TIME COURSE OF NEURONAL DEGENERATION IN THE PILOCARPINE MODEL OF EPILEPTOGENESIS

Julie Poirier

Department of Pharmacology & Therapeutics
McGill University, Montreal

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PREFACE

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include: A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners in made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.
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CONTRIBUTION OF AUTHORS

In accordance with McGill University’s “Guidelines For Thesis Preparation” this thesis is written in manuscript format. Therefore, Chapter 2 is composed of text and figures that have been duplicated from a manuscript about to be submitted for publication. The following statement lists the contribution made by each author:


I performed all of the experimental work and wrote the manuscript on which this chapter is based with revisions to the text and figures provided by Drs. De Koninck and Capek.
RÉSUMÉ

Dans le cadre d'un modèle d'épileptogénèse, on a examiné la progression de lésions cellulaires au sein de l'hippocampe en utilisant deux marqueurs de dégénérescence neuronale. On a utilisé la pilocarpine pour induire une période contrôlée (1h) de status epilepticus chez six groupes de rats. Après une période de recouvrement apparent de 1-2 semaines, ces animaux se mettent à avoir des crises d'épilepsie spontanées périodiques. On a utilisé une technique d'imprégnation d'argent servant à la détection de neurones soumis à un stress du cytosquelette, la coloration dite Dark Neuron, pour marquer les neurones de l'hippocampe à chacun de six intervalles de temps post-traumatique sélectionnés (de 3h à 3 semaines). On a ensuite comparé la proportion des cellules marquées avec celle obtenue par marquage au fluorochrome anionique Fluoro-Jade, réputé spécifique pour les neurones en dégénérescence.

Les résultats mettent en lumière un patron de marquage différentiel dans le temps. La coloration Dark Neuron révèle préférentiellement des cellules aux premiers intervalles de temps, alors que les colorations positives au Fluoro-Jade se retrouvent aux intervalles de temps plus tardifs. Il est intéressant de noter que le marquage au Fluoro-Jade atteignit son maximum à 1 semaine post-traumatisme initial, ce qui coïncide avec une perte significative de neurones, de même qu'une gliose prononcée dans la couche des cellules principales du CA3.

Les deux colorations semblent donc détecter des processus distincts de lésion cellulaire au cours de l'épileptogénèse. Ceci met en évidence le fait que des événements cellulaires distincts prennent place à différents stades de l'épileptogénèse. Chacun de ces changements cellulaires peut donc différer considérablement des changements permanents que l'on observe au sein du tissu chroniquement épileptique.

Comprendre la progression de ces lésions cellulaires peut donc être important en vue du développement de thérapies anti-épileptogéniques; thérapies complémentaires potentiellement précieuses face aux traitements conventionnels anti-épileptiques (à l'aide d'anticonvulsants) qui ne demeurent que palliatifs.
**ABSTRACT**

In this thesis, we investigated the progression of cellular injury using two markers of neuronal degeneration, in a model of hippocampal epileptogenesis. Pilocarpine was used to induce a controlled period of status epilepticus (SE) in six groups of rats. Following a 1-2 week period of apparent recovery, the animals developed spontaneous recurrent seizures. A silver impregnation technique for neurons subjected to cytoskeletal stress, the Dark Neuron stain, was used to label hippocampal neurons at each of six selected time points (from 3h-3weeks) following SE. The proportion of labelled cells was then compared to that obtained with the anionic fluorochrome Fluoro-Jade, reported to be specific for degenerating neurons.

Results revealed a differential temporal pattern of labelling. The Dark Neuron stain preferentially revealed cells at earlier time points, while with Fluoro-Jade staining was found at a later time point during epileptogenesis. Interestingly, maximal Fluoro-Jade labelling at 1 week post-pilocarpine injection coincided with significant cell loss and gliosis in the CA3 principal cell layer.

The two stains, therefore, appear to detect separate processes of neuronal damage during epileptogenesis. This finding highlights the fact that distinct cellular events take place at different stages of epileptogenesis, which may differ significantly from the permanent changes observed in chronically epileptic tissue. Understanding the progression of cellular injury may be important for the development of antiepileptogenic therapy, a potentially valuable complementary approach to anticonvulsant therapy for the treatment of chronic epilepsy.
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It is because of them that my life is truly complete.
Chapter 1. Research goals and literature review
INTRODUCTION

Epilepsy, in all its forms has plagued humans as far back as history can recount. The word epilepsy, which literally means “to take” or “to seize” was first used as early as 400 A.D. when Hippocrates’ writing gave insight into the cerebral nature of seizures. Throughout history, one can see the prevailing ignorance in the understanding of epilepsy. Over 150 years ago, it was strongly believed that epilepsy was associated with insanity, and it was a contagious disease contractable through a mother’s breast milk. Others believed that epileptics suffered from seizures because evil spirits overcame them.

John Hughlings Jackson more accurately described epilepsy as “…an occasional, an excessive, and a disorderly discharge of nerve tissue on muscles” (1870). He also introduced a classification scheme that divided seizures into two main categories: 1) generalised seizures in which both hemispheres are affected and 2) partial seizures where the abnormalities are more restricted to one side of the brain. Although the work of Hughlings Jackson represented a significant advance in the understanding of epilepsy, it was not until the 1930’s with the advent of electroencephalography (EEG) that the neurological nature of epileptic seizure disorders became widely accepted.

Over the past few decades, basic research has helped elucidate some of the main mechanisms underlying convulsive disorders. Electrophysiological studies have helped us gain insight into the functional consequences of seizure activity at the synaptic level, while morphological work revealed gross and microscopic pathological changes.

Traditional treatment with conventional antiepileptic drugs is largely symptomatic, aimed at controlling the initiation and spread of seizure activity by acting on one or more of the following targets: 1) voltage-dependent ion channels involved in action potential generation and transmitter release 2) glutamatergic excitatory neurotransmission, and 3) GABAergic (gamma-aminobutyric acid) inhibitory transmission. However, it has long been recognised that prevention of epilepsy would be preferable to chronic therapy once epilepsy has developed. Certainly, this strategy is limited to patients who have been diagnosed as being susceptible to
developing seizures following head trauma, strokes, neoplasms, and those with a history of extended febrile seizures. The rationale behind attempting to prevent epilepsy is to focus on the period during which plastic changes are taking place in the brain, referred to as epileptogenesis. This phase is commonly called the silent period, as there are no behavioural manifestations of epilepsy immediately following injury. Rather, there is a delay of months or years until the appearance of the first spontaneous seizure. Thus, it is during this interval that new therapeutic intervention can modify or halt synaptic reorganisation and prevent epileptogenesis.

The main goal of my thesis has been to follow the temporal evolution of cellular degeneration during epileptogenesis using 1) a reliable animal model of temporal lobe epilepsy 2) histological markers of neuronal stress and injury and 3) quantification of cell loss with respect to the time course of epileptogenesis.

1.1 EPILEPSY

Epilepsy comprises an enormous heterogeneity of disorders affecting approximately 1% of the general population (Hauser et al., 1993). For this reason, The International League Against Epilepsy (ILAE) has introduced a Classification of Epilepsies and Epileptic Syndromes (Anonymous1989). More than 30 types of epilepsy and epileptic syndromes have been identified according to a set of criteria that include: type of seizure, etiology, chronicity, electroencephalographic (EEG) features, and prognosis. The epilepsies are divided into 4 main groups: 1) focal or partial epilepsy, 2) generalised epilepsy, 3) syndromes undetermined whether focal or generalised, and 4) special syndromes. Epidemiological studies have found that the most common forms of epilepsy in adults are the focal or partial epilepsies which account for approximately two thirds of all cases (Bauer, 1994; Gastaut et al., 1975; Hauser et al., 1993). If the seizure is not accompanied by a loss of consciousness, it is classified as a simple partial seizure, while a seizure in which a loss of consciousness is associated is complex partial. Complex partial seizures are the single most common type of seizures in humans. Approximately 30% of patients with complex partial epilepsy continue to have seizures despite
optimal drug therapy (Bazil and Pedley, 1998) and less than 50% with partial and secondarily generalised seizures will remain seizure-free for longer than 12 months (Brodie and Dichter, 1996; Mattson et al., 1985). In general, once a patient fails two drugs in monotherapy, the likelihood that polytherapy will achieve complete control is small (Dasheiff et al., 1986; Schmidt and Richter, 1986).

1.2 TEMPORAL LOBE EPILEPSY

The relationship between temporal lobe epilepsy (TLE) and complex partial seizures is often misunderstood as the two terms are often assumed to be synonymous with one another. Although in most instances, the clinical pattern of TLE is complex partial seizures (Ojemann, 1997), complex partial seizures can arise from other parts of the brain. For the purpose of this thesis, the term temporal lobe epilepsy will be used to specifically describe complex partial seizures originating from the temporal lobe. The symptoms associated with TLE have been most described as psychomotor, including olfactory, visual, or auditory hallucinations. However, the most common features are an initial motionless stare, repetitive lip smacking, fumbling of the hands, and generally disorganised movements (Ojemann, 1997).

In the absence of a specific etiological understanding in any of the epilepsies or epileptic syndromes, approaches to drug therapy must therefore be directed at the control of symptoms, i.e. suppression of seizures (Loscher, 1998). In fact, all currently available drugs are anticonvulsant rather than antiepileptic. The drugs of choice for treating TLE include compounds such as phenytoin, carbamazepine, and valproate that were developed and introduced between 1910 and 1970. During the next 20 years, a family of 2nd generation drugs including gabapentin and lamotrigine have been introduced in treatment of TLE. It is interesting to note that most antiepileptic drugs were found by serendipity rather than design based on knowledge of processes involved in initiation, elaboration, and extension of seizures (Loscher, 1998). Apart from Phenobarbital and bromides which were found to be effective at suppressing seizures in the early part of the 20th century, the anticonvulsant effect of all standard anticonvulsant drugs was
first determined in animal models (Loscher and Schmidt, 1994). Therefore seizure models in laboratory animals are still the most important prerequisite in the preclinical search for new anticonvulsant drugs (Loscher, 1998).

1.3 TEMPORAL LOBE EPILEPSY: ANIMAL MODELS

Experimental research on epilepsy and antiepileptic drugs has been done mostly in mice and rats in which seizures are induced chemically or electrically. In his critical review of the relevance of animal models to the development of new antiepileptic rather than anticonvulsant drugs, W. Loscher (1985) describes the criteria for an ideal model of epilepsy: 1) the development of spontaneously occurring recurrent seizures, 2) a type of seizure similar in its clinical phenomenology to seizures occurring in human epilepsy, 3) an age-dependent onset of epilepsy similar to that observed in generalised epileptic syndromes in man, 4) the clinical seizures should be associated with epileptic-like activity in the EEG, 5) pharmacokinetics of antiepileptic drugs similar to those in humans thus allowing the maintenance of effective drug levels during chronic treatment, and 6) effective plasma concentrations of antiepileptic drugs similar to those required for control of the respective seizure type in humans.

There is no model that exists today which satisfies all of these requirements. The best option is to carefully select an animal model that satisfies as many of the criteria as possible, while being efficient and reproducible. Below, a select number of models relevant to the subject matter of this thesis are described, in particular, chemically-induced seizures.

1.3.1 Chemically-Induced Seizures

There are a number of chemicals that are capable of inducing seizures at toxic doses; however, only ones that have proven to be valuable tools for epilepsy research will be discussed. Chemoconvulsants induce seizures after both systemic and focal application. A commonly used convulsant to induce generalised seizures is sub-cutaneous administration of pentylenetetrazol (PTZ) (Olsen, 1981), a compound which acts as a gamma-aminobutyric acid (GABA)
antagonist. Other examples of drugs which act by decreasing inhibitory function include bicuculline, picrotoxin, and benzylpenicillin. In addition to compounds that induce seizures via an effect on GABAergic neurotransmission, there are other convulsants that exert selective effects on specific neurotransmitter systems, such as glycine antagonists (strychnine), excitatory amino acid receptor agonists (NMDA, Kainate), and cholinomimetics (pilocarpine) (Loscher and Schmidt, 1988).

In addition to chemically-induced seizures, focal seizures can also be induced by topical application of certain metals such as alumina cream (aluminum hydroxide), cobalt, and tungstic acid. It is also possible to produce epileptic cortical foci by briefly freezing a small area of cerebral cortex (Hanna and Stalmaster, 1973; Loiseau et al., 1987).

New animal models of epilepsy should be explored because each new method yields information on the nature of the convulsant process and the mechanisms of anticonvulsant drug action (Loscher and Schmidt, 1988).

