dedicated to my parents, Les and Connie Wallace. At the age of three I was seen writing my name as Dr. Amy Wallace. From that early point my parents motivated me to explore my desires and dreams. Without their encouragement and continued unwavering support, I would not have accomplished my masters degree.

1. Introduction

The International League Against Epilepsy (ILAE) defines epilepsy as a "condition characterized by two or more recurrent epileptic seizures over a period longer than 24 hours, unprovoked by any immediate identified cause" (Commission on epidemiology and prognosis: International league against epilepsy). In 1998, a population study using this definition estimated that the incidence of epilepsy in developed countries was between 30 and 50 per 100,000 with a prevalence of 5-8 per 1,000 (Brunetti, Pagano, & Garattini, 1998). This does not include the occurrence of non-epileptic seizures, of which there is no current estimate. Thus, it has been suggested that one in 20 people will experience a seizure in their lifetime (McNamara, 1992). Additionally, the cost of treating an individual epilepsy patient each year was estimated at $2,895 US dollars (Baker, Jacoby, Buck, Stalgis, & Monnet, 1997). Furthermore, epilepsy has also been related to low income, poor health, decreased quality of life, decreased ability to function in a family unit, difficulty with social support and an increased use of health care resources (Wiebe, Bellhouse, Fallahay, & Eliasziw, 1999). The high prevalence rates, treatment cost and effect on quality of life make research in the field of epilepsy crucial.

To provide directed research, the disorder of epilepsy has been separated into several distinct categories. Epilepsy is diagnosed based on the distinct behavioral and electrographic manifestations of the convulsion and seizure. When the discharge of seizure activity begins in a localized area, the seizures are classified as focal or partial. These seizures may or may not affect

consciousness, and when consciousness remains intact, the seizures are classified as simple partial seizures and are focal for the duration of seizure discharge. In contrast, complex partial seizures may include impairment of consciousness with seizure discharge beginning in a focal location and spreading to encompass other brain regions. When these complex partial seizures spread to diffuse and bilateral brain regions the seizure is considered a secondarily generalized partial seizure. Secondarily generalized partial seizures occur in 50-60% of the epileptic population and include temporal lobe epilepsy (TLE), epilepsy with a temporal lobe seizure focus (Engel, 1998; Gastaut, Gastaut, Gonzalves de Silva, & Fernandez-Sanchez, 1975; Houser et al., 1990; Penfield, 1975). Yet another type of seizure, generalized seizures, occur when seizure activity has no localized onset and appears to begin simultaneously in multiple regions of the brain, often bilaterally. Finally, status epilepticus (SE) is a seizure classification that can include all other seizure types with the addition of a period of continuous seizure activity. This classification system is beneficial in identification and treatment of seizures and to direct research foci. In considering research on TLE and the animal preparations used to study TLE as well as SE, one can begin to recognize that the molecular and cellular changes that underlie and occur concurrently with epilepsy are poorly understood. In order to better comprehend the molecular components of epilepsy and to promote focused treatment plans, the changes that occur during and after seizures have been investigated extensively.

TLE-like seizures and convulsions are induced in animals through two common techniques: chemoconvulsants and electrical stimulation. Chemoconvulsants include a variety of chemicals that when applied systemically or infused directly into the brain cause a period of seizure and convulsive activity. These seizures resemble seizure episodes of TLE patients and commonly continue on to SE. Common chemoconvulsants include pilocarpine, pentylenetetrazol (PTZ), bicuculline, tetanus-toxin, and kainic acid (KA). Seizures caused by electrical stimulation produce similarities to a variety of different seizure types. Electrical stimulation is applied to specific brain regions through implanted electrodes. This stimulation can be used to cause both status epilepticus (McIntyre & Edson, 1989) and progressive epileptic-like seizures. Both of these types of seizures can be established through kindling (Goddard, McIntyre, & Leech, 1969; Racine, 1972a, 1972b). Kindling involves repeated stimulations where the first application causes a brief electrographic seizure but no behavioral convulsions. However, over time the seizure develops into secondarily generalized partial seizures. Kindling provides a progressive seizure paradigm that allows for observation during the development of the epileptic state. Both chemoconvulsants and electrically generated seizure preparations along with analysis of resected tissue from TLE patients are characteristically used to analyze the molecular and cellular changes that happen during and following seizures.

A distinct pattern of changes in the hippocampus accompanies TLE. The hippocampus, arguably one of the most studied regions of the brain, is thought to

be involved in a variety of functions and disorders. The most common observation in resected human epileptic tissue is selective regional hippocampal cell loss (Peterson, Ribak, & Oertel, 1985), with 66% of patients with partial complex seizures show hippocampal sclerosis (Babb, Kupfer, Pretorius, Crandall, & Levesque, 1991). Moderate to severe cell loss is seen in fields CA1, CA3 and CA4 and the hilus of the hippocampus (Houser et al., 1990; Margerison & Corsellis, 1966; Meldrum, 1985). However, the dentate granule cells and the CA2 neurons appear to remain intact. Animal preparations show similar regional hippocampal cell loss. KA produces lesions comparable to human TLE (Nadler, 1981), most severely affecting fields CA1, CA3, and CA4 and the hilus (Nitecka et al., 1984; Sperk et al., 1983). Again, as in human TLE, field CA2 and the dentate gyrus incur less damage (Sperk et al., 1983). Pilocarpine-induced SE is associated with neuronal death in the hippocampus, mainly in field CA1 (Roux, Colicos, Barker, & Kennedy, 1999). It is suggested that most of the cell damage and necrosis following chemoconvulsants are caused by excess excitotoxicity produced by overstimulation of the glutamate receptor (Johnston, 1996; Rothman & Samaie, 1985, Shinozaki & Shibuya, 1974). However, chemoconvulsants have also been shown to cause apoptosis, the programmed deletion of single cells in the dentate gyrus (Fujikawa, 1996; Pollard, Khrestchatisky, Moreau, Ben-Ari, & Represa, 1994; Roux et al., 1999). Apoptosis occurs when a change in the neuronal environment causes the cell to put into action a suicidal process. Conversely, the kindled hippocampus displays far less damage when compared to other preparations and TLE patients. When seizures are kindled via

stimulation of the perforant path (a monosynaptic pathway to the hippocampus), apoptotic neuronal damage occurs in the hilar region (Cavazos, Das, & Sutula, 1994; Zhang, Smith, Li, Weiss, & Post, 1998). However, amygdaloid kindled rats do not show hippocampal cell loss (Tuunanen & Pitkanen, 2000). Nevertheless, following a single prolonged direct hippocampal electrical stimulation, apoptotic neurons have been found in the dentate gyrus (Bengzon et al., 1997). However, these data have failed to be replicated (personal communication, P. Mohapel, 2001). Thus, compared to SE in animals and human TLE, the kindled brain displays far less hippocampal damage. The hippocampal damage seen in both animal preparations and TLE has been suggested to be accompanied by abnormal hippocampal function that may contribute to the epileptic state (Houser et al., 1990).

In addition to cell loss, damage to and reduction of axonal processes occurs in the hippocampus concurrent with epilepsy. Human TLE patients show a reduction in the number of dendrites on the hippocampal granule cells and a loss of complex spines on both the apical and basilar proximal dendrites (Scheibel, Davies, & Scheibel, 1973). Additionally, both KA and tetanus-toxin treated animals show hippocampal atrophy along with dendritic degeneration (Cavazos et al., 1994; Colling, Man, Draguhn, & Jefferys, 1996; Jiang, Lee, Smith, & Swann, 1998). Rats kindled in the hippocampus also show a decrease in axospinous and some axodendritic synapses of the perforant path axons innervating the hippocampus (Geinisman, Morrell, & deToledo-Morrell, 1988). However, degeneration following kindling only occurs after direct stimulation of

the hippocampus. Similarly to the cell death that occurs in the hippocampus following seizures, this degeneration may also lead to abnormal hippocampal function.

In addition to hippocampal damage, damage is also commonly found in both temporal and neocortical areas following seizures. Molecular research in human TLE patients is limited to tissue resected from the seizure focus. However, along with the hippocampus, the amygdala is commonly resected. Therefore, it is known that amygdaloid sclerosis is commonly seen in human TLE patients (Miller, McLachlan, Bouwer, Hudson, & Munoz, 1994; Pitkanen, Tuunanen, Kalviainen, Partanen, & Salmenpera, 1998; Ragazzo & Galanopoulou, 2000). In fact, amygdaloid sclerosis in TLE has also been found in the absence of hippocampal sclerosis (Hudson et al., 1993; Miller et al., 1994). In animals, a greater amount of tissue can be assessed for damage. Thus, KA-induced SE has been associated with damage to the cortical and subcortical temporal lobe, the forebrain, and the mesencephalon (Sperk et al., 1983). The most intense damage has been described in the entorhinal and piriform cortices and the amygdala. Similarly, following pilocarpine-induced SE, rats display damage in both the piriform and the entorhinal cortices (Fujikawa, 1996). Conversely, amygdaloid kindled rats do not show cell loss in the amygdala (Tuunanen & Pitkanen, 2000). Moreover, I have found no studies that look outside the amygdala and hippocampus for cell damage in kindled animals. Damage and cell death following seizures differ in severity depending on the

tissue examined or preparation used; however, this damage is not the only change seen following seizure activity.

Beyond neuronal damage and death, there is also synaptic reorganization. In TLE, and many animal seizure preparations, granule cells display increases in the number of axons innervating the molecular layer of the dentate gyrus and forming synapses on granule cell dendrites (Babb et al., 1991; Cascino et al., 1994; Cronin & Dudek, 1988; Houser et al., 1990; Sutula, Cascino, Cavazos, Parada, & Ramirez, 1989). This structural change is called mossy fiber sprouting (MFS). Hippocampal mossy fibers form an excitatory pathway that innervates CA3 pyramidal cells and interneurons in the hilus. However, the new axons are hypothesized to form monosynaptic recurrent excitatory circuits within the inner molecular layer (IML) of the dentate gyrus, or to form additional projections to field CA3 stratum oriens. These changes have been hypothesized to be associated with epileptogenesis and the epileptic state. Specifically, MFS has been suggested to contribute to the creation of the seizure focus (Babb et al., 1991; de Lanerolle, Kim, Robbins, & Spencer, 1989). Furthermore, MFS is found in tissue resected from human patients, suggesting that it is a continual process accompanying the epileptic state (E.A. Proper et al., 2000). MFS has been shown in most animal preparations including kindling (Kato et al., 2001), KA-induced SE (Buckmaster & Dudek, 1999; Cronin & Dudek, 1988; Sutula, 1998), PTZ-induced SE (Golarai, Cavazos, & Sutula, 1992), and the spontaneously seizing tottering mouse (Stanfield, 1989). The chemoconvulsants produce more MFS than is found in kindled animals. The reorganization of the mossy fibers in

kindling occurs prior to development of generalized seizures, and appears permanent (Sutula, He, Cavazos, & Scott, 1988). Kindled rats also display increased sprouting in the CA3 stratum oriens (Cavazos, Golarai, & Sutula, 1991; Represa & Ben-Ari, 1992). However, MFS can be dissociated from epileptogenesis: the density of MFS is not correlated with the severity of the epilepsy or stage of the kindled seizures (Cronin & Dudek, 1988; Pitkanen et al., 2000), and can be found in the absence of hippocampal cell loss (Babb, Pretorius, Kupfer, & Brown, 1988; Cascino et al., 1994). Furthermore, seizures can be kindled in the absence of MFS (Armitage, Mohapel, Jenkins, Hannesson, & Corcoran, 1998). Hence, even after extensive research, the functional significance of this altered connectivity remains uncertain.

Although MFS is the most common structural change associated with seizures, other changes are also seen. Human TLE patients show a greater dendritic spine density on granule cells that exhibit MFS, as well as an increase in branches on apical dendrites (Isokawa, 1997). Similarly, following SE, rats show a correlated increase of granule cell dendritic spines and amount of MFS (Suzuki et al., 1997). Moreover, 30 days after rats have sustained SE, growth of novel basal dendrites on dentate granule cells is found (Represa, Jorquera, Le Gal La Salle, & Ben-Ari, 1993; Spigelman et al., 1998). These basal dendrites are a normal feature of granule cells in developing rats, adult primates, and humans, but are atypical in adult rats. High density on granule cell basal dendrites in human TLE patients have been commented on; however, this apparent change in density was not compared to controls (Frank et al., 1995). Furthermore, MAP2

immunoreactivity (IR), a marker of morphological changes of dendrites, increases in granule cells of the dentate gyrus of mice kindled to generalized seizures, demonstrating dendritic hypertrophy (Kato et al., 2001). Additional evaluation via electron microscopy revealed that there was an increase in dendritic width of long dendrites, but no difference in number of synapses in kindled mice (Kato et al., 2001). Moreover, MAP2 IR also increases in the amygdala and is suggested to reflect an overall increase in width and number of dendrites (Kato et al., 2001). To my knowledge, this is the only study to look outside the hippocampus for neuronal growth following an animal seizure preparation. These changes have unknown consequences on the seizures, and it is unknown if they are a cause or effect of the seizure activity. Thus, there appears to be a lack of understanding of the growth that occurs outside the hippocampus following seizure activity. However, there are changes in growth factors that suggest that growth outside the hippocampus may occur.

Neurotrophins change in conjunction with seizures and may provide important insight to the structural changes that occur. Neurotrophins play a key role in brain plasticity, and hence may play a critical role in the changes found after seizures. Indeed, following KA-induced SE, there is an increase in the message for nerve growth factor (NGF mRNA) in the dentate gyrus (Dugich-Djordjevic et al., 1992; Gall, Murray, & Isackson, 1991; Zafra, Hengerer, Leibrock, Thoenen, & Lindholm, 1990). Bicuculline-induced SE also produces a transient increase in basic fibroblast growth factor (bFGF) in both the dentate gyrus and the entorhinal cortex, but no changes were found in the frontal cortex or the striatum

(Riva, Gale, & Mocchetti, 1992). Furthermore, neurotrophic changes have been extensively examined during and after kindling. Both brain derived nerve growth factor (BDNF) and NGF mRNAs and NGF IR in the dentate gyrus, as well as in the parietal and piriform cortices have been found to increase between 30 min and 2 hr after a generalized kindled seizure. Furthermore, BDNF mRNA also increases in the amygdala. However, these changes are only transient (Bengzon et al., 1992; Ernfors, Bengzon, Kokaia, Persson, & Lindvall, 1991). In a more recent study after generalized kindled seizures, BDNF mRNA increased in the dentate gyrus and the perirhinal cortex, whereas changes in NGF mRNA were found in fields CA1 and CA3 of the hippocampus and the perirhinal and entorhinal cortices (Sato, Kashihara, Morimoto, & Hayabara, 1996). Again these changes were transient, and were only found after fully generalized kindled seizures. Sato et al. also examined acidic fibroblast growth factor (aFGF) and bFGF mRNA one hour after fully generalized kindled seizures and found no changes throughout the limbic system and the cortex. Similarly, neurotrophin 3 (NT3) mRNA reportedly is reduced (Bengzon et al., 1992), or remains unchanged (Ernfors et al., 1991), following generalized seizures. The changes in neurotrophin levels during and after seizures do not follow one clear pattern, and thus it remains unclear what overall effect they have on the seizure activity.

