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KINDLING AND ACTIVATION-INDUCED HIPPOCAMPAL PLASTICITY

By

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ABSTRACT

In the adult nervous system, neuroplasticity can be described as all modifications in neuronal structure or function in response to alterations in input. The kindling model of temporal lobe epilepsy represents one of the most widely studied models of neuroplasticity in the mature nervous system. Kindling can be defined as a progressive increase in evoked behavioural and electrographic seizure activity following electrical stimulation of forebrain structures. Recent research has shown that kindling produces a number of plastic changes in the brain, particularly in the hippocampal region. These changes include sprouting of axons, an increase in the size of the hilar area of the dentate gyrus, and reactive gliosis. To date, the relationship between kindling and kindling-induced hippocampal plasticity is not clear. The major emphasis of this thesis is to investigate whether kindling-induced hippocampal plasticity is induced by damage or whether kindling-induced plasticity can be induced by activation.

In Study 1, the neurotrophin nerve growth factor (NGF) was used as a tool to dissociate kindling-induced cell loss and kindling-induced mossy fiber sprouting. Intraventricular administration of NGF accelerated seizure development, enhanced mossy fiber sprouting and attenuated seizure-induced decreases in hilar cell density. These findings appear to dissociate kindling-induced mossy fiber sprouting and kindling-induced cell density effects. Moreover, these findings suggest that kindling-induced mossy fiber sprouting is due to the co-involvement of growth factors and activation. Study 2 showed that NGF may mediate kindling-induced mossy fiber sprouting via the cholinergic system, providing the first evidence that the cholinergic system plays a role in mossy fiber sprouting. Study 3 showed that mossy fiber sprouting can be

induced by non-epileptogenic stimulation, providing further evidence that mossy fiber sprouting can occur in the absence of neuronal damage. Study 4 showed that kindling-induced changes in hilar area and kindling-induced reactive gliosis follow a similar time course.

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LIST OF ABBREVIATIONS

AD	Afterdischarge
DG	Dentate Gyrus
EPSP	Excitatory Post-Synaptic Potential
FF	Fimbria Fornix
GFAP	Glial Fibrillary Acidic Protein
IML	Inner Molecular Layer
LTP	Long-Term Potentiation
MCID	Micro Computer Imaging Device
NGF	Nerve Growth Factor
PBS	Phosphate Buffered Saline

Chapter 1

1.0 Neuroplasticity

The adult mammalian central nervous system (CNS) is characterized by a large number of neurons that are specifically interconnected and function in a highly organized manner. In the past, it was assumed that these neuronal circuits were static (i.e., no change in the neuronal connections was possible in the adult brain once a specific termination pattern had been established during development) (Deller & Frotscher, 1997). This view has been replaced, however, by the concept of a CNS that is plastic (i.e., constantly changing and adapting). The term "neuroplasticity" is currently used to describe all modifications in neuronal structure or function in response to alterations in input (Shaw et al., 1994). To date, however, the mechanisms that underlie the structural and functional plastic changes that can occur in the adult CNS are not yet well understood.

One of the most widely studied areas of neuroplasticity is the brain's ability to reorganize following injury. For example, after a CNS trauma, surviving neurons form new synapses to replace those from neurons damaged by the lesion. This "reactive synaptogenesis" was first described in the brain by Raisman (1969). Since then, a number of other damage-induced forms of neuroplasticity have been demonstrated in a range of different species and in numerous brain regions (e.g., Cotman et al., 1981; Wells & Tripp, 1987; Schwegler et al., 1995). The dynamic nature of the brain to reorganize following damage has major implications for potential therapeutic strategies to restore function following brain injury. Specifically, by identifying factors involved in the reorganization of neuronal circuits following injury, it may be possible to

enhance neural compensation following brain damage and functional restoration (Deller & Frotscher, 1997).

Neuroplasticity does not only occur following damage. In some cases, neuroplasticity is activation-induced and may be a major mechanism which allows adult organisms to learn and to adapt to changing environments (Deller & Frotscher, 1997). For example, Greenough and colleagues have demonstrated alterations in dendritic branching patterns following maze training (Greenough & Volkmar, 1973) and training in a reaching task (Greenough et al., 1979). These findings demonstrate the capacity of the nervous system to undergo major reorganization after input manipulation, in the absence of any form of CNS damage.

The primary focus of this thesis will be on activation-induced structural plasticity in the adult rat hippocampus following kindling, an experimental epilepsy model which evokes seizure activity via electrical stimulation of the brain. This work will address issues of damage-induced neuroplasticity, activation-induced neuroplasticity, recovery of function, information storage, and epilepsy.

1.1 Relevant Anatomy

Given that this thesis focuses primarily on activation-induced plasticity in the hippocampus, a brief overview of hippocampal anatomy is presented here for background purposes.

Anatomy of the Hippocampal Formation

The hippocampal formation consists of two major subsystems: 1) the hippocampal gyrus and 2) the dentate gyrus.

Hippocampus Gyrus. Figure 1.0 shows a schematic of the rat hippocampus in the coronal plane. The hippocampus gyrus is divided into three subfields: the CA1, CA2 and CA3. The superior portion of the hippocampus corresponds to CA1, whereas area CA3 is bounded by the dentate gyrus and is the inferior portion of the hippocampus. Area CA2 serves as the transition between CA3 and CA1 (Amaral & Witter, 1995).

The hippocampus gyrus is divided into several different layers, referred to as strata (Amaral & Witter, 1995). Figure 1.1 outlines the strata of the hippocampus gyrus: the stratum oriens, the stratum pyramidale, the strata radiatum and the stratum lacunosum moleculare. The principle cell type in the hippocampus gyrus is the pyramidal cell and the cell bodies of these neurons form the stratum pyramidale. These pyramidal cells typically have large apical dendrites, along with several shorter basal dendrites and a single axon located at the opposite pole. These basal and apical dendrites of the pyramidal cells terminate in different strata on either side of the pyramidal cell layer: the basal dendrites of the pyramidal cells ramify in the stratum oriens, whereas the apical dendrites branch extensively into the stratum lacunosum-moleculare. The shafts of the apical dendrites, however, are located in the stratum radiatum. In CA3, there is also an additional stratum, referred to as the stratum lucidum, which is located between the pyramidal cell layer and the stratum radiatum.

Dentate Gyrus and Intrinsic Hippocampal Circuitry. The dentate gyrus can be divided into three layers: the molecular layer, granule cell layer and the polymorph layer (Amaral & Witter, 1995). The molecular layer consists of the granule cell dendrites. The granule cell bodies make up the the next layer, which consists of a narrow band of densely packed granule cells. The axons of the dentate granule cells are the mossy fibers. The mossy fiber pathway

projects to the CA3 region and represents the most significant input to CA3 pyramidal cells. In turn, the CA3 pyramidal cells are the major source of innervation to CA1, a pathway referred to as Schaffer's collaterals. The polymorph layer lies between the upper and lower blades of the granule cell layer, a region referred to as the hilus. The polymorph layer contains a range of different cell types, including interneurons, stellate cells, mossy cells, and basket cells (Amaral & Witter, 1995).

Extrinsic Innervation to the Dentate Gyrus. The major source of innervation to the dentate gyrus originates in layer II of the entorhinal cortex, which synapses with the outer two-thirds of the molecular layer (Steward & Scoville, 1976). The entorhinal cortex is connected to the hippocampus via a bundle of axons, known as the perforant path. The perforant path is topographically organized such that the medial entorhinal cortex projects to the middle molecular layer, whereas the lateral entorhinal cortex projects to the outer molecular layer (Wyss, 1981).

The granule cells of the dentate gyrus also receive inputs from contralateral (commissural) and ipsilateral (associational) hilar neurons (Van Groen & Wyss, 1988; Buckmaster et al., 1992). These commissural and associational projections terminate in the inner third of the molecular layer and are distinct from the entorhinal afferents in the outer molecular layer.

The dentate gyrus also receives sparse cholinergic and GABAergic inputs from the septum and the nucleus of the diagonal band of Broca. These projections terminate in the outer two-thirds of the molecular layer and in regions just above and below the granule cell layer (Kohler et al., 1984; Freund & Antel, 1988; Nyakas et al., 1987; Dutar et al., 1995).

Extrinsic Innervation to the Hippocampus Gyrus. The major extrinsic inputs to the

hippocampus gyrus arise from the entorhinal cortex, the septum, the nucleus of the diagonal band of Broca (NDB) and brain stem nuclei. The entorhinal input to the hippocampal formation appears to be glutamatergic (Nadler et al., 1976; White et al., 1977; Colbert & Levy, 1992). The stratum lacunosum moleculare of CA1 receives input from an entorhinal projection originating in the pyramidal cells of layer III of the entorhinal cortex (Steward & Scoville, 1976), whereas the stratum lacunosum moleculare of CA3 is innervated by stellate cells of layer II of the entorhinal cortex (Tamamaki & Nojyo, 1993). Cholinergic and GABAergic afferents emerging from the medial septum and the NDB project to the stratum oriens and the stratum radiatum of the CA1 and CA3 regions (Kohler et al., 1984; Gaykema et al., 1990; Mosko et al., 1973 & Nyakas et al., 1983). Both CA1 and CA3 also receive sparse inputs from serotonergic raphe (Moore & Halaris, 1975) and noradrenergic locus coeruleus (Jones & Moore, 1977) projections.

1.2 Damage-Induced Neuroplasticity

Examples of Neuroplastic Changes Following CNS Trauma

Following brain trauma, two opposing influences generally contribute to long-term functional outcome: 1) compensatory neural mechanisms which may improve recovery from damage, and 2) degenerative events secondary to the primary injury which may interfere with recovery (Schallert et al., 1997). To illustrate, consider the range of plastic changes that occurs following the axotomy of a neuron. These changes include neuronal loss, loss of afferent input, reactive growth and reactive gliosis.

Neuronal Loss. The axotomy of a neuron generally leads to the retrograde degeneration of the proximal axon and to the loss of the distal axon segment. In some cases, the

axotomized neuron atrophies and dies. An important factor influencing cell survival following axotomy, however, is the location of the lesion site. Cell death is more likely to occur if the axon is cut close to the cell body, whereas the loss of distal axon collaterals rarely leads to cell death (Sofroniew & Isaacson, 1988). The loss of distal axons may actually result in the growth of new axon collaterals in other target areas of the axotomized axons, a process referred to as "pruning" (Schneider et al., 1985).

Loss of Afferent Input. Following axotomy, the target neurons of the transected axon lose some of their inputs (i.e., loss of innervation). This loss of afferents could result in a range of functional and morphological changes in the target cell. Functional changes could include alterations in neuronal activity (Reeves & Steward, 1988), receptor composition, and receptor density (Zigmond et al., 1986), whereas morphological changes could include alterations in the shape of spines and dendrites (Frotscher 1991; 1993).

Reactive Growth. A number of other changes can also occur in the area deafferented by the lesion, which involve undamaged fibers in the denervated zone. These changes include reactive collateral sprouting (i.e., the growth of uninjured, neighbouring axons) and reactive synaptogenesis (i.e., the formation of new synapses). It is possible that reactive synaptogenesis and sprouting of undamaged fibers following CNS trauma may replace lost connections in an anatomically and functionally specific manner. In turn, these reactive growth processes may play a role in functional recovery following damage. Alternatively, it is possible that these reactive growth processes may form aberrant synaptic connections and may contribute to brain pathology. To date, however, the stimulus for the initiation and the factors controlling these reactive growth processes are unclear. It is also important to note that these reactive growth processes are

not examples of regeneration. Regeneration is a growth response of an injured axon, whereas collateral sprouting is growth characteristic of undamaged neurons.

Reactive Gliosis. The CNS is composed of several cell populations, including neurons, microglial cells and macroglial cells. Astrocytes and oligodendrocytes are the major macroglial cell types. One of the primary functions of glia is the preservation of the host tissue integrity following injury, which is referred to as reactive gliosis. Reactive gliosis is characterized by the hypertrophy and proliferation of microglial cells and astrocytes in the damaged region.

Reactive gliosis has been considered as a major impediment to axonal regrowth following injury because it results in the formation of a glial barrier around the lesion site (Ridet et al., 1997). More recently, however, it has been proposed that the formation of a glial barrier (i.e., glial scar) may be advantageous, because it isolates the intact CNS from secondary lesions (Ridet et al., 1997). Additional data also suggests that in certain conditions, reactive astrogliosis may actually provide a permissive substratum for neurite extension, which may play a role in post-lesion recovery (Ridet et al., 1997). To date, however, the complex role of reactive gliosis in the pathology of neurological events is poorly understood.

These examples of the consequences of axotomy clearly demonstrate the wide range of changes that can occur in the CNS following damage. Although some neuroplastic changes may actually improve recovery of function following injury, other forms of damage-induced plasticity may actually interfere with recovery of function.

Examples of Models of Damage-Induced Plasticity

Several well-established model systems exist for the analysis of damage-induced

plasticity in the brain. To illustrate, two models will be discussed: 1) global cerebral ischaemia and 2) use-dependent plasticity following CNS injury.

1) Global Cerebral Ischaemia . Global cerebral ischaemia induced by transient restriction of cerebral blood flow (CBF) during heart attack or coronary artery occlusion results in selective damage to particular groups of neurons in the brain, primarily in the CA1 and hilar regions of the hippocampus (Nunn & Hodges, 1994). The interruption of CBF can also result in profound anterograde amnesia and learning difficulties in humans (Nunn & Hodges, 1994).

In animal models of global ischaemia, similar patterns of brain damage and cognitive impairments have been identified (Davis & Volpe, 1990). With short durations of ischaemia, hippocampal cell loss is largely confined to the CA1 region (Davis & Volpe, 1990). Damage becomes more widespread in the hippocampus, however, with increasing durations of the arterial occlusions. Plastic changes, such as reactive gliosis and reactive neural growth in the hippocampal region, have also been reported following ischaemia in rodents (Onodera et al., 1990).

2) Use-Dependent Structural Changes Following CNS Injury. Unilateral electrolytic lesions of the forelimb representation area of the sensorimotor cortex cause rats to use their non-impaired forelimb preferentially. This increased use of the non-impaired forelimb is associated with transient increases in dendritic growth in the intact brain hemisphere (Jones & Schallert, 1992; 1994; Schallert & Jones, 1993). This enhanced dendritic growth may depend on both neural injury and behavioural pressure. Restricting use of the non-impaired forelimb during the period of dendritic growth prevents the enhanced growth response, whereas immobilization of a forelimb in sham-operated controls has no detectable effect on dendritic growth in the

hemisphere opposite to the non-immobilized forelimb (Schallert et al., 1997).

This suggests that brain damage may actually increase sensitivity to use-dependent compensatory neural growth (Schallert et al., 1997). These data have interesting implications for recovery of function following brain injury: while traditional experiments on mechanisms of recovery of function following damage focus on compensatory neural changes that may mediate behavioural outcome, these data suggest that behaviour may alter anatomical events.

1.3 Activation-Induced Plasticity

A number of structural and functional changes also occur in the brain following activation (i.e., in the absence of damage). In many cases, these changes are similar to those seen following damage-induced plasticity. To illustrate, two examples of activation-induced plasticity will be discussed: 1) neural changes induced by exposure to enriched environment and 2) long-term potentiation (LTP), which is a synaptic model for memory.

Enriched Environment Studies. An enriched environment is a large, complex environment with a range of objects for visual, motor, and cognitive stimulation. Animals are also housed in groups in the enriched environment to provide social stimulation.

Compared to subjects reared in isolation in a small cage (i.e., an impoverished environment), environmental enrichment produces a number of structural changes in the brain. These structural changes include increases in brain weight (Cummins et al., 1973; 1982), increases in cortical thickness (Diamond et al., 1976), increases in glial cell number (Szeligo et al., 1977), increases in hippocampal neuronal number (Kemperman et al., 1997), increases in hippocampal size (Kemperman et al., 1997), and increases in dendritic branching (Volkmar &

Greenough, 1972; Greenough et al., 1973). In addition to structural changes, environmental enrichment also produces functional changes in the brain. These functional changes include increases in levels of neurotrophin-3 (NT-3) in the visual cortex and hippocampus (Torasdotter et al., 1996), increases in glucocorticoid receptor expression in the hippocampus, and increases in the expression of the immediate early gene transcription factor NGFI-A in the hippocampus (Mohammed et al., 1993).

There is also evidence showing that environmental enrichment improves performance levels on a range of learning and memory tasks (e.g., Renner & Rosenzweig, 1987), which raises the possibility that enriched experience may help in recovery from brain damage. To date, however, the mechanisms underlying improvements in performance levels and changes in cerebral neurochemistry and neuroanatomy due to enriched experiences are not clear.