1.3.2 Cholinomimetics

The relationship of cholinergic mechanisms to epilepsy was suspected by neurologists by the turn of the 19th century (Langley and Kato, 1915). Decades later, it was suggested that acetylcholine (ACh) may be primarily involved in human convulsive disorders (Brenner and Merrit, 1942). In 1945, it was demonstrated experimentally in cats that intracisternal injection of acetylcholine resulted in motor seizures (Forster, 1945). An epileptogenic potential of ACh was discovered by Echlin when it was found that chronically isolated monkey cortex demonstrated an increased sensitivity to locally administered Ach (1959). In later years, evidence from electrophysiological experiments reinforced the idea that ACh may be involved in the cellular mechanisms of epilepsy (Dichter and Ayala, 1987). In terms of specific mechanisms, it has been shown experimentally that muscarinic cholinergic excitation in the brain occurs as a result of a reduced voltage-dependent and Ca\(^{2+}\)-dependent K\(^+\) conductance and is mediated by voltage-dependent Ca\(^{2+}\) and Na\(^+\) conductance (Benardo and Prince, 1982). Acetylcholine functions by
promoting the inward flow of $\text{Ca}^{2+}$ and $\text{Na}^+$ into neurons, which may be responsible for the membrane depolarisation that leads to epileptic events (Pumain et al., 1983). It should be noted that cholinergic mechanisms in epileptogenesis have not been given as much attention as it may seem, a primary reason being that anticholinergic drugs are not effective anticonvulsants, thereby unwarranting a further investigation into the role of ACh. However, a renewed interest in the relation of cholinergic mechanisms to epilepsy took place in the early 1980's when it was found that systemic injection of cholinergic agonists produced sustained seizure activity followed by widespread brain damage in rodents (Olney et al., 1986; Olney et al., 1983; Turski et al., 1983; Turski et al., 1983). In particular, injection of the muscarinic cholinergic agonist pilocarpine in rats and mice proved to be an interesting experimental model for studying TLE. This model will be discussed in a subsequent section. It should be mentioned that although administration of cholinergic agonists provokes intense seizure activity, the cholinergic system is not believed to have excitotoxic potential (Clifford et al., 1987), but rather plays a role in triggering and maintaining seizures, driving the excitatory mechanisms that are responsible for neuronal damage (ie. glutamate and aspartate-mediated) (Olney et al., 1986).

1.3.3 Pilocarpine

Pilocarpine is a potent cholinergic agonist originally isolated from the leaflets of South American shrubs, and until recently was commonly used in the treatment of acute glaucoma in man (Hardman et al., 1996). In 1983, Turski et al. introduced the use of a single systemic high-dose (350-400mg/kg) pilocarpine injection as a novel animal model of TLE (1983). They reported that systemic administration of this muscarinic cholinergic agonist produced electroencephalographic and behavioural seizures, accompanied by widespread brain damage similar to that observed in autopsied brains of human epileptics. Their electroencephalographic findings indicate that one of the most sensitive structures to the convulsant effect of pilocarpine is the hippocampus, while other structures remain unaffected or only slightly affected at early time points following injection. Although it is generally accepted that the hippocampus is indeed
one of the earliest structures affected following pilocarpine treatment, another prominent study by Clifford et al. (1987) reported the ventral forebrain as being the earliest region to be affected, in particular, the nucleus accumbens. The explanation provided is that this region is the most logical to be affected first since it has been shown to have the highest density of muscarinic receptors in the rat brain (Kobayashi et al., 1978; Nonaka and Moroji, 1984). In a later study, Turski et al. (1989) comment on this discrepancy between the two studies and reconfirm with their results that the hippocampus is the earliest structure to be activated according to their electroencephalographic recordings (Turski et al., 1989; Turski et al., 1983).

One of the main features of the pilocarpine model that makes it very relevant for comparison to the human epileptic condition is the reproducible occurrence of spontaneous recurrent seizures (SRS) in rats injected with pilocarpine following a delay or silent period of about 2 weeks (Cavalheiro et al., 1991; Mello et al., 1993; Turski et al., 1989; Turski et al., 1983). Spontaneity is one of the prominent signs of human epilepsy, therefore strengthening the clinical importance of this model (Loscher and Schmidt, 1988; Turski et al., 1983). Pilocarpine seizures also provide an opportunity to study the involvement of the cholinergic system in the onset, propagation and pathological consequences of limbic seizures (Clifford et al., 1987).

Behaviourally, pilocarpine seizures resemble other models of limbic seizures beginning with facial automatisms, head nodding, and progressing to forelimb clonus with rearing and falling (Clifford et al., 1987).

In terms of neuropathology, the cell damage that results from seizures is identical whether they are initiated with a high-dose pilocarpine injection, or a lower dose of pilocarpine administered with lithium (Clifford et al., 1987). It should be mentioned at this point that lithium-pilocarpine is an analogous model to pilocarpine injection alone, except that lithium in combination with pilocarpine has been reported to produce a 20-fold shift in the pilocarpine dose-response curve for producing seizures (Clifford et al., 1987), thereby permitting the use of a much lower dose of pilocarpine. It has been reported that lithium decreases noradrenaline and dopamine release at stimulated nerve terminals (Hardman et al., 1996). The decreased
noradrenaline release might predispose an animal to increased seizure susceptibility since others have found that blockade of noradrenergic function increases kindled seizure activity (Arnold et al., 1973). Lithium also directly influences cholinergic function by increasing the release of ACh (Haas and Ryall, 1977; Jope, 1979). In terms of cell damage reported at the light microscope level, pilocarpine-induced seizures consistently produce damage in the olfactory nucleus, pyriform cortex, entorhinal cortex, thalamus, amygdala, hippocampus, lateral septum, bed nucleus of stria terminalis, claustrum, substantia nigra, and neocortex (Clifford et al., 1987; Turski et al., 1989; Turski et al., 1983). In the hippocampus, the CA3 and CA1 regions are involved and damage has been noted to be greater in ventral as opposed to dorsal hippocampal regions. Interestingly, the highest cholinergic receptor densities are in CA1 and the dentate gyrus, while the region most consistently and severely damaged is CA3 (Clifford et al., 1987). This clearly indicates that the spread of seizure activity beyond the initial focus must entail activation of non-cholinergic pathways. At the level of the electron microscope, cellular changes include swelling of dendrites, swelling or vacuolar condensation of neuronal cell bodies, marked dilatation of astroglial elements with relative sparing of axonal components (Clifford et al., 1987). The neuropathology reported with the pilocarpine model is consistent with prolonged seizures produced by other means (Ben-Ari, 1985; Hajnal et al., 1997; Kapur et al., 1989; Khurgel et al., 1992; Sloviter, 1987).

Seizures produced by pilocarpine in rats are prevented by systemically given diazepam, clonazepam, phenobarbital, valproate, and trimethadione while diphenylhydantoin and carbamazepine are ineffective (Turski et al., 1989). Surprisingly, these latter two drugs are the drugs of choice for the treatment of temporal lobe epilepsy in humans. Therefore, a protection against seizures elicited by pilocarpine in rodents is not an invariant prediction of anticonvulsant activity in human forms of epilepsy (Turski et al., 1987).

The pilocarpine model has gained popularity in recent years since the studies by Turski et al in 1983. It is now one of the leading models of TLE along with kindling and kainic acid-induced lesions (Lemos and Cavalheiro, 1995; Liu et al., 1994; Longo and Mello, 1997;
Marcinkiewicz et al., 1997; Mello et al., 1996; Mello and Covolan, 1996; Morrisett et al., 1987; Priel et al., 1996; Sankar et al., 1998; Scharfman, 1993; Simmons et al., 1997; Walton and Treiman, 1988).

1.4 EPILEPTOGENESIS: THE DEVELOPMENT OF EPILEPSY

Despite many years of intensive study, the mechanisms underlying epileptogenesis are still unclear for most forms of epilepsy (Bausch and McNamara, 1999). The idea that cellular changes in the hippocampus may underlie epileptogenesis came about in part by EEG studies showing that the hippocampus is one of the earliest structures to be activated during seizures. In addition, the cure of epilepsy by surgical resection of the hippocampus in properly selected individuals led to the idea that hyperexcitability intrinsic to the hippocampus may contribute to the development of epilepsy (Bausch and McNamara, 1999). Thus, it is not surprising that from the perspective of mechanisms, the best studied form of seizure is the seizure activity in the hippocampus. The goal of this section is to provide an overview of morphological and functional changes that have been documented in the chronically epileptic brain and relate these documented permanent changes to epileptogenesis. However, despite these findings, it still remains unclear as to whether the pathological changes described are the cause of chronic seizures or rather the result of them. After more than 30 years of debate, this question cannot be answered: to do so requires first that we understand epileptogenesis.

1.4.1 Cell Loss

The most frequent lesion in patients with TLE is mesial temporal sclerosis or hippocampal sclerosis, consisting of gliosis and neuronal loss in the CA1, CA3, and the hilus of the dentate gyrus (Houser, 1990). This typical pattern of neuronal loss characteristic of hippocampal sclerosis (Kapur, 1999; Lewis, 1999) can be produced experimentally by repeated or prolonged seizures and results presumably from excitotoxic damage subsequent to excessive activation of glutamate receptors (Olney et al., 1986; Sloviter, 1994; Sloviter, 1994). There are
striking similarities between the pathology produced in experimental animals by prolonged
seizures (Sloviter, 1991) or head trauma (Coulter et al., 1996; Lowenstein et al., 1992) and the
pathological changes seen in the hippocampi of many patients with TLE (Meldrum and Bruton,
1992). As mentioned above, there is an extensive loss of dentate hilar neurons (Bausch and
Chavkin, 1997) and hippocampal pyramidal cells (DeGiorgio et al., 1992; Fujikawa et al., 1999;
Kapur, 1999). Recent data have also demonstrated cases where some granule cells of
experimental animals are also highly vulnerable (Sloviter et al., 1996). Seizure-induced
astrocytic damage has also been documented (Schmidt-Kastner and Ingvar, 1996). Interestingly,
in contrast to the many studies showing cell loss, a recent study described an increased
generation of hippocampal granule cells as a consequence of seizures (Parent et al., 1997). It
was shown that induction of limbic epilepsy resulted in an increased proliferation of granule cells
using bromodeoxyuridine labelling. Therefore, although death of certain cell populations may be
a main event during or as a result of epileptogenesis, there is also evidence of neurogenesis.

Mechanistically, neuronal loss can occur with either active or passive participation of
cellular constituents. This has been referred to as apoptosis or necrosis (Kerr et al., 1972).
Apoptosis is a form of gene-mediated death characterised by specific morphological features:
early nuclear chromatin condensation, cytoplasmic compaction with cell shrinkage,
endonuclease-mediated DNA fragmentation into oligonucleosomes, apoptotic body formation
and well-preserved organelles. In contrast, necrosis resulting from sudden injury with the cell
unable to maintain homeostasis is characterised by early cytoplasmic vacuolization before any
nuclear changes occur and is associated with an inflammatory response (Tomei and Cope, 1991).

It appears that epileptic and ischemic neuronal death is primarily but not exclusively
apoptotic (Charriaut-Marlangue and Ben-Ari, 1995; Charriaut-Marlangue et al., 1996; Pollard et
al., 1994; Pollard et al., 1994). Long-term repetitive stimulation of the perforant path induced
apoptosis in the granule cells but necrosis in the hilar and pyramidal cells (Sloviter et al., 1996).
The surviving granule cells showed dendritic deformations and shrinkage (Isokawa, 1996a;
1.4.2 Axon Sprouting

In addition to the neuronal loss, the second morphological change induced in the hippocampus by seizures is sprouting of dentate granule cell axons, which are commonly referred to as mossy fibres. This occurs in both animal models of epilepsy (Bausch and Chavkin, 1997; Cronin et al., 1992; Tauck and Nadler, 1985) as well as in human epilepsy (Babb et al., 1991; de Lanerolle et al., 1989; Sutula et al., 1994). Denervation of the inner molecular layer secondary to hilar cell loss is believed to constitute the initial stimulus for sprouting (Tauck and Nadler, 1985). The sprouted mossy fibre axons appear to make synaptic contacts with granule cells and GABAergic basket cells. However, the functional effects of mossy fibre sprouting and synaptic reorganisation are debated: does it serve to enhance excitability or diminish it by enhancing inhibition (Kapur, 1999)? In addition, the importance that has been attributed to axon sprouting has also been questioned. Studies which prevented mossy fibre sprouting by knocking out c-fos (Watanabe et al., 1996) or intraventricular administration of antibodies to nerve growth factor (NGF) retards epileptogenesis but does not prevent it (Rashid et al., 1995; Van der Zee et al., 1995). Another group reported that blockade or pilocarpine- or kainate-induced mossy fibre sprouting by cycloheximide did not prevent epileptogenesis (Longo and Mello, 1997). Therefore, the significance of mossy fibre sprouting is unclear as well as the cause of it, whether it be cell loss, growth factors, or generation of new cells. It has been proposed that seizure-induced expression of neurotropic genes may underlie the sprouting of axons of the granule cell layer (Sutula et al., 1996). It has been shown that NGF protein levels in dentate granule cells are increased by seizure activity (Gall and Isackson, 1989). Whether axon sprouting is a necessary event in epileptogenesis is a question that remains to be answered.

1.4.3 Gliosis.

Reactive gliosis occurs in response to injury, including pilocarpine-induced seizures, in the mature central nervous system (CNS). A salient manifestation of reactive gliosis is an increase in glial fibrillary acidic protein (GFAP), a protein subunit of glial intermediate filaments.
found exclusively in astrocytes in the CNS (Amaducci et al., 1981; Eng et al., 1971). Glial proliferation characteristically accompanies neuronal loss seen in Ammon's horn sclerosis and after various insults, including status epilepticus, and contributes to epileptogenesis.