The effects of neurotrophic administration during kindling have also been studied. BDNF infusion into the hippocampus delays hippocampal kindling and blocking BDNF accelerates hippocampal kindling (Reibel et al., 2000). However, hippocampal infused BDNF does not affect amygdaloid kindling (Reibel et al.,

2000). Contrarily, NGF infusion into the hippocampus facilitates amygdaloid kindling and increases MFS (Adams et al., 1997) whereas anti-NGF both delays amygdaloid kindling and blocks MFS seen in CA1 stratum oriens (Van Der Zee, 1995). Though the effects of manipulating of neurotrophins on kindling are inconsistent, they still suggest that structural changes outside the hippocampus are occurring.

Additionally, changes are seen in the receptors that bind neurotrophins. The tyrosine protein kinase (*trk*) family of receptors has a high affinity for neurotrophins. Following PTZ-induced SE and generalized kindled seizures, there is a transient increase of *trk*B (binds BDNF) mRNA in the dentate gyrus (Bengzon et al., 1993; Humpel, Wetmore, & Olson, 1993). Similarly, *trk*C (binds NT3) increases in the dentate gyrus, peaking 12 hr following KA-induced SE and 3 hr following bicuculline-induced SE (Mudo et al., 1995). Furthermore, the neurotrophin receptor p75 (p75NTR), which bind all known neurotrophins, has been shown to increase after pilocarpine seizures (Roux et al., 1999). Both protein and mRNA levels for p75NTR have been found to increase in field CA1 and the piriform and entorhinal cortices (Roux et al., 1999). However, p75 has recently demonstrated a proapoptotic role and could potentially promote cell death. Hence, the increase in p75 after pilocarpine-induced seizures is found to correlate 85% with neurons marked as apoptotic (Roux et al., 1999). Therefore, the increases in the neurotrophins cannot be classified as either a trophic effect or proapoptotic mechanism. Thus, the effects of changes in neurotropic levels and receptors following seizures remain unclear.

Neurogenesis has been demonstrated to occur in the adult human dentate gyrus and therefore warrants study following seizures (Eriksson et al., 1998). Following this rationale, neurogenesis has also been demonstrated to occur in many animal preparations of seizures. Electroconvulsive seizures, a preparation involving triggering of several tonic-clonic seizures to mimic the use of electroconvulsive treatment given for depression, produce increased neurogenesis in the dentate gyrus of the rat (Scott, Wojtowicz, & Burnham, 2000). Furthermore, neurogenesis in the dentate gyrus granule cell layer is also produced by pilocarpine and KA-induced SE (Gray & Sundstrom, 1998; Parent et al., 1997). Following kindling, there is an increase in neurogenesis in the dentate gyrus (Parent, Janumpalli, McNamara, & Lowenstein, 1998; Scott, Wang, Burnham, De Boni, & Wojtowicz, 1998). Indeed one seizure provoking stimulation has been shown sufficient to cause increased neurogenesis (Bengzon et al., 1997). Thus, in the rat dentate gyrus, neurogenesis appears to occur commonly with seizure activity. However, it is unclear how many of these new neurons survive and become functional. Two studies have demonstrated that new neurons developed in the adult hippocampus do send axonal projections to field CA3, and vesicle markers have shown the projections to be surrounded by synaptic vesicles (Markakis & Gage, 1999; Stanfield & Trice, 1988). Nonetheless, it remains unclear as to whether these neurons serve any particular function.

The abundance of information concerning cell death and damage, sprouting and neurogenesis and growth factors have primarily focused on the

hippocampus. Cell death and damage as well as growth factors have been investigated to some degree in the temporal lobe and neocortex. The changes seen in growth factors outside the hippocampus and the abundance of changes in sprouting and neurogenesis in the hippocampus suggest that there may be undiscovered changes in sprouting in regions outside the hippocampus.

The objectives of the studies in this thesis are to determine wether axonal sprouting occurs both within and outside of the hippocampus in association with epileptogenesis. Both kindling and kainic acid seizures were used, and I measured changes in Timm granules, synaptophysin, and GAP-43 protein levels in a variety of limbic and cortical structures.

2. Experiment 1

2.1. Introduction

Animal preparations for the study of TLE demonstrate patterns of hippocampal changes following seizure activity that are similar to these associated with human TLE. These preparations may be useful for identifying changes occurring outside of the hippocampus following seizure activity. Using a chemoconvulsant that induces SE is one way to study the changes that occur with a massive seizure episode. After SE, dramatic changes in both neuronal death and damage as well as MFS are seen. Changes seen following KA-induced SE most closely resemble those seen in resected human TLE tissue. Therefore to elucidate the changes outside the hippocampus and to further clarify the changes that occur in the hippocampus following a massive seizure event, I employed KA-induced SE will be in this experiment.

Kainic acid is a neurotoxin isolated from Japanese seaweed *Digenea simplex* and is a rigid analogue of the excitatory neurotransmitter glutamate (Johnston, 1996; Nitecka et al., 1984; Shinozaki & Shibuya, 1974; Sperk et al., 1983) that acts as an agonist at the AMPA-kainate receptor (London & Coyle, 1979; Popoviciu, Arseni, Tudosie, Bagathai, & Roman, 1988; Sonnenberg et al., 1989). The effects of KA administration on rats closely resemble human TLE in behavioral convulsive profile and distinct neuropathology. Following initial exposure to KA rats develop progressive limbic behavioral convulsions that culminate in severe limbic SE as early as 90 min following the injection (Heggli, Aamodt, & Malthe-Sorenssen, 1981). The seizures progress to chronic,

spontaneous and recurrent episodes (Ben-Ari, 1985; Fisher, 1989; Lothman, Bertram, & Stringer, 1991; Nadler, 1981).

Following KA-induced SE, MFS is found in the hippocampus. Terminals of the mossy fibers contain high levels of zinc, and therefore can be visualized at the light microscopic level via labeling with a histological silver staining technique called the Timm stain (Danscher, 1981; Danscher, Norgaard, & Baatrup, 1987; Sloviter, 1982). Furthermore, the Timm stain reliably stains the ectopic MFs that are found following KA-induced SE. It remains unknown if the Timm stain detects changes that occur outside the hippocampus. Therefore, in order to evaluate and replicate changes following KA-induced SE, I used the Timm histological method.

Synaptophysin, also known as BM89 (Gaitanou, Mamalaki, Merkouri, & Matas, 1997) and p38 (Jahn, Schiebler, Ouimet, & Greengard, 1985), is a well characterized synaptic vesicle protein (Alford, Masliah, Hansen, & Terry, 1994; Eastwood, Burnet, & Harrison, 1994; Eastwood, Burnet, McDonald, Clinton, & Harrison, 1994; Okada, Erickson, & Hendrickson, 1994). Present in all regions of the central nervous system and 95% of cortical synaptic terminals (Jahn et al., 1985), immunoreactivity of synaptophysin protein is an effective tool for characterizing the changes in synaptic populations (Calhoun et al., 1996). Synaptophysin is a 38-kd membrane protein found in small synaptic vesicles and appears to be necessary for calcium-dependent synaptic transmission (Alder, Kanki, Valtorta, Greengard, & Poo, 1995; Wiedenmann & Franke, 1985). Exclusively found in synaptic vesicles (Cutler & Cramer, 1990; Volknandt, 1995; Walch-Solimena, Jahn, & Sudhof, 1993; Wiedenmann, Rehm, Knierim, & Becker,

1988), synaptophysin has shown to be a reliable measure of synaptic density (Masliah et al., 1991). However, an increase in synaptophysin levels can also indicate an increased number of vesicles per synapse rather than a greater number of synapses (Eastwood, Burnet, & Harrison, 1995).

Synaptophysin IR has been employed to identify changes in synaptic density associated with many different disorders. Changes in synaptophysin IR have been found in human schizophrenia patients (Glantz & Lewis, 1997), and are correlated with cognitive decline in Alzheimer disease (Hamos, DeGennaro, & Drachman, 1989; Sze et al., 1997). Within the field of epilepsy research, synaptophysin IR has been correlated with human hippocampal sclerosis. With an increase in sclerosis there is a decrease in synaptophysin IR in fields CA1 and CA4 of the hippocampus, and an increase in the IML in the dentate gyrus, where ectopic MFS occurs (Davies et al., 1998; Looney et al., 1999; E. A. Proper et al., 2000). However, following KA-induced SE and PTZ kindling, there were no changes found in synaptophysin mRNA within the hippocampus (Mahata et al., 1992). Following a focal cortical seizure model using KA, increases in synaptophysin IR have been shown in the cortex contralateral to the KA infusion (Chen, Wong, Banerjee, & Snead, 1996). Therefore, changes in synaptophysin IR have been detected in a variety of models including human TLE, with changes occurring in the hippocampus as well as in the cortex.

Growth associated phosphoprotein-43 (GAP-43), also known as F1, B50, neuromodulin, p57, and pp46, has been linked to synaptic plasticity and nerve regeneration in the adult brain (Benowitz & Routtenberg, 1997). GAP-43 has

intrinsic growth properties was and that does not require facilitation by neurotrophic factors (Benowitz & Routtenberg, 1997). GAP-43 is a 26 kd protein that is attached to the presynaptic terminal membrane or growth cone (Kosik et al., 1988; Skene, 1989), and functions in both immature and adult plasticity. It falls to nearly undetectable levels after maturation, and rises dramatically in neurons that are undergoing axonal regeneration (Benowitz, Shashoua, & Yoon, 1981; Benowitz, Yoon, & Lewis, 1983; Redshaw & Bisby, 1984; Skene, 1989) Furthermore, axons that fail to regenerate are also unable to express the GAP-43 protein (Skene, 1984; Skene & Willard, 1981). Only discrete areas and some neurotransmitter pathways continue to express GAP-43 in adult life including the hippocampus and parts of the limbic system (Benowitz, Apostolides, Perrone-Bizzozero, Finklestein, & Zwiers, 1988; Nelson, Friedman, O'Neill, Mishkin, & Routtenberg, 1987, Cantallops, 1999, Neve et al., 1987).

Changes of GAP-43 protein levels have been related to axonal plasticity in the adult brain. The magnitude and duration of long term potentiation are correlated with increased GAP-43 IR in the hippocampus (Lovinger, Colley, Akers, Nelson, & Routtenberg, 1986). Furthermore, after sustained pilocarpine-induced SE, an increase in GAP-43 IR occurs in the IML of the dentate gyrus, where ectopic MFS also occurs (Naffah-Mazzacoratti, Funke, Sanabria, & Cavalheiro, 1999). Following KA-induced SE there is a transient upregulation of GAP-43 mRNA as well as a long-lasting increase in GAP-43 IR in MF neurons (Bendotti et al., 1997; Bendotti, Pende, & Samanin, 1994; McNamara & Routtenberg, 1995). Bendotti et al have also evaluated changes in GAP-43

mRNA during and after kindling. Following a stage 2 dorsal hippocampal kindled seizure, there was a transient increase of GAP-43 mRNA in the hilus of the dentate gyrus and field CA3 pyramidal cells that did not occur following a generalized stage 5 kindled seizure (Bendotti, Vezzani, Tarizzo, & Samanin, 1993). Furthermore, using a fast hippocampal kindling paradigm, a transient increase of GAP-43 mRNA was found in the dentate granule cell layer at 12 and 24 hr after generalized seizure (Elmer, Kokaia, Kokaia, Ferencz, & Lindvall, 1996). Minor transient increases of GAP-43 mRNA were also seen in the CA1 and CA3 pyramidal layers and the amygdala and piriform cortex, with no changes being found in the parietal cortex (Elmer et al., 1996). Consequently, changes in GAP-43 IR have been seen following seizures in the hippocampus, and changes in GAP-43 mRNA have been identified in cortical regions. This suggests that it will prove to be a valuable marker for identification of axonal growth both within and outside the hippocampus following KA-induced SE.

Utilizing the Timm stain and immunohistochemical techniques for GAP-43 and synaptophysin IR, I examined tissue from several regions following KA-induced SE. The hippocampus, amygdala, piriform cortex and perirhinal cortex were inspected in detail. The hippocampal region will be examined to replicate previous work as well as to identify any other changes that might be found. The hippocampus is divided into four fields: CA1- CA4. The anatomical region also includes the dentate gyrus, subiculum and entorhinal cortex. The hippocampus proper receives input from the entorhinal cortex via the perforant path. The perforant path neurons predominantly make excitatory connections with the

dentate granule cells. The granule cells of the dentate project via mossy fiber axons through the hilus and form excitatory synaptic contacts with the field CA3 pyramidal cells. From there, the axons of the pyramidal neurons in the CA3 project to the pyramidal neurons of the CA1 which in turn project back to the entorhinal cortex. Although there are several other circuits within the hippocampus, this tri-synaptic loop is our primary interest. Many changes following seizure activity have been found within the tri-synaptic circuit. Specifically, the mossy fibers have been found to develop new axon terminals and ectopic connections within the IML of the dentate gyrus as well as in the CA3 stratum oriens (Babb et al., 1991; Ben-Ari, 1985; Sutula et al., 1989). It is these ectopic connections that are of interest in the current investigation. Identifying changes in GAP-43 and synaptophysin IR within the MFS may further clarify the specificity of the changes.

Furthermore, the amygdala will be analyzed due to its strong involvement in temporal lobe epilepsy and sclerosis. The amygdala is located in the medial temporal lobe and includes a group of nuclear and cortical structures (Amaral & Insausti, 1992; Pitkanen & Amaral, 1991). The amygdala is often involved in limbic driven seizure activity (Goddard et al., 1969), and TLE patients can have amygdaloid sclerosis (Hudson et al., 1993; Miller et al., 1994). Furthermore, anatomical evidence has suggested that the lateral, basolateral and accessory basal nuclei of the amygdala project to the entorhinal cortex (Amaral & Insausti, 1992; Krettek & Price, 1974). Thus, analyzing the amygdala following KA-

induced SE with GAP-43 and synaptophysin antibodies will refine the amygdala's involvement with seizures.