Long-Term Potentiation. One of the most widely studied models of activity-dependent neural plasticity in the adult preparation is long-term potentiation (LTP), a synaptic model of learning and memory (Bliss & Lynch, 1988). In general, LTP can be defined as a stable, long-lasting increase in the amplitude of post-synaptic responses evoked in a neural pathway following activation of that pathway via brief tetanic stimulation (Teyler & DiScenna, 1987; Malenka, 1994). In the hippocampus, these increases can be observed in the population excitatory post-synaptic potential (population EPSP), which is a field measure of the strength of synaptic drive, and also in the population spike, which is a field measure of the number of cells generating action potentials (Lomo, 1971). Population EPSP and population spike amplitudes can remain increased for hours in acute preparations (Bliss & Lomo, 1973; Bliss & Gardner-Medwin, 1983) and for days, or even months in chronic preparations following LTP induction (Racine et al.,

1983). These LTP effects, however, are not permanent and progressively decay back to baseline levels over time (Racine et al., 1983). Figure 1.2 shows a representative example of population EPSP and population spike recorded from a bipolar electrode implanted in the rat hippocampal dentate gyrus following perforant path stimulation before (solid line) and after LTP induction (dotted line). It remains to be determined, however, whether long-term potentiation is due to changes that take place in the pre-synaptic cell (e.g., an increase in neurotransmitter release), the post-synaptic cell (e.g., an increase in receptor density), or to some combination of pre- and post-synaptic modifications (Barinaga, 1990; Malenka, 1994).

Although LTP can be induced in a number of different brain sites (Bliss & Lomo, 1973; Racine et al., 1983), the majority of experimental work to date has focused on the hippocampus (Bliss & Lomo, 1973; Teyler & DiScenna, 1987; Brown et al., 1988; Gustafsson & Wigstrom, 1988). The hippocampus is a structure known to be involved in certain forms of memory storage, and hippocampal LTP exhibits several properties required for a memory mechanism: 1) LTP is specific to the activated pathway, 2) it is dependent on neural firing patterns, and 3) it is associative (i.e., temporally pairing a weak input that is incapable of generating LTP itself with activation of a strong input can result in LTP of the weak input (Gustafsson & Wigstrom, 1989; Malenka, 1994)). In the hippocampus, there are two forms of LTP that differ regarding their requirement for activation of N-methyl-d-aspartate (NMDA) glutamate receptors for LTP induction (Nicoll & Malenka, 1995). These forms of LTP are typically referred to as NMDA-dependent or non-NMDA-dependent LTP. Different synapses within the hippocampus (i.e., mossy fiber synapses in the CA3 region and the Schaffer collaterals in the CA1 region) exhibit either of the two forms of LTP (Nicoll & Malenka, 1995).

Recent research has also shown that LTP can induce structural changes in the brain, and it has been suggested that the long-lasting increase in the efficacy of synaptic transmission observed in LTP may be supported by structural synaptic modifications (Lynch et al., 1988; Lynch & Baudry, 1991; Bliss & Collingridge, 1993). Examples of LTP-induced structural modifications include: 1) increases in synaptic numbers in the dentate gyrus of both young and aged rats (Geinisman et al., 1991; 1992), 2) increases in the size of dendritic spines (Van Harreveld & Fifkova, 1975; Fifkova & Van Harreveld, 1977), and 3) increases in the size of dendritic spine heads (Desmond & Levy, 1982). Furthermore, it has been demonstrated that induction of LTP in the dentate gyrus is followed by a significant increase in the number of axospinous synapses with multiple, completely partitioned zones (Geinisman et al., 1993), and increased hippocampal mossy fiber sprouting (Adams et al., 1997b; Chapter 4). Although it is possible that LTP-induced synaptic modifications may represent a structural substrate of the persistent enhancement of synaptic responses that define LTP, additional studies demonstrating a causal relationship between these structural modifications and the long-term increases in synaptic efficacy observed in LTP are required.

In summary, these two models of activation-dependent neuroplasticity show that a wide range of functional and structural changes can occur in the brain in the absence of damage. Moreover, these examples of activation-dependent plasticity clearly demonstrate the brain's dynamic ability to reorganize following alterations in input.

1.4 The Kindling Model of Temporal Lobe Epilepsy: Damage-Induced Plasticity or Activation-Induced Plasticity?

The remainder of this introduction will focus on plasticity in the brain following seizure activity using the kindling model of temporal lobe epilepsy. Although kindling is one of the most widely studied experimental models of epilepsy, it also represents an excellent model of neuroplasticity. It is currently controversial, however, whether kindling is a model of damage-induced or activation-induced plasticity. For background purposes, a brief summary of epilepsy and different animal models of epilepsy will be discussed here before describing kindling.

General Description of Epilepsy

The epilepsies are a heterogeneous group of disorders of the nervous system with diverse etiologies, electrographical and behavioural seizure patterns, and pharmacological sensitivities. Epilepsy affects approximately 1% of the general population (Shin & McNamara, 1994).

Seizure Classification. In 1981, the International League Against Epilepsy (ILAE) proposed a classification scheme of epileptic seizures based on clinical and electroencephalographic criteria. According to this widely accepted classification system, seizures are divided into two major categories: partial and generalized. Partial seizures are defined by epileptic activity (i.e., transient, hypersynchronous episodes of neuronal activity) that is limited to one focal region of the brain. Partial seizures are subdivided into three groups: simple, complex, and secondarily generalized. With simple partial seizures, patients remain conscious, whereas with complex partial seizures, patients show impaired consciousness. Secondarily generalized seizures are partial seizures that progress to encompass the entire brain

bilaterally, causing a generalized seizure which involves motor convulsions.

With generalized seizures, epileptic activity encompasses the entire brain and patients lose consciousness. Two typical examples are absence seizures and grand mal seizures. Absence seizures are characterized by a sudden lapse in consciousness and may be accompanied by minor motor movements, whereas grand mal seizures involve a total loss of consciousness and major motor convulsions.

Temporal Lobe Epilepsy (TLE). Complex partial seizures originating in the temporal lobe are the most common seizure type and account for approximately 40% of all cases in adults (Engel, 1989). Moreover, complex partial seizures are a particularly devastating form of epilepsy because they are generally quite resistant to anticonvulsant drugs: approximately only 25% of adults suffering from complex partial seizures achieve seizure control despite optimal contemporaneous treatment (Mattson et al., 1985).

Temporal lobe epilepsy is also often accompanied by hippocampal or endfolium sclerosis, a pathological lesion which refers to neuronal loss and gliosis of the hilus of the dentate gyrus, CA1, and CA3 regions of the hippocampus (Cavazos & Sutula, 1990). In addition, morphological studies have revealed that hippocampal sclerosis in humans is also associated with aberrant sprouting of the axons of the hippocampal dentate granule cells (i.e., hippocampal mossy fibers) (Babb et al., 1991). Figure 1.3 shows a schematic of the hippocampal region outlining the mossy fiber pathway. Although it is not known whether this synaptic reorganization is adaptive or maladaptive, this area of investigation targets a potentially novel site for therapeutic intervention in epileptogenesis in humans.

Use of Animal Models to Study Epilepsy

The basic mechanisms underlying seizure development, propagation, and maintenance in the different forms of human epilepsy are not well understood. Moreover, the amount of experimental data that researchers can realistically obtain from human epileptics is limited by both ethical and technical constraints. It is also unlikely that data from post-mortem studies using brain tissue from chronic epileptics will provide a great deal of insight into the mechanisms underlying epileptogenesis because these patients have suffered from multiple seizures and different drug regimens which may confound attempts to determine the original source of the epilepsy.

To address these limitations, many researchers rely on experimental animal models. A major advantage of using animal models to study features of epileptogenesis is that a large number of relevant variables can be controlled directly by the researcher. Examples include the nature of the stimuli used to initiate the epileptogenic process, the site and the rate of stimulation, and the total amount of seizure activity induced. Because rodent and human nervous systems respond similarly to seizure-provoking stimuli, researchers may then be able relate these findings back to the study of human epilepsy.

Examples of Models of Epilepsy Other Than Kindling

Status Epilepticus. Status epilepticus is a state of continuing or recurring seizures, whereby recovery between attacks is incomplete. In many cases, status epilepticus can result in death. Following exposure to a status-inducing agent (e.g., kainic acid), rats experience chronic, spontaneous, recurrent seizures that can last for hours (Cronin & Dudek, 1988). The kainate-

treated rat is a well-established model of status epilepticus (Nadler, 1981; Ben-Ari, 1985)

Kainate-treated rats display neuropathological abnormalities (i.e., neuronal loss, mossy fiber sprouting, gliosis) that are strikingly similar to those reported for human temporal lobe epilepsy (Buckmaster & Dudek, 1997). Moreover, there is a significant correlation between the extent of mossy fiber sprouting and hippocampal neuronal loss in kainate-treated rats, which suggests that either the neuronal loss or the axon reorganization may play an important role in epileptogenesis (Buckmaster & Dudek, 1997). Thus, the kainate-treated rat represents a model of damage-induced plasticity and is a useful tool for investigating the morphological and functional changes that occur in the brain following seizure activity.

Genetic Models of Epilepsy. Genetic models of epilepsy represent a unique approach for studying the mechanisms underlying seizure predisposition and provide an opportunity to elucidate the underlying neurological dysfunctions that distinguish epileptogenesis from normal neurological activity. For example, two strains of genetically epilepsy prone rats (GEPRs) have been developed to study factors affecting seizure-predisposition: the GEPR-3 (a moderate seizure strain) and the GEPR-9 (a severe seizure strain). Both types of GEPRs can exhibit spontaneous seizures and can exhibit exaggerated responses to seizures induced by different modalities (e.g., sound) (Dailey et al., 1989). Other examples of genetic models of epilepsy include the DBA/2 mouse, which experiences audiogenic seizures (Chapman et al., 1987), the *el* (epilepsy like) mouse, which experiences seizures induced by vestibular stimulation (Mori, 1988), the tottering mouse, which displays focal motor seizures, and the epileptic baboon (*Papio Papio),* which experiences seizures induced by an intermittent light stimulus.

The Kindling Model of Temporal Lobe Epilepsy

To date, "kindling" represents one of the most widely studied animal models used to study epileptogenesis. The term kindling was proposed by Graham Goddard in 1969, and much of the early experimental kindling work was conducted by Goddard and his students. Although they originally studied kindling as a potential model of memory, it is utilized primarily as a model of temporal lobe epilepsy, particularly complex partial seizures with secondary generalization. Kindling can be defined as a progressive and permanent increase in electroencephalographic and behavioural seizure activity produced by spaced and repeated electrical stimulation of certain forebrain structures (Goddard et al., 1969). Kindling is also widely studied as a model of neuroplasticity. It leads to neuronal sprouting and synaptogenesis. As mentioned, however, there is some debate as to whether these growth effects are damage- or activation-induced.

A Brief Summary of Kindling Basics

Electrographic Correlates of Kindling. Electrical kindling typically involves the application of brief (1 or 2 second), low-intensity trains of biphasic electrical pulses at a frequency of 60 Hz once daily through a chronically implanted bipolar wire electrode (Goddard et al., 1969). These brief, low intensity pulses (generally ranging from 40-400 μA) are capable of triggering epileptiform afterdischarges (AD), which are recorded with an electroencephalograph (EEG).

The afterdischarges evoked in the early stages of kindling are weak: the amplitude of AD spikes is small, the AD duration is short, and there is little propagation to other brain sites

(Racine, 1972). With repeated kindling stimulations, however, there is an increase in the AD amplitude, duration, frequency, spike complexity, and propagation in both the primary (stimulated site) and in secondary brain sites (sites that are synaptically connected to the primary site) (Racine, 1972). Figure 1.4 shows representative examples of an AD evoked in the early (AD #3) and late (AD # 18) stages of amygdaloid kindling.

Moreover, as kindling progresses, animals also exhibit a lowering of the afterdischarge threshold (Racine, 1972). Afterdischarge threshold refers to the minimum amount of current required to elicit an afterdischarge. In addition, it has been shown that the occurrence of these epileptiform afterdischarges is critical for the development of kindling. Neural activity triggered by subthreshold (for AD) stimulation fails to produce kindling (Racine, 1972), although some facilitation of subsequent kindling can result from prior induction of LTP.

Behavioural Seizure Activity. Kindling also produces increases in the strength of the behavioural response (Goddard et al., 1969; Racine, 1972). The behavioural progression of kindling generally develops through 5 well-defined stages in the rat according to Racine's (1972) seizure classification scale: 1) chewing, 2) head clonus, 3) forelimb clonus, 4) clonic rearing and bilateral forelimb clonus, and 5) loss of postural control. An animal is generally considered to be fully kindled following two to three consecutive stage 5 seizures, and a common measure to evaluate the behavioural progression of kindling is the number of afterdischarges required to reach this fully kindled state (i.e., three consecutive stage 5 seizures) (Racine, 1972). Amygdaloid kindling, for example, typically requires 12-16 afterdischarges to reach the fully kindled state (Cain, 1992).

What is the effect of interstimulus interval? Interstimulus interval affects the rate of the

development of kindling. For example, interstimulus intervals ranging from 30 minutes (Racine et al., 1973) to up to seven days (Goddard et al., 1969) can be used to reliably produce kindling effects. Additional stimulations are invariably required to produce kindling if the interstimulus interval is 30 minutes compared to interstimulus intervals of 60 minutes or greater. Moreover, if interstimulus intervals are as brief as 15 minutes or less, kindling cannot be reliably induced with standard train parameters (Racine et al., 1973).

Does age affect kindling? The rate of kindling is also affected by age: very young rats (18 days old) kindle significantly faster than young adult rats (90 days old) (Moshe et al., 1983). By contrast, aged rats (at least one year old) require significantly greater number of stimulations to establish kindling compared to young adult rats (Fanelli & McNamara, 1986).

Is kindling permanent? The kindling-induced electrographic and behavioural changes outlined above are considered to be permanent (Racine, 1972). For example, it has been demonstrated that amygdaloid kindling persists with relatively little loss following intervals of up to twelve months (Goddard et al., 1969; Wada et al., 1974; Wada & Osawa, 1976). Moreover, following these long delays, kindled animals rarely require more than one or two stimulations to show maximal behavioural and electrographic responses.

Does kindling result in spontaneous epileptic events? Kindling also results in both ictal and interictal spontaneous events. Interictal spikes, an index of increasing epileptogenesis, can develop after only a few stimulations in many different brain structures, including the piriform and perirhinal cortices, amygdala, entorhinal cortex, ventral hippocampus, septal area and dorsal hippocampus (Racine et al., 1998). It is currently unclear, however, how long interictal spikes persist following termination of kindling. Although interictal spikes tend to decrease in

frequency following the last afterdischarge, they can appear in very relaxed animals up to six weeks following the last kindling stimulation (Racine, 1978).

Pinel and Rovner (1978) have also demonstrated that the development of an evoked generalized motor seizure (i.e., stage 5 seizures) is not the final stage of development of epileptogenesis during kindling. If kindling is continued beyond this stage, the electrographic and behavioural responses become increasingly stable, there is an increase in the frequency of rearing and falling during the motor convulsion, running and jumping fits often occur, interictal spiking increase in frequency, and spontaneous motor seizures eventually develop. It is important to note, however, that many stimulations (several hundred) are typically required to reach this degree of responsivity.

Species Differences. Kindling has been reliably demonstrated in a wide range of different species, including the rat, frog, reptile, mouse, rabbit, cat, monkey and baboon (Racine, 1978). To date, however, the rat is the most widely used species for kindling experiments.

Do different brain regions differ in reactivity? Goddard et al. (1969) have demonstrated kindling effects in a range of different brain structures and found that brain sites varied in the number of stimulations required to develop generalized seizures. Most recently identified pattern of reactivity is as follows: olfactory bulb and perirhinal cortex (fastest), pyriform cortex, amygdala, globus pallidus, entorhinal cortex, septal area, caudate putamen, ventral hippocampus, and dorsal hippocampus (slowest) (Racine et al., 1998). Kindling does not occur with stimulation of the superior colliculus, reticular formation or cerebellum (Racine, 1972). Sato et al. (1990) suggested that differences in kindling rates between different sites could be due to the following factors: 1) differences in their connections to the motor systems responsible for

generating the convulsive response, 2) differences in their connections to other forebrain regions that are critical for propagating the seizure discharge, or 3) differences in the reactivity of the stimulation sites themselves.

Differences also exist in the electrographic and behavioural patterns of the seizure activity evoked by the stimulation of the different brain regions. For example, an initial amygdala stimulation evokes a brief afterdischarge (10-15 seconds), whereas an initial dorsal hippocampal stimulation evokes a longer afterdischarge (25-60 seconds). To date, the amygdala is the most commonly used stimulation site, partly because it requires a small number of stimulations to produce fully kindled animals (Cain, 1992).