1.4.4 Dendritic Changes

Dendritic degeneration is another common pathological finding in TLE and its animal models (Isokawa, 1998). When neurons from the hippocampus and neocortex are examined from patients with chronic focal epilepsy, they show dramatic dendritic abnormalities. Dendritic spine loss has been repeatedly reported and has been suggested to be more severe with an increased duration of a seizure disorder (Multani et al., 1994). Dendrites of pyramidal cells have also been reported to have varicose swellings at irregular intervals along their length (Muller et al., 1993). Isokawa (1998) claims that the reported dendritic degeneration that is identified later in the chronic phase of epilepsy is not the direct outcome of initial acute seizures. Instead, following initial acute seizures, surviving neurons undergo substantial changes in the morphology and density of dendrites and spines in the chronic phase, during which the gradual development of spontaneous seizures is established (Isokawa, 1998). In the pilocarpine animal model of epilepsy, the membrane time constant of neurons, which can assess a cell’s total surface area and geographic extent of dendritic branches, was reported to be significantly reduced in rats that experienced many spontaneous seizures in the chronic phase (Isokawa, 1996b). This suggests that the higher the frequency of spontaneous seizures, the more severe the local dendritic shrinkage.

1.4.5 Impaired Inhibition.

Repeated intense seizures caused an attenuation of gamma-aminobutyric acid (GABA)-mediated inhibition of the granule cells and in the pyramidal cells of the hippocampus (Coulter et al., 1996; Sloviter, 1994; Tauck and Nadler, 1985). This change cannot be explained by a selective loss of GABAergic inhibitory interneurons, since the GABA immunoreactive neurons
were shown to be more resistant to seizure-induced injury than other hippocampal neurons (Sloviter, 1987). As well, relative preservation of GABAergic cells in surgical specimens from patients with epilepsy was confirmed (Babb et al., 1989). The neurons among the most sensitive to the seizure-induced neuronal death are the mossy cells in the dentate hilus (Lowenstein et al., 1992; Sloviter, 1989). These cells receive synaptic input from granule cells via collaterals of mossy fibres, and from the entorhinal cortex via the perforant path. Their axons project to the molecular layer of the dentate gyrus. Functionally, mossy cells are excitatory.

To account for the paradoxical loss of GABA-mediated inhibition with preservation of GABAergic neurons, the dormant basket cell hypothesis (Sloviter, 1987) suggests that the seizure-induced loss of hilar excitatory neurons removes tonic excitatory projection to GABAergic basket cells, the inhibitory interneurons in the dentate hilus. Being deafferented, these cells then lie dormant with the end result being disinhibition (Sloviter, 1987). Loss of mossy cells which govern lateral inhibition in the dentate area may cause functional delamination of the granule cell layer and result in synchronous multilamellar discharges in response to excitatory input (Sloviter, 1994). Therefore, there are 3 premises to this theory: 1) the general preservation of the inhibitory network, 2) the loss of excitatory afferents onto GABAergic interneurons, 3) decreased inhibition on principal cells (disinhibition) (Bernard et al., 1998).

Jeffereys and Traub (1998) also present evidence for the existence of functionally dormant interneurons in the tetanus toxin model of chronic epilepsy. From their results, they suggest that the term "dormant" is a useful operational description of a situation in which inhibitory interneurons are present and excitable by direct, strong stimulation, but fail to be recruited by normal synaptic input onto them. However, a recent critical review of the dormant basket cell hypothesis calls into question some of the concepts and warns against a blind acceptance of the theory (Bernard et al., 1998). First, there is strong morphological evidence showing that the inhibitory network is more fragile than as stated in the hypothesis; some GABAergic interneurons do degenerate in both human TLE (de Lanerolle et al., 1989; Marco et
The second part of the hypothesis regarding the apparent loss of major excitatory inputs onto interneurons in TLE is also debated. The mossy cells that are vulnerable to seizure-induced damage only account for a small percentage of excitatory afferents onto basket cells. Most of the glutamatergic inputs to basket cells originate from the entorhinal cortex, from granule cells and from CA3 pyramidal cells (Kneisler and Dingledine, 1995; Ribak and Seress, 1983; Ribak and Peterson, 1991; Seress and Ribak, 1990a; Seress and Ribak, 1990b; Zipp et al., 1989). Therefore, there is clearly far from unanimous support for the dormant basket cell hypothesis. In fact, as time progresses, the model seems to provide an inadequate picture of what may be occurring during epileptogenesis. As discussed by Bernard et al. (1998), their findings indicate that the concept of dormant interneurons as an explanation for epileptiform activity is certainly not universally acceptable.

1.5 Anatomy of Temporal Lobe: The Hippocampal Formation as a Focus of Epileptogenesis

In the human, the temporal lobe forms a major part of the basal surface of the cerebral hemisphere. The Sylvian fissure demarcates it from the rest of the cerebral hemisphere but posteriorly, no obvious boundary separates it from the occipital lobe. Morphologically, the temporal lobe is the most heterogeneous of all cerebral lobes, with the limbic and non-limbic components intimately welded together (Gloor, 1997). A significant amount of the temporal lobe consists of the archicortex, characterised by 3 cell layers as compared to the neocortex which is 6-layered. The archicortex is confined to the mesial and basal layers of the hemispheres, where it forms prepiriform, periamygdaloid, and hippocampal cortices. While the neocortex typically shows an alternation between layers dominated by pyramidal cells and layers dominated by non-pyramidal cells, the archicortex consists primarily of large pyramidal neurons. The hippocampus is considered to be a central component of the limbic system which
is connected in both afferent and efferent directions with other parts of the brain (Gloor, 1997). The hippocampus has several principal subcortical connections including the amygdala, thalamus, hypothalamus, brainstem, striatum, claustrum, and precommissural septum.

1.5.1 Hippocampal Formation.

The hippocampal formation can be divided into 4 cortical regions: the hippocampus proper (CA1, CA2, CA3/CA4), the dentate gyrus, and subiculum. The fields of the hippocampus are linked by unique and largely unidirectional connections. Of the 3 layers which comprise the hippocampal archicortex, the principal cell layer is Layer II. Layer I is a molecular layer composed largely of apical dendrites of principal cells and Layer III is referred to as the Stratum Oriens, a polymorphic layer of cells. It is difficult to make a distinction in Nissl-stained sections between the end of CA3/CA4 and the polymorphic layer of the dentate gyrus. Lorente de No's (de No, 1934) system of subdividing the hippocampus into sectors is almost universally accepted by experimental workers in neuroanatomy and electrophysiology.

1.5.2 Principal Neurons

As is the case for the neocortex, the hippocampus contains two main populations of neurons: the excitatory principal (output) neurons, and the local circuit inhibitory interneurons. The main output neurons of the hippocampus are the pyramidal cells of CA1 and CA3, the granule cells of the dentate gyrus, and the mossy cells of the dentate hilus. These cells receive excitatory input mostly from the entorhinal cortex as well as other sectors of the hippocampal formation. Principal neurons emit axons that project to adjacent hippocampal sectors and to subcortical targets, or through commissural connections to the contralateral hippocampal formation. The pyramidal cells differ slightly in morphology and connectivity depending on the sector of interest, however, the description given below provides a general overview of the morphological characteristics of these neurons. Pyramidal cells are characterised by a triangular/pear-shaped cell body with a single apical dendrite ramifying in the molecular layer.
The peripheral branches of the apical dendrite receive synaptic contact from the perforant path and from hippocampal commissural and association fibres. The basal dendrites penetrate the stratum oriens, as well as the axons of pyramidal cells which also exit into the stratum oriens or polymorph layer and head towards the alveus or fimbria. Mossy cells are another population of principal cells that occupy the polymorphic layer/hilus of the dentate gyrus. They have a bipolar or multipolar morphology, the proximal dendrites covered in thorny excrences. The thorns, like those found on CA3 pyramidal cells, are part of the synaptic structures they form with mossy fibre axons that pass through the hilus on their way from the granule cells to the CA3 region.

1.5.3 Local Circuit Interneurons

Cells of this type are found in all layers of the hippocampus proper and dentate gyrus (Gloor, 1997). These cells are much less densely distributed than pyramidal cells and granule cells, which accounts for the transparent appearance of layers such as the stratum oriens, which is sparsely populated by interneurons. In terms of basic morphology, interneurons are generally smaller than pyramidal cells or granule cells, are round/ovoid in shape, and are multipolar. Most local circuit interneurons are GABAergic as their cell bodies have been shown to be immunoreactive to GABA or the GABA-synthesising enzyme glutamic acid dehydrogenase (GAD) (Braak et al., 1986; Kosaka et al., 1985; Ribak et al., 1978). Peptides are located in the cell bodies and processes of interneurons, and are often co-localised in the same cell with GABA/GAD immunoreactivity (Kosaka et al., 1985; Sloviter and Nilaver, 1987). However, some peptide-containing neurons are not necessarily GABAergic, and more than one peptide may be found in the same neuron. Below, is a brief summary of some peptides which have been identified in hippocampal neurons.

Cholecystokinin (CCK) is widely distributed among local circuit interneurons in all parts of the hippocampus, but has been associated in particular with a large number of basket cells in the dentate gyrus. CCK has been reported to be co-localised with GABA/GAD in some basket
cells (Kosaka et al., 1985). It is also reported to be contained in other local circuit interneurons in the stratum oriens and stratum lacunosum-moleculare. It is rare in the dentate hilus however (Sloviter and Nilaver, 1987), where most cells are GABAergic.

Somatostatin (SS) is another common peptide found to be localised in interneurons. Like CCK, some, but not all SS-containing neurons are GABAergic. In addition, SS tends to be colocalised with Neuropeptide Y in many cells (Chan-Palay, 1987; Kohler et al., 1987). In the hippocampus, SS has a striking presence in many polymorphic cells and axons of the hilus of the dentate gyrus (Amaral et al., 1988; Johansson et al., 1984; Sloviter and Nilaver, 1987).

Neuropeptide Y (NPY) is contained in relatively large neurons that show a distribution throughout the hippocampus paralleling that of SS-containing neurons. The two peptides are often colocalised in the same cell, but SS-immunoreactive neurons are much more abundant than those containing NPY (Gloor, 1997).

Vasoactive intestinal peptide (VIP) is contained in some basket cells located in the molecular layer of the dentate gyrus, stratum radiatum, and stratum lacunosum-moleculare of the hippocampus (Kohler, 1983; Roberts et al., 1984; Sloviter and Nilaver, 1987).

Immunoreactivity for substance P (SP) has been reported in the stratum oriens of CA3 in cells that are morphologically similar to SS-and NPY-immunoreactive cells. The terminals of the axons originating from these cells are scattered through all the hippocampus (Davies and Kohler, 1985; Del Fiacco et al., 1987). However, it should be noted that such immunoreactivity may correspond to neurokinin B rather than substance P immunoreactivity.

Enkephalin (ENK) and dynorphin (DYN) are examples of opioid peptides that are contained in some interneurons of the stratum radiatum and lacunosum-moleculare of the hippocampus. ENK has also been found in the short circuit interneurons in the molecular layer of the dentate gyrus (Gall et al., 1981; McGinty et al., 1983). It should be mentioned that opioid peptides are not confined to interneurons; they are also found to be in principal neurons such as granule cells (Finley et al., 1981).
Some hippocampal nonpyramidal neurons contain one of three types of calcium-binding proteins; parvalbumin (PV), calbindin-D_{128K}, and calretinin (CR) (Celio, 1986; Jacobowitz and Winsky, 1991; Sloviter, 1989). Of the PV-immunoreactive neurons in the hippocampus, 97.1% are also GAD-immunoreactive (Norman et al., 1982). Many hippocampal interneurons contain a type of calcium-binding protein most likely because these proteins provide a protective mechanism that buffers excess damaging calcium that may enter the cells when they are firing at a high rate without accommodation. The majority of local circuit interneurons function as inhibitory interneurons, as revealed by their GABA immunoreactivity.

1.5.4 Hippocampal Sectors

**Sector CA1** This is the largest hippocampal sector in the human brain and contains the largest number of neurons among all hippocampal sectors. There is species variation i.e. In the rat, CA1 is narrow with the cells densely packed while in primates it is wider with more loosely packed cells. N-methyl-D-aspartate (NMDA) receptors are the most abundant glutamate receptor in the CA1 stratum radiatum and stratum oriens; in fact they are more concentrated in this region that anywhere else in the brain (Gloor, 1997). Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors are also in abundance but more in the stratum pyramidale. Kainate (K) receptors are not abundant in the CA1 region. The presence of calcium-binding proteins in this sector is very low, with some interspecies differences: there are reports of low concentrations of calbindin D in rat pyramidal CA1 neurons (Baimbridge et al., 1982; Baimbridge and Miller, 1982; Sloviter, 1989) while there are virtually none in humans (Sloviter et al., 1991). This combination of high NMDA receptor levels and low Ca^{2+}-binding protein concentrations may explain the high degree of vulnerability to excitotoxic insults.

**Sector CA2** This represents the narrowest sector of the hippocampus. The pyramidal cells are more densely packed than in CA1, but are not easily delineated from those of CA1. The main difference between CA2 and CA3 is that sector CA2 lacks mossy fibre terminals and therefore
has no stratum lucidum. These two sections are often lumped together as CA3 because it is so
difficult to discern it from either CA1 or CA3.