Cortical regions that have strong relationships with seizures as well as the amygdala and the hippocampus will be examined. The perirhinal cortex not only has a low seizure threshold for kindled seizures, but also has robust connections with the amygdala (Mohapel & McIntyre, 1998; Suzuki et al., 1997) and the hippocampus via the entorhinal cortex (Suzuki, 1996). Furthermore, changes in BDNF mRNA increase in the amygdala following kindled seizures (Bengzon et al., 1993; Sato et al., 1996) suggesting that there are connectivity changes. Due to its strong connections to regions that develop sclerosis following seizure activity, and the changes in BDNF neurotrophin levels following seizures, analyzing both GAP-43 and synaptophysin IR following KA-induced SE will further clarify any connectivity changes that do occur.

Lastly, the piriform will be analyzed for changes due to its sensitivity to kindled seizures as well as its connection to the amygdala and the entorhinal cortex (Mohapel, Dufresne, Kelly, & McIntyre, 1996). The piriform cortex consists of 3 layers and the endopiriform nucleus, sometimes described as layer 4 (Haberly, 1990). All piriform layers will be examined for changes following KA-induced SE. The anatomical connections of the piriform cortex are closely related to other seizure sensitive regions. The piriform cortex projects to the entorhinal cortex and the amygdala among other projections (Haberly, 1990). The piriform cortex has also shown changes in BDNF, NGF and *trkB* mRNA following seizure activity (Bengzon et al., 1992; Ernfors et al., 1991). These neurotrophic changes

in the piriform cortex following seizures suggest that there are anatomical transformations being made. This study will attempt to visualize and characterize some of these changes using GAP-43 and synaptophysin IR.

Changes in GAP-43 and synaptophysin are quantifiable under light microscope and will further elucidate the regional changes in proteins involved with seizure activity. The changes in these areas were examined at two time periods following KA-induced SE. Observing the changes 5 days after seizures allows for identification of changes in protein level that occur shortly after the seizure event and may or may not be permanent. To assess changes that may be more permanent, or long-lasting, the changes seen at 30 days following the seizure activity were also be quantified. With the analysis of these two time points further information will be gained about changes in synaptosomal and axonal proteins that may in turn help clarify changes found after seizure activity.

2.2. Experiment 1: Methods and Materials

2.2.1 Animals

Forty Long-Evans hooded rats (Charles River, Montreal, Quebec, Canada) were maintained on a 12:12 light:dark schedule at a temperature of 19 to 21 °C and were group housed in bedded plexiglass cages with free access to food and water. During the first week following arrival they were handled daily. This handling regimen was implemented to habituate the rats to the experimenters and experimental manipulations so as to decrease undue stress that can affect seizures (Arnold 1973). The rats typically weighted between 350 to 450g at the time of kainic acid (KA) injection. Procedures involving animals were conducted in accordance to the guidelines of the Canada Committee on Animal Care and were approved and monitored by the University of Saskatchewan Committee on Animal Care and Supply.

2.2.2 KA Injection

One week after arrival, rats were separated into control and KA injection groups. KA treated rats received an intraperitoneal (i.p.) injection of 12mg/kg KA (Sigma, dissolved in 0.9% saline). Control rats were given an injection of 1mg/kg vehicle i.p. All rats received an additional i.p. injection of 10ml 0.9% saline as per University of Saskatchewan Animal Care guidelines. Rats were observed for convulsions in their home cages for 3 hours or until convulsions had subsided. Convulsions were rated according to the following scale (Zhang, Gelowitz, Lai,

Boulton, & Yu, 1997): stage 1, freezing behavior; stage 2, wet dog shakes; stage 3, head nodding and unilateral forelimb clonus; stage 4, bilateral forelimb clonus; stage 5, loss of postural balance. Stage 6 is the development of epileptic status, characterized by continuous clonic-tonic convulsions involving the whole body, salivation, rearing, and falling. Following the observation period, rats were returned to the colony room, and monitored twice daily for one week, during which time they received 1ml saline to maintain hydration.

2.2.3 Perfusion

Five or 30 days after injection of KA, rats were deeply anaesthetized with an overdose of sodium pentobarbital and perfused intracardially with 0.9% saline (154 mM NaCl), followed by 0.37% sodium sulfide (4.87mM Na_2S*9H_2O, 7.97mM $NaH_2PO_4*H_2O$), saline and 4% paraformaldehyde (3.1M paraformaldehyde, 7.75mM Na_2HPO_4, 2.46mM $NaH_2PO_4*H_2O$). Twelve to twenty hr later brains were removed and placed in paraformaldehyde solution for 2 hr, followed by at least 24 hr refrigerated incubation (4°C) in 25% sucrose buffered solution. The brains were then flash frozen at -45°C in isopentane for 30 sec and stored at –70°C until sectioned.

2.2.4 Histology

2.2.4.1 Sectioning

Brains were sectioned coronally in a cryostat at –22°C into 40μm sections, beginning just prior to the hippocampus and continuing through until the end of the hippocampus. Sections for Timm histochemistry were thaw mounted directly onto poly-l-lysine coated slides. Slides were treated within 0.1% poly-l-lysine solution in distilled water for five min and allowed to dry at 60°C, and then treated again for five min and dried. The slides were stored at 4°C until used. Sections for Immunohistochemistry were immersed in 0.9% phosphate buffered saline (PBS) and left free floating at –4°C until processed for immunohistochemistry.. All slides were stored in the dark until Timm staining, typically 2-3 days later.

2.2.4.2 Timm Staining

Mounted sections were stained 2-3 days after sectioning using Sloviter's modified Timm stain. Slides were dehydrated in ascending ethanol concentrations followed by xylenes. They were then rehydrated in descending ethanol concentrations and placed in the developer for 50-60 min. The developer was 120:60:20:1mls mixture of gum arabic (50g/100mls), hydroquinone (512.62mM $C_6H_6O_2$), citrate solution (799.04mM $C_6H_5Na_3O_7{*}2H_2O$, 2113.91mM $C_6H_8O_7{*}H_2O$) and silver nitrate (1M $AgNO_3$). Slides were then washed in distilled water, dehydrated in ascending ethanol concentrations, cleared in xylenes, and cover slipped with permount. Stained slides were kept at room temperature in the dark until microscopic quantification.

2.2.4.3 Immunohistochemistry

Four rats were perfused with paraformaldehyde and 4 rats were perfused using the method described in 2.2.3 to serve as pilot tissue. This tissue was stained following the standard immunohistological methods. Following comparison between the two tissue types, immunohistological methods were adapted for the sodium sulfide perfused tissue. This process was repeated until the tissue perfused with sodium sulfide displayed virtually identical immunological staining as the tissue perfused with paraformaldehyde. The following methods are the adapted immunohistological procedure.

2.2.4.4 Synaptophysin Immunohistochemistry

Free-floating sections were stored at –4°C in PBS until processed. Sections were incubated in 0.2% H_2O_2 in methanol for 30 minutes to inactivate endogenous peroxidase activity, washed for 5 min in PBS, and blocked in 5% Carnation Instant skim milk powder in PBS and 10% Triton x 100 (milk PBS-T) for one hour. Following this incubation, the sections were put into the polyclonal anti-synaptophysin (SP15, generously supplied by Dr. W.G. Horner, University of British Columbia, Vancouver, BC, Canada) at a concentration of 1:5 in milk PBS-T for 48 hours. GAP-43 protein was harvested from goat, and the antibody to the goat protein was raised in mouse. The tissue was then washed in 5% milk-PBS-T twice for 10 min each, and incubated in the secondary antibody (Horseradish peroxidase conjugated antimouse IgG +IgM H+L, raised in goat) at a concentration of 1:250 overnight. Subsequently, the tissue was washed 3 times for 10 min each in milk PBS-T followed by incubation for 10 min. in Tris HCl

(40mls), diaminobenzodine (1.5mls) and H_2O_2 (4.5 microliters). To verify the specificity of the antibodies, two sections were processed without primary antibodies in the incubation. After staining was complete, the sections were washed in PBS, mounted on poly-l-lysine coated slides, and dried over night. The following day the tissue was dehydrated in ascending ethanol concentrations, cleared in xylenes, and cover slipped with permount. The slides were kept in the dark until image analysis. This procedure was adapted from previous procedures to ensure proper staining levels following sodium sulfide perfusion.

2.2.4.5 GAP-43 Immunohistochemistry

Tissue was stored free floating in PBS at 4^0C until processed. Nonspecific binding was blocked using 0.5% bovine serum alum and 2% horse serum in PBS-T for one hr at 4^0C. The tissue was then incubated in the primary antibody, monoclonal anti-GAP-43 (generously provided by Dr. D. Schreyer, University of Saskatchewan, Saskatoon, Saskatchewan, Canada), at a concentration of 1:10,000 in the above solution overnight at 4^0C. Following incubation the tissue was then washed in PBS for the following times: 30min, 20min, 45 min At this point the tissue was incubated in the secondary antibody (Biotin conjugated goat anti-mouse IgG) at a concentration of 1:200 diluted in the initial blocking solution for 3 hr at room temperature. Subsequently, the tissue was washed 3 times for 15min each in PBS-T. The tissue was then incubated in Avidin/Peroxidase (Sigma) diluted 1:400 in PBS for 3 hr at room temperature, followed by incubation in diaminobenzodine and H_2O_2 in DDH_2O for twenty min at room temperature.

Sections were then mounted on poly-l-lysine coated slides and dried overnight. The following day, the tissue was dehydrated in ascending concentrations of ethanol, cleared in xylenes, and cover slipped. The slides were stored in the dark until image analysis. This immunohistochemical procedure was modified to ensure proper IR following sodium sulfide perfusions.

2.2.5 Data Collection

Using a computerized imaging program (Northern Eclipse, Toronto, Ontario, Canada), a Sony camera captured images from a Zeiss microscope at 20 times magnification. All analysis was performed on a gray scale version of the image. The optical density (OD) of the area of interest was found by computer analysis of a line drawn through the darkest region. The OD of the background was found by computer analysis of a square drawn in the region identified as background. For each subject, samples were drawn from multiple sections and alternate sides of the brain. All numbers were saved to a spread sheet for further statistical analysis. Background measurements were taken from fibers of passage close to the area of interest because sparse population of cell terminals and dendrites in these regions suggests that staining found there is nonpositive background staining of DAB (Calhoun et al., 1996). For the hippocampal regions, corpus callosum was chosen as background and depicted in Figure 1. For the piriform cortex, perirhinal cortex, and amygdala, the optic tract or the internal capsule was used for the background measurement (Figure 2). Analysis of the hippocampus was taken in the dorsal region because previous work has suggested that most of

the MFS occurs here (Represa & Ben-Ari, 1992). Both ventral and dorsal blades of the IML of the dentate gyrus were analyzed. Furthermore, changes have been previously found in the stratum oriens of the CA3 field (Represa & Ben-Ari, 1992), and this area was also analyzed for sprouting. In the piriform cortex, all three layers were analyzed separately, as well as layers 1, 2, and 3 in the perirhinal cortex. The basolateral amygdala has displayed changes in other proteins in previous studies, and therefore was analyzed for changes (Kato et al., 2001).

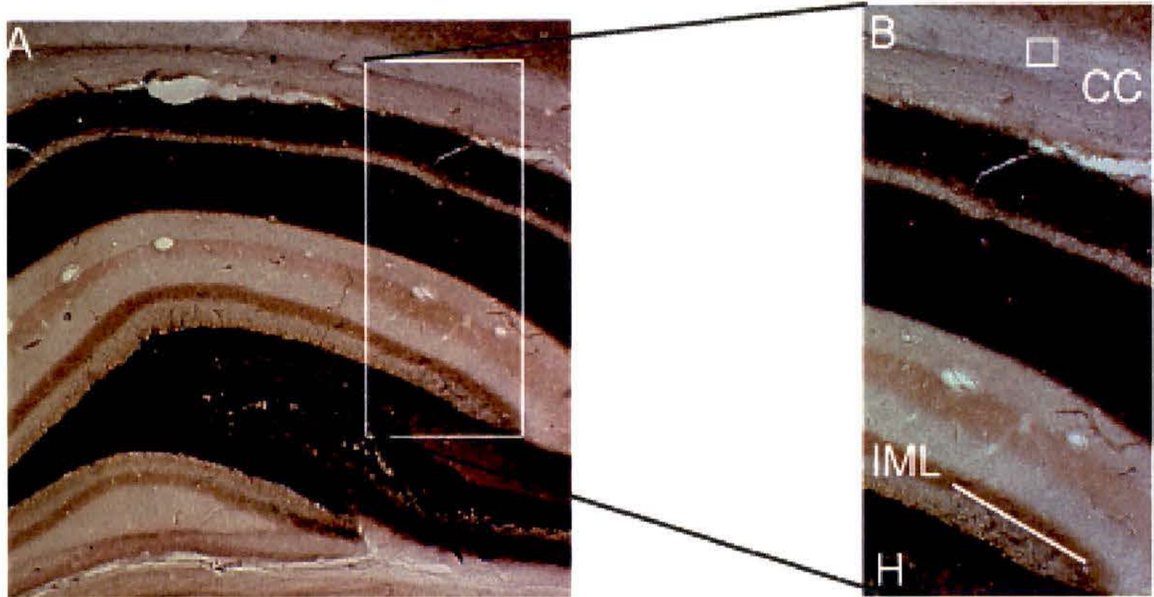
2.2.6 Statistical analysis

Optical density data obtained from the areas of interest were applied to an analysis equation yielding percent different scores between areas of interest and background.

$$\left[\frac{background - area_of_interest}{(background + area_of_interest) \div 2}\right] \times 100$$

For each section analyzed, a percent difference score was calculated. The scores from each rat were then averaged across sections, with at least 2 scores from each hemisphere. This score was the average percent difference score for the rat. The average percent difference scores obtained were then compared between control and experimental groups using independent t tests.

Figure 1: Photomicrograph representation of how data were obtained from the hippocampus. A is a sample of a Timm stained hippocampus. B is a magnified section from picture A depicting how the data from IML of the dentate gyrus were obtained. C is a representative example of how the data were obtained from the stratum oriens in the CA3 of the hippocampus. In B and C, the white squares represent the background measurement and the white line represents the site at which the optical density measurement was taken. (CC - Corpus callosum, IML - IML of the dentate gyrus, H - hilus of the dentate gyrus, CA3 - CA3 region of the hippocampus, SO - stratum oriens layer of the CA3).