Transfer Kindling. After generalized motor responses develop as a result of kindling the primary site, synaptically connected brain sites require very few afterdischarges before generalized convulsions develop. This *transfer* effect was initially described by Goddard et al. (1969). They kindled the amygdala in the rat and then tested the contralateral amygdala or the ipsilateral septal area. Both test regions kindled with significantly fewer stimulations than required for the appropriate control groups. Transfer effects appear to be general phenomena and have been demonstrated in a variety of limbic and cortical sites (Racine, 1972; Racine, 1975; Cain, 1985).

Are there epileptogenic trigger areas during kindling? An epileptogenic trigger area refers to a site in the brain that is capable of evoking seizure activity following focal stimulation or is vulnerable to the development of epileptic activity because of its anatomical inputs and/or its intrinsic circuitry. To date, studies investigating the pyriform and perirhinal cortices have demonstrated that these structures are important in epileptogenesis. For example, Tseng and

Haberly (1988) studied cells in layer III of the pyriform cortex with regenerative depolarizing potentials and suggested that these cells may serve as pace makers for spontaneous discharge. Moreover, McIntyre et al. (1993) found similar burst-mode cells in the perirhinal cortex, and Kairiss et al. (1984) demonstrated that the pyriform cortex generates spontaneous interictal spikes during the early stages of kindling even when it is not the primary site. As noted earlier, other brain structures are also capable of generating interictal spikes. After the pyriform cortex, their order of reactivity is the amygdala, entorhinal cortex, ventral hippocampus, septal area and dorsal hippocampus (Racine et al., 1988).

Other studies have used *in vitro* brain slice preparations to investigate whether tissue from kindled brains is more reactive than tissue from non-kindled controls. For example, Kairiss et al (1984) tested hippocampal slices from kindled and non-kindled rats. They found that, in some slices from kindled rats, there was an increased tendency to show burst responses in a high potassium medium. By contrast, McIntyre and Wong (1986) found burst responses generated by stimulation were greatly enhanced in kindled slices compared to control tissue when they tested amygdala/pyriform slices. These data suggest that kindling produces greater increases in the inherent excitability of the amygdala/pyriform area compared to the hippocampal area.

1.5 What Are The Mechanisms Underlying Kindling?

Although it is becoming increasingly apparent that epileptogenic processes derive from an imbalance between excitatory and inhibitory controls in selected brain regions, there is still very little agreement regarding the mechanisms underlying kindling. Part of the difficulty arises from the fact that various patterns of neural activation, such as epileptiform events, can trigger a

range of different effects, any of which could contribute to the development and/or the maintenance of the epileptogenic state (Racine et al., 1998). These effects can be grouped in to three major categories: 1) enhanced connectivity within excitatory systems, 2) decreased or increased levels of function within inhibitory systems, and 3) changes in the intrinsic response properties of neurons that render them more susceptible to firing in the burst mode (Racine et al., 1998). Although all of these effects have been implicated in kindling epileptogenesis, no one effect has been shown to be critical. A brief summary of each of these effects is outlined below.

1) Enhanced Connectivity Within Excitatory Systems

Kindling-Induced Potentiation. It has been well-established that kindling can produce potentiation effects (Racine et al., 1983; Savage et al., 1984). For example, if stimulation pulses are applied to the amygdala before and after the completion of kindling, there will be an increase in the amplitude of the responses evoked in target structures. This increase in evoked response amplitude is referred to as kindling-induced potentiation or KIP (Racine et al., 1975; 1983). To date, KIP effects have been reported in several different pathways in the brain (Douglas & Goddard, 1975; Racine et al., 1975; Racine et al., 1983). Furthermore, it has been demonstrated that kindling in the hippocampus can lead to a saturation of potentiation, since it becomes difficult to produce LTP following induction of potentiation by kindling (Racine et al., 1983). This saturation effect suggests that KIP and LTP are non-additive. These findings raise the possibility that kindling may depend upon enhanced connectivity within excitatory systems.

Interactions Between KIP, LTP, and Kindling. Despite these apparent similarities, there are also substantial differences between KIP and LTP and between kindling and LTP. To begin

with, LTP in most subcortical sites has been shown to decay over a period of weeks, whereas KIP appears to be more robust and long-lasting (Racine et al., 1983). Also, pathways that do not readily support LTP have been shown to support KIP (Racine et al., 1983; Giacchino et al., 1984; Steward & Sutula, 1986). The lateral olfactory tract, for example, shows a reliable KIP effect and kindles rapidly, but does not show LTP in response to standard paradigms. By contrast, pathways both into and out of the hippocampus support high levels of LTP but they kindle slowly (Racine et al., 1983). It should be kept in mind, however, that these differences might simply reflect an optimal choice of parameters for KIP induction and a less optimal choice for LTP induction.

It has also been demonstrated that kindling and LTP can differentially affect components of the evoked response, such as the population EPSP and population spike in the dentate gyrus. The EPSP remains enhanced throughout the kindling procedure, whereas the population spike initially increases and then decreases in amplitude as kindling progresses (DeJonge & Racine, 1987; Racine et al., 1991). By contrast, LTP produces a greater enhancement of the population spike than of the population EPSP in this system (Abraham et al., 1985).

Although it has been shown that prior induction of LTP can significantly facilitate the rate of subsequent kindling (Sutula & Steward, 1987), the maximum savings is only about 50% (Racine et al., 1976; Sutula & Steward, 1987; Cain, 1989). The term "savings" refers to the fact that previously stimulated animals require significantly fewer stimulations to reach the fully kindled state compared to naive animals. Repeated LTP in the absence of afterdischarges, however, will not lead to a kindled state (Racine, 1978).

Permanence of LTP and Kindling. The major problem with LTP, as it is usually

expressed, as a kindling mechanism, is that it is too short lived (Cain, 1989). It has been demonstrated that LTP in most systems decays quickly (Racine et al., 1983; Racine et al., 1986; Sutula & Steward, 1987). As described earlier, kindled animals can be left unstimulated for up to several months and show little decay of the kindled state. By contrast, LTP decays to baseline levels, and no residual effects or savings have been reported following re-induction of LTP (DeJonge & Racine, 1985). As mentioned above, it is possible that non-optimal parameters have been used thus far in LTP experiments. There is at least one report suggesting that LTP in area CA1 of the hippocampus can remain stable for long periods of time (Staubli & Lynch, 1989), and LTP in the neocortex has recently been shown to be long-lasting.

In summary, these findings suggest that these potentiation effects may play a secondary role in the development of kindling. It remains to be seen whether some form of synaptic potentiation may also serve as a primary kindling mechanism.

Kindling and Mossy Fiber Sprouting. One way in which connectivity can be enhanced is via the growth of new connections. As outlined earlier, it has recently been demonstrated that kindling produces a reorganization of the synaptic connections of the hippocampal mossy fiber pathway (Sutula et al., 1988). It has been suggested that mossy fiber synaptic reorganization may influence the development and/or the maintenance of kindling by creating recurrent excitatory connections among granule cells. As a result, functional synaptic transmission in these synapses could increase recurrent excitation among granule cells, which may result in abnormal excitation and the generation of seizures (Tauk & Nadler, 1985). This possibility will be discussed further in a later section in this introduction.

2) Kindling and Inhibition

GABA is the primary inhibitory neurotransmitter in the mammalian brain, and it has been suggested that the generation of epileptiform activity may involve alterations in GABA-mediated synaptic inhibition (Taylor, 1988; Jefferys, 1990). Several researchers have investigated the possibility that kindling may be the result of a decrease in inhibitory transmission or a failure of inhibition, thus resulting in increased excitatory neurotransmission (Cornish & Wheal, 1989; Morimoto, 1989; Sloviter, 1987). This hypothesis is controversial because a clear reduction in GABAergic inhibition has not been reliably observed in kindling.

The GABA Hypothesis of Kindling. The GABA hypothesis of kindling states that kindling causes a permanent change in some part of the $GABA_A$ inhibitory system. This leads to a chronic, localized, and perhaps subtle change in GABAergic function, which produces some or all of the physiological abnormalities which characterize the kindled state (Burnham, 1989). There are two pieces of evidence supporting this hypothesis: 1) pharmacological blockade of the GABA system accelerates the rate of kindling, whereas pharmacological enhancement of GABA activity retards kindling, and 2) kindling has been reported to produce abnormalities in the GABA system (e.g., a decrease in glutamic acid decarboxylase (GAD), GABA levels are selectively decreased, GABA binding is decreased) (Burnham, 1989). By contrast, however, a large number of assay studies have actually showed no change or *increased* activity in GABA systems (Burnham, 1989).

Paired-Pulse Test. A common method used to measure neuronal inhibition in the intact preparation is the paired-pulse test. In this technique, the first pulse (conditioning pulse) activates inhibitory circuitry which causes a depression of the response to the second (i.e., test)

stimulus. The extent to which the second pulse is reduced relative to the first response indicates the amount of inhibition present. A lack of paired pulse depression during specific intervals of the conditioning and test stimuli suggests a reduction in synaptic inhibition (Tasker & Dudek, 1991).

Kindling-induced decreases in paired pulse inhibition have been consistently observed in the CA1 region of the hippocampus (King & Dingledine, 1985; Kapur et al., 1989; Kapur & Lothman, 1989; Kamphuis & Lopes da Silva, 1990; Kamphuis et al., 1991; Michelson et al., 1989). In addition to decreased paired pulse inhibition, Kamphuis et al. (1991) found increases in GABA exocytosis in CA1 region following kindling. Based on these results, they suggested that changes at the level of the GABA receptor complex may be involved in kindling-induced enhanced seizure susceptibility in the CA1 region. In addition, Kapur et al. (1989) also reported that kindling-induced GABAergic inhibition in the CA1 region was long-lasting, if not permanent. Reduced inhibition has also been reported in the amygdala (Rainnie et al., 1992), which is consistent with data showing a long-lasting decrease in the number of GABA-immunoreactive neurons in amygdaloid kindled rats (Callahan et al., 1991).

Inhibition actually appears to be potentiated, however, in several brain sites following kindling. These sites include the dentate gyrus (Tuff et al., 1983; Oliver & Miller, 1985; Maru & Goddard, 1987; Stringer & Lothman, 1989), the CA3 (Racine et al., 1983) and the pyriform cortex (Racine et al., 1991). Moreover, Milgram et al. (1995) have reported that paired-pulse inhibition remained elevated in the dentate gyrus until the kindling stimulations were stopped, even in animals that had received 300 kindling stimulations and had developed spontaneous seizures.

Taken together, data from paired pulse studies clearly show that inhibition is increased in some brain sites following kindling, whereas it is decreased in other brain regions following kindling.

Inhibition and Cell Loss. Recent research has suggested that seizure activity may produce the selective loss of some hippocampal cell populations, which may disturb the balance between excitation and inhibition in the remaining cells. For example, Sloviter (1987) proposed that hippocampal granule cell seizure activity irreversibly damages hippocampal interneurons and causes a persistent loss of recurrent inhibition. Although immunocytochemical staining showed that GABA containing neurons (i.e., basket cells) actually survived, there was a dramatic loss of somatostatin-containing interneurons and mossy cells (Sloviter, 1987). Sloviter proposed that some of these cells normally activate inhibitory neurons. A seizure-induced loss of this basket cell-activating system, then, would lead to a reduction of inhibition and could play a role in the development of the epileptic state. Cell loss and inhibition will be discussed in more detail in a later section.

In summary, although inhibition appears to be decreased by kindling in certain areas, it is increased in others. To account for these findings, it has been suggested that decreased inhibition could be the basis for epileptogenesis, whereas the increased inhibition may represent a compensatory mechanism in response to the seizure activity (Tasker & Dudek, 1991).

3) Intrinsic Properties of Neurons

It has been suggested that kindling produces changes in the intrinsic response characteristics of the affected cells, leaving them hypereactive to even normal levels of input

(i.e., neurons begin responding in an epileptiform burst mode) (King et al., 1985; Yamada et al., 1991). To date, the majority of work done on the burst response has focused on the hippocampus (Schwartzkroin, 1975; 1977; Schwartzkroin & Prince, 1977; 1978; Schwartzkroin & Wyler, 1980). For example, King et al. (1985) demonstrated that there is an increase in spontaneous epileptiform bursts recorded from the CA3/CA3 region in kindled rats compared to controls at 1 day and at 28 days following the last kindled seizure. They suggest that this long-lasting increase in burst firing may represent a mechanism for the initiation or the propagation of kindled seizures. Moreover, Zhao and Leung (1991) have shown that partial hippocampal kindling increases paired pulse facilitation and burst frequency in hippocampal CA1 neurons , which may contribute to an increase in seizure susceptibility

Aside from the hippocampus, epileptiform bursting has also been examined in other regions of the brain. For example, McIntyre and Wong (1986) found that electrical stimulation of the amygdala nuclei elicited burst responses in pyriform cells in slices from both control and kindled animals. The mean duration of the burst, however, was increased in the cells from the kindled animals compared to the controls. These data suggest that the increased excitability of the chronic epileptic site developed in vivo was retained in the excised amygdala-pyriform slice.

To date, the mechanisms underlying epileptiform bursting are not clear. It has been suggested that the synchronization of epileptiform bursts derives from recurrent excitatory synapses in the hippocampal region (Dudek et al., 1986; Wong et al., 1986; Traub & Jefferys, 1994). These excitatory connections may be sufficiently powerful that bursting activity may spread between synaptically connected neurons (Wong et al., 1986). An increase in the number or strength of these connections would represent a mechanism that falls into the first category

covered above. Computer simulation studies using neuronal networks where each cell is connected to more than one post-synaptic neuron have shown that: 1) bursting in one cell excites all its follower cells, and 2) the sequential recruitment of an increasing number of neurons leads to a simultaneous discharge of the population (Wong et al., 1986). Moreover, there is experimental evidence to support this proposed scheme for neuronal synchrony. For example, simultaneous paired intracellular recordings reveal that a burst of action potentials in a pre-synaptic cell can activate action potentials in a post-synaptic cell and the rhythm of spontaneous discharge in a neuronal population can be affected by the activity of a single neuron within the population (Wong et al., 1986).

It has also been suggested that calcium and calcium dependent currents may participate in the generation of epileptiform burst responses (Schwartzkroin & Wyler, 1980; Speckman et al., 1993) For example, measurements of stimulus-induced decreases in extracellular calcium in the CA1 region with ion-selective electrodes have shown an enhanced calcium influx into pyramidal neurons after kindling, indicating the involvement of calcium currents in kindling epileptogenesis. Moreover, a blockade of calcium currents can reduce or block convulsions. These findings raise the possibility that the development of a burst response may involve alterations in the intrinsic response characteristics of the membrane itself, although such responses could also result from enhanced connectivity between cells or reduced inhibition (Racine et al., 1982). To date, very little attention has been paid to the membrane response properties of neurons in kindled tissue.

1.6 More Recent Hypotheses Regarding Kindling Mechanisms

More recently, research has focused on neuronal loss and neural growth as potential mechanisms underlying kindling. These are specific examples of the more general categories reviewed above. Neuron loss, for example, would be expected to shift the balance towards excess excitation if a substantial portion of the lost neurons were inhibitory.

1) Neuronal Loss and Kindling

Cell Loss and TLE. Selective cell death associated with hippocampal sclerosis is observed in the majority of individuals with temporal lobe complex partial seizures. Although the cause of seizure-induced neuronal loss is unclear, three major hypotheses have been proposed in the clinical literature. One hypothesis is that neuronal loss is a benign consequence of seizures but is mechanistically unrelated to their cause. A second hypothesis is that neuronal loss pre-dates and causes the development of seizures. A third hypothesis is that neuronal loss is progressive and develops concurrently with temporal lobe epilepsy. Once produced, however, the lesion may become incorporated into and may become a necessary part of the seizure focus (Franck, 1993). To date, however, the functional significance of cell loss in the development and maintenance of epilepsy is unclear.

Cell Loss and Kindling. Although kindling has been reported to induce hippocampal neuronal loss, particularly in the hilar region of the dentate gyrus (Cavazos & Sutula, 1990; Cavazos et al., 1994), other researchers have failed to find any form of kindling-induced neuronal loss or degeneration (e.g., Khurgel et al., 1995). To address this issue, Bertram and Lothman (1993) measured both hilar cell counts and hilar volume in both kindled and control rats.

Although they found that hilar cell density was decreased following kindling, this decrease in cell density was due to an expansion of hilar volume. These findings provide support for the notion that kindling may not produce actual neuronal loss. Rather, kindling may produce an increase in hippocampal volume, which has been previously misinterpreted as neuronal loss. Additional support for this possibility is provided by Watanabe et al. (1996), who found a significant increase in hilar volume with no evidence of cell loss using mice that carry a null mutation of the immediate early gene c-fos . It has been suggested that the transient expression of c-fos following neuronal activity is linked to lasting modifications in neuronal structure and function in the mammalian nervous system (Watanabe et al., 1996).