**Sectors CA3/CA4**  This sector contains large pyramidal neurons which are slightly less
packed than CA2 neurons. The CA3 region has a stratum lucidum, while not visible in Nissl-
stained sections, appears as a clear band in AChE-stained sections. The stratum pyramidale of
CA3 stains very darkly with the Timm stain (Timm, 1958) for heavy metals because of the high
zinc content of mossy fibres in the stratum (Haug, 1976; Haug et al., 1971; Swanson et al.,
1978). As CA3 penetrates the hilus of the dentate gyrus, the pyramidal neurons become less
packed and assume a multipolar morphology. Some of the large neurons seen here may
represent the terminal blade of CA3, but others, the mossy cells, are regarded as a constituent of
the hilus (Amaral, 1978). Investigators agree that it is difficult to make a clear distinction
between dentate gyrus and the hippocampus proper, with special reference to the transitional
zone between CA3/CA4 and the hilus (Amaral et al., 1990; Green and Mesulam, 1988; Seress.
1988). It is generally accepted that the term CA4 be avoided (Amaral, 1978; Amaral et al.,
1990). There are relatively low levels of NMDA and AMPA receptors in CA3 as compared to
CA1, while the concentrations of kainate (K) receptors reach the highest levels anywhere in the
brain in the stratum lucidum (Gloor, 1997). Principal neurons of CA3 do not stain for calbindin
D (Baimbridge et al., 1982; Baimbridge and Miller, 1982; Seress et al., 1991; Sloviter, 1989;
Sloviter et al., 1991) or chromogranin A. Like the CA1 region, the lack of Ca\(^{2+}\)-binding proteins
may explain its increased vulnerability to a variety of insults such as epileptic seizures and
ischemia.

**Dentate Gyrus**  Like the hippocampus proper, the dentate gyrus consists of a trilaminated
archicortex. The granule cell layer is analogous to the pyramidal cell layer of the hippocampus
proper in that it contains the principal output cells of the region, namely the granule cells. These
neurons possess a tuft of profusely branching apical dendrites, and in contrast to the pyramidal
cells, the granule cells have no basal dendrites. In coronal sections of non-primates, the dentate
 gyrus assumes a characteristic horseshoe shape, while in primates, it becomes more undulated.
Above the granule cell layer is the molecular layer which represents layer I of the dentate archicortex, which contains the apical dendrites of the granule cells and some small neurons. The deepest layer, layer III, is analogous to the statum oriens of the hippocampus proper. While the hippocampal counterpart of this layer is largely acellular, the polymorphic layer, as it is called in the dentate gyrus, contains multiple cell types, with both excitatory and inhibitory functions (Amaral, 1978). There have been reports of high levels of alpha-amino-3-hydroxy-5-methyl-isoxazole-4-propionate receptors (AMPA receptors) (Hampson et al., 1992), in the dentate gyrus, while kainate receptors are also in abundance in the polymorphic layer, or the hilus (Gloor, 1997).

1.6. NEURONAL DEGENERATION DURING EPILEPTOGENESIS: HISTOLOGICAL MARKERS

The main objective of this thesis is to document the progression of cellular stress in the hippocampus during epileptogenesis. We decided to use a classical silver degeneration stain developed by Gallyas, the Dark Neuron Stain (Gallyas et al., 1992; Gallyas et al., 1990; van den Pol and Gallyas, 1990) as well as a modern complement, Fluoro-Jade, reported by Schmued et al in 1997 (Bowyer et al., 1998; Eisch et al., 1998; Eisch and Marshall, 1998; Schmued et al., 1997; Schmued and Bowyer, 1997) to document the temporal evolution of stressed neurons at different time points following pilocarpine-induced SE. An advantage to using histological markers of cellular stress as opposed to following the levels of peptide expression with immunocytochemistry is that the degeneration stains do not rely on peptide expression. With immunocytochemical techniques, the question remains as to whether a cell population has actually disappeared following injury, or rather has downregulated expression of a given peptide. As we will discuss in the following section, the Dark Neuron Stain and Fluoro-Jade are reported to be selective for specific cellular components that are altered following injury to the brain.
1.6.1 Histological Stains: Principles

The purpose of this section is to provide a brief overview of the concepts behind histological stains, with a focus on silver impregnation. Subsequent sections will elaborate on a particular silver stain, the Dark Neuron stain, as well as the anionic dye Fluoro-Jade.

In histological stains, dyes are used to give colour to the tissue component of interest. In some cases, the dye is coloured, and directly used as a reagent, while others are colourless until they react with a tissue component to form a coloured product (Kieman, 1981).

A dye molecule is composed of two main parts: a chromophore, which is an arrangement of atoms which absorbs light in the visible part of the spectrum, and one or more auxochromes, which is the part that attaches to the substrate. A fluorochrome (i.e. Fluoro-Jade, which will be discussed later) is a dye which absorbs UV, blue, or green light and emits light of a longer wavelength.

The discovery of silver stains has been attributed to Camillo Golgi (1843-1926) who discovered his famous Golgi method more or less by accident in 1873. He observed the darkening of some whole neurons in dichromate-fixed blocks of tissue which had been subsequently immersed in a solution of silver nitrate. Silver nitrate is an essential reagent in most of the methods discussed in this section. The Golgi technique has since been modified into 3 techniques: the traditional Golgi stain, the rapid Golgi stain, and the Golgi-Cox technique (Kemali, 1976; Kieman and Berry, 1975; Ramon-Moliner, 1970). Although the mechanism behind the spontaneous labelling of some neurons is not understood for any of the Golgi methods, they remain useful for illustrating neuronal morphology. The Golgi stain colours neurons completely in a dark brown or black colour.

It is clear that since the time of Golgi, silver stains have formed an essential foundation for neuroanatomy. Throughout this century, different silver staining techniques have been developed to label whole neurons, axons, terminals, or glial cells. A few select stains will be reviewed in subsequent paragraphs.
Impregnation is a condition where an unreduced metal (i.e. silver) is taken up from a solution of salt or other compound and deposited in a colloidal state on a tissue element (Baker, 1958). After this, the tissue is removed to a reducing solution and the metal is reduced to the elementary state, usually in the form of a black deposit. Silver impregnation can become quite complicated because of the many factors involved to make it specific for various tissue elements. For example, temperature is one of the most important factors to consider because a higher temperature increases the kinetic energy of the particles and permits a greater number of collisions of particles against the tissue surface (Baker, 1958). An example of a silver stain is Holmes's silver method for axons (Holmes, 1943), in which axons are selectively stained black while cell bodies and nuclei are unstained. The Nauta & Gygax method (Nauta and Gygax, 1951) has been a popular method for silver impregnation of degenerating axon terminals in the central nervous system. Lafarga et al. (Lafarga et al., 1986) described a procedure for the demonstration of "neuronal nuclear bodies", which is a distinct organelle of the cell nucleus.

The scientist who, in the latter part of this century, has contributed the most to the development of modern silver impregnation techniques is F. Gallyas. In 1970, he introduced a simple and reproducible technique for demonstrating fibrous neuroglia (1970a). In the same year he also described another method for the specific labelling of micro- and oligodendroglia (1970b). A decade later, Gallyas published methods to localize terminal degeneration in the central nervous system (Gallyas et al., 1980a), as well as axonal degeneration following axotomy (Gallyas et al., 1980b). For our study, we used the Dark Neuron stain (Gallyas et al., 1990; van den Pol and Gallyas, 1990), which Gallyas describes as a "trauma-induced Golgi-like staining of neurons" (van den Pol and Gallyas, 1990). Gallyas has detailed the rationale behind his techniques in several publications (1982; 1979; 1992; 1992; 1992). The Dark Neuron stain is discussed in detail in section 1.6.2.

In conclusion, a short review of the principles of nuclear staining is also relevant for Chapter 2, where we use Propidium Iodide, a commonly used fluorescent nuclear stain. The nucleus of a eukaryotic cell contains nucleic acids, namely DNA and RNA. These are associated
with nucleoproteins that are rich in the basic amino acids arginine and lysine. The dyes used as nuclear stains impart colour to the chromatin by binding either to the nucleic acids or to the nucleoprotein (Kiernan, 1981). Fluorochromes that intercalate within the nucleic acid double helix are widely used in fluorescence microscopy (Biggiogera and Biggiogera, 1989). Propidium Iodide is an example of a nuclear stain which we used for our study. RNAse treatment is recommended for specific quantification of nuclei, as it will abolish staining of the ribosomes.

Another nuclear stain which does not intercalate into DNA but rather is an example of a cationic dye is the Nissl stain. Nuclei of cells of the nervous system, along with the cytoplasmic Nissl bodies (RNA) of neurons are easily stained with basic dyes (Kiernan, 1981). Basic dyes are coloured cations which attach by electrostatic forces to anionic groups in the substrate. Examples of cationic dyes include toluidine blue, cresyl violet acetate, neutral red, and thionine.

1.6.2 Dark Neuron Stain : Marker of Reversible Cellular Damage

The phenomenon of "Dark Neurons" in experimental neuropathology has been reported in many studies. "Dark Neurons" exhibit shrunken outlines, corkscrew-like dendrites, pyenotic nuclei and show an unusually high affinity for various histological stains (Gallyas et al., 1990). In addition to their artifactual production by post-mortem trauma to incompletely fixed nervous tissue (Cammermeyer, 1978; Cammermeyer, 1961). Dark Neurons can also be formed in vivo as a consequence of pathological conditions including cerebral ischemia, insulin hypoglycemia, brain injury, status epilepticus, excessive stimulation, deafferentation, and poisoning with various chemicals; i.e. 6-hydroxydopamine (6-OHDA), kainic acid, and colchicines. The large number of pathological conditions that result in the formation of Dark Neurons illustrates the general applicability of this method. In fact, the Dark Neuron stain described by Gallyas et al. (1992; 1990; van den Pol and Gallyas, 1990; 1992) has been used to assess neuropathological damage in recent studies (Du et al., 1998; Pollard et al., 1994; Sloviter et al., 1996). The selective affinity for silver is increased rapidly in affected neurons, allowing detection of traumatized cells fixed minutes after injury (van den Pol and Gallyas, 1990). Axons, although
they are labelled less frequently than dendrites, do stain with this silver technique. The obvious advantage in using the Dark Neuron stain is that not only is one able to quickly identify affected brain regions resulting from a specific injury, but it also permits the identification of cell types based on morphology.

Silver stains have been used for decades to detect dying and dead axons and boutons several days after injury (Fink and Heimer, 1967; Nauta and Gygax, 1951). They were not used to study dendritic trees however, and a long interval of several days between the time of trauma and the time of fixation was required to maximize the labelling. The Dark Neuron stain is capable of detecting traumatized neurons within intervals of a few minutes between the time of insult and fixation (van den Pol and Gallyas, 1990). It must be noted that only damaged neurons are impregnated with silver; normal neurons do not stain.

The silver-staining of neurons using the technique of Gallyas (1992; 1990; van den Pol and Gallyas, 1990; 1992) consists of 3 main steps: (1) dehydration, (2) esterification, and (3) development. Gallyas discusses the kinetics of metallic silver formation in the developer (1990), which is characterised by an induction period, during which crystallisation nuclei are produced, and an acceleration period, during which these nuclei are enlarged to microscopic visibility. Sections in the developer turn pale yellow at the end of the induction period, then keep darkening until they become brown and finally black.

Esterification with anhydrous 1-propanol serves to increase the affinity for silver (argyrophilia) of both mitochondria and dark neurons (ie. the induction period is shortened). Because mitochondrial labelling is not desired, a small amount of water is added to the esterification solution (4%) which serves to decrease the extent of argyrophilia in mitochondria. Another strategy used to decrease mitochondrial staining is during treatment with acetic acid prior to developing. It also helps to increase the induction period of mitochondria, as compared to dark neurons, in order to preferentially stain the latter.

Gallyas et al. (1992) discuss their hypothesis for a common mechanism of formation of dark neurons as a result of neuropathological insults. The argyrophilic labelling appears uniformly
throughout the neuron after injury, affecting not only the soma, but a large proportion of the dendrites as well. They postulate that some intraneuronal focal disturbance caused spreads through the soma and dendritic tree, resulting in argyrophilia. In addition, this spread of argyrophilia appears to occur independent of metabolic processes, as successful silver staining was achieved by concussive head injury inflicted 30min after the onset of perfusion-fixation (Gallyas et al., 1992; Gallyas et al., 1992b). This raises an interesting question as to how the signal that a cell is damaged travels through the affected neuron under such conditions. Fast intraneuronal transport and the bioelectrical activity of the plasma membrane stop functioning in mammals at about 10°C and under anoxid conditions. Intracellular diffusion of some signaling substance by the traumatized elements would also be inconceivable because of the low rate of diffusion and the progressive dilution of the substance as it moved.