A
B
CC
IML
H

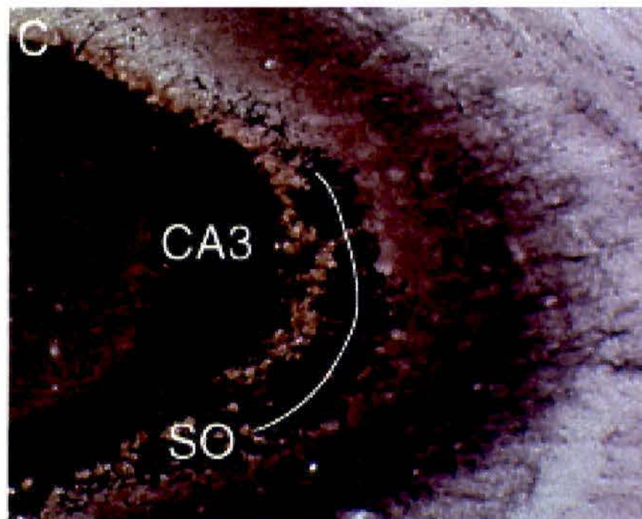
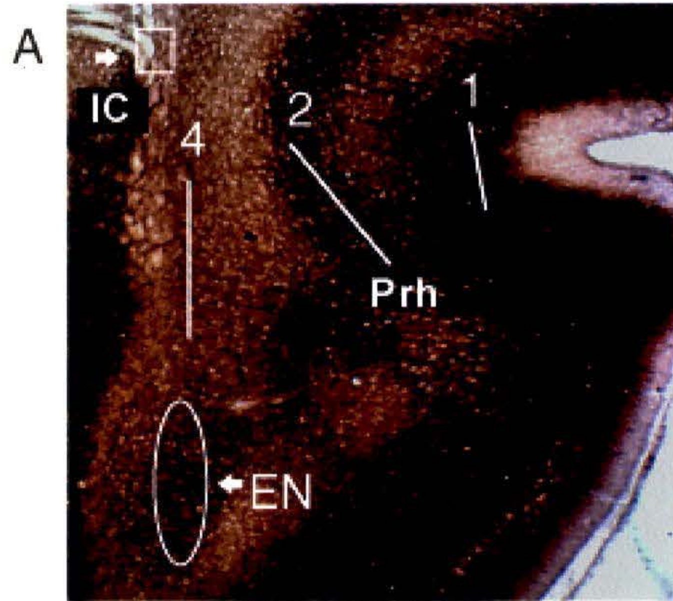
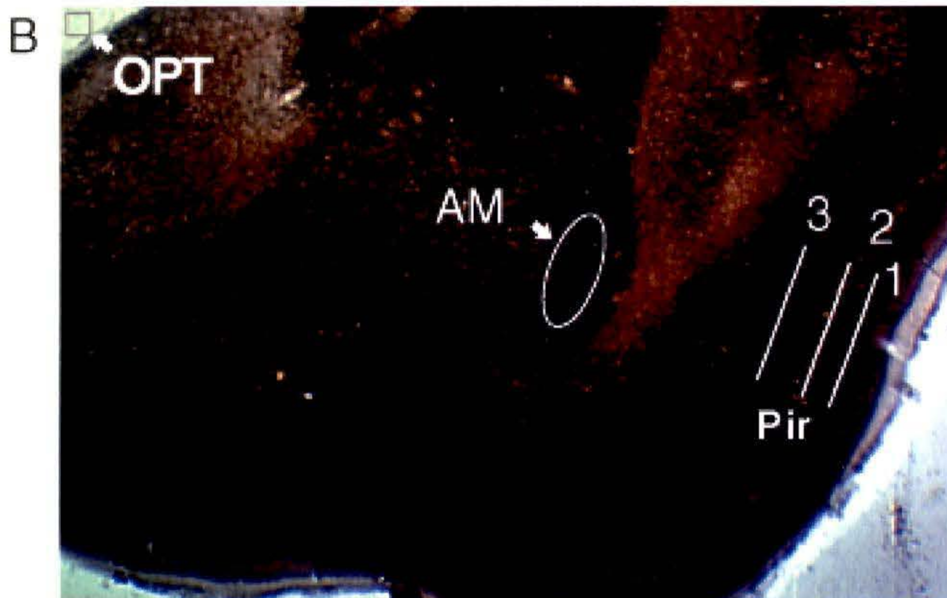

C
CA3
SO

Figure 2: Photomicrograph representation of how data were obtained from the piriform and perirhinal cortexes. A is a sample of a Timm-stained example of how data were obtained from the perirhinal cortex and the endopiriform nucleus. B is a representative example of how the data were obtained from the piriform cortex and the amygdala. In A and B, the white squares represent the background measurement and the white line represents the area from which the optical density measurement was taken for each layer of cortex. The ovals represent the area from which optical density measurements were taken for the endopiriform nucleus and basal lateral nucleus of the amygdala. (IC - Internal capsule, PRH - Perirhinal cortex, EN - Endopiriform nucleus, OPT - Optical tract, AM -Amygdala, PIR - Piriform cortex)

A



B



2.3 Experiment 1: Results

2.3.1 Kainic acid seizures

The 5 and 30 day groups were further divided into subgroups according to the maximum stage of seizure. See Tables 1 and 2 for number of rats used in each comparison. Four rats were not included because they died within 24 hr of KA administration.

2.3.2 Hippocampus

2.3.2.1 IML of the dentate gyrus:

Both the 5 and 30-day groups displayed a greater amount of Timm granules in the IML of the dorsal blade of the dentate gyrus as compared to controls. Tissue from the rats that had experienced stage 5 KA seizures and a 30 day interval ($n=5$) displayed significantly higher percent difference scores than the control rats ($n=4$) ($t(7)=2.7$, $p<0.05$). Similarly, tissue from the rats that had experienced 5 stage 5 seizures and a 5 day interval ($n=4$) displayed significantly more Timm granules in the IML of the dentate gyrus than the control group ($n=4$) ($t(6)=1.7$, $p<0.05$). Means are displayed in Table 1 and 2. A one-way ANOVA comparing all seizure stages failed to reveal significant differences in Timm staining between the different seizure stages.

Synaptophysin IR was significantly higher in the IML of the dentate gyrus in the 5 day interval group ($n=2$) ($t(8)=1.75$, $p<0.05$). There was no difference in synaptophysin IR the IML of the dentate gyrus in the 30 day group. Furthermore,

there was no difference from control in the IML of the dentate gyrus in GAP-43 IR in the 5 day group (see Tables 1 and 2 for means). The 30 day group was not analyzed with GAP-43 due to tissue loss. See Figures 3 and 4 for photomicrographs representative of staining for both groups.

2.3.2.2 CA3

There were no significant differences in Timm staining or synaptophysin IR in the stratum oriens in layer CA3 of the hippocampus at either time interval or in GAP-43 IR at the 5 day interval.

2.3.3 Perirhinal Cortex

There were no significant differences detected in layers 1, 2 or 4 of the perirhinal cortex in Timm stain or synaptophysin IR in either the 30 day group or the 5 day group and in GAP-43 at the 5 day interval (means reported in Tables 1 and 2).

2.3.4 Piriform Cortex

There were no significant differences in Timm staining or synaptophysin IR in layers 1, 2, 3 of the piriform cortex or in the endopiriform nucleus at either time interval, and there were no differences in GAP-43 at the 5 day interval (means are reported in Tables 1 and 2).

2.3.5 Amygdala

There were no significant differences in Timm staining or synaptophysin IR in the basal lateral nucleus of the amygdala at either time interval. There were no significant differences in GAP-43 IR found in the basolateral nucleus of the amygdala at the 5 day interval. Means are reported in Tables 1 and 2.

Figure 3: Representative photomicrographs of the IML of the dentate gyrus from the 5 day KA group and their controls.

KA 5 days

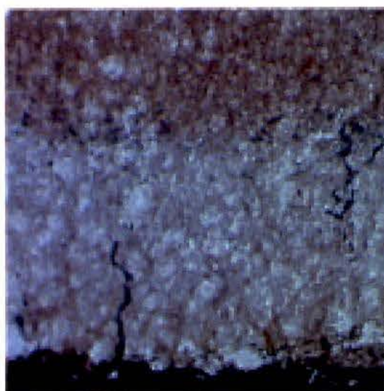
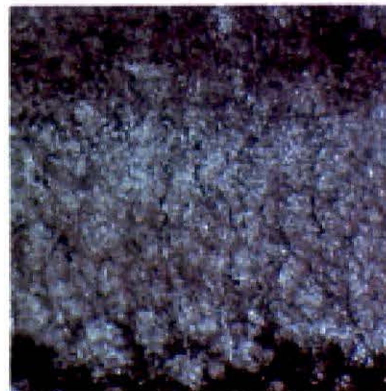
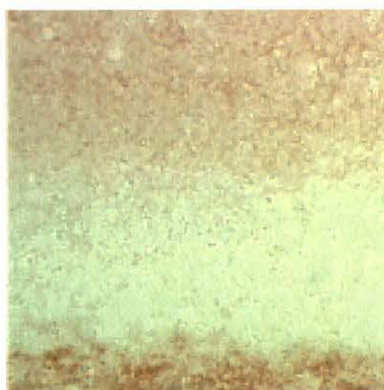
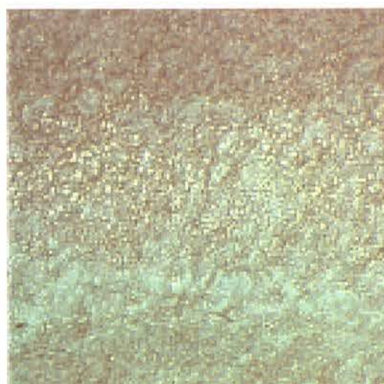
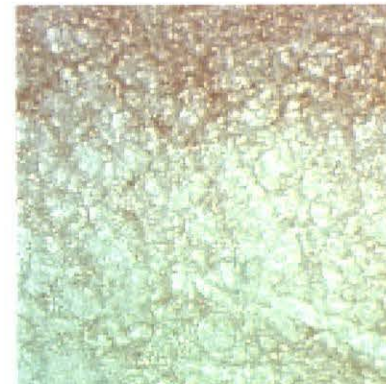
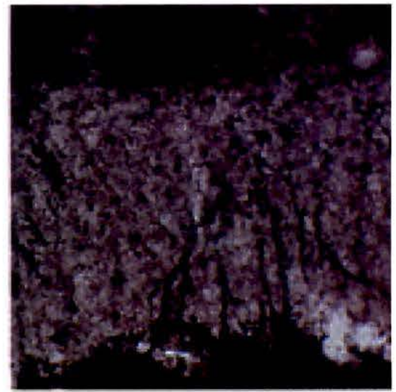
	Control	KA
Timm		
Synaptophysin		
GAP-43		

Figure 4: Representative photomicrographs of the IML of the dentate gyrus from the 30 day KA group and their controls.

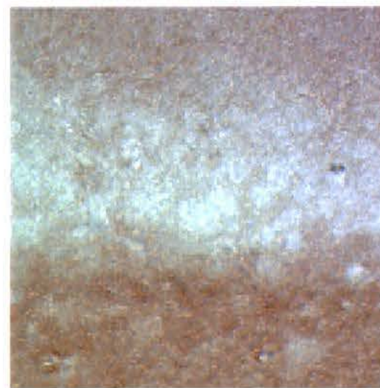
KA 30 days

Control KA

Timm



Synaptophysin



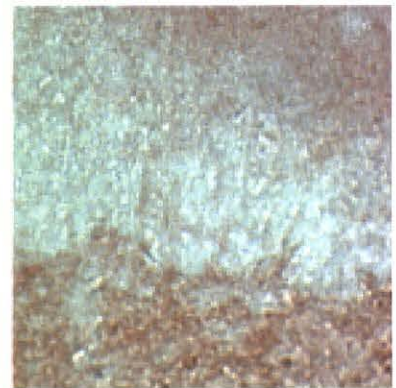
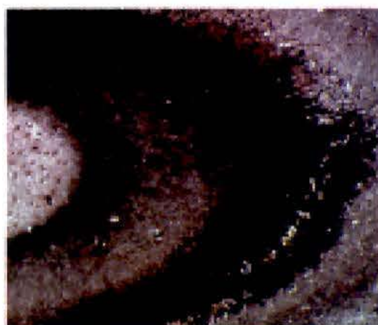


Figure 5: Representative photomicrographs of the CA3 region of the hippocampus in animals treated with KA and allowed to survive for 5 days and controls.

KA 5 days
Control
KA
Timm
Synaptophysin
GAP-43



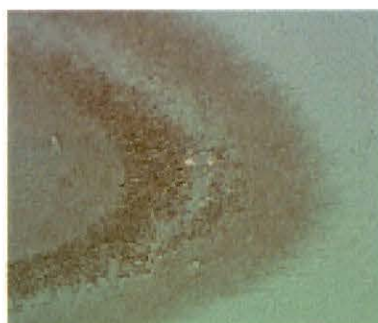


Figure 6: Representative photomicrographs of the CA3 region of the hippocampus in animals treated with KA and allowed to survive for 30 days and controls.

KA 30 days

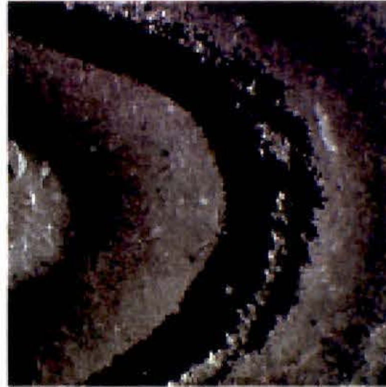
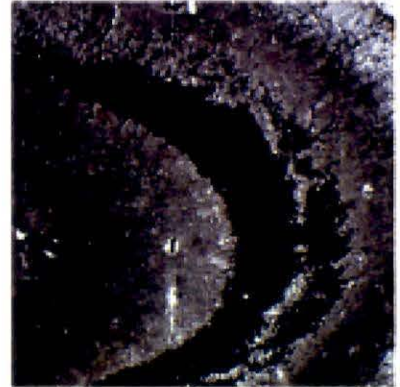
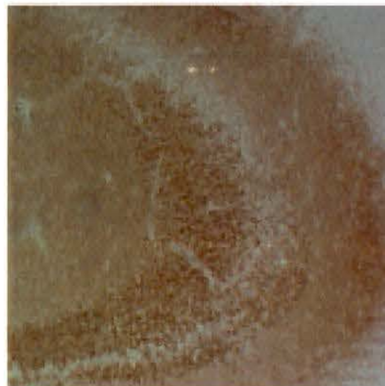
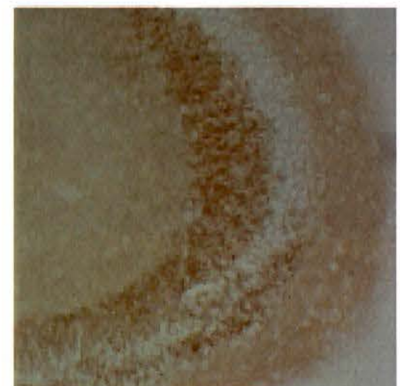
	Control	KA
TImm		
Synaptophysin		

Figure 7: Photomicrographs of the perirhinal cortex from KA treated rats with 5 days before perfusion and their respective controls.