Although the cause of these kindling-induced changes in hilar volume is currently unknown, a few possibilities have been proposed such as changes in glial cell size or changes in glial cell number (Bertram & Lothman, 1993). Moreover, Khurgel et al. (1995) showed that kindling can activate astrocytes in the absence of neuronal degeneration. These data suggest that astrocytes can be activated by factors that are not related to neuronal degeneration and that some forms of seizure-induced plasticity may be activation-induced. To date, however, very little research has been done on these kindling-induced changes in hilar area. No one has quantitatively examined whether reactive gliosis can account for hilar area changes, and the time course for kindling-induced hilar area changes is not known (i.e., it is not known whether kindling-induced changes in hilar area are transient or permanent). If reactive gliosis can account for kindling-induced changes in hilar area and if both follow a similar time course, this would provide additional support for the notion that kindling does not produce neuronal degeneration. To address this, we investigated the time course for kindling-induced changes in hilar area and

gliosis in Study 4 (Adams et al., 1998).

2) Mossy Fiber Sprouting and Kindling

As outlined earlier, both TLE and kindling have been reported to induce a reorganization of synaptic connections of the hippocampal mossy fiber pathway. The mossy fibers are the axons of the hippocampal dentate granule cells. In the normal brain, the mossy fibers establish synaptic contacts with interneurons in the inferior region and with a limited region of the apical dendrites of the CA3 pyramidal neurons. Following kindling, the mossy fibers form an aberrant band of terminations in both the supra granular layer of the fascia dentata and the CA3 infrapyramidal layer (stratum oriens).

The mossy fiber terminals are rich in zinc and are most commonly studied using the Timm stain, a histochemical technique used for the visualization of heavy metals. Kindling-induced alterations in Timm granule density are apparent early in the course of kindling, increase with repeated stimulations, and are permanent (Cavazos et al., 1991). Aside from the Timm stain, other histochemical markers of the mossy fiber pathway include high affinity kainic acid receptors (Represa et al., 1989) and dynorphin-A immunoreactivity (de Lanerolle et al., 1989; Houser et al., 1990).

Although the functional significance of kindling-induced mossy fiber sprouting is not clear, two major hypotheses exist. One hypothesis is that mossy fiber synaptic reorganization may influence the development and or the maintenance of kindling by creating excitatory synapses on dendrites of other granule cells (Cronin et al., 1992). As a result, functional synaptic transmission in these synapses could increase recurrent excitation among granule cells,

which may result in abnormal excitation and the generation of seizures (Tauck & Nadler, 1985). Three pieces of evidence support this hypothesis: 1) following entorhinal cortex lesions or in epileptic tissue, granule cell axons form synaptic contacts with granule cell dendrites (Frotscher & Zimmer, 1983; Babb et al., 1991; Represa et al., 1993; Franck et al., 1995; Okazaki et al., 1995), 2) following sprouting, antidromic stimulation of granule cell axons evokes multiple discharges, consistent with recurrent excitation (Tauk & Nadler, 1985; Cronin et al., 1992; Masukawa et al., 1992; Wuarin & Dudek, 1996), and 3) current source density analysis shows evidence for recurrent excitation among granule cells following axon reorganization (Golarai & Sutula, 1996).

Alternatively, it is possible that sprouting granule cell axons target interneurons (Sloviter, 1992). In this case, granule cell axon reorganization would not result in recurrent excitatory circuits, but in recurrent inhibition (Sloviter, 1992). This seems unlikely, however, given the evidence for recurrent excitation outlined above.

Another possibility is that kindling-induced mossy fiber sprouting may simply be a reactive alteration that parallels the primary causal events that are responsible for the emergence and the progression of kindling. By this argument, kindling-induced mossy fiber sprouting may simply be an epiphenomenon of the kindling process and may have no functional role in either the development and/or the maintenance of the kindled state.

3) Cell Loss, Mossy Fiber Sprouting, and Kindling

More recently, research has focused on the relationship between kindling, kindling-induced neuronal loss, and kindling-induced mossy fiber sprouting. Specifically, it has been

suggested that kindling-induced neuronal loss may be a trigger for kindling-induced mossy fiber sprouting. This possibility is not unreasonable, given that the neural growth is elicited in response to neuron loss in the mature CNS (Cotman, 1985) and that selective elimination of an afferent pathway can result in sprouting of uninjured neighbouring neurons in the brain (e.g., Raisman, 1969; Lynch et al., 1973; Zimmer, 1974).

As noted earlier, however, kindling has been reported in the absence of neuronal loss or degeneration (e.g., Racine et al., 1975; Bertram & Lothman, 1993; Qiao & Noebels, 1993; Watanabe et al., 1996). This raises the possibility that cell death may not be necessary for hippocampal mossy fiber sprouting and provides support for the hypothesis that mossy fiber sprouting may be activation-induced. To investigate the relationship between neuronal death and mossy fiber sprouting, Stringer et al. (1997) elicited repeated seizures of the hippocampal - parahippocampal circuit, a paradigm known as maximal dentate activation. A silver impregnation stain was used to assess neuronal loss, an assay for damaged DNA was used to detect damaged or dying neurons, and immunohistochemistry for 72 kDa heat shock protein was used to detect any neurons that had suffered potentially injurious stress. Mossy fiber sprouting was assessed using the Timm stain. Stringer et al. (1997) found that repeated seizures of the hippocampal-parahippocampal circuit can cause mossy fiber sprouting in the absence of any evidence of cell death. These findings suggest that, using the maximal dentate activation paradigm, alterations in neuronal excitability in the hippocampus can result in the growth of axonal processes in the absence of neuronal damage.

These data raise a number of interesting issues. Can cell loss and mossy fiber sprouting similarly be dissociated using the *kindling* model of epileptogenesis? Maximal dentate activation

involves reverbatory seizure activity within the hippocampal-parahippocampal circuit, whereas kindling involves partial complex seizures with secondary generalization. It is possible that different mechanisms may underlie mossy fiber sprouting in the two paradigms. To date, no research has convincingly demonstrated that kindling-induced mossy fiber sprouting can proceed in the absence of damage. This issue was addressed in Study 1 (Adams et al., 1997a).

The data from the Stringer et al (1997) study also raise another interesting question: if mossy fiber sprouting is activation-induced, can other forms of neural activation (i.e., non-epileptogenic stimulation) also trigger mossy fiber sprouting? If so, this would provide additional evidence that seizure-induced mossy fiber sprouting could occur in the absence of neural damage. We addressed this question in Study 3 by investigating whether LTP trains, which do not produce neural damage, could trigger mossy fiber sprouting (Adams et al., 1997b).

1.7 If Seizure Activity Induces Neural Growth, What Triggers This Response?

There is a growing body of evidence showing that high levels of neuronal activity are associated with changes in gene expression. It has been well-established that seizure activity induces the transcriptional activation of immediate early genes (IEGs), such as c-fos (Morgan & Curran, 1991; Keissling & Gass, 1993; Labiner et al., 1993). Moreover, there is evidence that immediate early genes, c-fos in particular, are a part of a chain of molecular events that results in the sprouting of the mossy fibers in seizure models (Watanabe et al., 1995). This seizure-induced transcriptional activation of c-fos and other IEGs is followed by the expression of genes encoding neurotrophic factors (e.g., nerve growth factor [NGF], brain-derived neurotrophic

factor [BDNF], basic fibroblast growth factor [bFGF]) (Gall & Isackson, 1989; Enfors et al., 1991; Gall, 1993; Gall et al., 1994), neurotrophic factor receptors (Bengzon et al., 1993; Bugra et al., 1994) and axonal growth associated proteins such as GAP-43 (Bendotti et al., 1993; Meberg et al., 1993). Given that neurotrophic factors exert morphoregulatory effects on hippocampal neurons (Walicke et al., 1986; Mattson et al., 1989; Ip et al., 1993; Patel & McNamara, 1995), it has been proposed that seizure-induced expression of these genes may produce mossy fiber sprouting (Watanabe et al., 1996).

NGF is a Member of the Neurotrophin Family

The Neurotrophins. The neurotrophins are a family of neurotrophic polypeptides that promote the growth, survival and differentiation of selected neurons in the peripheral and central nervous systems (Korsching, 1986). Members of the neurotrophin family include nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3 (NT-3), neurotrophin-4/5. (NT-4/5), and neurotrophin-6 (NT-6), which was recently discovered in fish (Lindholm, 1997). In general, these neurotrophic factors are all structurally related and act on distinct, but overlapping, populations of neurons to regulate neurotransmitter synthesis, promote cell survival, and cause neurite outgrowth (Levi-Montalcini, 1987; Korsching, 1986). Many of these neurotrophic factors are not only critical for the normal development and maintenance of the nervous system, but also play a regenerative role in the nervous system's ability to respond to injury (Ip & Yancopoulos, 1994). For present purposes, the discussion will be limited primarily to NGF in the CNS. NGF is currently recognized as a trophic factor for neostriatal and basal forebrain cholinergic neurons.

The Neurotrophin Receptors. There are two different types of neurotrophin receptor: the

low affinity p75 receptor and the high-affinity tyrosine kinase (trk) receptor (Hempstead et al., 1991). The p75 receptor is a 75 kDa transmembrane glycoprotein (Radeke et al., 1987). All members of the neurotrophin family bind to p75 with similar low affinity dissociation constants ($Kd=10^{-9}$).

The neurotrophins show high-affinity interactions ($Kd=10^{-11}$) with a family of tyrosine kinase receptors known as trk A, trk B, and trk C. The three known trk receptors display overlapping specificities for the neurotrophins. NGF mediates its effects via trk A. BDNF and NT-4/5 activate the Trk B receptor, and NT-3 binds preferentially to Trk C, although it can also activate the Trk B receptor in certain cell types (Ip et al., 1993). The Trk proteins possess a tripartite structure consisting of an extracellular domain for ligand recognition, a single transmembrane domain, and a cytoplasmic tail which contains the tyrosine kinase for initiating the signaling cascade (Schneider & Schweiger, 1991).

NGF and Its Receptors. Receptor dimerization is believed to be necessary for high-affinity neurotrophin binding and the resultant trophic activity (Kolbeck et al., 1994). Although both p75 and Trk A receptors are capable of homo- and hetero- dimerization, the role of p75 in the formation of the high-affinity receptor and the resultant trophic activity remains unclear. Although it has been postulated that the high-affinity binding of NGF requires the co-expression of p75 (Hempstead et al., 1991), other studies have demonstrated that the p75 receptor is not necessary for signaling (e.g., Meakin & Shooter, 1992). Thus, although Trk A is clearly a functional NGF receptor that mediates some of the signal transduction processes initiated by NGF, the precise functions of the p75 neurotrophin receptor in NGF-mediated signaling is not known.

Distribution of Trk A in the CNS. As outlined earlier, it is believed that NGF mediates its biological effects via Trk A receptors. It has been suggested that Trk expression is key to neurotrophin responsiveness and that the localization of Trk expression may be used to define the biological functions of neurotrophins, such as NGF.

In the CNS, it has been well-established that Trk A expression is localized primarily to the cholinergic neurons in the basal forebrain and the neostriatum (Holtzman et al., 1992; Steininger et al., 1993). Other populations of noncholinergic neurons in the CNS have also been reported to express Trk A (i.e., the paraventricular anterior and reuniens thalamic nuclei, the rostral and intermediate subnuclei of the interpenducular nucleus, the prepositus hypoglossal nucleus, and the area postrema) (Holtzman et al., 1995). It should be noted that, aside from Trk A receptors present on the cholinergic projections from the basal forebrain to the hippocampus, there is little evidence for Trk A expression in the hippocampus

NGF and Its Role in the Basal Forebrain Cholinergic System. In the brain, NGF is widely recognized as a trophic factor for the basal forebrain cholinergic neurons. In the rat, the basal forebrain cholinergic system consists of acetylcholine-synthesizing neurons that are distributed across several brain regions, including the medial septal nucleus, the diagonal band of Broca, the magnocellular preoptic area, substantia innominata, the nucleus basalis of Meynert and the nucleus of the ansa lenticularis (Paxinos & Butcher, 1985). Cholinergic neurons of the medial septum and the diagonal band of Broca project predominantly to the hippocampus and olfactory brain regions (Woolf et al., 1984; Zaborsky et al., 1986), whereas neurons of the nucleus basalis of Meynert project primarily to the neocortex (Paxinos & Butcher, 1985).

It has been well-established that NGF plays a critical role in the survival and function of

the cholinergic neurons in the basal forebrain. For example, the application of exogenous NGF increases both the activity of choline acetyltransferase (ChAT) (i.e., the enzyme that catalyzes the formation of acetylcholine) (Gnahn et al., 1983; Mobley et al., 1986; Williams & Rylett, 1990) and the levels of mRNA for ChAT in basal forebrain cholinergic neurons (Higgins et al., 1989). Chronic treatment with NGF also enhances high-affinity choline uptake and acetylcholine release in the forebrain (Williams & Rylett, 1990; Rylett & Williams, 1990). Moreover, infusions of NGF prevents the loss of cholinergic neurons that occurs when their projections to the hippocampus are transected (Hefti et al., 1986; Williams et al., 1986; Kromer, 1987).

It has also been shown that the basal forebrain cholinergic system is dependent upon distant cortical targets as the source of NGF (Lauterborn et al., 1991). NGF is generally considered to be a target-derived molecule, indicating that it is synthesized by the target cells of projecting axons and binds to specific receptors localized in the terminal membranes of these axons. The NGF receptor complex is then internalized and transported retrogradely back to the cell body where it initiates response cascades. As outlined earlier, basal forebrain cholinergic neurons project to both the hippocampus and the neocortex. It has been established that these target fields contain the highest levels of NGF mRNA and protein in the brain (Korsching et al., 1985; Shelton & Reichardt, 1987; Whittemore et al., 1986). By contrast, the basal forebrain has relatively low levels of NGF mRNA and higher levels of NGF protein. This suggests that NGF protein may be synthesized in target brain regions and may then be retrogradely transported to the basal forebrain (Korsching et al., 1985; Shelton & Reichardt, 1986). Evidence to support this possibility is provided by data showing that when injected into the hippocampus, labeled NGF is retrogradely transported by medial septum/diagonal band cholinergic neurons (Schwab et al.,

1979), by nucleus basalis of Meynert cholinergic neurons when injected into the neocortex (Seiler & Schwab, 1984), and by diagonal band neurons when injected into the olfactory bulb (Altar & Bakhit, 1991). Moreover, transection of the fimbria fornix (a projection from the hippocampus to the basal forebrain) leads to increased levels of NGF within the hippocampus (Gasser et al., 1986; Larkfors et al., 1987).

NGF is also involved in damage-induced sprouting in the adult CNS. For example, when the fimbria fornix (FF) is transected or the medial septal area is lesioned, the cholinergic fibers from the basal forebrain which normally innervate the hippocampus degenerate. As a result, the sympathetic fibers of the superior cervical ganglion (SCG) penetrate the CNS to innervate the terminal zone of the mossy fibers, which coincides with the terminal area of the cholinergic fibers eliminated by the FF lesion (Loy & Moore, 1977; Stenevi & Bjorklund, 1978). NGF has been implicated in this sprouting response (Crutcher, 1987). As described earlier, NGF levels are increased in the hippocampus following FF lesions. It has been suggested that this NGF accumulation in the hippocampus following FF transection or medial septal lesions may set up a gradient that promotes the growth of sympathetic fibers toward the source of NGF. Evidence to support this possibility is provided by the finding that the ingrowth of sympathetic fibers from the SCG can be retarded by anti-NGF infusions (Springer & Loy, 1985).

In addition, transection of the perforant path (the major pathway connecting the entorhinal cortex and the hippocampus) destroys the entorhinal inputs to the outer two thirds of the molecular zone of the dentate granule cells (Lynch et al., 1972), and triggers the cholinergic fibers from the inner one third of the molecular zone to sprout and fill in the deafferented outer zone (Lynch et al., 1972). It has been established that NGF activity is increased in the dentate

gyrus following perforant path lesions, which suggests that the sprouting observed in the outer molecular zone could be mediated by NGF. Additional support for this possibility is provided by data showing that the administration of exogenous NGF evokes axonal growth within the lesioned septohippocampal pathway (Hagg et al., 1990; Junard et al., 1990), whereas anti-NGF inhibits collateral sprouting of the septohippocampal fibers following entorhinal cortex lesions (Van der Zee et al., 1992).

Taken together, these findings show that NGF plays a critical role in the maintenance and functioning of the basal forebrain cholinergic neurons. More specifically, NGF is not only important for the survival of adult cholinergic neurons, but it can also induce sprouting following damage in the mature CNS.