A reasonable hypothesis which explains the rapid spread of intraneuronal damage lies in the concept of neurofilaments, elements of a continuous cytoskeletal network, which are capable of storing mechanical energy. Neurofilaments are intermediate filaments which occur in neurons throughout the nervous system and are generally more abundant in axons than dendrites. The mechanical energy is stored in the form of a metastable inner structure of individual neurofilaments and the discharge of stored mechanical energy can spread throughout the neurofilamentous network, after focal initiation, like a row of dominoes (Gallyas et al., 1992). This theory also accounts for the shrinkage of cells which is often a hallmark of damaged neurons. The stored mechanical energy released in the whole network of neurofilaments may also damage cellular components attached to them, as well as the nuclear membrane and cytoplasm, resulting in release of ions and proteins, leading to shrinkage. In addition, the disruption of cytoskeletal elements could lead to a re-arrangement of negative and positive side groups of amino acids, resulting in the increased affinity for basic dyes and silver.

Although the term Dark Neuron is widely accepted to describe an injured neuron, Gallyas (1992) cautions the accuracy of this word. He suggests the term “collapsed” neuron which more accurately describes the morphopathological phenomena, but also accounts for the possibility
that the neuron may recover. This idea is supported by others who also suggest that the Dark Neuron state is compatible with cell survival due to the apparent preservation of organelles and membranes (Chang and Baram, 1994). This study, along with previous work, provide evidence that Dark Neurons represent acute, reversible neuronal injury in aldehyde-fixed tissue (Auer et al., 1985a; Evans et al., 1984). However, it has been speculated that Dark Neurons persisting after 2h of recovery following seizures may be lethally injured (Soderfeldt et al., 1983).

1.6.3 Anionic Dyes: Principles

The Dark Neuron stain has been a commonly used marker of cellular stress in the brain for many years (Gallyas et al., 1992; Gallyas et al., 1992; Gallyas et al., 1990; Gallyas et al., 1992a; Pollard et al., 1994; Toth et al., 1997; van den Pol and Gallyas, 1990). Obvious advantages to using silver-staining techniques are that they have been tested under a variety of pathological conditions, and they provide the observer with excellent morphological resolution of affected cell types. However, there are technical limitations to the use of the Dark Neuron stain, which prompted us to explore complementary, yet more flexible markers of cellular degeneration. From a purely technical standpoint, silver staining techniques are notorious for their capricious nature; the traditional Golgi stain being the most famous example. In addition, because of the specific tissue fixation requirements such as long post-fixation delays, and subsequent harsh tissue processing, the technique does not lend itself well to multiple labelling protocols with immunocytochemical markers. It is for these reasons that we also selected Fluoro-Jade, a member of the anionic dye family. Furthermore, using Fluoro-Jade allowed us to complete our time-dependent profile of cell degeneration during epileptogenesis as it revealed a different population of affected cells as did the Dark Neuron stain. Below, a discussion of the nature of anionic dyes will be presented, with specific reference to Fluoro-Jade.

The cell body of a healthy neuron is recognizable under the light microscope (LM) by its vesicular nucleus and large nucleolus, and the presence in the cytoplasm of granular Nissl substance which contains ribosomal RNA. This classical appearance is visible in sections
stained with basic dyes, whose coloured cations are attracted to the negatively charged phosphate groups of DNA and RNA (Kiernan et al., 1998). In addition, it has long been known that in sections stained with hematoxylin and eosin (H&E), dead and dying neurons are revealed as they display strong nuclear and cytoplasmic eosinophilia (Sankar et al., 1998). Eosin is not the only anionic dye with this staining property. Dying neurons can also be detected with Luxol fast blue (Brown, 1977) or acid fuschin (Auer et al., 1985a; Chang and Baram, 1994; Ingvar et al., 1988). Using acid fuschin, Kiernan et al. (1998) demonstrated the presence, in the kainic acid model of epilepsy, of abnormal hippocampal CA1, CA3, and hilar acidophilic neurons which stood out as bright red objects against a pink background. This result is in contrast to what is seen in normal neurons where the only stained components were the blue nucleoli. Therefore, it is clear that acidophilia, like the argyrophilia discussed in the previous section, is a useful indicator of the extent of cellular damage.

Recently, the principals behind the affinity of anionic dyes for injured neurons has been reviewed (Kiernan et al., 1998). Anionic dyes are attracted to macromolecular cations, which exist principally in proteins. The intensity of staining is proportional to the local concentration of side-chain lysine and arginine groups. Arginine-rich basic proteins are most abundant in the histones associated with DNA and RNA. Therefore, two hypotheses are put forth to explain the increased staining typically seen in a dying neuron. A separation of nucleic acids from their histones would unmask the arginine side chains of the histones, resulting in a higher intensity of staining. The second hypothesis is that a dying cell may synthesize large amounts of a lysine or arginine-rich basic protein before it dies. The results of this study (Kiernan et al., 1998) indicate that the material in the nuclei of neurons damaged by kainic acid administration owed most of their acidophilia to high concentrations of arginine, while the cytoplasmic acidophilia is likely due to high concentrations of lysine-containing proteins. Therefore, their work is in support of the second hypothesis; cytoplasmic acidophilia in dying neurons is due to an increased production of basic proteins at some stage prior to cell death. This hypothesis is shared by Schmued et al. (1997).
There is no clear consensus as to whether acidophilic neurons are irreversibly damaged, although a greater number of studies suggest irreversible damage (Auer et al., 1985a; Auer et al., 1985b; Ingvar et al., 1988; Kieman et al., 1998) rather than reversible (Chang and Baram, 1994). Auer et al. (1985a; 1988) provides substantial evidence in support of acidophilic neurons undergoing degeneration using a model of hypoglycemic brain damage and of fluorothyl-induced status epilepticus. They found that the acidophilic cells detected at the LM level corresponded in location, number, and time point of appearance to the morphological changes reported at the EM level which are hallmarks of irreversible cellular injury: coarse clumping of hyperchromatic nuclear chromatin, amorphous cytoplasm lacking endoplasmic reticulum and Golgi apparatus, gross discontinuities of the plasma membrane and nuclear membrane, and flocculent densities in mitochondria. These cells were considered to be dead due to their lack of architecture and integrity of the cell membrane.

1.6.4 Fluoro-Jade: Marker of Irreversible Cellular Damage

In comparison with other anionic dyes, the recently developed anionic fluorochrome called Fluoro-Jade was the most interesting for our purposes. As a complement to the Dark Neuron stain, Fluoro-Jade was best-suited because it is very simple and efficient to use and is a fluorescent marker, thereby enabling us to perform a multilabelling protocol with immunofluorescence and examine the sections under a confocal microscope. Like acid fuschin, Fluoro-Jade has been reported to selectively label degenerating neuronal cell bodies and a large extent of their processes.

As was done in previous work using the Dark Neuron Stain, the specificity of Fluoro-Jade for dying neurons was tested using a variety of insults to the brain including kainic acid, the seafood contaminant domoic acid, MK-801 which is an NMDA receptor antagonist, and stereotaxic injection of multivalent metals such as iron and manganese salts (Schmued et al., 1997). This diverse selection of neurotoxic insults was shown to validate Fluoro-Jade’s ability to detect neuronal degeneration. Therefore, it was proposed that regardless of the mechanism by
which a neuron dies, it can be labeled with this anionic dye. In contrast to traditional silver staining techniques such as the *Dark Neuron Stain*, *Fluoro-Jade* is fast and simple to use. The stain can be performed in a couple of hours, and a large number of mounted sections can be processed simultaneously. Because *Fluoro-Jade* is a fluorescent marker of cell degeneration, it lends itself well to multiple labelling studies; the extensive histochemical processing required for silver staining tends to mask other brightfield labels (Schmued et al., 1997).
Preface to Chapter 2

Despite many years of intensive study, the mechanisms underlying epileptogenesis are still unclear for most forms of epilepsy (Bausch and McNamara, 1999). Although the hippocampus is known to be a central structure in the development of TLE, there is no clear consensus as to what cellular changes constitute epileptogenesis, and what are defined as the permanent changes resulting from it.

One way we begin to define a clearer picture of epileptogenesis is to establish a temporal profile of injured cell populations in the hippocampus from immediately following status epilepticus (SE), to the occurrence of the first spontaneous recurrent seizure (SRS). Knowledge of cell types affected at early time points after SE may indicate targets for antiepileptogenic therapy, an approach potentially preferable to current anticonvulsant treatment.

We also report in Chapter 2, for the first time, a relationship between two leading markers of neuronal degeneration, the argyrophilic Dark Neuron stain, and the anionic fluorochrome, Fluoro-Jade.
Chapter 2. Differential progression of Dark Neuron and Fluoro-Jade labelling in the rat hippocampus following pilocarpine-induced status epilepticus
2.1 ABSTRACT

We have followed the time-dependent progression of neuronal degeneration in the rat hippocampus throughout a defined period of epileptogenesis. Pilocarpine was administered systemically (380mg/kg i.p.) to induce status epilepticus. Status epilepticus (SE) continued for 1h before it was stopped with diazepam (4 mg/kg i.p.). Two histochemical methods reported to specifically label injured neurons following insult, the Dark Neuron stain and Fluoro-Jade, were used to follow the progression of cellular damage at 6 specific time points following the initial pilocarpine-induced insult: 3h, 6h, 12h, 24h, 1 week, and 3 weeks. The neuronal nuclear protein, NeuN, was used as a specific marker of neurons, allowing us to assess in parallel, neuronal loss in specific hippocampal regions throughout epileptogenesis.

Results revealed a different time-dependent progression of Dark Neuron and Fluoro-Jade labelling: a significantly greater proportion of silver impregnated cells labelled by the Dark Neuron stain was quantified in the stratum radiatum and stratum pyramidale of CA1 at the early time points of 3h and 24h in the stratum radiatum of CA1 and CA3 as compared with the proportion of Fluoro-Jade labelling in adjacent sections. In contrast, the maximal staining with Fluoro-Jade appeared at a later stage during epileptogenesis (between 24h and 1 week), with a significantly greater proportion of neurons labelled than with the Dark Neuron stain in the stratum radiatum of CA1, stratum pyramidale of CA1, and the polymorphic layer of the dentate gyrus. Neurons from control animals were not significantly labelled by either of the two staining methods. Specific quantification of neuron numbers using immunodetection of NeuN revealed a significant decrease in cell number (approximately half) in the stratum pyramidale of CA3 by 1 week post-pilocarpine-induced SE.

Our results suggest that the Dark Neuron and Fluoro-Jade stains are labelling neurons at different stages of post-traumatic epileptogenesis induced by pilocarpine. The Dark Neuron stain reveals early cellular injury (ie. within hours of trauma), while Fluoro-Jade marks neurons...
at later time points (i.e. days). Thus, these two stains reveal distinct injury-related cellular events during epileptogenesis.
2.2 INTRODUCTION

Several silver impregnation techniques (Gallyas et al., 1980a; Gallyas et al., 1980b; Gallyas et al., 1993; Gallyas et al., 1990; Nauta and Gygax, 1951; Vaidya et al., 1999; van den Pol and Gallyas, 1990) and anionic dyes (Auer et al., 1985a; Chang and Baram, 1994; Ingvar et al., 1988; Kieman et al., 1998; Sankar et al., 1998; Schmued et al., 1997) have been used to label the cell body and dendritic processes of degenerating neurons. It has long been recognised that many dying and dead neurons, seen in a variety of diseases, exhibit an increased affinity for various silver stains (argyrophilia) and/or strong nuclear and cytoplasmic eosinophilia, which can be detected with anionic stains. In fact, a recent study revealed a previously unknown, strong anion-dependence of the Golgi stain (Stefanovic et al., 1998).

In the adult rat, pilocarpine-induced status epilepticus (SE) causes neuronal death in the CA1, CA3, and hilar regions (Olney et al., 1983; Turski et al., 1989; Turski et al., 1983; Turski et al., 1983). SE is followed by a 2-3 week silent period before the appearance of spontaneous recurrent seizures (SRS) (Olney et al., 1983). The behaviour, and electroencephalographic findings using this model show some resemblance to the human condition (Turski et al., 1983). At the electron microscope level, it has been found that pilocarpine-induced seizures result in swelling of dendrites and cell bodies, and dilatation of astroglial elements (Olney et al., 1986). In addition, dendritic spine loss has recently been reported and suggested to be more severe with increasing duration of seizures (Jiang et al., 1998; Muller et al., 1993; Multani et al., 1994).

We have used the pilocarpine model to study the cascade of cellular injury throughout epileptogenesis. An advantage to studying epileptogenesis as opposed to the chronic condition that characterises epilepsy is the potential for therapeutic intervention before permanent brain damage. However, data are lacking on the early cascade of cellular events during epileptogenesis. One approach to begin answering this question is to determine the temporal pattern of neuronal injury following SE in the hippocampus. Therefore, we investigated the time-dependent relationship between two leading markers of neuronal degeneration: the Dark
Neuron stain (Gallyas et al., 1992; Gallyas et al., 1993; Gallyas et al., 1990; van den Pol and Gallyas, 1990) and Fluoro-Jade (Bowyer et al., 1998; Eisch et al., 1998; Eisch and Marshall, 1998; Schmued et al., 1997; Schmued and Bowyer, 1997).

2.3 EXPERIMENTAL PROCEDURES

A total of 53 adult male Sprague Dawley rats (100-150g) were used. They were divided into six groups which were sacrificed at 3h, 6h, 12h, 24h, 1 week and 3 weeks following pilocarpine injections. All rats were housed in individual cages on a standard 12/12-h light/dark cycle with free access to food and water. All animal experiments were approved and conducted in accordance with University animal care committee guidelines.