KA 5 days

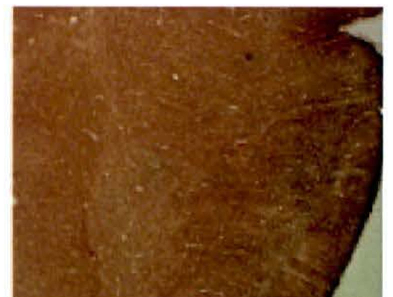
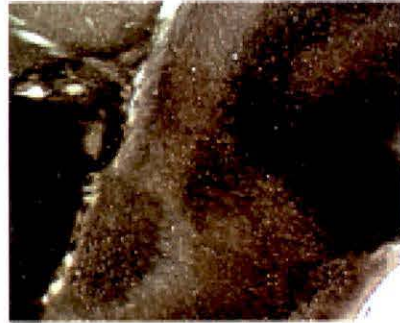
	Control	KA
Timm		
Synaptophysin		
GAP-43		

Figure 8: Photomicrographs of the perirhinal cortex from KA treated rats with 30 days before perfusion and their respective controls

KA 30 days

Control KA

Timm



Synaptophysin

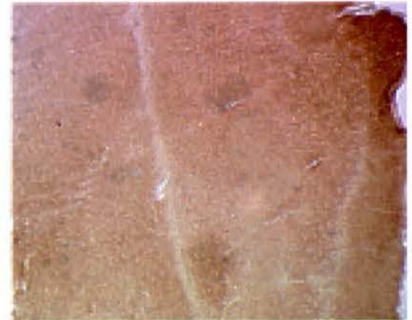


Figure 9: Photomicrographs of the piriform cortex from rats treated with KA and sustained stage 5 seizures with 5 days off before perfusion, and their respective controls.

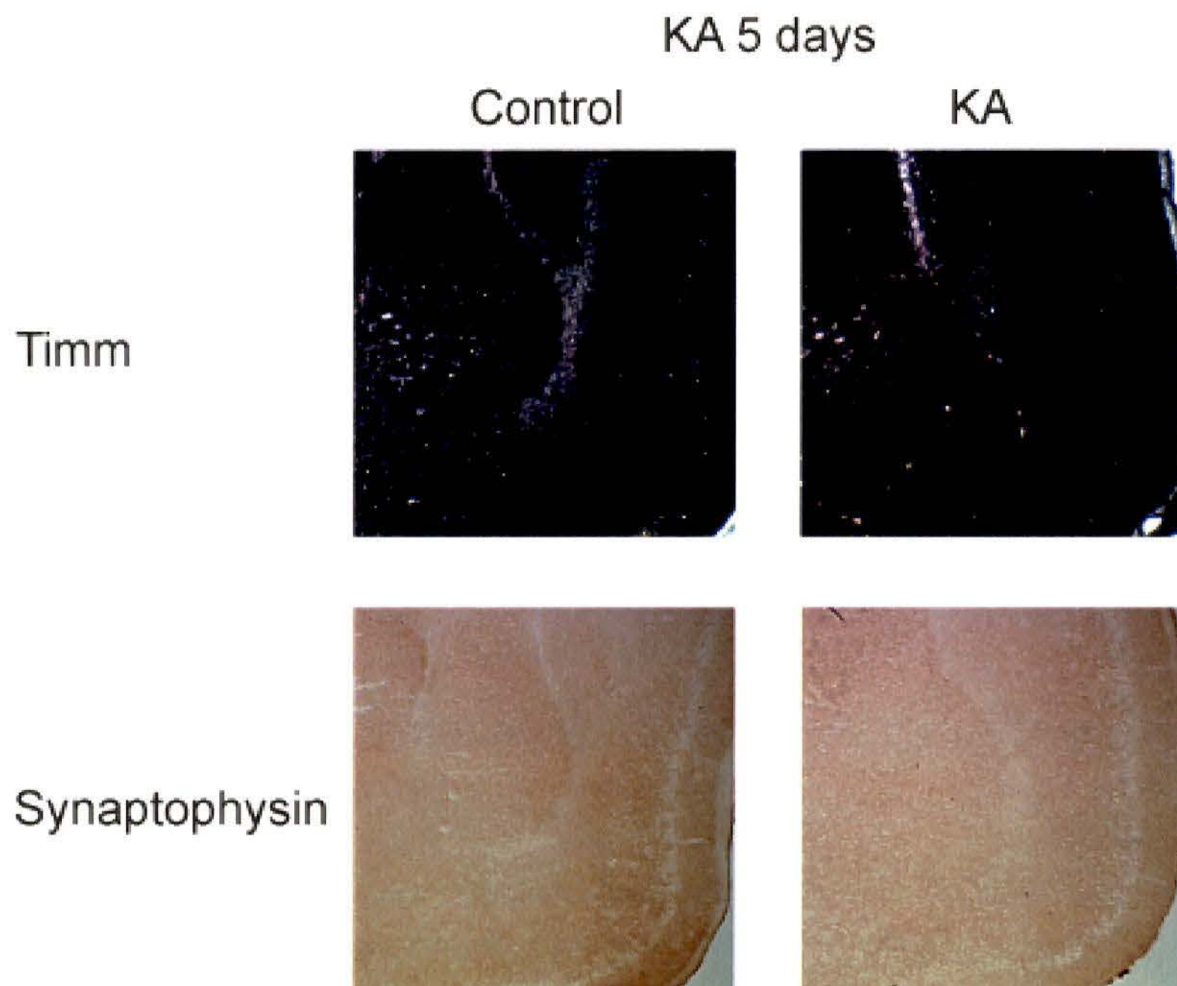
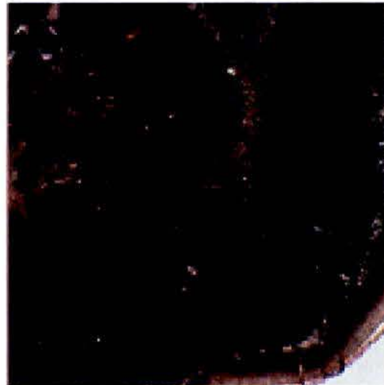

KA 5 days
Control
KA
Timm
Synaptophysin

Figure 10: Photomicrographs of the piriform cortex in tissue 30 days after KA-induced seizures, and their respective controls.

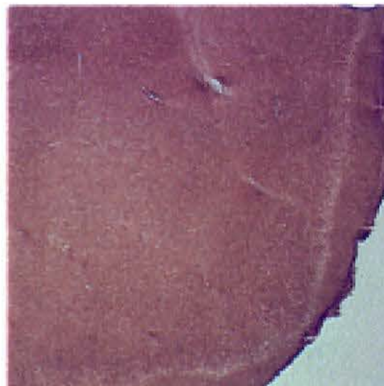
KA 30 days

Control | KA

Timm



Synaptophysin



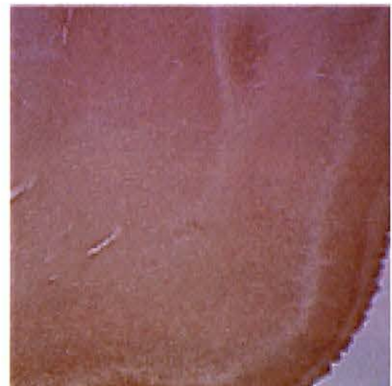


Table 1: Regional percent difference scores (±SEM) in optical density of Timm stain, synaptophysin IR and GAP-43 IR in rats killed 5 days after KA or saline treatment (n=number of rats).

Table 1

	Timm Stain Control	KA	GAP-43 Control	KA	synaptophysin Control	KA
IML of the Dentate Gyrus	n=4 15.07±6.75	*n=4 64.10±10.26	n=6 -10.65±4.36	n=4 0.072±4.48	n=2 2.19±2.13	*n=8 12.08±2.68
CA3	n=3 109±64.90	n=7 93.37±21.64	n=5 12.29±1.39	n=3 10.67±3.34	n=4 10.07±7.15	n=8 14.06±5.60
Perirhinal Cortex Layer 1	n=5 164.97±9.85	n=5 172.72±9.81	n=3 39.38±3.15	n=2 56.95±22.32	n=4 46.55±9.33	n=2 39.76±14.81
Perirhinal Cortex Layer 2	n=5 128.85±25.60	n=5 133.79±26.53	n=3 23.31±1.07	n=2 30.82±7.8	n=4 16.51±4.38	n=2 22.20±2.74
Perirhinal Cortex Layer 4	n=5 113.03±25.14	n=5 105.21±22.48	n=3 18.34±2.18	n=2 21.10±0.69	n=4 10.42±2.88	n=2 10.97±1.06
Piriform Cortex Layer 1	n=3 167.99±13.25	n=8 145.99±17.55	n=3 39.38±3.15	n=3 58.87±13.03	n=4 25.03±7.20	n=3 26.11±7.04
Piriform Cortex Layer 2	n=3 129.17±20.97	n=5 102.275±17.48	n=3 23.31±1.07	n=3 34.29±5.69	n=4 9.44±4.67	n=3 17.01±6.13
Piriform Cortex Layer 3	n=3 175.78±11.33	n=5 174.80±7.33	n=3 18.34±2.82	n=3 23.31±2.25	n=4 19.72±7.45	n=3 17.39±5.77
Endo-piriform Nucleus	n=5 129.39±19.85	n=5 92.17±25.50	n=3 24.85±5.21	n=2 31.86±9.37	n=4 23.11±6.84	n=2 19.58±7.27
Amygdala	n=3 179.86±10.94	n=5 165.93±13.38	n=3 20.24±3.68	n=2 21.85±7.71	n=4 12.09±4.85	n=3 12.53±3.94

Table 2: Regional percent difference scores (±SEM) in optical density of Timm stain and synaptophysin IR in rats killed 30 days after KA or saline treatment (n=number of rats).

Table 2

	Timm Stain		Synaptophysin	
	Control	KA	Control	KA
IML of the Dentate Gyrus	n=4 17.02±16.49	*n=5 64.91±8.73	n=4 5.50±4.52	n=9 9.44±2.77
CA3	n=4 129.84±11.67	n=7 146.94±12.50	n=5 21.60±2.25	n=7 20.69±3.27
Perirhinal Cortex Layer 1	n=4 174.76±4.46	n=5 193.49±3.14	n=3 44.83±6.68	n=6 41.45±6.82
Perirhinal Cortex Layer 2	n=4 164.12±6.80	n=5 153.47±11.56	n=3 10.36±0.79	n=6 14.26±1.74
Perirhinal Cortex Layer 4	n=4 106.63±15.44	n=5 128.58±11.37	n=3 6.44±4.64	n=6 8.58±0.95
Piriform Cortex Layer 1	n=4 176.84±2.4	n=4 147.19±16.10	n=3 38.50±7374	n=5 26.61±7.01
Piriform Cortex Layer 2	n=4 153.21±8.83	n=4 114.45±19.22	n=3 16.59±5.61	n=5 6.31±2.36
Piriform Cortex Layer 3	n=4 184.21±1.60	n=5 170.31±8.63	n=3 25.98±6.30	n=5 14.17±4.87
Endo-piriform Nucleus	n=4 160.11±12.07	n=4 140.00±8.00	n=3 26.67±7.50	n=5 21.25±7.44
Amygdala	n=4 185.61±1.92	n=4 176.79±4.24	n=3 20.34±8.34	n=5 15.76±8.56

5. Experiment 1: Summary

The objective of this experiment was to determine the nature of the sprouting that occurs in the hippocampus and to ascertain wether there is sprouting outside of the hippocampus following a massive seizure event. The results obtained from the hippocampal tissue replicated what has been reported with the Timm stain. There was more MFS as demonstrated by a greater number of Timm granules in the IML of the dentate gyrus following both a 5 day and a 30 day interval. As shown before, this change in the MFS appears to be stable, lasting beyond 30 days (Buckmaster & Dudek, 1999; Cronin & Dudek, 1988). However, my results do not show the same changes as typically seen in field CA3. Previously, studies have found sprouting in CA3 stratum oriens after KA-induced SE (Buckmaster & Dudek, 1999; Cronin & Dudek, 1988). On further inspection of published photomicrographs from previous studies, I conclude that the CA3 stratum oriens has a much lower baseline than found in the present study. It is unclear why the baselines are variable, but with higher baseline measurements, changes would be difficult to detect.

Synaptophysin IR was also increased in the IML 5 days following SE. However, the changes were not detected at the 30 day interval. This suggests that the MFs display an increase in vesicles following the seizure event, but that this change returns to baseline by 30 days. It has been speculated that there are changes occurring that will facilitate the spontaneous seizures that develop after KA-induced SE. The change in synaptophysin IR occurs between the time when SE was induced and the time when spontaneous seizures develop (Bragin,

Wilson, & Engel, 2000; Cavalheiro, Riche, & Le Gal La Salle, 1982). Therefore, it could be a process related to spontaneous seizure generation.

I found no changes in GAP-43 IR in the hippocampus after SE; however other studies have found changes after SE. Naffah-Mazzacoratti et al found increases in GAP-45 IR in the IML of the dentate gyrus following pilocarpine-induced SE at both 5 days and 30 day interval (Naffah-Mazzacoratti et al., 1999). Other studies have reported upregulation of GAP-43 IR in the IML following KA-induced SE; however their data are unquantified (Bendotti et al., 1997; Bendotti et al., 1994). Furthermore, there are upregulation found in GAP-43 mRNA following KA-induced SE, with a peak at 24 hr after seizure (McNamara & Routtenberg, 1995). The present study is the first to quantify GAP-43 IR following KA-induced SE, and in my hands there were no changes in the IML of the dentate gyrus. Furthermore, as previous studies have shown, following KA-induced SE no changes in GAP-43 IR are seen in any other parts of the hippocampus.

There were no differences found in any of the other areas analyzed. The lack of differences can be explained several different ways. Primarily, there could simply be no quantifiable changes in axonal growth and synaptic changes following KA-induced SE. However, in my opinion this is unlikely. Most likely there are either no changes in these specific proteins, or else our technique is not sensitive enough to measure the changes that do occur. These changes may be apparent at the electron microscopic level. Furthermore, the changes might be transient and did not fall within the time intervals investigated. Lastly, there might

be an overall change in synaptic and axonal organization where there is both an increase and a decrease in protein levels, resulting in no net change.

Altogether, these data suggest that the MFS following SE is still the most commonly observed change. It is dramatic in the Timm stained tissue at both intervals and detected by synaptophysin IR at the 5 day interval. Furthermore, finding no changes outside the hippocampus suggests that although there may be changes that occur, they are less dramatic than MFS and may be difficult to detect.