Neurotrophins, Neuroplasticity, and Kindling

Neurotrophins Play a Role in Neuroplasticity. One of the first indications that neurotrophins may play a role in neuronal plasticity in the intact adult central nervous system came from observations that BDNF and NGF are regulated by neuronal activity in hippocampal neurons. For example, levels of NGF and BDNF mRNA are upregulated by depolarization (Zafra et al., 1990), following seizure activity (Gall & Isackson, 1989; Enfors et al., 1991) and following stimulation of neurons with glutamate receptor agonists (Zafra et al., 1990; 1991).

Neurotrophin levels in the brain are also regulated by other forms of stimuli. For example, exposure to light rapidly upregulates BDNF mRNA levels in the rat visual cortex, whereas rearing rat pups in darkness decreases levels of BDNF in the visual cortex (Castren et al., 1992). Environmental enrichment increases levels of NT-3 in the visual cortex and

hippocampus (Torasdotter et al., 1996). Kindling and the induction of LTP also lead to increased levels of BDNF and NGF in the rat hippocampus (Castren et al., 1993; Patterson et al., 1992). Taken together, these findings demonstrate that the concept of an activity-dependent regulation of neurotrophic factors is well established.

Neurotrophic Factors and Their Receptors are Involved in Kindling. Seizure activity transiently increases NGF and BDNF mRNA expression in cortical and hippocampal neurons (Gall & Isackson, 1989; Isackson et al., 1991). Moreover, *in situ* hybridization has shown that hippocampal kindling leads to an increase in NGF and BDNF mRNA in the dentate gyrus and in the parietal and pyriform cortices (Enfors et al., 1991). There is also an increase in NGF protein in the rat forebrain after kindling-induced seizures (Bengzon et al., 1992). In addition, Trk B and Trk C mRNA expression is increased in the hippocampus following kindling, whereas Trk A mRNA levels were unaffected by kindling (Bengzon et al., 1993).

Intraventricular administration of an antibody to NGF has been shown to delay amygdaloid kindling (Funabashi et al., 1988; Van der Zee et al., 1995) and block mossy fiber sprouting (Van der Zee et al., 1995). In addition, intraventricular administration of BDNF blocks hippocampal kindling (Larmet et al., 1995). Taken together, these data provide support for the hypothesis that neurotrophins are regulated by seizure activity, particularly NGF, and raise the possibility that they may mediate kindling-induced mossy fiber sprouting. Given that neurotrophins can promote cell survival and enhance neural sprouting, it is possible that neurotrophin administration (i.e., NGF) during kindling may protect hilar cells from kindling-induced damage while enhancing mossy fiber sprouting. If so, this may provide a way to dissociate kindling-induced mossy fiber sprouting from kindling-induced cell loss. This

possibility was addressed in Study 1 (Adams et al., 1997a). If NGF does play a role in kindling-induced mossy fiber sprouting, it is possible that this response is mediated by the cholinergic system. This possibility was addressed in Study 2.

1.8 Experimental Rationale

The general objective of this thesis was to gain a better understanding of hippocampal plasticity as expressed by kindling-induced structural changes in the dentate gyrus and mossy fiber system.

Specific Objectives and Rationales:

Study 1 (Chapter 2); Adams et al. (1997a)

The objective for Study 1 was to determine whether cell loss is necessary for mossy fiber sprouting in kindling by using the neurotrophin, NGF, as a tool to dissociate kindling-induced mossy fiber sprouting and kindling-induced neuronal loss.

Rationale. Given that neurotrophins can promote cell survival and axonal sprouting, there is the possibility that neurotrophin administration during kindling may protect hilar cells from damage and thereby provide a tool for dissociating kindling-induced hilar cell loss and kindling-induced mossy fiber sprouting. In this study, we tested the hypothesis that the pairing of activation and NGF would enhance mossy fiber sprouting while reducing decreases in hilar cell density associated with kindling in adult male rats. We also evaluated hilar area measurements to determine whether kindling-induced decreases in hilar cell density could be due to kindling-induced increases in hilar area.

Throughout this thesis, mossy fiber sprouting was investigated using the Timm staining

method coupled with computer-assisted, semi-quantitative densitometry. Hilar neuronal density was evaluated using Cresyl Violet staining and a camera lucida attached to a light microscope. Hilar area measurements were evaluated using Cresyl Violet staining and computer-assisted imaging.

Study 2 (Chapter 3)

Study 1 showed that the intraventricular administration of NGF accelerated the progression of kindling and increased mossy fiber sprouting. The objective of Study 2 was to investigate how NGF mediated these effects. Specifically, did NGF increase kindling rates and increase kindling-induced mossy fiber sprouting via the cholinergic system?

Rationale. As outlined earlier, it has been well-established that NGF mediates its biological effects primarily via its high-affinity receptor, Trk A. Also, given that there is very little evidence for the existence of Trk A receptors in the hippocampus, it is unlikely that NGF can mediate its effects directly on hippocampal neurons. Alternatively, it is possible that NGF acts indirectly via the high-affinity Trk A receptors on cholinergic neurons in the basal forebrain. Two pieces of evidence support this possibility: it has been well-established that these neurons are sensitive to NGF, and these same cholinergic systems are involved in kindling (i.e., it has been shown that cholinergic agonists can produce kindled seizures (e.g., Wasterlain et al., 1982) whereas cholinergic antagonists retard the development of kindling (e.g., Westerberg & Corcoran, 1987)). To address this possibility, we evaluated the effects of a cholinergic agonist (10 mg/kg; pilocarpine hydrochloride) and antagonist (15 mg/kg; scopolamine hydrochloride) on kindling-induced mossy fiber sprouting following perforant path kindling.

Study 3 (Chapter 3); Adams et al. (1997b)

The objective for Study 3 was to investigate whether mossy fiber sprouting is activation-induced as opposed to damage-induced using LTP trains (LTP trains are a form of non-epileptogenic stimulation that do not produce damage).

Rationale. To address this question, we investigated whether non-epileptogenic, non-damaging LTP stimulation delivered to the perforant path could induce mossy fiber sprouting compared to implanted, match-handled control rats. Stimulation parameters were selected that reliably induce LTP in the dentate gyrus.

Study 5 (Chapter 4); Adams et al. (1998)

The objective for Study 4 was to determine whether reactive gliosis is a potential mechanism underlying kindling-induced changes in hilar area.

Rationale. As reported in the literature, we observed kindling-induced changes in hilar area in Study 1. To date, very little research has been done to investigate this form of kindling-induced plasticity. The purpose of this study was to investigate whether reactive gliosis could account for kindling-induced changes in hilar area. To accomplish this, we investigated the time course for kindling-induced changes in reactive gliosis and kindling-induced changes in hilar area following perforant path kindling. This would allow us to evaluate whether kindling-induced reactive gliosis occurs with a time course consistent with kindling-induced structural changes. To accomplish this, hilar area and reactive gliosis were evaluated at 7 days, 1 month, and 2 months post-kindling compared to controls.

To evaluate kindling-induced reactive gliosis, the density of glial fibrillary acidic protein

(GFAP) immunostaining levels in the hilar region was evaluated at 7 days, 1 month and 2 months post-kindling compared to controls. Density of GFAP immunostaining levels was quantified using computer-assisted imaging.

FIGURES and CAPTIONS

Chapter 1

Figure 1.0 Schematic of the rat hippocampus in the coronal plane. The hippocampal gyrus is divided into three subfields: the CA1, CA2 and CA3. The superior portion of the hippocampus corresponds to CA1, whereas area CA3 is partly bounded by the dentate gyrus and represents the inferior portion of the hippocampus. Area CA2 serves as the transition between CA3 and CA1 (Amaral & Witter, 1995).

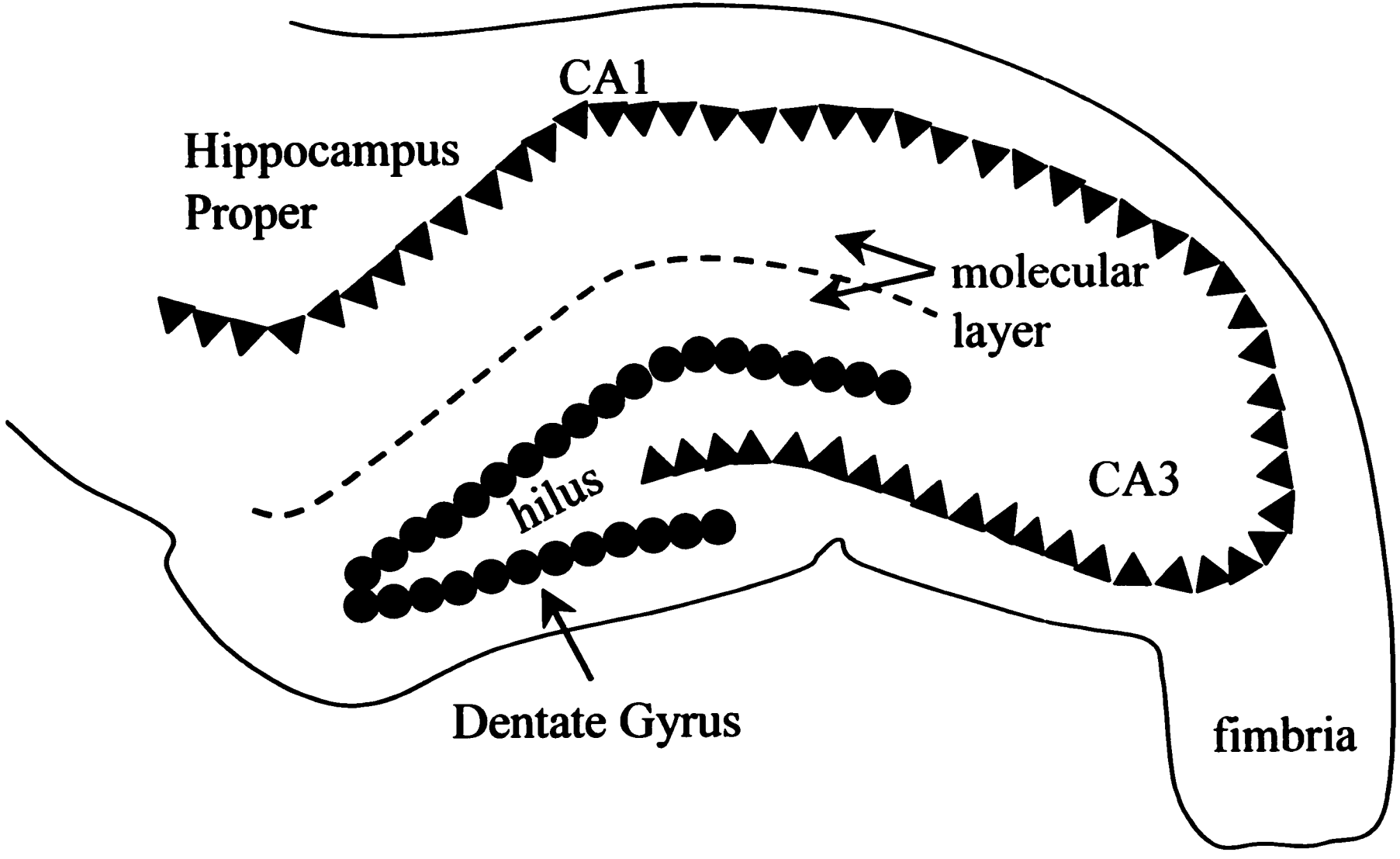

CA1
Hippocampus
Proper
molecular
layer
hilus
CA3
Dentate Gyrus
fimbria

Figure 1.1 (Above the dotted line) Schematic of the strata of the hippocampal gyrus: the stratum oriens, the stratum pyramidale, the strata radiatum and the stratum lacunosum moleculare. The principle cell type in the hippocampal gyrus is the pyramidal cell and the cell bodies of these neurons form the stratum pyramidale. These pyramidal cells typically have large apical dendrites, along with several shorter basal dendrites and a single axon located at the opposite pole. These basal and apical dendrites of the pyramidal cells terminate in different strata on either side of the pyramidal cell layer: the basal dendrites of the pyramidal cells ramify in the stratum oriens, whereas the apical dendrites branch extensively into the stratum lacunosum-moleculare. The shafts of the apical dendrites, however, are located in the stratum radiatum. In CA3, there is also an additional stratum, referred to as the stratum lucidum, which is located between the pyramidal cell layer the stratum radiatum.

(Below the dotted line) The dentate gyrus can be divided into three layers: the molecular layer, granule cell layer and the polymorph layer (Amaral & Witter, 1995). The molecular layer consists of the granule cell dendrites. The granule cells constitute the next layer, which consists of a narrow band of densely packed granule cells. The axons of the dentate granule cells are the mossy fibers. The polymorph layer lies between the upper and lower blades of the granule cell layer, a region referred to as the hilus. The polymorph layer contains a range of different cell types, including interneurons, stellate cells, mossy cells, and basket cells (Amaral & Witter, 1995).

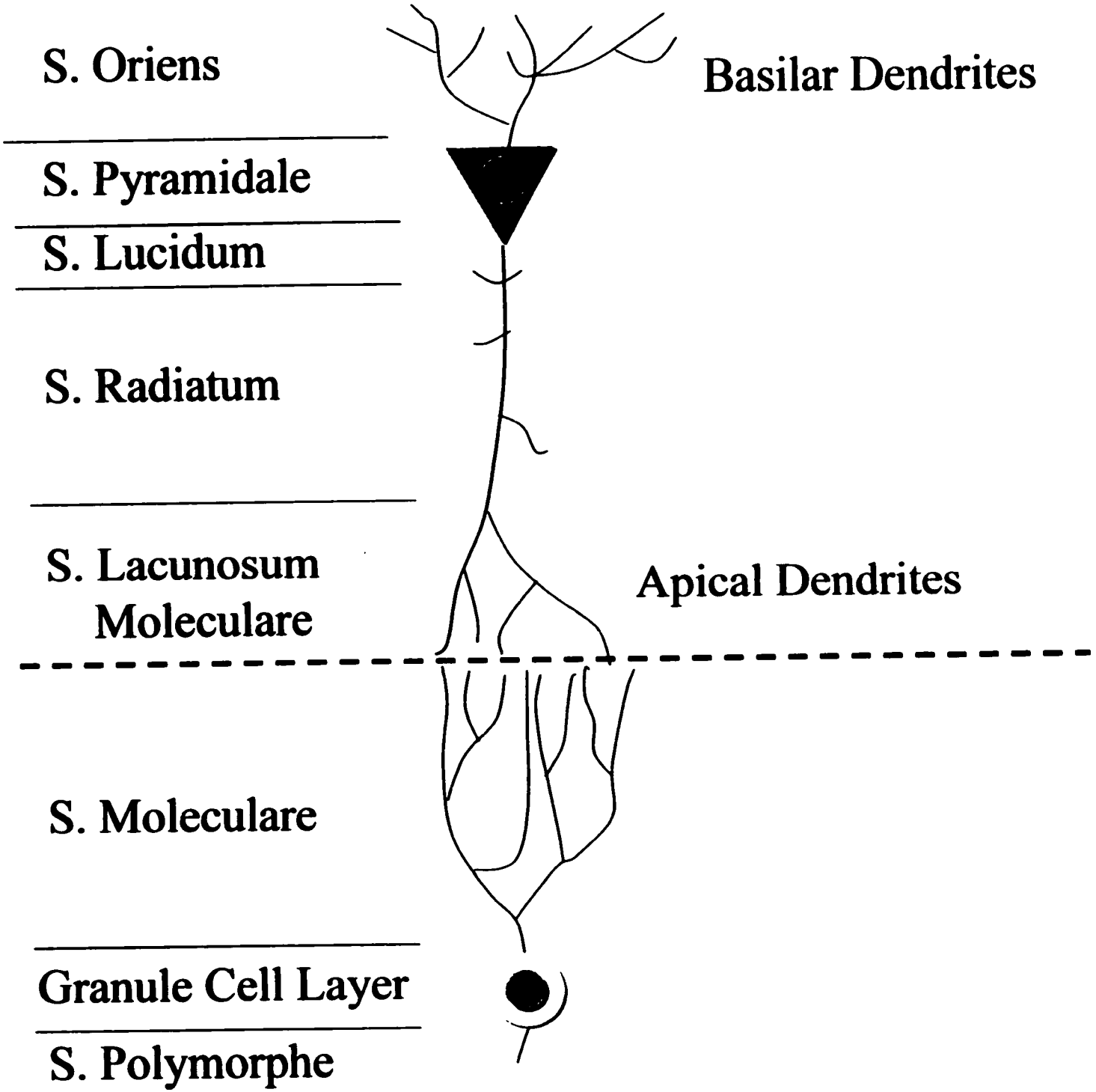

S. Oriens
Basilar Dendrites
S. Pyramidale
S. Lucidum
S. Radiatum
S. Lacunosum
Moleculare
Apical Dendrites
S. Moleculare
Granule Cell Layer
S. Polymorphe

Figure 1.2 Representative example of a field potential, including a population EPSP and population spike, recorded from a bipolar electrode implanted in the rat hippocampal dentate gyrus following perforant path stimulation before (solid line) and after LTP induction (dotted line). Vertical calibration= 2.0 mV; horizontal calibration= 5.0 msec.