2.3.1 Pilocarpine induced status epilepticus

Pilocarpine hydrochloride (Sigma, St. Louis, MO, USA) was freshly dissolved in 0.9% saline and administered intraperitoneally in the dose of 380-mg/kg to animals in each experimental group, excluding control animals. Scopolamine methylbromide (Sigma, St. Louis, MO, USA), an analogue that does not cross the blood brain barrier, was injected subcutaneously in the dose of 1mg/kg 30min prior to all dosages of pilocarpine to suppress peripheral cholinergic effects. Control animals received an equivalent dose of scopolamine, followed by 0.9% saline. Diazepam (4mg/kg i.p.) was administered 1h after the onset of SE in pilocarpine-treated rats to stop SE and thus standardise the duration of seizure activity in all pilocarpine-treated animals. Control animals also received diazepam injections. Additional injections of diazepam were given as needed to prevent further seizures.

2.3.2 Behavioural Monitoring

At the dose of 380mg/kg, pilocarpine produced a sequence of behavioural alterations including staring spells, and motor limbic seizures that built up progressively into limbic SE. The limbic seizures were rated on a five-point scale according to strength (1) mouth and facial movements (2) head nodding (3) forelimb clonus (4) rearing (5) rearing and falling. A full motor
seizure, with loss of postural control, was classified as a Stage 5 motor seizure (Racine, 1972). Rats were monitored continuously for the first 8 hours following pilocarpine injections.

2.3.3 Fixation and Sectioning

For the histological and immunocytochemical experiments, the animals were perfused transcardially with a fixative containing 4% paraformaldehyde. In order to avoid artifactual production of Dark Neurons due to mechanical disruption, brains were removed from the skull after a delay of at least 24 hours following perfusion-fixation (Gallyas et al., 1990) (van den Pol and Gallyas, 1990). Upon removal from the skull, brains were placed in a solution of 30% sucrose in 0.1M phosphate buffer for at least 16h prior to sectioning. Serial 50μm-thick sections were obtained and processed for the Dark Neuron stain, Fluoro-Jade, and immunocytochemistry. The sections were taken between bregma –2.30mm and –4.90mm. These co-ordinates were chosen as they included all hippocampal regions of interest. A total of 36 sections were cut for each rat and arranged to preserve their serial order.

2.3.4 Dark Neuron Stain

For the Dark Neuron stain, the procedure was similar to that described earlier (Gallyas et al., 1992) (Gallyas et al., 1990) (van den Pol and Gallyas, 1990). The sections were placed in 50%, 75%, and 100% 1-propanol for 5 min each and then in 1-propanol containing 1% sulphuric acid and 1% distilled water for 16 h at 56°C. The sections were then rehydrated in 50% and 25% 1-propanol, washed in distilled water, and treated with 8% acetic acid for 10 min. Subsequently, the sections were placed in a silicotungstate physical developer which contained silver nitrate for ~20 min. dehydrated, and mounted.

2.3.5 Fluoro-Jade

Fluoro-Jade (Histochem Inc, Jefferson AR, USA) is an anionic fluorochrome reported to selectively stain degenerating neurons in slices (Schmued et al., 1997) (Bowyer et al., 1998) (Eisch and Marshall, 1998) (Eisch et al., 1998) (Schmued and Bowyer, 1997). The sections were
immersed in 100% ethanol for 5 min, followed by 2 min in 70% ethanol and two 1-minute-long rinses in distilled water. Slides were then transferred to a solution of potassium permanganate for 17 min, gently shaken. After two more rinses, sections were placed in Fluoro-Jade staining solution for 30 min at room temperature. Following staining, the sections were rinsed three times with distilled water. The slides were dried, dehydrated in xylene and mounted. Sections were examined under a fluorescence microscope using FITC filter sets.

2.3.6 Neuron and Glial specific labelling

The neuron specific nuclear marker NeuN was used to quantify cell loss in the hippocampus, while immunostaining for Glial Fibrillary Acidic Protein (GFAP) was used to assess the extent of gliosis in the same region. Sections were incubated in 10% normal horse serum for 1hr. and then for 48-72 hours in a mixture of primary antibodies consisting of a rabbit polyclonal GFAP antibody (Vector: 1:200) and a mouse monoclonal NeuN antibody (Chemicon: 1:1000). After several rinses in PBS+T, the sections were incubated in biotinylated horse anti-mouse IgG (Vector: 1:200) for two hours at room temperature. Following further rinses, the sections were incubated in a mixture of streptavidin conjugated to Texas Red (Vector: 1:50), and sheep anti-rabbit IgG conjugated to FITC (ICN: 1:20) for two hours. All antibodies were diluted in PBS+T containing 5% normal horse serum.

2.3.7 Quantification

To count neurons labelled with the Dark Neuron stain, a BH-2 Olympus microscope equipped with a 40X objective and a 10X projection lens was used. The microscope was equipped with a CDD video camera, connected to a MCID-M4 image analysis system (Imaging Research Inc., St. Catharines, Ontario, Canada). The labelled neuron profiles were detected by the image analysis system using software designed for silver grain counting. Measurements were made on a single focal plane. Shading error correction was performed before measurements to correct for irregularities in illumination in the microscopic field. Segmentation
values were selected, using a trial and error method, on the basis of those which would provide the most accurate measurements when compared to the direct visual counting of profiles on the computer screen. Once the ideal thresholds for detection were found, their values were saved and kept at the same levels for all samples. All counting was done in the right hippocampus. Profiles in 3 randomly selected fields from each of CA1, CA3, and dentate gyrus were counted in a known area, for a total of 27 fields per hippocampus. From these values, the total profile area stained per total area of the field was calculated and reported as the percentage of neuron profiles. All cell bodies, blood vessels and cortical tissue that were not in focus were excluded. This areal density measurement has proven an efficient method of quantifying the proportion of labelled cells (Gundersen et al., 1985). A similar protocol was followed for the analysis of Fluoro-Jade stained sections except for the capturing of images, which was done using an Axioplan 2 Zeiss fluorescent microscope. Digital images were saved and subsequently retrieved using the image analysis program.

2.3.8 Dissector Method

The sections used for quantification of neuronal loss were immunoreacted with antibodies against NeuN. (Biggiogera and Biggiogera, 1989) (Suzuki et al., 1997). As indicated previously (Todd et al., 1998), although NeuN-immunoreactivity was sometimes restricted to the nucleus, it frequently extended into the cytoplasm, and in these cells NeuN-staining alone could not be used to clearly identify nuclear boundaries. Therefore, Propidium Iodide was also used to clearly delineate the nuclei. Sections were incubated in a mouse monoclonal antibody against NeuN (Chemicon: 1:1000) for 48-72 hours at 4°C, and following several PBS+T washes, the sections were incubated in a goat anti-mouse IgG conjugated to Alexa 488 (Molecular Probes: 1:50) for 2 hours at room temperature, and subsequently stained with propidium iodide (PI) (Sigma; 0.1% in PBS) in the presence of RNAse (Boehringer-Mannheim; 10 mg/ml) to reveal cell nuclei. Sections stained with fluorescent protocols were examined under a Zeiss LSM 410 confocal scanning laser microscope.
For quantitative analysis of neuron loss in the hippocampus as revealed by NeuN immunofluorescence, the optical dissector method was carried out with confocal microscopy (Todd et al., 1998) (Gundersen et al., 1988) (West, 1993). The sections were scanned sequentially with each of the two lines of the laser through a 63X oil-immersion lens, and z-series consisting of 2 optical sections separated by 7μm were obtained. Each series was started at the most superficial plane for which the tissue was present throughout the field of view. For each series of optical sections, the 1st was designated as the reference section and the 2nd as the lookup section. The separation of 7μm was chosen as it was unlikely that neuronal nuclei could fall entirely between the reference and lookup sections. Neuronal nuclei which were present within the lookup section but were not apparent in the reference section (tops) were counted, while nuclei which appeared in both the reference and lookup sections were eliminated from the cell counts.

2.3.9 Statistical tests

To test for significant differences among the proportions of neuron profiles that were labelled with the Dark Neuron or Fluoro-Jade stains, a 1-way analysis of variance (ANOVA) was used followed by multiple student t-tests (with a Bonferonni correction) to determine whether there was a significant difference between the two distributions at any time point selected. The data was transformed using a square root transformation \( x' = \sqrt{x + 3/8} \) to normalize the underlying distribution (Zar 1984). The critical value for significance was set at \( p < 0.05 \). Data are expressed as mean ± SEM.
2.4 RESULTS

2.4.1 Behavioural

At the dose of 380 mg/kg, pilocarpine produced a sequence of behavioural alterations including staring spells, and motor limbic seizures that built up progressively into limbic SE. Four rats that received pilocarpine injections did not develop visibly detectable seizures, while two rats out of 37 died as a result of the pilocarpine-induced SE. Periodic examination of the animals revealed that they were epileptic by 3 weeks. The present data are therefore based on the analysis of 35 animals that received pilocarpine injections and 18 control animals that received saline injections.

2.4.2 Pattern of Dark Neuron labelling

Quantification of the density of labelled neuronal profiles was performed at all time intervals. The results are expressed as the percentage of labelled neuronal profiles in a given region. As early as 3h post-pilocarpine-induced SE, a significant number of positively-stained profiles using the Dark Neuron stain were detected in the stratum pyramidale of CA1 (0.83%±0.21), of CA3 (1.47%±0.86), as well as the polymorphic region of the dentate gyrus (1.94%±0.50) (Fig. 1). Although quantification was performed unilaterally, both hippocampi were inspected in each section to ensure that the pattern of staining was uniform bilaterally.

The types of neurons labelled with the Dark Neuron stain included hilar interneurons, and pyramidal neurons of the CA1, and CA3 regions. These results are consistent with other reports of degenerating cell populations following SE (Freund et al., 1992) (Turski et al., 1984) (Turski et al., 1983) (Olney et al., 1986) (Sloviter, 1991). The Dark Neuron stain impregnated the soma as well as a large proportion of the dendritic tree, facilitating the identification of labelled cell types (Fig. 1). Sections from control animals did not reveal significant staining when compared to pilocarpine-treated animal at all time points (Fig. 1). At 3h following pilocarpine injection, an average of 0.07%±0.01 neuronal profiles appeared labelled in the polymorphic region of control
animals. In contrast, in treated animals, an average of 1.94%±0.50 neuronal profiles was reported using the Dark Neuron stain. In the polymorphic layer in sections from treated animals sacrificed at 6h, an average of 2.10%±0.74 neuronal profiles was quantified, while only 0.02%±0.02 was measured in control sections. Figure 2 illustrates representative cell types revealed using the Dark Neuron stain which included pyramidal cells from CA1 and CA3, and neurons of the dentate gyrus including mossy cells, oviform cells, stellate cells, dentate pyramidal basket cells, and some granule cells.

The Dark Neuron and Fluoro-Jade stains labelled neurons from the same hippocampal regions, with significant staining detectable using Fluoro-Jade by 24h in the stratum radiatum of CA1 (0.65%±0.16), and CA3 (0.63%±0.14), and the stratum pyramidale of CA1 (0.55%±0.08) as compared to sections stained with Fluoro-Jade from control animals, where no detectable staining was seen at any time point selected (Fig. 3). Figure 3 illustrates a representative staining in a control and a pilocarpine-treated animal at 24h following vehicle or pilocarpine injection respectively. With both the Dark Neuron and Fluoro-Jade stains, labelling of cell bodies and dendrites was sufficient to allow identification of stained cell types (Fig. 4).

Due to the extensive gliosis that occurs at later stages in epileptogenesis, and the concomitant increase in Fluoro-Jade labelling of cells at this time, we wanted to test whether Fluoro-Jade labelled neurons or glial cells. We performed double-labelling studies using either Fluoro-Jade and an anti-NeuN antibody, or Fluoro-Jade and an anti-GFAP antibody on adjacent serial sections from the same rat. Figure 5 is a representative illustration of the relationship between Fluoro-Jade and GFAP immunoreactivity. The two stains did not overlap as shown in this example, and this result was consistent throughout the hippocampus. In contrast, Fluoro-Jade coincided with cells labelled with NeuN immunoreactivity (not shown).
2.4.3 Time Course of Dark Neuron and Fluoro-Jade labelling

The time course of labelling was clearly different between the Dark Neuron and Fluoro-Jade stains. A significantly larger percentage of Dark Neuron-marked profiles was found at 3h in the stratum radiatum of CA1 (0.52%±0.10, p<0.01), and in the stratum pyramidale of CA1 (0.83%±0.20, p<0.05) than with Fluoro-Jade labelling at the same time point (stratum radiatum: 0.01%±0.007, stratum pyramidale: 0.04%±0.03) (Fig. 6). In contrast, at 24h following pilocarpine injection in the stratum radiatum of the CA1, the percentage of neuronal profiles labelled with Fluoro-Jade was significantly greater (0.65%±0.12, p<0.05) than with the Dark Neuron stain (0.02%±0.01). Similarly, we observed this result in the stratum radiatum of CA3 (Fluoro-Jade 0.63%±0.15 vs. Dark Neuron 0.03%±0.02, p<0.01). This trend continued to be apparent by 1 week, particularly in the stratum radiatum of CA1 (Fluoro-Jade 0.03%±0.17 vs. Dark Neuron 0.002%±0.002, p<0.05), the stratum pyramidale of CA1 (Fluoro-Jade 2.51%±1.3 vs. Dark Neuron 0.06%±0.03, p<0.05) and the polymorphic layer of dentate gyrus (Fluoro-Jade 2.38%±0.31 vs. Dark Neuron 0.01%±0.01, p<0.01).