3. Experiment 2

3.1 Introduction

Kindling is a preparation that appears to have features similar to TLE. However, unlike KA treatment, kindling is a progressive process that cumulates in complex partial seizures with secondary generalization. Therefore, it allows study not only of the epileptic state, but also of the development of the state as well. Kindling occurs with application of brief trains of electrical pulses into a region, evoking a short electrographic seizure. Depending on the site of stimulation, this first seizure may or may not be associated with convulsive activity. Evoking multiple sessions of focal discharge is both necessary and sufficient for kindling to occur (Goddard et al., 1969; Racine, Okujava, & Chipashvili, 1972; Racine, 1972a). Thus, through a period of daily stimulations, convulsions will progressively move through a set of well defined stages. Racine defined a behavioral convulsive scale of 6 consecutive steps with limbic kindling in rats. These stages closely follow those of the behavioral patterns seen in human TLE patients. The stages are immobility (stage 0), mastication (stage 1), head movement (stage 2), forelimb clonus (stage 3 and 4), and a fully generalized seizure characterized by loss of posture and balance (stage 5). The duration of the time spent in each seizure stage, and the number of daily stimulations needed to evoke each stage, depend on the site that the electrical stimulation is applied to. However, any site that shows initial afterdischarge will continue to kindle, generating an epileptic state. Consequently, kindling provides a way of studying not only an end epileptic state but also the process that occurs during the

development of that state. Furthermore, kindling also evokes changes throughout the brain similar to both KA and TLE. In this study, the site of electrical stimulation was the amygdala, the most commonly kindled site (Cain, 1992).

As with the previous study, GAP-43 and synaptophysin IR as well as the Timm stain were used to look for changes outside the hippocampus as well as to replicate the changes found inside the hippocampus. With kindling, it is possible to include a group of rats that are analyzed during the process of seizure development. This group received 5 stimulations. Furthermore, a group of rats that displayed 5 stage 5 seizures with a 5 day interval before sacrifice was analyzed. The same areas included in the previous study were analyzed in this experiment.

3.2. Experiment 2: Methods

3.2.1 Animals

Sixty male Long-Evans hooded rats (Charles River, Montreal, Quebec, Canada) were group housed on arrival. Rats were handled daily for one week and then individually housed immediately following surgery. All rats had free access to food and water and were kept on a 12:12 light:dark cycle. The colony room was maintained at 19-21 °C. All procedures were carried out during the light portion of the cycle and according to the guidelines of the Canadian Committee on Animal Care and were approved and monitored by the University of Saskatchewan Committee on Animal Care and Supply.

3.2.2 Surgery

Rats weighed 250-300g at the time of surgery. Rats were deeply anesthetized with Somnotol® (10 mg/kg sodium pentobarbital i.p.) and placed into a sterotaxic apparatus. Two bipolar stimulating/recording electrodes were implanted into the amygdala at –2.6 anterior-posterior (AP), 4.5 medial-lateral (ML), -9.1 dorsal-ventral (DV), coordinates relative to bregma, taken from a flat skull. Coordinates were based on Paxinos and Watson rat brain atlas (Paxinos & Watson, 1986). Four dental screws and a reference electrode were attached to the skull using dental acrylic. The electrode pins were inserted into a plastic holder. Rats were monitored until fully awake and returned to the colony room in a clean individual

plexiglass cage and were allowed to recover for one week prior to the onset of kindling.

3.2.3 Kindling

3.2.3.1 Afterdischarge

Each experimental rat was paired with a yoked control. The pairs were treated similarly in respect to surgery, kindling, perfusion, and histology. The only difference was that the control rats did not receive any electrical stimulation. The day before afterdischarges (ADs) were assessed, the rats were habituated to the room in which they would be kindled. The following day, the rats were again transported in their home cages to the kindling room. The AD threshold (ADT) was assessed by passing a one sec train of constant current balanced biphasic square wave pulses (1-ms duration) at 60pps through the stimulating/recording electrode. An initial current of 30μA was passed, and stimulation was raised by 10μA at 1 min intervals until five sec of AD was evoked. The lowest effective intensity was arbitrarily defined as the ADT. Control rats were placed in the treatment box for a similar amount of time.

3.2.3.2 Kindling

Kindling stimulation was delivered once daily at the initial ADT beginning 24 hr following the ADT assessment. The behavioral stages were rated according to a 6 point scale (Racine, 1972a) in which stage 0 involves freezing, stage 1 indicates mastication, stage 2 indicates mastication and rhythmic head nodding, stage 3

indicates unilateral forelimb clonus, stage 4 indicates bilateral forelimb clonus and rearing, and stage 5 indicates rearing and falling. The duration of AD was also recorded. The experimental rat of each yoked pair was kindled to either 5 ADs (including ADT) or 5 stage 5 seizures.

3.2.4 Perfusion

Rats in which 5 ADs were triggered were perfused two days later, whereas the rats in which 5 stage 5 seizures were triggered experienced a 5 day interval before perfusion. At the time of perfusion, rats were deeply anesthetized and perfused according to the Timm perfusion method described in Experiment 1(3.3).

3.2.5 Histology

3.2.5.1 Sectioning

Sections were taken according to the methods from Experiment 1 (3.4.1) with the following difference. During sectioning, when the electrode tracks were reached, the sections were mounted on poly-l-lysine coated slides for cresyl violet staining and electrode placement assessment. Following cresyl violet staining, the tissue was microscopically assessed to confirm that electrode placements were in the amygdala.

3.2.5.2 Cresyl violet staining

Sections were dried overnight, and then were dehydrated in slowly descending ethanol concentrations and washed in distilled water, followed by 5-10 min in 1% Cresyl Violet. Slides were washed again in distilled water and then differentiated with 1% acetic acid in 95% ETOH. The slides were washed in distilled water, followed by ascending ethanol concentrations. They were then cleared in xylenes and cover slipped with permount.

3.2.5.3 Timm Staining

See description Experiment 1 (3.4.2).

3.2.5.4 Synaptophysin immunohistochemistry

See description Experiment 1 (3.4.3).

3.2.5.5 Gap-43

See description in Experiment 1 (3.4.4).

3.2.6 Image Analysis and data collection

See description in Experiment 1 (3.5).

3.2.7 Statistical analysis

See methods in Experiment 1 (3.6).

3.3 Experiment 2: Results

3.3.1 Kindling

Kindled rats were included in the data analysis only if the electrode tip was located in the amygdala.

3.3.2 Hippocampus

3.3.2.1 IML of the dentate gyrus

The rats kindled to 5 stage 5 seizures displayed more Timm granules in the IML of the dentate gyrus than controls. That is, tissue from kindled rats (n=2) was significantly darker than tissue from control rats (n=3) ($t(3) = 2.43$, $p<0.05$) (means reported in Table 3). However, there were no differences in Timm stain in the IML in the 5AD group (Table 4). Additionally, there were no changes found in either GAP-43 or synaptophysin IR in the IML of the dentate gyrus in either the 5 stage 5 group or the 5AD group.

3.3.2.2 CA3

There were no significant differences in Timm staining, synaptophysin IR, or GAP-43 IR in either the 5AD or 5 stage 5 groups in stratum oriens in the CA3 region of the hippocampus.

3.3.3 Perirhinal Cortex

There were no significant differences in Timm staining, synaptophysin IR, or gap-43 IR in either the 5AD group or the 5 stage 5 group in layers 1, 2 or 4 of the perirhinal cortex (means reported in Tables 3 and 4).

3.3.4 Piriform Cortex

There were no significant differences in Timm staining, synaptophysin IR, or GAP-43 IR in either the 5AD group or the 5 stage 5 group in layers 1, 2 or 3 or in the endopiriform nucleus (means reported in Tables 3 and 4).

3.3.5 Amygdala

There were no significant differences in Timm staining, synaptophysin IR, or GAP-43 IR in either the 5AD group or the 5 stage 5 group in the basolateral nucleus of the amygdala (means reported in Tables 3 and 4).

Figure 11: Representative photomicrographs of the MFS in the IML of the dentate gyrus in the 5 stage 5 group and the 5AD group and their controls.

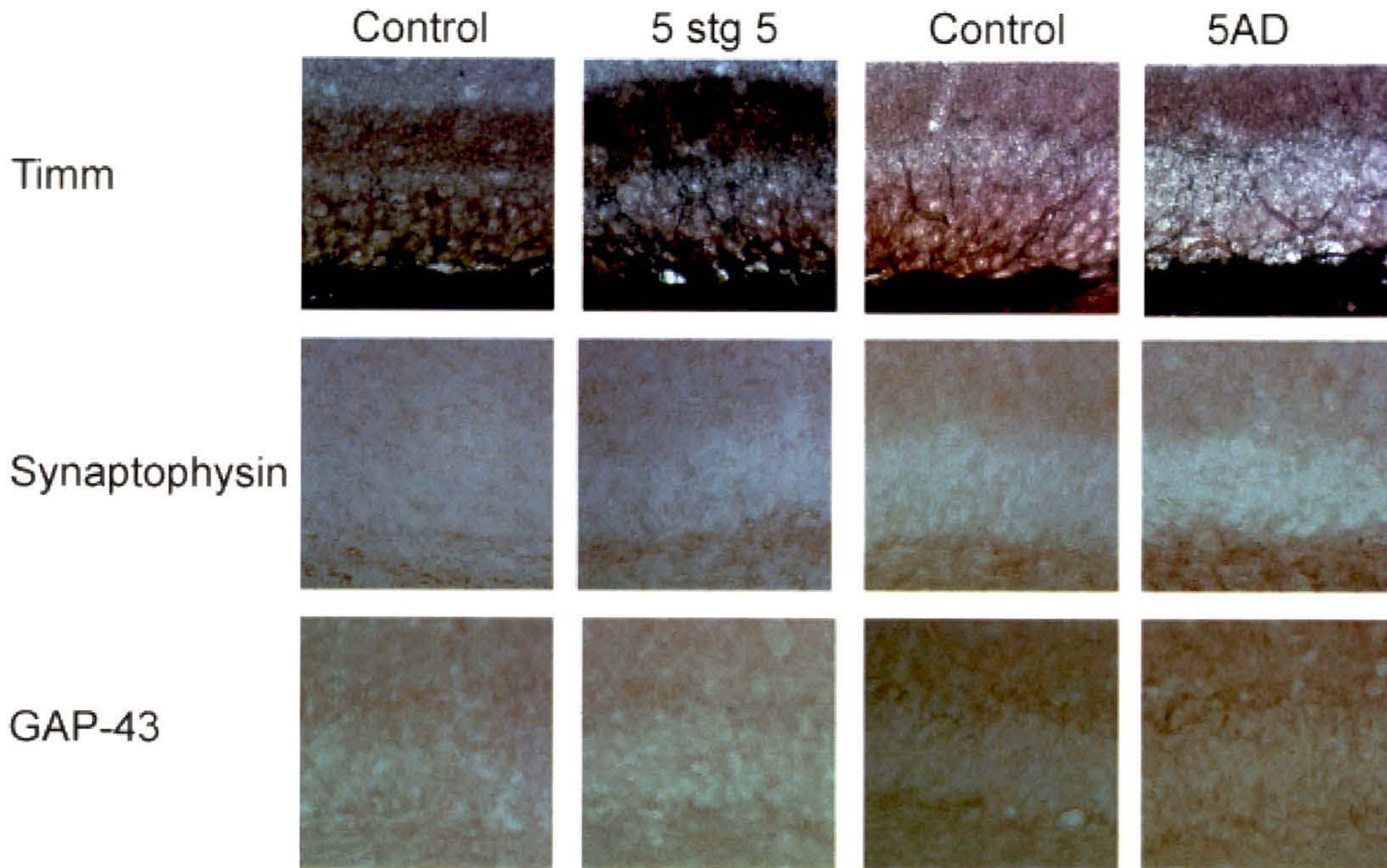

Kindled seizures
Control
5 stg 5
Control
5AD
Timm
Synaptophysin
GAP-43

Figure 12: Representative photomicrographs of field CA3 in the 5 stage 5 group and the 5AD group and their controls.

Kindled seizures

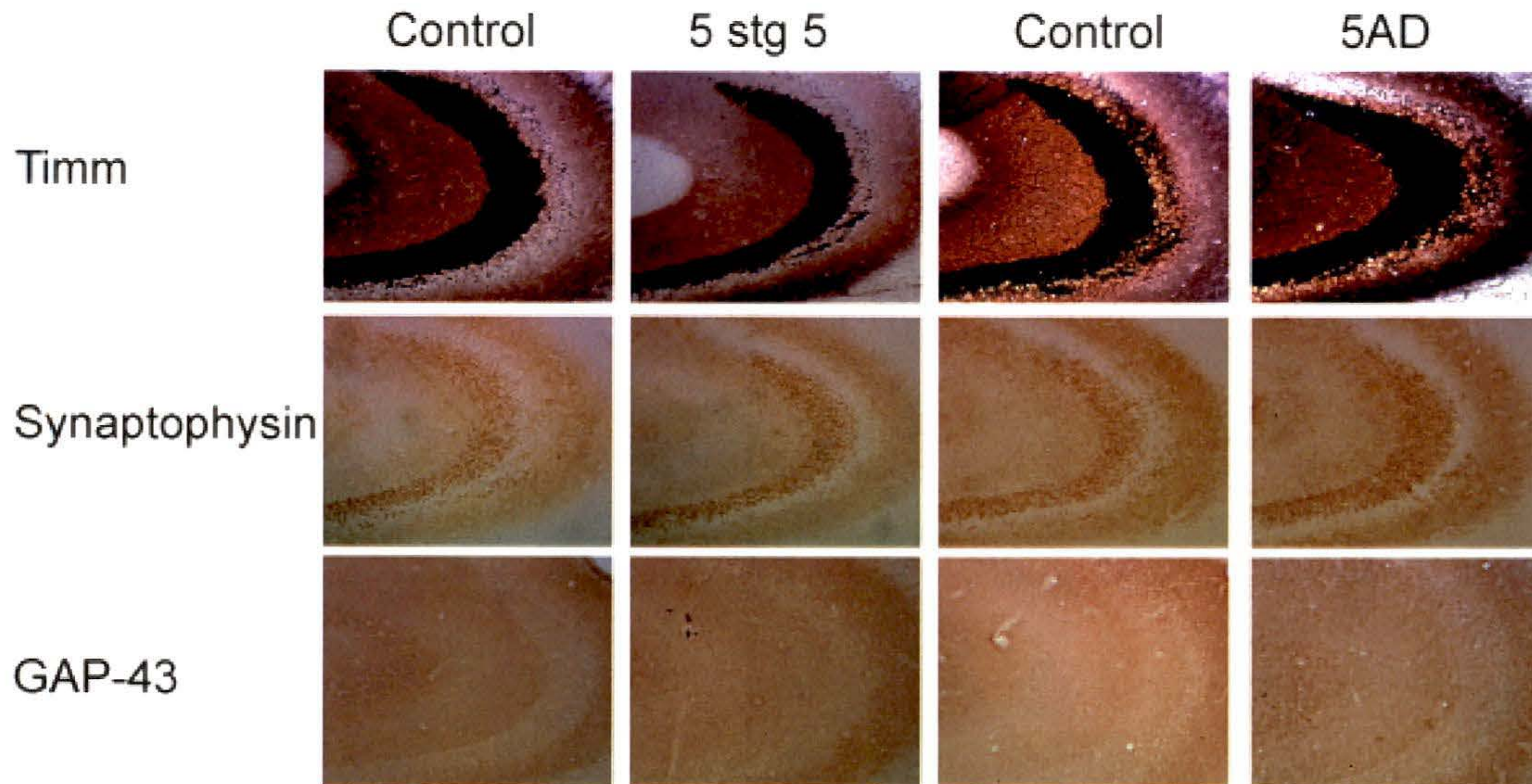
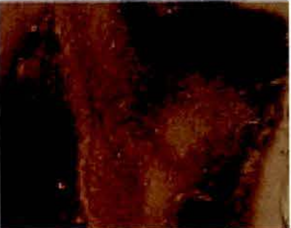


Figure 13: Microphotographs from perirhinal cortex in rats kindled to 5ADs and 5 stage 5 seizures.