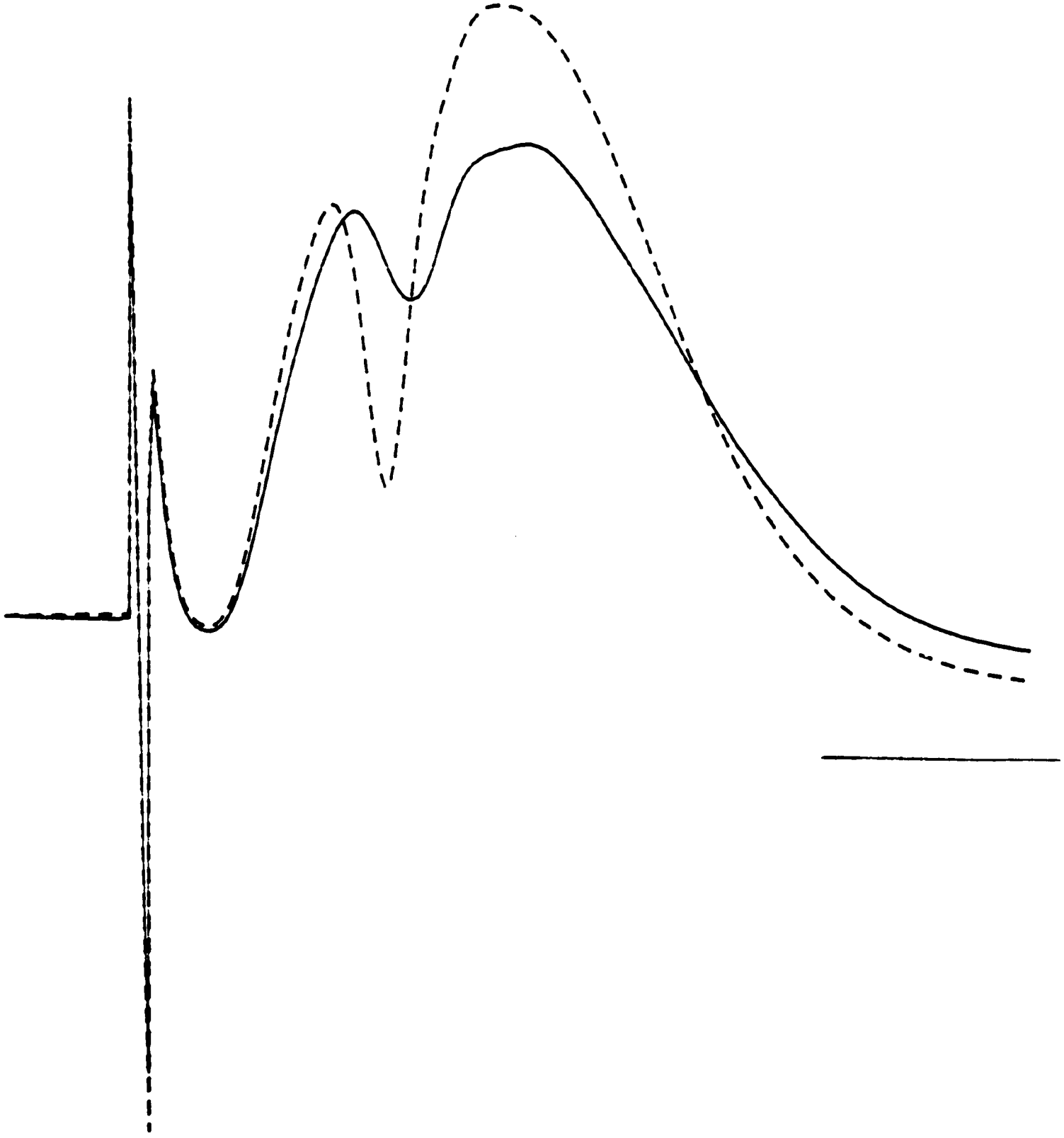


Figure 1.3 Schematic of a portion of the hippocampal region, including the dentate gyrus and area CA3. In the normal brain, axons of the dentate granule cells (mossy fibers) synapse with the apical dendrites of the CA3 pyramidal cells. Seizure activity in human temporal lobe epilepsy is associated with aberrant sprouting of the mossy fiber axons of the hippocampal dentate granule cells (Babb et al., 1991).

(mf= mossy fibers; gc= granule cell; pyr= pyramidal cell)

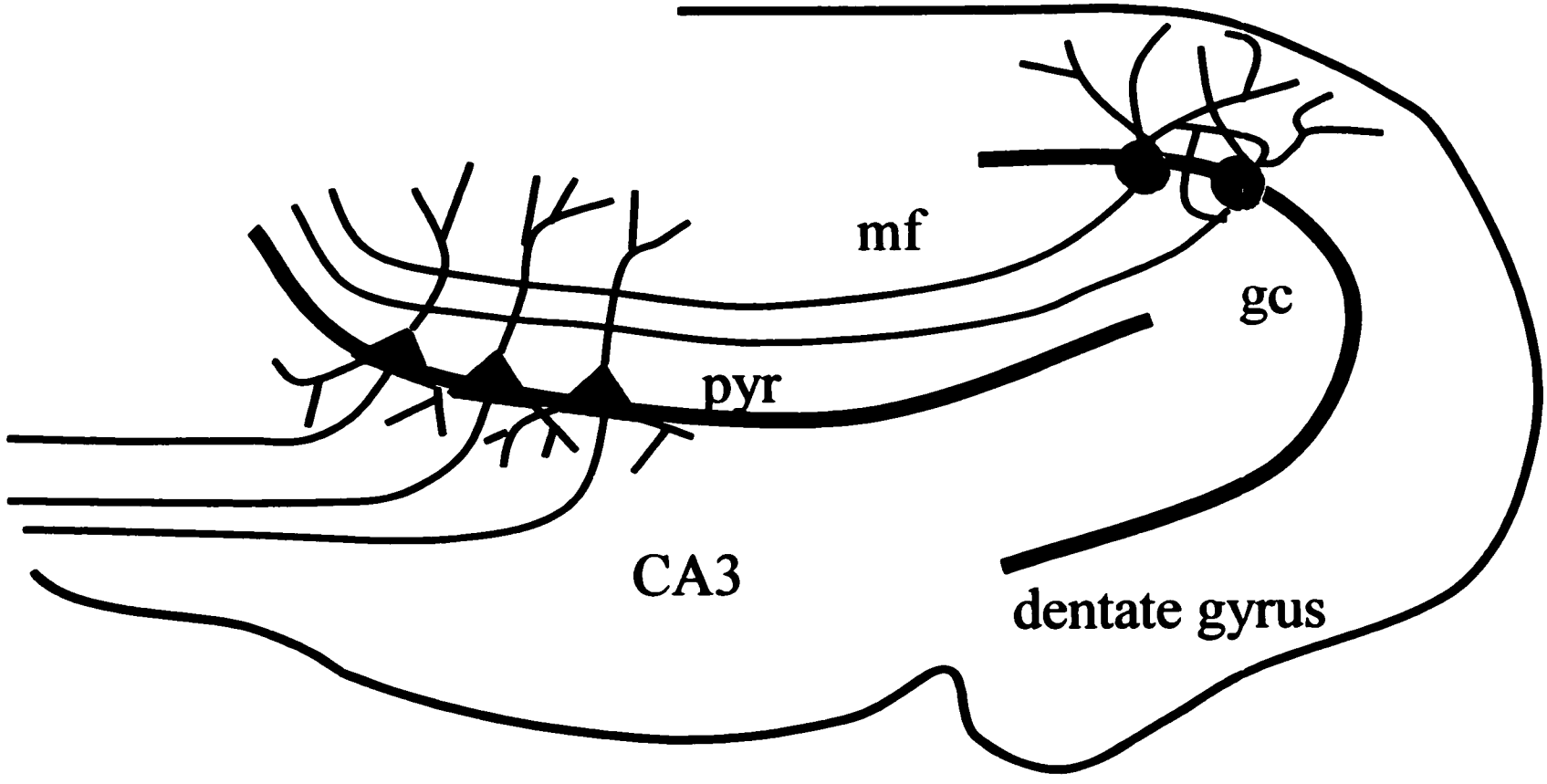
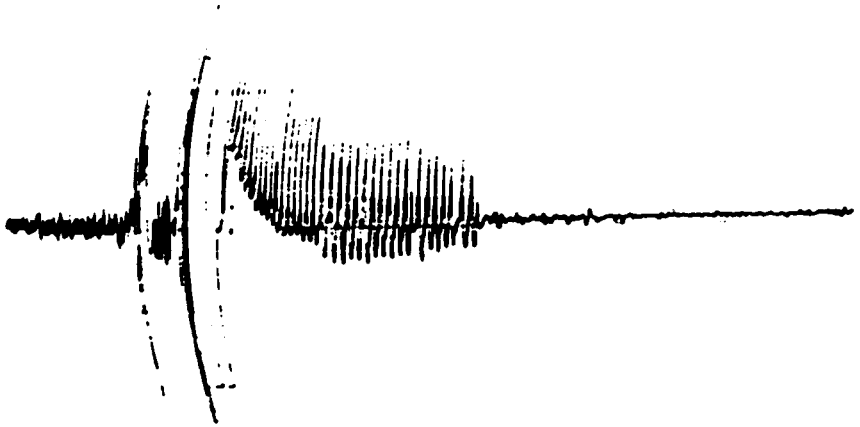
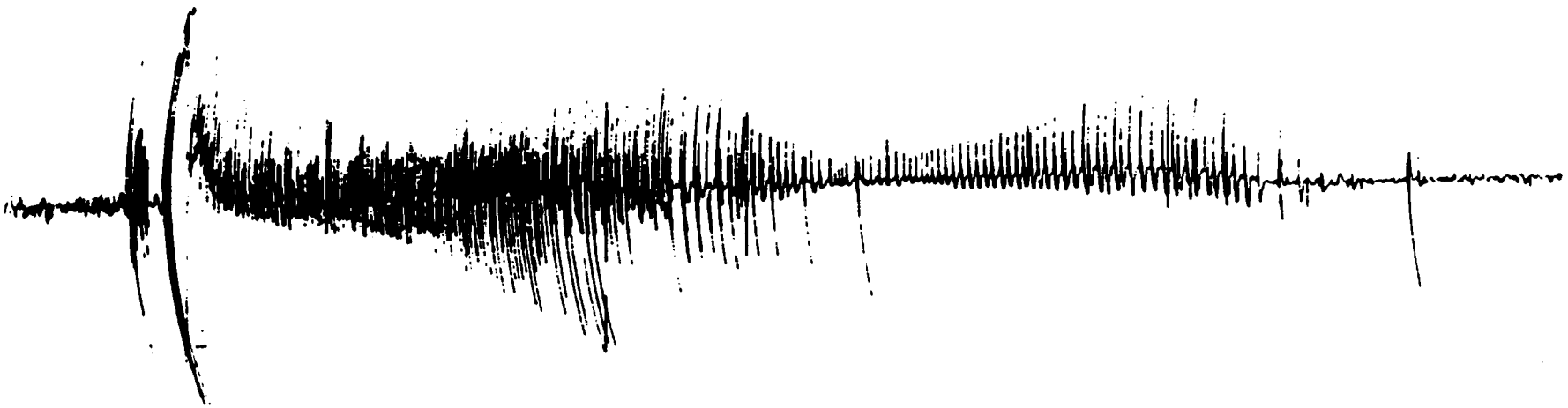

mf
gc
pyr
CA3
dentate gyrus

Figure 1.4 Representative example of afterdischarges evoked from a bipolar electrode implanted in the perforant path following three (AD#3) and eighteen (AD#18) kindling stimulations. Note that with repeated kindling stimulations, there is an increase in the AD amplitude, duration, frequency and spike complexity.

AD # 3



AD # 18



Chapter 2

Is it possible to dissociate kindling-induced mossy fiber sprouting from kindling-induced neuronal loss using NGF?

Kindling recently has been shown to induce a variety of permanent changes in the brain, including axonal sprouting of the mossy fiber pathway that originates from the hippocampal dentate gyrus granule cells (Sutula et al., 1988) and neuronal loss in specific populations of limbic neurons, particularly of hilar cells in the hippocampus (Cavazos & Sutula, 1990; Cavazos et al., 1994). To date, the relationship between kindling-induced sprouting and kindling-induced neuronal loss is unclear (Sutula et al., 1992). It has been suggested that kindling produces hilar cell loss and that the mossy fibers may sprout as a secondary consequence of axonal degeneration in their target regions (Cronin et al., 1994). In this case, hippocampal neuronal loss may be a cause and an effect of recurrent seizures by promoting the development of recurrent excitatory connections in dentate granule cells. However, kindling-induced mossy fiber sprouting also has been reported in the absence of any noticeable hippocampal neuronal loss or degeneration (Represa et al., 1989; 1993; Represa & Ben-Ari, 1992). Alternatively, it is possible that kindling-induced mossy fiber sprouting may be triggered by neuronal activation. In either case, cell loss or activation, sprouting may be regulated by growth factors (Diamond et al., 1992).

To address these issues, we have capitalized on recent evidence implicating neurotrophic factors in kindling epileptogenesis and kindling-induced mossy fiber sprouting. Because neurotrophic factors can exert morphoregulatory effects on hippocampal neurons (Mattson et al., 1989; Ip et al., 1993; Patel & McNamara, 1995), it has been proposed that seizure-induced expression of neurotrophic genes may underlie the sprouting of the axons of the dentate granule

cells (Watanabe et al., 1996). Certainly, seizure activity can increase nerve growth factor mRNA expression (Gall & Isackson, 1989; Enfors et al., 1991) and NGF protein levels (Bengzon et al., 1992) in cortical and hippocampal neurons. Furthermore, blocking NGF activity not only retards amygdaloid kindling (Funabashi et al., 1988; Rashid et al., 1995; Van der Zee et al., 1995) but also inhibits mossy fiber sprouting (Rashid et al., 1995; Van der Zee et al., 1995). Together, these findings strongly support the possibility that increased levels of NGF in the target region of the mossy fibers may play a role in kindling-induced mossy fiber sprouting and may contribute to further seizure development. However, kindling-induced neuronal loss was not investigated in these studies.

Increased levels of NGF could occur as a consequence of partial deafferentation of the granule cells, a concept well-established in other sprouting paradigms (Diamond et al., 1976; Diamond, 1982). Because neurotrophins can promote cell survival (Eide et al., 1993), there is the possibility that neurotrophin administration might protect hilar cells from damage and thereby provide a tool for dissociating kindling-induced hilar cell loss and kindling-induced sprouting. In this study, we tested the hypothesis that the pairing of activation and NGF would enhance mossy fiber sprouting while reducing the decreases in hilar cell density associated with kindling.

MATERIALS AND METHODS

Animals and surgical procedures

Adult male Long-Evans hooded rats (n=36) weighing between 300-400 g were used. Rats were maintained on an *ad lib* feeding schedule, housed individually, and kept on a 12 hour on/12 hour off light cycle. Using stereotaxic procedures, rats were anesthetized with sodium

pentobarbitol (65 mg/kg) and implanted in the right amygdala with a bipolar electrode made from teflon-coated, stainless steel wires (diameter 190 μm). Stereotaxic coordinates (Paxinos & Watson, 1985) were 2.8 mm posterior and 4.8 mm lateral to bregma, and 8.6 mm ventral to brain surface. Following electrode implantation, a cannula was implanted into the right lateral ventricle, at 0.6 mm posterior and 1.3 mm lateral to bregma and 5 mm below the skull surface. To confirm correct placement of the electrode and cannula, histological examination of coronal sections containing the lateral ventricle or the amygdala were conducted at the beginning of the experiment (n=4). Both the electrode and the cannula were held in place by dental acrylic and three stainless steel screws inserted into the skull.