2.4.4 Progression of Cell Loss and Gliosis

To contrast with results obtained with Dark Neuron and Fluoro-Jade, the progression of cell loss that occurred throughout the period of epileptogenesis was also quantified. Figure 7 illustrates NeuN immunolabelling to specifically detect neurons (left panel), while GFAP immunoreactivity consistently labelled glial cells (right panel). This labelling was used to determine where neuronal death and gliosis occurred at various time intervals following the initial pilocarpine insult. Significant neuronal death and gliosis was observed at 1 and 3 weeks post-pilocarpine injection. Figure 8 illustrates the cell loss that occurred by 1 week following the onset of SE, specifically, at the tip of the dorsal blade of the granule cell layer. Increased proliferation of astrocytes, as shown by GFAP immunolabelling was present in the same region where cell loss had occurred. Cell death and gliosis was also detected in the stratum pyramidale of CA3. This cell loss and increased glial proliferation was reproduced in 5 out of 9 pilocarpine-
treated rats sacrificed 1 week after pilocarpine-induced SE, and 4 out of 7 of rats sacrificed 3 weeks following injection.

Using combined NeuN immunoreactivity for labelling neurons and PI for detection of nuclei (Figure 9), estimates of neuronal loss were obtained. Detailed quantification was performed in CA3. A significant decrease in cell density (expressed as number of cells/mm²) \((p<0.01)\) was observed in the stratum pyramidale of CA3 in pilocarpine-treated rats \((n=2)\) versus control rats \((n=2)\), sacrificed 1 week following treatment. Figure 10 illustrates a comparison between Dark Neuron and Fluoro-Jade labelling versus neuronal counts at an early time point \((3h)\) versus that at 1 week. A significant decrease in the proportion of cells labelled by the Dark Neuron stain was observed at 1 week \((n=9\) rats) compared to 3h \((n=4)(p<0.05)\). In contrast, there was a significant increase in the proportion of cells revealed with Fluoro-Jade by 1 week \((n=4)\) as compared to the same region at 3h post-pilocarpine \((n=5; p<0.05)\).

2.5 DISCUSSION

Our results suggest that the Dark Neuron and Fluoro-Jade stains are labelling neurons at different stages of post-traumatic epileptogenesis induced by pilocarpine. Therefore, the two markers reveal different steps in the degeneration process of neurons, and each of these two steps has a very distinct time course. Pilocarpine-treated rats had a significantly increased percentage of Dark neuron profiles at earlier time points \((<24h)\) compared with Fluoro-Jade positive cells stained at the same time in adjacent brain sections. In contrast, the incidence of Fluoro-Jade labelled neuron profiles dramatically increased in CA1, CA3, and polymorphic layer at the later time points \((1\) week).

Van den Pol et al. (1990) showed that increased argyrophilia can be induced in neurons through a varied array of insults. Because Dark Neurons consistently appeared in treated animals and not in controls, the authors postulated that all neurons proceed through a common mechanism of cellular response to a stressful insult, leading the soma and dendrites to become argyrophilic at early time points following injury. They conclude that each causative mechanism
converts the metastable inner structure of neurofilaments in the cytoskeleton into a low-energy structure at a single focus within each affected soma-dendrite complex (Gallyas et al., 1992). From this primary focus the structural change propagates throughout the rest of the cell and dendrites. This phenomenon could explain the very uniform argyrophilia that is seen in affected neurons, a property of the stain that has made it very appealing to use in several studies (Mello and Covolan, 1996; Pollard et al., 1994; Toth et al., 1997).

Schmued et al. (1997) discuss their inferences regarding the specificity of Fluoro-Jade for degenerating neurons. They conclude that a degenerating neuron expresses a strongly basic molecule as Fluoro-Jade is very acidic. In fact, it is well-documented that other anionic dyes, such as acid fuschin, strongly stain the nuclei and cytoplasm of neurons severely damaged by injury or disease (Auer et al., 1985a; Auer et al., 1985b; Chang and Baram, 1994; Fujikawa, 1996; Ingvar et al., 1988; Kieman et al., 1998). Recently, it was reported by Kieman et al. (1998) that kainate-induced cell death is preceded by increased production of basic proteins, which become concentrated in the nucleus and perikaryon, and that the cytoplasmic acidophilia of degenerating hippocampal neurons is due to a protein rich in lysine. Our results suggest that Fluoro-Jade is marking neurons that are in an injured state presumably as a result of limbic seizures (no staining was detected in control rats at any time point), however, the cascade of cellular changes leading to increased affinity for Fluoro-Jade must be the result of a different causative mechanism than what takes place with the Dark Neuron stain, as it occurred at a later stage during epileptogenesis. Maximal Fluoro-Jade staining of hippocampal cells occurred at delays longer than 24 hours after pilocarpine injection.

Schmued et al. (1997) found that in animals treated with kainic acid, cellular degeneration as revealed with Fluoro-Jade, was at its peak 1-4 days following SE, while terminal degeneration was optimally seen after 4-7 days. Our study reports peak Fluoro-Jade labelling in the CA1, CA3, and hilus was approximately 7 days following the onset of SE. In addition to this, however, we have shown that although the appearance of Dark Neurons and
Fluoro-Jade positive neurons may coincide at some time points, they are clearly not revealing the same steps in the degeneration process.

It has been shown by Liu et al. (1994) that pilocarpine-treated rats receiving a dose of 380 mg/kg pilocarpine showed a significant decrease in neuronal density and total neuron number in CA1 and CA3 at 3 weeks following pilocarpine injection. Additional SRS did not contribute to any further significant cell loss 6 to 12 weeks later. They concluded that the bulk of the cell loss was attributed to the original insult resulting from SE, and not from the SRS. In fact, results from other studies support the notion that there is a strong positive correlation between the duration of the initial period of SE and the extent of hippocampal damage (Lemos and Cavalheiro, 1995) (Olney et al., 1986). Sustained limbic seizure activity consistently results in cellular damage if allowed to continue for at least 1h. Therefore, it was suggested in one study that the duration and intensity of the initial insult will have direct consequences on the extent of the cytopathology (Siesjo and Wieloch, 1986). In the work by Liu et al., the exact time point during the first 3 weeks where the degeneration occurs most was not examined, therefore warranting a closer examination of cell degeneration and death at earlier time points. Pollard et al. (1994) using the kainic acid model of epilepsy, demonstrated the appearance of Dark neurons in the amygdala and hippocampal CA3 region. Consistent with our results, Pollard et al. reported positively stained cells in the hippocampus as early as 6h post-kainic acid treatment. At 48h, the maximal number of silver-stained neurons was detected as compared to their longest sample survival time of 72h. While they did not study time points beyond 72h, it remains clear from their results that Dark neurons are representative of early neuronal stress, indicating that the use of this marker is optimal as an indicator of initial cellular response to a traumatic insult. In the present study, longer sample survival times up to 3 weeks confirmed the disappearance of Dark neurons. Although Pollard et al. did confirm that some Dark neurons were indeed apoptotic, it remains to be confirmed that most of the cell death is due to apoptosis or another mechanism. Sloviter (1996) has reported that intermittent perforant path stimulation for 24h induced acute pathological features in granule cells that were characteristic of apoptosis, whereas
the same treatment simultaneously induced the pathological features of necrosis in dentate hilar neurons and hippocampal pyramidal cells. Therefore, further information will be needed to determine relative contribution of apoptosis and necrosis throughout the period of epileptogenesis for each cell population in other experimental models of epilepsy.

A complementary study by Du et al., (1998) investigated the vulnerability of layer III neurons of the medial entorhinal cortex to prolonged seizure activity. An injection of the indirect excitotoxin aminooxyacetic acid (AOAA) into the rat entorhinal cortex results in acute behavioural seizures and subsequent neuronal damage. A temporal study of affected neurons in the entorhinal cortex displayed a similar pattern of degeneration with time using the Dark Neuron stain (Gallyas et al., 1990), with silver-impregnated neurons in the CA1 region detected as early as 3h post-injection. Although this study focused primarily on the neuronal damage within the entorhinal cortex, it is reported that Dark Neurons could only be detected up to 5 days following the insult. Our results suggest that neurons from within the same hippocampal cell populations are affected as a result of SE, however, the time-dependent profile of cellular injury as revealed by the two stains appears to be different. A possible explanation is that Dark Neurons are cells subjected to a reversible cytoskeletal challenge while Fluoro-Jade labels neurons that are irreversibly damaged. This idea is supported by previous studies (Auer et al., 1985a; Auer et al., 1985b; Ingvar et al., 1988) where morphologically, at the electron microscope level, Dark Neurons were found to be compatible with cell survival while acidophilic cells were accompanied by the morphological hallmarks of cell death, including clumping of nuclear chromatin, cytoplasm lacking endoplasmic reticulum/Golgi, flocculent densities in mitochondria and gross discontinuities of the plasma membrane and nuclear membrane. Since Fluoro-Jade is presumed to have affinity for degenerating cells in a similar manner to acid fuchsin (Schmued et al., 1997), it is reasonable to suggest that Fluoro-Jade and acid fuchsin label cells that are irreversibly damaged.

For quantification of cell loss in the hippocampus, it was important to consider neuron loss rather than total cell loss, including glial cells. As previous studies have reported (Babb et
al., 1984: Cavazos and Sutula, 1990; Grisar, 1986; Khurgel et al., 1992) gliosis in the epileptic hippocampus. (Babb et al., 1984; Cavazos and Sutula, 1990; Grisar, 1986; Khurgel et al., 1992) a rise in the number of glial cells could counterbalance the actual neuron loss that has taken place. We used stereological estimates of cell loss throughout epileptogenesis using a combined NeuN/PI labelling, ensuring that counts were restricted to neurons. Our results revealed significant neuron loss in the pilocarpine model of epileptogenesis at 1 week post-pilocarpine injection. It is interesting to note that this trend of cell loss corresponds to the general pattern of *Fluoro-Jade* labelling seen in some hippocampal regions with time. Our results thus suggest that in addition to displaying different time progressions of labelling, the *Dark Neuron* stain and *Fluoro-Jade* may be indicating that distinct cellular changes occur at different stages of epileptogenesis. The appearance of *Fluoro-Jade*-positive cells corresponds to a period where cell loss has already occurred, therefore positive staining at later time points (1–3 weeks) may indicate a second population of neurons undergoing stress due to secondary changes. The subsequent decrease in *Fluoro-Jade* staining by 3 weeks may be indicative that the critical period of cellular degeneration may be earlier, at 1 week following SE.