Kindled Seizures

Control 5 stg 5 Control 5AD

Timm



Synaptophysin

GAP-43

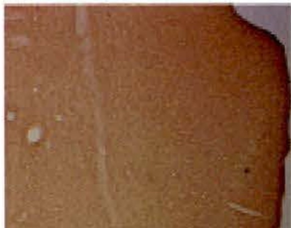


Figure 14: Photomicrographs of the piriform cortex from rats kindled to 5ADs and rats kindled to 5 stage 5 seizures.

Kindled seizures

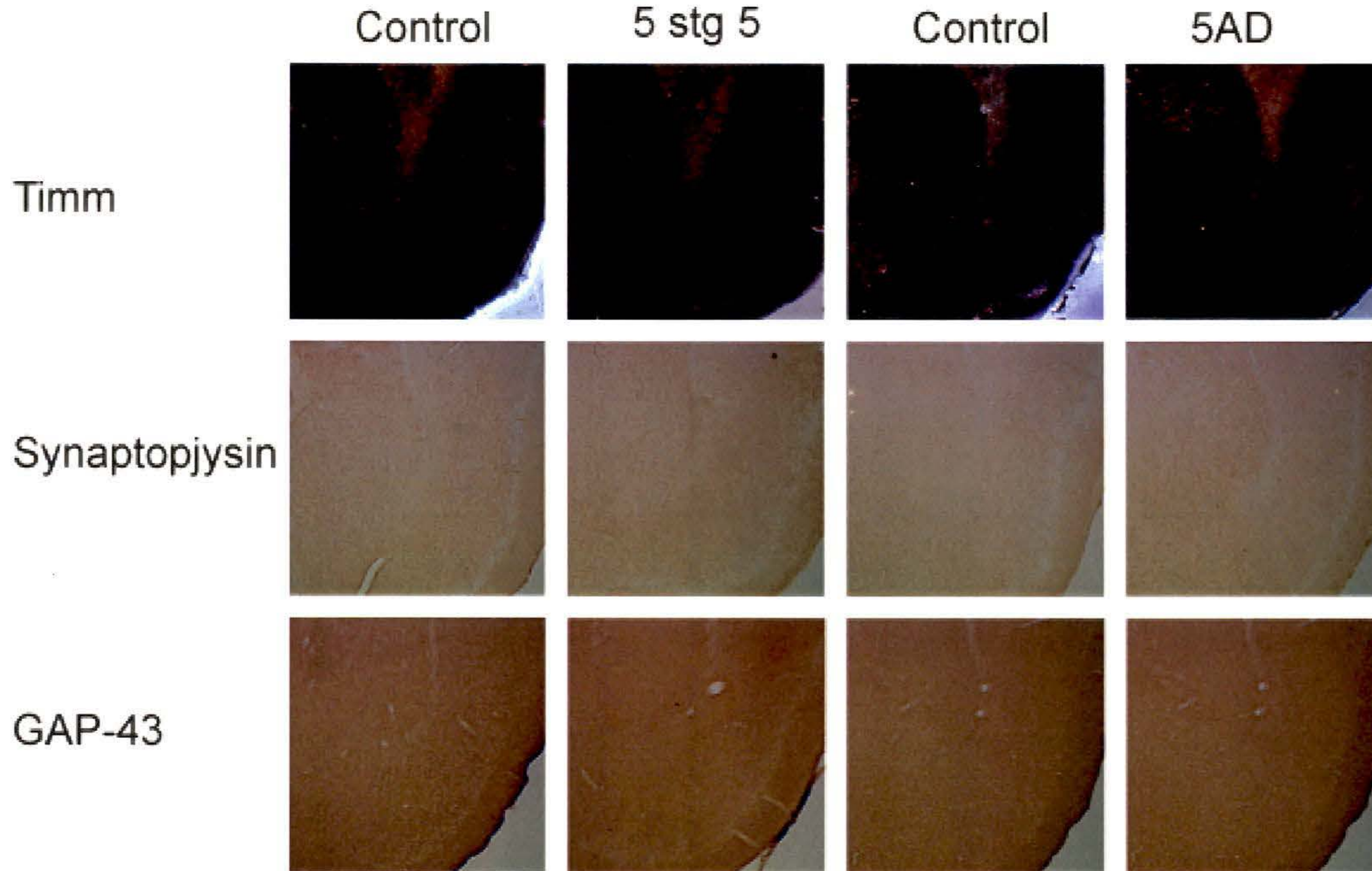


Table 3: Regional percent difference scores (±SEM) in optical density of Timm stain, synaptophysin IR, and GAP-43 IR in rats killed 5 days after 5 stage 5 seizures or sham stimulation (n=number of rats).

Table 4: Regional percent difference scores (±SEM) in optical density of Timm stain, synaptophysin IR, and GAP-43 IR in rats killed 2 days after 5ADs or sham stimulation (n=number of rats).

Table 4

	Timm Stain		Gap-43		Synaptophysin Immunoreactivity	
	Control	Kindled	Control	Kindled	Control	Kindled
IML of the Dentate Gyrus	n=6 23.53±8.04	n=4 21.18±6.70	n=12 26.26±2.67	n=8 9.79±0.59	n=7 26.39±3.62	n=5 8.89±1.24
CA3	n=6 100.08±18.00	n=7 100.31±21.83	n=11 15.30±2.73	n=9 12.47±2.60	n=12 12.95±1.46	n=8 13.36±2.67
Perirhinal Cortex Layer 1	n=4 173.18±8.79	n=5 162.10±9.98	n=11 56.49±3.38	n=9 54.63±1.81	n=12 53.41±4.59	n=9 47.16±3.92
Perirhinal Cortex Layer 2	n=4 123.70±22.86	n=5 118.61±9.03	n=11 26.34±1.75	n=9 27.86±1.90	n=12 19.96±2.05	n=9 20.31±3.17
Perirhinal Cortex Layer 4	n=4 60.67±17.94	n=5 61.83±12.86	n=11 25.70±1.81	n=9 29.02±2.14	n=12 14.16±1.81	n=9 16.52±2.19
Piriform Cortex Layer 1	n=5 117.02±27.24	n=5 145.64±8.73	n=11 50.15±3.21	n=10 45.30±5.10	n=11 40.33±3.56	n=8 35.36±5.73
Piriform Cortex Layer 2	n=5 107.63±22.92	n=5 132.83±15.16	n=11 35.37±2.88	n=10 32.33±4.21	n=11 22.98±2.05	n=8 18.28±3.30
Piriform Cortex Layer 3	n=5 148.59±15.05	n=5 160.38±6.86	n=11 37.09±2.67	n=10 32.61±4.07	n=11 27.92±2.90	n=8 22.85±3.27
Endo-piriform Nucleus	n=4 65.77±32.72	n=5 86.74±21.61	n=10 25.90±3.17	n=10 23.31±4.96	n=11 16.62±3.93	n=8 12.47±3.13
Amygdala	n=5 126.58±25.23	n=5 144.89±11.31	n=11 18.52±2.97	n=9 21.15±3.24	n=11 14.46±3.12	n=8 9.74±2.75

3.4. Experiment 2: Summary

The objective of this study was to determine the axonal and synaptic changes that occur during and following kindling. Similar to previous studies, I found increased MFS in the IML of the dentate gyrus after kindling of 5 stage 5 seizures with a 5 day interval. However, there were no differences found in the MFS after 5ADs. This is comparable to the results of Cavazos et al, where following 5ADs and a 24 hr interval in amygdaloid kindled rats there was an elevated but not statistically significant difference in MFS (Cavazos et al., 1991). However, unlike pervious studies, increases in Timm stain were not evident in the field CA3 stratum oriens. Again, my baseline measurements in control rats were above those previously reported, and may have resulted in any differences remaining undetected.

Furthermore, there were no changes found in either GAP-43 or synaptophysin IR in the hippocampus. Previous studies have found changes in GAP-43 mRNA following 5ADs, but did not examine protein levels (Bendotti et al., 1993). However, it could be that after 5ADs there would not yet be increased protein levels. As well, following PTZ kindling, there are no changes found in the hippocampus of synaptophysin mRNA at 2 days following 5 stage 5 seizures (Mahata et al., 1992). Therefore, the lack of changes found in protein levels could indicate that sprouting does not involve these proteins, or that the time points selected were not at the intervals when the changes could have been detected.

There were no significant changes found outside of the hippocampus in Timm, GAP-43, or synaptophysin staining. It is unknown whether this simply means that there is no sprouting outside the hippocampus or whether the techniques are not sensitive enough to detect the changes.

4. Experiment 3

4.1. Rationale

Given the results of the two previous studies, it is necessary to establish that my staining technique can detect changes that occur outside the hippocampus. Chen at al. used KA infusion to induce synaptophysin IR in the cortex adjacent to the infusion site (Chen et al., 1996). The objective of experiment 3 was to produce similar lesions as Chen et al and examine the cortical tissue surrounding the lesion for changes in Timm stain, and synaptophysin IR.

4.2. Experiment 3: Methods

4.2.1 Animals

Ten Long-Evans rats obtained from Charles River Montreal, Quebec, Canada were used for this experiment. The rats were housed in pairs before and after surgery. Rats were kept on a 12:12 light:dark schedule and had free access to food and water. The temperature was maintained between 19-21°C. Rats were handled daily following arrival and weighed between 350 and 400 grams at the time of surgery.

4.2.2 Surgery

Rats were deeply anesthetized with 5% Isofluorene, followed by placement into a sterotaxic apparatus, where they were maintained under anesthesia with 2% Isofluorene. A 20 gage guide cannula was inserted into the anterior neocortex at the following coordinates: AP –0.4, ML +4.0, DV –9.1 based on Paxinos and

Watson (Paxinos & Watson, 1986). Fifty mM KA in artificial cerebrospinal fluid (ACSF; 124mM NaCl, 5mM KCl, 2 mM $CaCl_2$*H_2O, 1 mM $MgCl_2$, 1 mM NaH_2PO4, 24 mM $NaHCO_3$ and 11 mM D-glucose) or ACSF was infused at 1µl/min, to a total of 10µl. A microinfusion pump was used to inject 10µl of solution from a 100µl Hamilton syringe through PE10 tubing, through a cannula made of a 20g blunt end needle. The cannula was left in place for 2 min following infusion to allow for complete diffusion of the solution. Following infusion, the incision was sutured, and rats were monitored until fully awake, observed for convulsive behavior, and returned to the colony room.

4.2.3 Perfusion

Following a 30 day interval, the rats were given a lethal dose of sodium pentobarbital and perfused intracardially using the Timm procedure outlined in Experiment 1 (3.3).

4.2.4 Histology

4.2.4.1 Sectioning

Refer to methods from Experiment 1, with the following additions (3.4.2). Sections were taken for histological assessment of infusion induced damage.

4.2.4.2 Cresyl Violet Staining

Sections taken to assess infusion induced damage were stained with cresyl violet. See Experiment 2 (7.5.2).

4.2.4.3 Infusion placement:

Infusion placements were mapped on to the appropriate brain map. Damage was assessed using a light microscope and was based on gliosis and loss of tissue.

4.2.4.5 Synaptophysin Immunohistochemistry

See methods in Experiment 1 (3.4.3).

4.2.4.6 Gap-43 Immunohistochemistry

See methods in Experiment 1 (3.4.4).

4.2.5 Data collection

See description in Experiment 1 for quantification of synaptophysin IR (3.5). The Timm stained sections were quantified using a counting technique. The images were captured using the methods mentioned before (3.5). On a grey scale image, background was taken from the corpus callosum, which was the closest white matter to the area of interest. The area of interest was outlined on the computer, and the number of dark clusters was counted. To obtain a number corrected for background, the number of clusters found in the white matter background selection was subtracted from the number found in the region of interest.

4.2.6 Statistical analysis

See methods in Experiment 1 for the statistical analysis of the synaptophysin IR

(3.6). For the Timm stained tissue, corrected counts were averaged across at least 4 sections from each area. The sections were not averaged across hemispheres because comparisons between hemispheres were performed. An individual t-test was performed on the average corrected counts.

4.3 Experiment 3: Results

4.3.1 Animals

Eight rats were included for analysis. One KA-infused rat died during post operative recovery and one saline-infused rat died during surgery. Tissue from the infusion site of the KA showed the expected amount of damage with consistent damage lateral to the internal capsule extending throughout the entire dorsal/ventral plane. The damage began just anterior to 1.7 AP and continued posterior until -3.8 AP (Paxinos & Watson, 1986). Because, the rationale of the study was to determine whether the methods used in the previous sections were sensitive to changes occurring in the cortex, a stringent analysis of the lesion was not performed.

4.3.1 Sensorimotor cortex

Rats infused with KA displayed patchy and punctate clusters of Timm staining in the area surrounding the infusion site. Tissue adjacent to the site of infusion of KA (n=4) displayed greater Timm staining when compared to tissue from control rats that were infused with ACSF (n=4) ($t(14)=7.23$, $p<0.001$). The KA infused rats also displayed greater staining in the infused cortex as opposed to the homeotopic contralateral cortex ($t(14) = 6.99$, $p<0.001$), whereas the rats infused with ACSF did not display this difference ($t(14) = 0.19$, $p>0.05$) (Means reported in Table 5).