A flow-regulated mini-osmotic pump (Alzet model 2002) was connected to the cannula via 3.5 cm of polyethylene tubing. To prolong the effectiveness of the pump, the bottom third of each pump was coated in paraffin prior to implantation (Vahlsing et al., 1989). This served to reduce the pump's flow rate from 12 μl/day to approximately 9 μl/day, making the pump effective for an additional seven days. The pump was placed subcutaneously in the dorsal neck/back region and delivered either phosphate buffered saline (PBS; pH 7.2) or 2.5 S NGF (1 mg/ml in PBS). An additional control group received cytochrome C (1 mg/ml in PBS), a protein similar in size and charge to NGF. Animals were given 7 days to recover from surgery before the kindling protocol was initiated. Solutions were infused at a rate of 9 μg/day for 18 days, beginning on the day of surgery and ending on the last day of the kindling protocol. Thus, animals in the kindled NGF-infused group were pre-treated with a total of 63 μg of NGF (9 μg/day for 7 days) before the initiation of kindling. Due to a lack of availability of NGF, the NGF-infused non-kindled group was not run during the same experimental period as the other 5

groups.

Preparation of 2.5 S NGF

2.5 S NGF was isolated from male mouse salivary glands according the procedure of Mobley et al. (1976). The purified protein migrated as a doublet at 13.5 kDa in SDS-PAGE gels. Biological activity was measured in a dissociated cell assay (Coughlin and Collins, 1985) using neonatal mouse dorsal root ganglion neurons. This procedure was done by a technician in Jack Diamond's laboratory in the Department of Biomedical Sciences at McMaster University.

Kindling paradigm

Rats were stimulated twice daily, with interstimulus intervals of at least 6 hours, for a total of 11 days. Each stimulation comprised a one-second train of one-millisecond pulses at a frequency of 60 Hz and a pulse intensity ranging from 500-600 μA. This was sufficient to trigger epileptiform afterdischarges (AD) of greater than five seconds following each stimulation. The durations of the afterdischarges were recorded in electroencephalograph (EEG) recordings from the amygdala electrode. Although it may have been useful to directly record AD duration in the hippocampus as compared to the amygdala, the damage caused by a recording electrode in the hippocampus would have substantially interfered with subsequent histologic analyses. Furthermore, there is evidence demonstrating that electrophysiological data recorded from the amygdala accurately reflects electrographic seizure propagation in the hippocampus (Racine, 1972; Watanabe et al.,1996). An experimenter blind to the experimental conditions monitored the progression of kindling by recording the behavioral seizure stage after each stimulation according

to Racine's classification (1972): (1) mouth and facial twitches, (2) clonic head movements, (3) unilateral forelimb clonus followed by contralateral clonus, (4) clonic rearing, and (5) loss of postural control. Animals were regarded as fully kindled when they exhibited three consecutive stage 5 seizures. Non-kindled, implanted controls remained in the colony for 18 days.

Histologic analyses

At Day 18 post-surgery, rats were anaesthetised with sodium pentobarbitol (65 mg/kg) and were perfused transcardially with 50 mL of a sodium sulfide solution (8.9 g $Na_2S.\ 9H_2O$, 10.9 g sucrose, 1.19 g $Na_2PO_4.H_2O$ per 100 mL dH_2O) at room temperature. Kindled rats were perfused immediately following the last kindling stimulation. Following perfusion, the brains were removed, covered with tissue-Tek® (Miles, Inc. Diagnostics Division, Elkhart, IN), and immediately frozen on dry ice. Forty-micron sections were taken through the region of the brain containing the hippocampus using a cryostat at -18°C. The sections were mounted on chromium potassium sulphate-coated slides. To ensure that brain sections included in the data analysis were from comparable levels, we matched major landmarks in the sections to those found in the Paxinos and Watson (1986) rat brain atlas. Horizontal sections were taken with the ventral surface up, and the first section was taken at a plane that corresponded to a depth of 7.6 mm ventral to bregma. Eighty-four serial sections were taken. Alternate sections were stained using either a modified Timm method (Sutula et al., 1988; Van der Zee et al., 1995) for the analysis of mossy fiber sprouting or cresyl violet for the determination of neuronal cell number. Six Timm-stained and adjacent cresyl violet-stained sections were analyzed, corresponding to depths of 4.94-7.34 mm ventral to bregma. The pairs of sections were separated by 400 μm. Each section was

matched against the landmarks in the corresponding atlas sections. To ensure objectivity in data analysis, slides were coded and all subsequent analyses were done by an observer who was unaware of the treatment of the animal.

The Timm method stains neural elements containing heavy metals (i.e., the high Zn^{2+} content of the terminals of the mossy fiber axons of the dentate granule cells). To minimize variability in Timm staining between groups, sections from animals from different groups were processed simultaneously. Slides for the NGF-infused non-kindled group were processed at a later time.

Horizontal sections from the dorsal dentate gyrus were examined at 50x magnification by creating a digitized image using an MCID image analysis system (Micro Computer Imaging Device, Brock University, St. Catherines, Ontario, Canada) attached to a light microscope (Zeiss Axioskop) with a high resolution CCD camera (MTI CCD 72). Sites of measurement of Timm granule density were determined at geographically predetermined sites (Fig. 2.0). The density of Timm granules in the CA3 region was measured by placing an open circle cursor (0.013 cm^2) at 16 adjacent positions along the stratum oriens of the CA3 as described by Van der Zee et al. (1995) (Fig. 2.0), and the density of Timm granules in the inner molecular layer (IML) was measured at 9 adjacent cursor positions by placing one cursor position above the genu of the hilus and 4 cursor positions to the right and left of this cursor (Fig. 2.1). Background values were provided by readings at 8 cursor placements in the stratum radiatum of the CA3 as described by Van der Zee et al. (1995) (Fig. 2.0). To control for variations in background Timm staining density from section to section, the density readings in the stratum oriens and the IML were divided by the background density values from the stratum radiatum for each section. This

provided a ratio between the stratum oriens or the IML density value and the stratum radiatum density value per section for all experimental groups. Density measurements were evaluated from 6 brain sections per rat at different section levels for both the right and left sides of the brain.

Cresyl violet staining and measurement of hilar cell density and hilar area

Cresyl violet selectively stains Nissl substance, a characteristic granular substance found in the nerve cell body. Hilar cell density was evaluated using a light microscope (Zeiss Axioskop) with a camera lucida attachment. Horizontal sections from the dentate gyrus were examined at 400x magnification. Using the camera lucida attachment, an unbiased counting grid (200 x 265 μ) was positioned in the hilus perpendicular to the CA3/CA4 region, and cells with visible nuclei containing a nucleolus within the grid were manually circled and counted. Neuronal numbers within the grid were evaluated from 6 brain sections per rat at different section depths for both the right and left sides of the brain. Focus was varied as required to count all cells within the grid.

Recent experimental evidence suggests that the observed reduction in hilar neuron density may not be due to actual neuronal loss, but instead may be attributable to a kindling-induced increase in hilar area (Bertram and Lothman, 1993; Watanabe et al., 1996). To examine this possibility, digitized images of the horizontal cresyl violet stained sections used for the evaluation of hilar neuronal density were examined at 50x magnification using the MCID imaging system attached to a light microscope with a CCD camera, and area measurements of each hilus were calculated. Hilar area was defined by the inner edge of the granule cell layer and the lines connecting the tips of the two granule cell blades to the beginning of the pyramidal cell layer of Ammon's horn (Bertram and Lothman, 1993) (Fig. 2.2).

RESULTS

Behavioural Progression of Kindling

A repeated measures ANOVA was conducted to evaluate the behavioural progression of kindling in the PBS-, cytochrome C-, and NGF-infused kindled groups as a function of stimulation number. There was a marked acceleration in the behavioural progression of kindling in the NGF-infused rats relative to PBS and cytochrome C-infused rats ($p<0.001$) (Fig. 2.3). Post-hoc analyses revealed no difference in the behavioural progression of kindling between the PBS and cytochrome-C groups ($p>0.05$). The mean number of stimulations to reach a stage 5 seizure was also calculated for all groups and data were subjected to a 1-way ANOVA with post-hoc Tukey tests. On average, NGF-treated rats required approximately 45% fewer stimulations (mean=8.28 ± 1.01) to reach a stage 5 seizure compared to the kindled PBS and cytochrome C groups (combined mean=14.92 ± 0.91) (Fig. 2.4). By the end of the kindling paradigm, all rats had shown at least three stage 5 seizures.

Afterdischarge Duration Analyses

A repeated measures ANOVA was conducted to evaluate afterdischarge duration as a function of stimulation number. As expected, afterdischarge (AD) duration significantly increased as a function of stimulation number across all groups ($p< 0.001$) (data not shown). Afterdischarge duration as a function of stimulation number did not differ between the groups ($p>0.05$). In addition, a 1-way ANOVA revealed that there were no differences in the cumulative durations of afterdischarges between the groups ($p>0.05$) (data not shown).

Mossy Fiber Sprouting Analyses

NGF infusions increased Timm staining in the stratum oriens (Fig. 2.5) and IML of kindled animals (Fig. 2.6). A 3-way ANOVA (3x(2x 16)) with one between variable (Group) and 2 within variables (Brain Hemisphere [left or right] and Cursor Position [1-16; starting at the hilus]) was conducted for the analysis of Timm densitometry in the CA3 region. Analyses were done on both raw densitometry measures and on measures corrected for background density. The results were nearly identical. Statistical analyses revealed no differences in density of background staining in the stratum radiatum across all groups, indicating that there was no influence of seizure activity on the staining in the stratum radiatum. There was a main effect for Cursor Position ($p<0.001$), showing that the density of Timm granules was greatest in the hippocampal CA3 area near the hilus and decreased with increasing distance from the hilus in all animals (Fig. 2.5). This main effect was further qualified by a significant Group x Cursor Position interaction ($p<0.001$). Post-hoc analyses revealed that Timm granule density was significantly enhanced in the kindled groups (upper curves; Fig. 2.7) relative to all non-kindled control groups (lower curves; Fig 2.7) ($p<0.05$). This enhancement was further increased in the NGF-kindled group (uppermost curve; Fig 2.7) compared to the kindled-PBS and cytochrome C groups ($p<0.05$). Timm granule density in the CA3 region of the NGF-infused non-kindled condition was decreased relative to the other non-kindled infused controls. However, because the Timm staining of this group was completed at a later date than the other 5 groups, these results may not be representative and should be interpreted with caution.

A 4-way ANOVA (6x(6x2x9)) with one between variable (Group) and 3 within variables (Section[1-6 ventral to dorsal], Brain Hemisphere [left or right], and Cursor Position[1-9]) was

conducted for the analysis of Timm density in the IML region. There was a main effect for Group ($p<0.05$). Preliminary analyses revealed no differences between the control-infused kindled groups (PBS- and cytochrome C-) or between all non-kindled groups (NGF, PBS and cytochrome C). Thus, for graphical presentation, data for these respective groups was combined and analysed (Fig. 2.8). Post-hoc analyses revealed increased Timm granule density in the NGF-infused kindled group compared to the control-infused kindled groups ($p<0.05$) and all non-kindled groups ($p<0.01$). However, there were no significant differences in Timm granule density in the IML region between the control-infused kindled groups and non-kindled groups ($p>0.05$).

Hilar Neuronal Density Analyses

For analysis of hilar neuronal density, a 3-way ANOVA (3x(2x6)) with 1 between variable (Group) and 2 within variables (Brain Hemisphere [left or right] and Brain Section Depth [1-6; ventral to dorsal]) was conducted. Evaluations of hilar neuronal density were performed at the same horizontal levels as the Timm analyses. Analyses revealed a main effect for Brain Section Depth ($p<0.001$), confirming the findings of Spiller and Racine (1994), showing that neuronal density is higher in more ventral brain sections than in more dorsal brain sections. For data presentation, however, cell density data were collapsed across section level and presented as a measure of mean neuronal density. Orthogonal comparisons revealed that mean neuronal densities were approximately 15% lower in the kindled control-infused rats compared to all the non-kindled rats and the kindled NGF-infused rats ($p<0.05$) (Fig. 2.9). There was also a main effect for Group ($p<0.02$) (data not shown).

Morphometric Analyses

To evaluate the possibility that the observed reduction in mean hilar neuron density may be attributable to a kindling-induced increase in hilar area rather than an actual neuron loss, a 3-way ANOVA (3x(2x6)) with 1 between variable (Group) and 2 within variables (Brain Hemisphere [left or right] and Brain Section Depth [1-6; ventral to dorsal]) was conducted to evaluate hilar area. Area was defined as shown in Fig. 2.2. There was a main effect for Group ($p<0.05$), and post-hoc analyses showed that seizure activity increased the area of the hilus in the kindled PBS and cytochrome C-infused groups compared to the PBS non-kindled condition ($p<0.05$) (Fig. 2.10). There was also a significant main effect for section level: $F(5,160)=22.97$, $p<0.001$, indicating that hilar area was greater in more ventral sections compared to more dorsal sections (data not shown). These findings implicate kindling-induced increases in hilar area rather than hilar cell loss as an explanation for the decrease in hilar cell density. In any event, the administration of NGF prior to and during kindling appeared to attenuate these kindling-induced hilar changes, whether measured by neuronal density (Fig. 2.9) or by mean hilar area ($p>0.05$) (Fig. 2.10).

DISCUSSION

Summary of Results and Implications

Intraventricular administration of nerve growth factor was shown to accelerate epileptogenesis and enhance kindling-induced sprouting of mossy fibers from dentate granule cells in the hippocampus, without any evidence of an associated loss in hilar neuron numbers. These findings appear to exclude the possibility that the mossy fiber sprouting is triggered by a

partial deafferentation of the target region. Instead, we favour the interpretation that kindling-induced mossy fiber sprouting is dependent on the co-involvement of neuronal activation and growth factors such as NGF.

Co-involvement of Neuronal Activity and Activation-Induced Upregulation of Growth Factors in the Regulation of Kindling-Induced Mossy Fiber Sprouting

There is evidence that the high levels of neuronal activity occurring during seizures are associated with changes in gene expression (Morgan and Curran, 1991). As outlined earlier, kindling a long-lasting increase in excitatory synaptic transmission (Sutula & Steward, 1986) which is mediated in part by the NMDA family of glutamate-gated ion channels in the granule cells of the dentate gyrus (Mody & Heinemann, 1987; Modey et al., 1988). This increase in excitatory synaptic transmission is accompanied by a complex sequence of gene expression which includes the transcriptional activation of a number of immediate early genes (IEGs) (Morgan and Curran, 1991; Kiessling and Gass, 1993; Labiner et al., 1993), followed by the expression in the dentate granule cells of genes encoding neurotrophic factors (Gall and Isackson, 1989; Ernfors et al., 1991; Gall, 1993; Gall et al., 1994), neurotrophic factor receptors (Bengzon et al., 1993; Bugra et al., 1994) and axonal growth-associated proteins (Bendotti et al., 1993; Meberg et al., 1993). This raises the possibility that activation-induced transcriptional regulation of gene expression in the dentate granule cells may play a critical role in the development of mossy fiber sprouting by initiating a chain of molecular events culminating in neural growth (Morgan and Curran, 1991). In the present study, NGF alone does not appear to be sufficient to trigger sprouting in this system.

This concept of a co-involvement of neuronal activation and growth factors in the

regulation of sprouting has been well-documented in the peripheral nervous system (Diamond et al., 1992). Specifically, it has been demonstrated that NGF was responsible for the initiation and maintenance of the collateral sprouting of cutaneous nociceptive sensory axons in rats, that impulse activity in the same neurons dramatically accelerated the onset of the NGF-driven sprouting, and that this action of nerve impulses was dependent on the presence of endogenous NGF (Diamond et al., 1992). This suggests that the action of nerve impulses combined with the presence of endogenous NGF may be to prime the cellular mechanisms that initiate the sprouting response (Diamond et al., 1992). The results obtained in the present study parallel these earlier findings, and raise the possibility that a comparable mechanism may exist in the central nervous system (CNS). Seizure activity upregulates a number of different neurotrophic factors, however, and it is unlikely that NGF is the only growth factor contributing to activation-induced mossy fiber sprouting in the kindling model.

Kindling-Induced Decreases in Neuronal Density versus Kindling-Induced Increases in Hilar Area

There is some debate in the literature regarding whether kindling produces genuine hilar neuronal loss (Cavazos and Sutula, 1990) or whether kindling, by causing an increase in hilar area with a corresponding decrease in neuronal density, leads to only an apparent hilar neuronal loss (Bertram and Lothman, 1993; Watanabe et al., 1996). In the present study, we showed that, although amygdala kindling twice a day for 11 days significantly decreased neuronal density in the hilus by approximately 15 %, the hilar area was also significantly increased by approximately the same amount. This result fails to support the hypothesis that kindling produces hilar cell loss. It

is more likely that amygdaloid kindling induces a structural change that leads to an increase in hilar area. It remains to be determined whether or not this hilar change is permanent.