2.6 ACKNOWLEDGEMENTS

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Figure 1. Example of neurons labelled with the Dark Neuron stain in a pilocarpine-treated animal versus a control animal treated with saline. The sections were taken from rats sacrificed 3h following initial injection of pilocarpine (treatment group) or saline (control group). The right panels illustrate Dark Neuron staining of the CA1 pyramidal cells (top), CA3 pyramidal cells and interneurons (middle), and hilar neurons (bottom) in sections from pilocarpine-treated rats. The left panels show no evidence of labelled neurons in the corresponding regions in sections from control animals. Scale bar = 100μm. DG=dentate gyrus
Figure 2. *Camera lucida* drawings of representative cell types labelled with the *Dark Neuron* stain from pilocarpine-treated rats. This silver stain provides a complete labelling of the neurons, facilitating their morphological identification. A CA1 pyramidal cell (*top drawing*); a CA3 pyramidal cell (*middle drawing*); various cell types of the dentate gyrus: 1. Mossy cell 2. Spheroid cell of the dentate/hilus border 3. Large spiny stellate cell 4. Dentate pyramidal basket cell 5. Granule cell  
All scale bars = 100μm. Sr = stratum radiatum. So = stratum oriens.
Figure 3. Confocal micrographs of *Fluoro-Jade* labelling of the CA3 region in a control rat hippocampus (*left*) versus a pilocarpine-treated rat (*right*). The micrographs represent reconstructions from 25 serial optical sections (1.0\(\mu\)m apart). Both animals were sacrificed 24h following initial pilocarpine injection. Scale bar = 100\(\mu\)m.
Figure 4. Confocal micrographs of Fluoro-Jade labelled hippocampal neurons in a pilocarpine-treated rat sacrificed 24h after injection. The micrographs represent reconstructions from 25 serial optical sections (1.0µm apart). Fluoro-Jade labelled not only the cell body, but a significant proportion of the dendrites as well. Note the presence of fluorescent cells in the principal cell layer of CA1 (top), the principal cell layer of CA3 (middle), and the polymorphic layer of the dentate gyrus (bottom). Scale bar = 100µm.
Figure 5. Confirmation that Fluoro-Jade specifically labels neurons in pilocarpine-treated rats. This confocal image of the CA3 region of a rat sacrificed 1 week following pilocarpine-induced SE clearly illustrates that GFAP, a specific marker for astrocytes, does not overlap with Fluoro-Jade labelling. Scale bar=50μm.
Figure 6. Comparison of the relative distribution of labelled neuronal profiles with the Dark Neuron and Fluoro-Jade stains at 6 time points following pilocarpine injection. Means ±SEM are shown. There is a difference in the time-dependent profile of labelled cell using the two methods: the proportion of Dark Neurons is significantly higher at earlier time points (i.e. < 24h) in the stratum radiatum of CA1 and the stratum pyramidale of CA1: the density decreases to very little by 3 weeks. On adjacent serial sections processed with Fluoro-Jade, a different pattern arises. Between 3 and 12h following pilocarpine injection, the percentage of Fluoro-Jade positive neurons remains lower than Dark Neuron-labelled profiles until 1 week when there is a significantly greater proportion of profiles labelled with Fluoro-Jade. (p<0.05*: p<0.01**)
Figure 7. Confocal micrographs of double-immunofluorescence labelling of NeuN and GFAP in the granule cell layer of a pilocarpine-treated rat sacrificed 3 weeks following the onset of status epilepticus. The micrographs represent reconstructions from 19 serial optical sections (1.0μm apart). The lack of overlap between the two stains indicates that NeuN is a specific neuronal marker. Scale bar=50μm.
Figure 8. Confocal micrographs illustrating profound granule cell loss in the dentate gyrus of a rat sacrificed 3 weeks following pilocarpine-induced status epilepticus as revealed by NeuN (left) and GFAP (right) immunoreactivity. The micrographs represent reconstructions from 19 serial optical sections (1.0 μm apart). Arrows point to regions of significant neuron loss (NeuN, left). The section shows extensive gliosis over the region where cell loss occurred (arrows, right panel). Neuron specific labelling therefore presents the advantage, over conventional Nissl staining, of avoiding confounding results due to gliosis for quantification of neuronal loss. Scale bar=50 μm.
Figure 9. Confocal micrographs showing NeuN-immunoreactivity in relation to nuclear staining with PI. Note that neurons immunoreactive to NeuN have a diffuse labelling throughout the nucleus and cytoplasm. The same sections are also stained with propidium iodide, which is specific for nuclei of neurons and glia. Note the clear delineation of the nuclei, which facilitates accurate stereological estimates of cell number. Scale bar = 50μm.
Figure 10. Representative depiction of the labelling trends in the CA3 region at 3h and 1 week following pilocarpine injection. The results show a significant decrease in Dark Neuron staining (expressed as % neuronal profiles), and a gradual increase in Fluoro-Jade labelling throughout this period of epileptogenesis (3h-1 week). Quantification of cell number revealed a decrease (expressed as neurons/mm²) in the CA3 region as shown. (*p<0.05. **p<0.01)
Chapter 3. Discussion
3.1 Summary of Findings

We have investigated the relationship between two markers of neuronal degeneration: the argyrophilic Dark Neuron stain, and the anionic fluorochrome Fluoro-Jade. Our results suggest that each stain reveals a separate component of cellular reaction to injury. The Dark Neuron stain labelled hippocampal neurons at early time points (within hours) following pilocarpine-induced status epilepticus, while Fluoro-Jade revealed neurons after longer delays (days).

The Dark Neuron and Fluoro-Jade stains labelled neurons from the same hippocampal regions such as the stratum radiatum and pyramidale of CA1 and CA3, and the polymorphic region of the dentate gyrus. Both stains allowed for morphological identification of cell types as there was complete labelling of the cell body as well as a large proportion of the dendritic tree.

We also reported significant cell loss in the CA3 region at 1 and 3 weeks following pilocarpine injection using NeuN as a specific marker of neurons. No significant neuronal loss was detected at the earlier time points studied in pilocarpine-treated animals. In addition, qualitative results describing patches of neuron loss in the granule cell layer were presented. Gliosis which accompanied cell death at 1 and 3 weeks in the CA3 and granule cell layers was revealed by GFAP immunostaining.

It was also confirmed that the Fluoro-Jade stain labelled neurons and not glial cells. Double-labelling studies using Fluoro-Jade and an antibody against either GFAP or NeuN showed an overlap between Fluoro-Jade and NeuN in neurons. In contrast, GFAP-positive cells were not labelled with Fluoro-Jade anywhere in the hippocampus.

In addition to the main result of the thesis, we have elaborated on the use of an antibody against neuronal nuclear protein (NeuN) and propidium iodide (PI) as accurate tools for quantification of neuron number in the hippocampus, using the optical dissector method for serial confocal images. This method proved to be superior over conventional Nissl staining in
the hippocampus where glial nuclei are stained as well, thus avoiding confounding results due to the extensive gliosis that accompanies neuronal loss.

3.2 Scope of our findings

The pattern of cell loss and gliosis characteristic of human temporal lobe epilepsy can be produced experimentally as we have demonstrated using the pilocarpine model. This profile of cell loss appears to result in a large part from excitotoxic damage subsequent to excessive activation of glutamate receptors (Olney et al., 1986; Sloviter, 1994; Sloviter, 1994). The cellular changes which develop as a consequence of this excitotoxicity include swelling of dendrites, vacuolar condensation of neuronal cell bodies, and dilatation of astroglial elements (Clifford et al., 1987). Mechanistically, the cell loss can occur with either active or passive participation of cellular components, referred to as either apoptosis and necrosis, respectively (Kerr et al., 1972; Yakovlev et al., 1997). Studies of apoptosis and necrosis are complementary to our work, as we will discuss below.

The bulk of the data about epilepsy focuses on treatment of the permanent chronic seizures, and all currently available therapy is symptomatic, aimed at decreasing seizure frequency. However, as it was introduced in Chapter 1, it has long been recognised that prevention of epilepsy in susceptible individuals would certainly be more beneficial for patients. Therefore, our rationale behind selecting stains that reveal neurons at different stages of cellular stress is based upon the premise that strategies to developing preventative therapy are superior to palliative treatment.

As we outlined in detail in Section 1.6, the Dark Neuron stain is presumed to be a marker of early, reversible cytoskeletal disruption in neurons. In fact, this stain is often used to assess the general pattern of cellular injury in tissue (Gallyas et al., 1990; Ingvar et al., 1988; Mello and Covolan, 1996; Pollard et al., 1994; Toth et al., 1997; van den Pol and Gallyas, 1990). It is hypothesized that a traumatic insult to the brain disrupts the stability of the cytoskeleton. An end result of this process is the increased affinity for silver (argyrophilia) which can be detected with
the Dark Neuron stain. Although we have shown the early appearance of Dark Neurons within hours of status epilepticus, the fate of these neurons is unclear. Strategies to answer this question will be discussed further in the subsequent section.

In contrast to the Dark Neuron stain, the occurrence of labelling with anionic dyes such as acid fuchsin has been shown to correspond with the morphological features of cell death, including chromatin clumping, amorphous cytoplasm lacking organelles, and discontinuities in the plasma membrane (Kieman et al., 1998). The cytoplasmic eosinophilia seen in these cells is attributed to the increased production of lysine-containing proteins. Fluoro-Jade is an example of an anionic dye which, from our results, labels neurons at a time point during epileptogenesis when there is significant cell loss. This finding also raises the question regarding the fate of a Fluoro-Jade-labelled neuron. Knowing the survival outcome of a marked neuron using each staining method increases the value of these techniques as predictors of cellular injury and death.

There are a sparse number of publications that address the subject of apoptosis and necrosis in the hippocampus. One study (Sloviter et al., 1996) reports apoptosis in granule cells and necrosis in pyramidal cells and dentate hilar neurons following 24h of perforant path stimulation. However, it is also mentioned that the degree of overlap between the two processes of cell death is unknown. Another study (Pollard et al., 1994) compares the pattern of Dark Neuron staining with the pattern of apoptosis seen after several time points following kainic acid injection, from 3h-72h. They reported the coincidence of Dark Neuron labelling and the features of apoptosis in hippocampal cells as early as 6h following status epilepticus. However, they claim that the Dark Neuron stain detects dying neurons, and the appearance of Dark Neurons occurs in parallel with cell death. Their interpretation of the use of the Dark Neuron stain as an indicator of cell death may be premature, as several studies suggest that it coincides with signs of reversible cellular injury (Auer et al., 1985a; Evans et al., 1984; Gallyas et al., 1992). Nevertheless, the occurrence of apoptosis at early time points such as 6h after status epilepticus indicates that active cell death does not occur solely at later time points during epileptogenesis.
If we are to address the subject of the fate of neurons revealed with the *Dark Neuron* stain, then the literature highlights two avenues. A first hypothesis could be that since *Dark Neurons* have appeared at the same time as apoptotic cells, then the stain could be signalling that labelled cells are dying cells (Pollard et al., 1994). However, the second hypothesis is based on morphological data that tells us that *Dark Neurons* may be compatible with cell survival due to the preservation of organelles and membranes (Chang and Baram, 1994; Ingvar et al., 1988).

Regarding the fate of a *Fluoro-Jade* labelled neuron, the story is also unclear. One study reports that acidophilic neurons detected after a traumatic brain insult have disruption of the cell membrane and mitochondrial flocculent densities (Ingvar et al., 1988). These features do not signal apoptosis, but rather necrosis, where hallmarks include loss of membrane integrity, nuclear flocculation, cell swelling, and lysis (Yakovlev et al., 1997). Does this mean that *Fluoro-Jade* exclusively reveals necrotic neurons? These questions about the fate of *Dark Neuron* and *Fluoro-Jade* labelled neurons warrant further investigation, and will be addressed in Section 3.3.

### 3.3 Future Directions

Certainly, one of the most important questions arising from our study is the fate of cells labelled by either the *Dark Neuron* stain or *Fluoro-Jade*. We feel that the key to answering this question is to follow the progression of apoptosis and necrosis in time. This can be done in serial sections, using a terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) (Charriaut-Marlangue and Ben-Ari, 1995; Labat-Moleur et al., 1998) assay to detect apoptotic cells, and either *Dark Neuron* or *Fluoro-Jade* stain applied to the subsequent section. In this manner, the temporal profile of cell death could be used to draw some conclusions about the fate of neurons labelled with the *Dark Neuron* and *Fluoro-Jade* stains.

The results from these experiments would lead to the next question of what cell types are most vulnerable to seizure-induced injury. Either the *Dark Neuron* or *Fluoro-Jade* stain could
be used for double-labelling studies with immunocytochemical markers of specific cell types. For example, neurons of the dentate gyrus have been characterised on the basis of the peptides they express: CCK, SS, GAD, NPY, and VIP (see Section 1.5.3). The ultimate aim of our work is to identify cell types which can be used as targets for preventative therapy. If we know, for example, that somatostatin (SS)-positive cells are revealed by the Dark Neuron stain at an early time point during epileptogenesis, and through our studies of cell death it is confirmed that SS cells are among the principal populations of cells that die, then this cell type could be a primary target for rescue therapy.

The concept of rescue therapy is broad, and recently, different strategies have been developed with the goal of blocking one or more pathophysiological steps that occur following brain injury. One example is a study which has demonstrated the implication of caspase-3-like proteases in neuronal apoptosis induced by traumatic brain injury and suggests that the blockade of such caspases can reduce post-traumatic apoptosis and associated neurological dysfunction {20464}. In recent years the focus for preventative therapy has been on neurotrophins. Neurotrophins are structurally related polypeptides that make up one family of growth factors (Thoenen, 1991). Nerve growth factor (NGF) is the best characterised member of this family, along with brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5. They are expressed in the adult central nervous system where they play a critical role in the survival and maintenance of neurons.

A study (Holtzman and Lowenstein, 1995) demonstrated the attenuation of cholinergic axonal sprouting after intraventricular infusions of an anti-NGF antibody in the pilocarpine model of epilepsy. Others have confirmed that limbic seizures do cause a rapid and pronounced increase in the expression of mRNA for NGF in the granule cells. We could elaborate on this idea by administering an anti-NGF antibody following the onset of status epilepticus and determining its effect on the pattern of Dark Neuron and Fluoro-Jade labelling. An effect would indicate that neurotrophins are playing a functional role in the remodelling of the network which results in cellular stress and degeneration.
Pursuing any of these experiments would provide valuable information as to what cellular changes during epileptogenesis are important to target for therapeutic purposes.

3.4 Conclusion

The results of this thesis emphasize the fact that epileptogenesis is a complex process of reorganisation and remodelling of brain network processes. We are brought back to the most confounding problem facing epilepsy researchers: Are the cellular changes described in temporal lobe epilepsy a cause or consequence of the chronic seizures? It is difficult, if not impossible to answer by studying chronically epileptic tissue where the changes are permanent. We have attempted to begin answering this question by showing that some cellular stress and/or degeneration occurs immediately after status epilepticus (revealed by the Dark Neuron stain) and some occurs at a time point closer to the development of chronic seizures (shown by Fluoro-Jade labelling). It is likely that these markers highlight crucial processes which contribute to the development of permanent seizures. By dissecting the process of epileptogenesis we may thus be better able to answer the question of how temporal lobe epilepsy develops.
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