There was significant synaptophysin IR in the sensorimotor cortex surrounding the KA infusion site exhibited by a darker, punctate positive staining. Rats

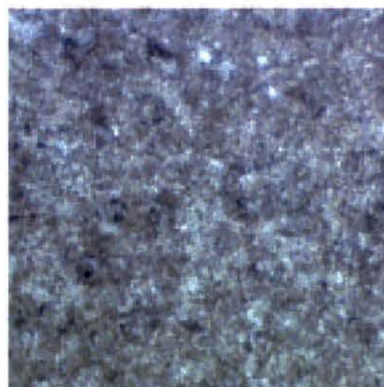
infused with KA (n=4) displayed greater synaptophysin IR than rats infused with ACSF (n=4), ($t(26) = 4.06$, $p<0.05$). Within the rats infused with KA, the cortex ipsilateral to the lesion displayed more synaptophysin IR than the contralateral homeotopic cortex ($t(9) = 2.76$, $p<0.05$), whereas the ACSF infused rats displayed no differences between the infusion site and the homeotopic contralateral cortex ($t(41) = -1.88$, $p>0.05$). (Figure 10, Table 5)

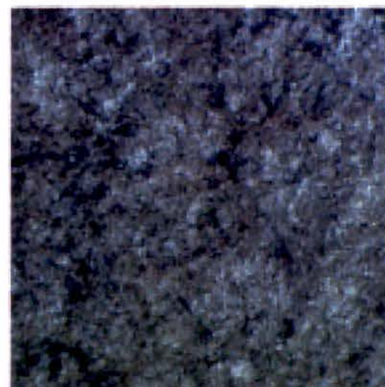
Figure 15: Photomicrographs of representative sections from KA and ACSF infusion rats.

KA infusion

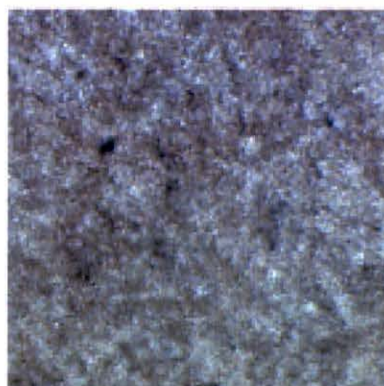
Contralateral | infusion

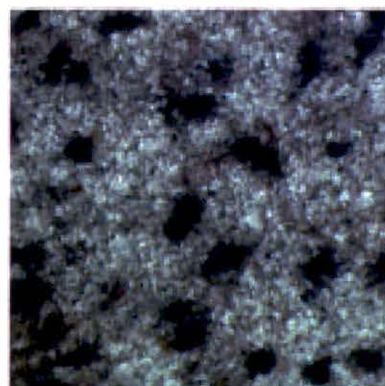
Timm
ACSF infusion





Timm
KA infusion





Synaptophysin
ACSF infusion

Synaptophysin
KA infusion

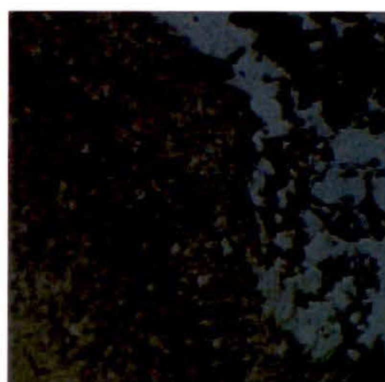


Table 5: Regional counts of Timm stained neurons in the somatosensory cortex (±SEM) and optical density (±SEM) of synaptophysin stained tissue in rats 30 days after KA or ACSF infusion. Each cell is based on analysis of 4 rats.

Table 5

Side of brain	Timm		Synaptophysin IR	
	ACSF	KA	ACSF	KA
Ipsilateral	19.38±11.01	254.88±30.66	3.67±2.64	53.02±11.86
Contralateral	16.88±6.48	29.13±10.14	13.02±4.23	6.66±11.60

4.4. Experiment 3: Summary

The objective of this study was to ascertain if my staining techniques can detect changes in Timm granules and synaptophysin IR outside the hippocampus. Increased Timm granules and synaptophysin IR detected in the sensorimotor cortex adjacent to the lesion. The changes in synaptophysin IR are consistent with those found by Chen at al, described changes in synaptophysin IR in the cortex near the KA lesion (Chen et al., 1996). This suggests that my staining techniques can indeed detect changes in synaptophysin IR outside the hippocampus. However, these changes were detected only in tissue adjacent to damaged tissue. This implies that the immunohistochemical technique for synaptophysin may not be sensitive enough to detect smaller changes that might occur following seizures or, perhaps equally plausible, changes do not occur outside the hippocampus following seizures.

The changes in Timm histochemistry appeared to form in clusters and exhibited properties different from staining associated with MFS. Following KA-induced seizures and kindling, the Timm staining occurring in the ILM of the dentate gyrus appears as small grains. However, following KA infusion, the cortical Timm staining occurs in large clusters. It is unknown whether these clusters are evidence of sprouting stained by the Timm stain, or wether they represent another event that may be occurring in the area around the infusion damage.

Overall, it has been determined that my staining techniques can detect changes in synaptophysin IR following KA infusion. Furthermore, I replicated the

results of Chen et al., verifying that synaptic reorganization does occur in the cortex adjacent to KA infusion damage (Chen et al., 1996).

5. General Discussion

The purpose of my thesis was to determine whether axonal sprouting occurs following seizures both outside and within the hippocampus. The objectives were to ascertain whether proteins associated with axonal sprouting (synaptophysin, GAP-43), as well as Timm staining, change in association with KA-induced SE or kindling. Furthermore, to gain a detailed understanding of the time course of any changes, I took tissue at several intervals after seizures or kindling.

In agreement with previous investigators, I found that MFS as indicated by the Timm stain occurs following SE and kindling (Cavazos et al., 1991; Represa & Ben-Ari, 1992; Sutula et al., 1988). Furthermore, I found increased synaptophysin IR in the IML of the dentate gyrus following induction of SE. This suggests either that there are increased vesicles in the MFs at 5 days after SE, or that there are increased numbers of MFs at this time. However, staining for synaptophysin returned to baseline after 30 days, indicative of a transient change and arguing against increased numbers of fibers. Furthermore, I found no changes in GAP-43 IR in the IML in association with either KA-induced SE or kindling, suggesting either that MFS is not dependent on increased expression of GAP-43 or that the changes are too small to be quantified. Overall my findings are similar to other observations of MFS.

It was surprising to me to discover that neither KA-induced SE or kindling is associated with evidence of axonal sprouting in regions outside the hippocampus, including the cortex and the amygdala. It is unlikely that the

negative results are due to inadequacy of my staining techniques, in view of the positive control contained in Experiment 3. There I found that I could detect increased Timm granules and synaptophysin IR in the cortex after focal infusion of KA. So the question remains, why are changes not seen in the cortex after systemic injection of KA or after kindling. One possibility is that axonal sprouting does occur in non-hippocampal regions, but that there are both increases and decreases in protein levels. If relatively balanced increases and decreases in protein occur, this would result in no change in staining. To determine whether this explanation is likely, tissue would have to be analysed with electron microscopy, with synapses and axons evaluated. For example, Kato et al (2001) characterized changes in dendrites in the amygdalohippocampal area after kindling. Although they found no significant changes in MAP-2 IR, they did find an overall increase in dendritic width. Therefore it is possible that opposing changes in both GAP-43 and synaptophysin do indeed occur but are undetectable at the light microscopic level.

Another possibility is that sprouting outside the hippocampus does occur in association with kindling or KA-induced SE, but that the changes are very small and fail to be detected via immunohistochemistry and densitometric quantification. That such sprouting might occur is suggested by the observations of changes in neurotrophins associated with seizures. With the evidence that manipulating neurotrophins affects seizures (Adams et al., 1998; Reibel et al., 2000; Van Der Zee, 1995), as well as observations that changes in neurotrophins occur (Dugich-Djordjevic et al., 1992; Gall et al., 1991; Riva et al., 1992; Zafra et

al., 1990), it might be suggested that extra-hippocampal axonal sprouting does occur. According to this hypothesis, however, any changes in GAP-43 and synaptophysin must be too small to be observable.

Additionally, it is possible that axonal sprouting does *not* occur outside the hippocampus in association with seizure activity. Extra-hippocampal morphological changes associated with seizures are, according to this hypothesis, more subtle in nature, such as changes in presynaptic release mechanisms or postsynaptic receptors. Such mechanisms alone could be sufficient to account for seizure progression and the kindled state. They would, therefore, be undetectable via quantification of proteins indicative of sprouting. Indeed, changes have been found in several neurotransmitter systems that are linked to seizures (Kang et al., 2001; Kokaia & Kokaia, 2001). Furthermore, structural changes have been described in the amygdalohippocampal area following kindling in the absence of changes in the number of synapses (Kato et al., 2001). Thus my data are consistent with the possibility that extra-hippocampal axonal sprouting does not occur in association with seizures.

Finally, it is also possible that the intervals I chose for analysis were not optimal for detecting changes in the levels of protein. Changes in GAP-43 mRNA in the MFs after KA-induced SE occur at 24 hr, with no change seen at 48 hr or 7 days (Bendotti et al., 1994). Furthermore, changes in GAP-43 IR after KA-induced SE can be detected at 2 months (Bengzon et al., 1997). Therefore, it is possible that GAP-43 IR changes at intervals other than the 2 I sampled after KA-induced SE. Furthermore, mRNA for GAP-43 has been observed to increase after 5 kindling

stimulations and returns to baseline after development of fully generalized seizures (Bendotti et al., 1993). Again, the time course for increased synthesis of protein after increase of mRNA is unknown, as is its convergence with the sampling intervals I employed. Finally, to my knowledge changes in mRNA for synaptophysin and in the protein itself have not been assessed following KA or kindling. Thus, it is unclear whether changes would have been detected at other intervals or during seizures.

In summary, the results of the studies reported in my thesis do not argue conclusively against the hypothesis that extra-hippocampal axonal sprouting is associated with kindling or KA-induced SE, although they certainly are consistent with the conclusion that such sprouting does not occur. I readily acknowledge that my studies were limited to SE and kindling as well as to only 2 synaptic proteins, and hence that there are many additional lines of research that should be performed to further investigate seizure-related sprouting outside the hippocampus. Given the robust nature of the electrographic and behavioral events associated with these seizure conditions, the idea of axonal sprouting is intuitively plausible, although the possibility remains, as argued above, that more subtle mechanisms are involved.

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7. Appendix
7.1 Curriculum Vitae

Amy E. Wallace

A. Personal History

Business Address:
University of Saskatchewan
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Home Address:
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Home Phone: (306) 955-9212
Place of Birth: Fort Collins, Colorado
Birth Date: September 29, 1973
Citizenship: United States of America

B. Educational History

1. University of Saskatchewan
   Saskatoon, SK Canada

Position: Visiting Scholar, 1997-Present
Department of Psychology: Behavioral Neuroscience
Department of Psychiatry: Neuropsychiatry Research Unit
Research:
The Anatomical Changes Related to the Kindling Process
Research Advisor: Michael E. Corcoran, Ph.D.

2. University of Victoria
   Victoria, BC Canada

Degree: Masters of Arts, In Progress
Department: Psychology; Behavioral Neuroscience
GPA: 3.92/4.0

Master Thesis:
Does extra-hippocampal sprouting accompany epileptogenesis?
Thesis Advisor: Michael E. Corcoran, Ph.D.

3. University of Colorado
Boulder, Colorado USA

Degree: Bachelors of Arts, Magma Cum Laude
Major: Psychology, Behavioral Neuroscience
Major GPA: 3.5/4.0

Honors:	Psy Chi National Honors Fraternity	1992-1996
	Golden Key National Honors Society	1994-1996
	Honors Program	1994-1996

Honors Thesis:
The Anticonvulsant Vigabatrin: Effects on Recovery
Thesis Advisor: Theresa D. Hernandez, Ph.D.

C. Professional Positions

1. Undergraduate Research Assistant 1994-1996
   University of Colorado
   Hernandez Laboratory
   Supervisor: Theresa Hernandez, Ph.D., Assistant Professor

2. Child Care Assistant Summer 1994
   High Plains Elementary School
   Englewood, Colorado
   Part Time Summer Position
   Supervisor: Van Schweilton

3. Project management Summer 1993
   John Snow Incorporated
   Denver, Colorado
   Full Time Summer Position
   Supervisor: Christy Crosser

D. Volunteer Positions

1. Epilepsy Saskatoon	1997-2000
2. Saskatoon Schizophrenia Society	1997-2000
3. Girl Guides of Canada	1998-2000
4. Brain Awareness Week	1997-1999
5. Life Sciences Research Day	1997-1999

E. Membership in Professional Associations

1. Society for Neuroscience	Student Member	1997-Present
2. American Epilepsy Society	Student Member	1999-Present
3. Saskatchewan Neuroscience Network	Student Member	1997-Present
4. Psi Chi National Honors Fraternity	Student Member	1993-1997
5. Golden Key National Honors Society	Student Member	1994-1997

F. Grants / Funds Awarded

1. Research Assistanceship
   Awarded by M.E. Corcoran
   $14,400 Annually, 1997-Present

2. Graduate Teaching Fellowship
   University of Victoria
   $2000 Annually, 1997-1999

3. Undergraduate Research Apprenticeship Program
   Howard Hughes Medical Association
   "The Effects of GAMMA-Vinyl-GABA on Recovery from Brain Damage"
   $1000, Spring 1995

4. Summer Undergraduate Research Fellowship (SURF)
   Howard Hughes Medical Association
   "The Effect of the Anti-Convulsant Vigabatrin on Behavioral Recovery form an Anteromedial Cortex Lesion in Rats"
   $1000, Summer 1995

5. Marion Merrell Dow (Funds awarded to T.D. Hernandez)
   Student Research Grant, matched by SURF grant
   "The Effect of the Anti-Convulsant Vigabatrin on Behavioral Recovery form an Anteromedial Cortex Lesion in Rats"
   $1000, Summer 1995

6. Undergraduate Research Opportunities Program (UROP)
   "The Effect of the Aniti-Convulsant Vigabatrin on Exploratory Behavior and Functional Recovery in Rats Following Anteromedial Cortex Lesion"
   $750, Academic year, 1995-1996

7. Hoechst Marion Roussel
   (Formerly Marion Merell Dow, Funds Awarded to TDH)
   Student Research Grant, Matching UROP Grant
   "The Effect of the Anti-Convulsant Vigabatrin on Exploratory Behavior and Functional Recovery in Rats Following Anteromedial Cortex Lesion"
   $1250, Academic year, 1995-1996

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Wallace, A.E., Montanez, S., Lorio, M., Hernandez, T.D. (1996). Vigabatrin, Unlike Some Anti-Convulsants, Dose Not Hinder Recovery of Function.Annual Meeting of the American Epilepsy Society Abstracts, 37, 5 : 27.

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Title of Thesis:

Does extra-hippocampal sprouting accompany epileptogenesis?

Autho

Amy Elizabeth Wallace
April 2, 2002