The possibility that there is a more subtle form of kindling-induced denervation of synaptic targets cannot be excluded. One other major source of input to the inner molecular layer is the cholinergic septo-dentate afferents. Given that TrkA expression in the hippocampus is limited primarily to these cholinergic fibers (Holtzman et al., 1994; 1995), they would not be expected to suffer as a consequence of the NGF infusions. Although the cause of the kindling-induced hilar area increases is unclear (Bertram & Lothman, 1993; Watanabe et al., 1996), it has been suggested that they could result from a number of causes, including increases in the dendritic tree, branching of axon terminals, increases in the size or number of glial cells (Bertram and Lothman, 1993; Watanabe et al., 1996), or an accumulation of fluid in the extracellular or intracellular space (Watanabe et al., 1996). In fact, astrocyte hypertrophy has been reported in the hilar region as a result of amygdala kindling (Khurgel et al., 1992). Thus, it is possible that glial cell changes (i.e., hypertrophy or proliferation) may underlie the observed kindling-induced increases in hilar area. This possibility is investigated in Study 4. Interestingly, in the present study, we found no kindling-induced decrease in neuronal density or increase in hilar area in the kindled group infused with NGF. These findings suggest that NGF may exert an overall stabilizing or protective role in the hilus.

Mossy Fiber Sprouting as a Potential Mechanism For Epileptogenesis

As outlined earlier, kindling produces a permanently enhanced sensitivity to electrical stimulation that is accompanied by lasting mossy fiber sprouting (Sutula et al., 1988; Represa et

al., 1993). Kindling-induced mossy fiber sprouting has been reported in both the CA3 (Represa & Ben-Ari, 1992; Van der Zee et al., 1995) and the IML regions of the hippocampus (Sutula et al., 1988; Watanabe et al., 1996). In the present study, there was no evidence of kindling-induced mossy fiber sprouting in the IML region in the kindled control groups compared to the non-kindled controls. The appearance of IML sprouting in the group that received both kindling stimulations and NGF suggests that our procedures may be near threshold for kindling-induced IML sprouting. This indicates that CA3 sprouting may show a reliably lower threshold than IML sprouting, at least in Long-Evans rats following amygdaloid kindling. By contrast, Spiller and Racine (1994) report kindling-induced mossy fiber sprouting in the IML region but not in the CA3 region following perforant path kindling in Long-Evans rats.

To date, the consequences of kindling-induced mossy fiber sprouting are unclear. It has been proposed, however, that this sprouting could explain the development and maintenance of epileptogenesis by the creation of recurrent excitatory circuits that promote the progression and the maintenance of kindling (Sutula et al., 1988; Cronin et al., 1992; Okazaki et al., 1995). Although our results are consistent with such a mechanism, there is evidence that mossy fiber sprouting is neither necessary nor sufficient for kindling. For example, Longo and Mello (1997) showed that a protein synthesis inhibitor called cycloheximide can block kainate- or pilocarpine-induced mossy fiber sprouting without interfering with subsequent epileptogenesis. In addition, using rapid kindling, rats are repeatedly stimulated at short time intervals (e.g., 10 times per day for 2 days) and can become fully kindled within hours (Lothman et al., 1985; Lothman & Williamson, 1993). It is unlikely that mossy fiber sprouting (i.e., the growth of new fibers) can occur so quickly, which suggests that mossy fiber sprouting isn't a necessary component of

kindling epileptogenesis. Rather, these findings suggest that kindling can proceed in the absence of mossy fiber sprouting. This issue will be discussed again in a later section.

In summary, our findings indicate that NGF plays an important role in the development of kindling and kindling-induced mossy fiber sprouting. We suggest that kindling-induced mossy fiber sprouting is attributable to the co-involvement of neuronal activation and activation-induced upregulation of growth factors, as opposed to kindling-induced cell loss, and that this sprouting contributes to lasting modifications of neural structure and function in the epileptic brain.

FIGURES and CAPTIONS

Chapter 2

Figure 2.0 Digitized image of the hippocampal CA3 region. The density measurements of Timm granules were performed by placing an open circle cursor at 16 adjacent positions along the stratum oriens starting adjacent to the hilar region. Eight cursor placements were placed in the stratum radiatum adjacent to the hilar region provided the background staining density. Note that cursor windows for background measures in the stratum radiatum were clearly out of the mossy fiber tracts.

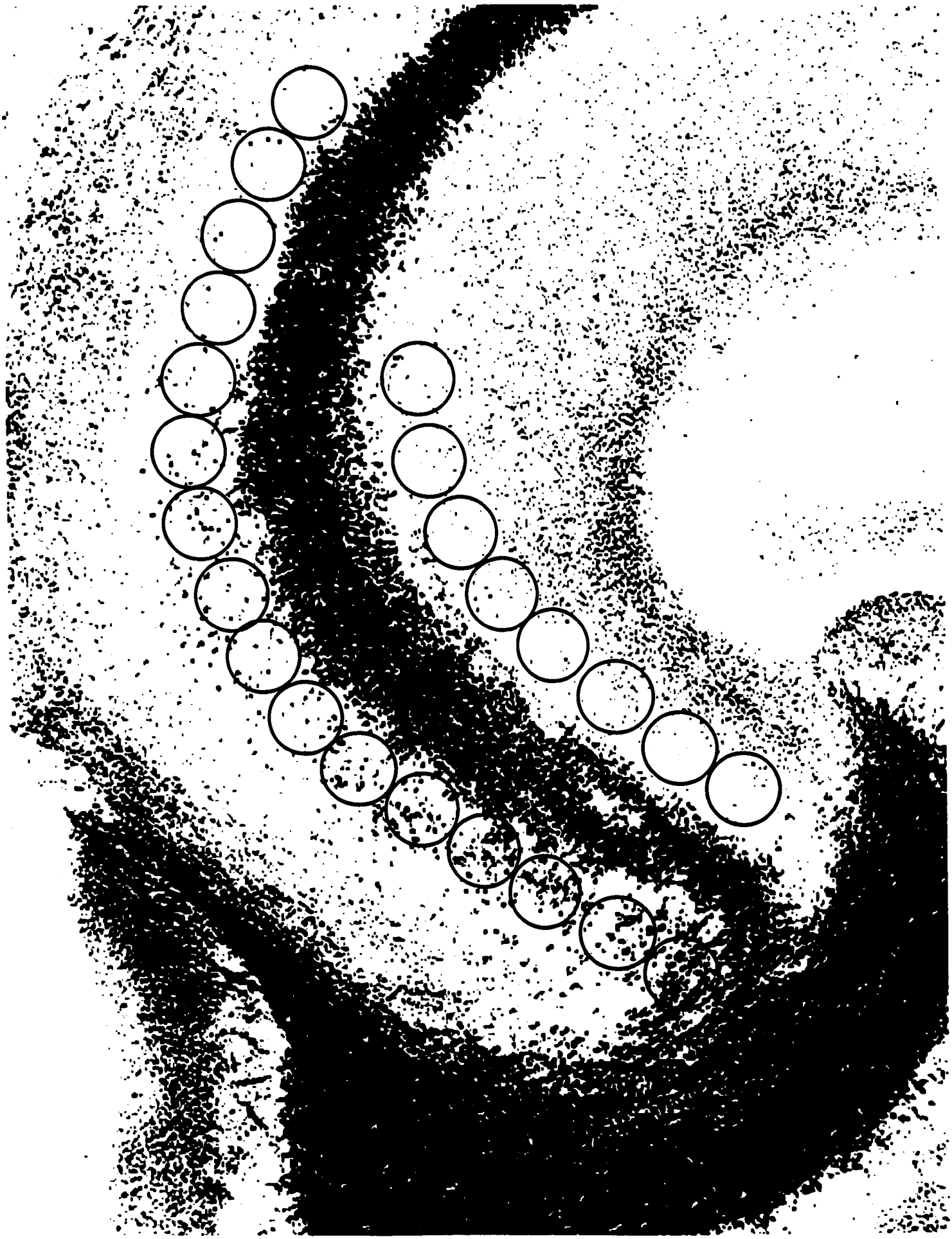


Figure 2.1 Digitized image of the IML region. Density of Timm granules in IML region was measured at 9 adjacent cursor positions by placing one cursor above the genu of the hilus and 4 cursor positions to the right and left of this cursor. Background values were provided as described in Fig 2.0.

Figure 2.2 Hilar area measurements. Hilar area as outlined using MCID image analysis system. Hilar area was defined by the inner edge of the granule cell layer and the lines connecting the tips of the two granule cell blades to the beginning of the pyramidal cell layer of Ammon's horn. Cresyl violet stained sections used for the determination of neuronal density were also used for hilar area measurements.

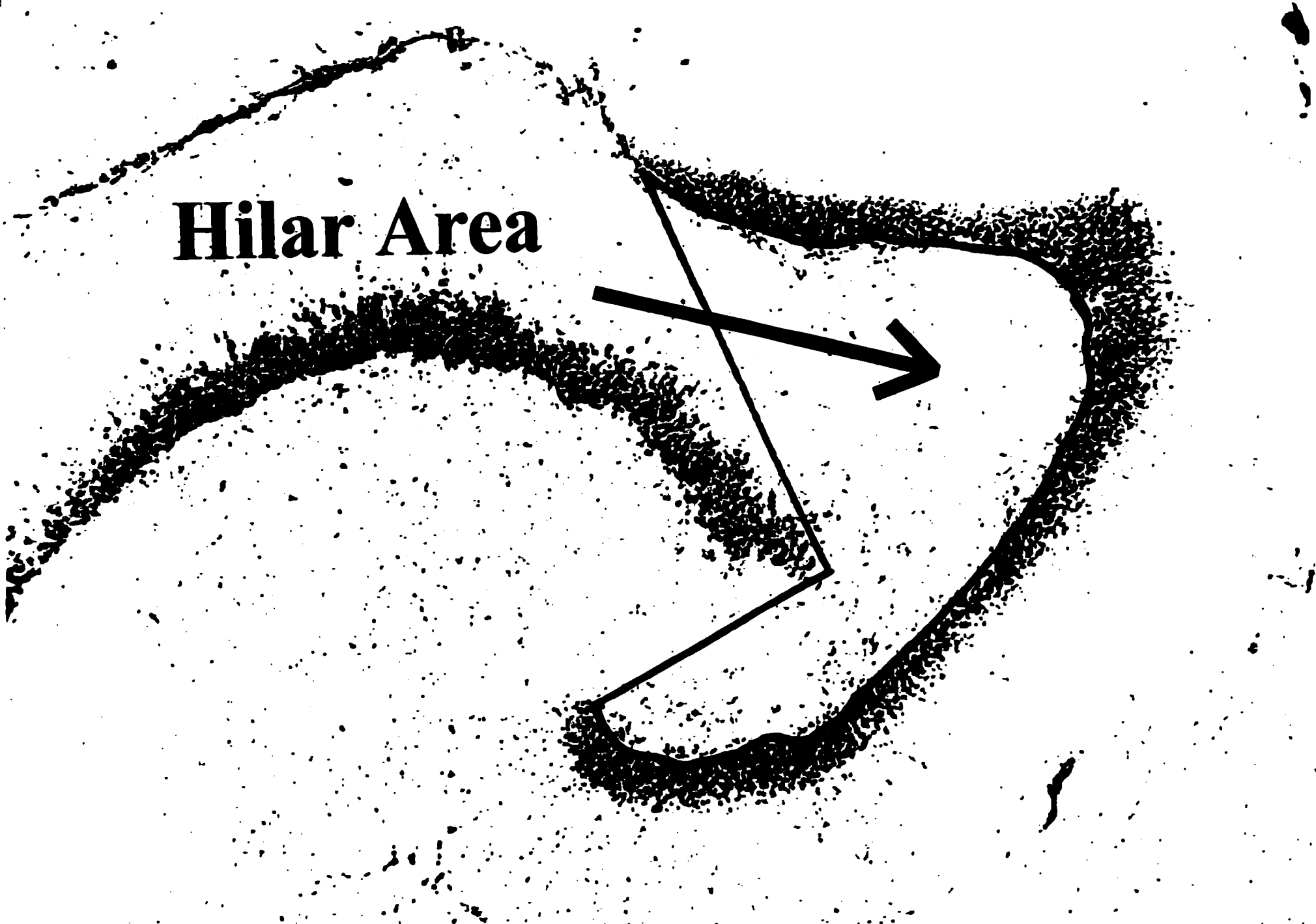

Hilar Area

Figure 2.3 Behavioral progression of seizure activity. NGF administration accelerates the behavioral progression of kindling. Values represent mean seizure stage ± standard error of the mean (S.E.M.) for NGF-kindled (n=7), cytochrome C-kindled (n=5) and PBS-kindled (n=6) animals.

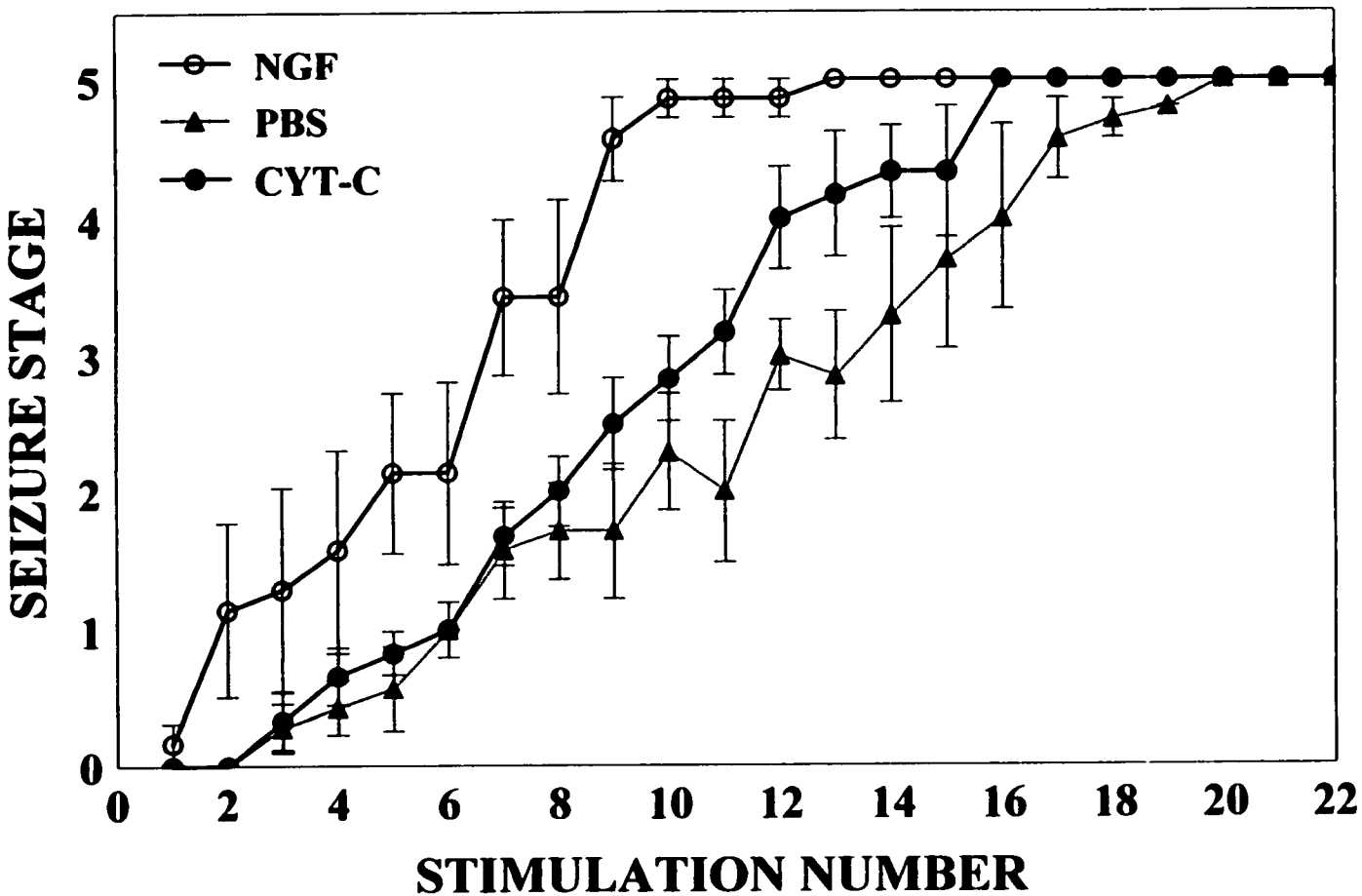

NGF
PBS
CYT-C
SEIZURE STAGE
0
1
2
3
4
5
0
2
4
6
8
10
12
14
16
18
20
22
STIMULATION NUMBER

Figure 2.4 Behavioural progression of kindling. NGF-infused rats (n=7) require 45% fewer stimulations to reach a stage 5 seizure compared to rats infused with PBS (n=6) or cytochrome C (n=5). Values represent mean number of stimulations ± S.E.M. required to reach 3 consecutive stage 5 seizures. * indicates $p<0.05$

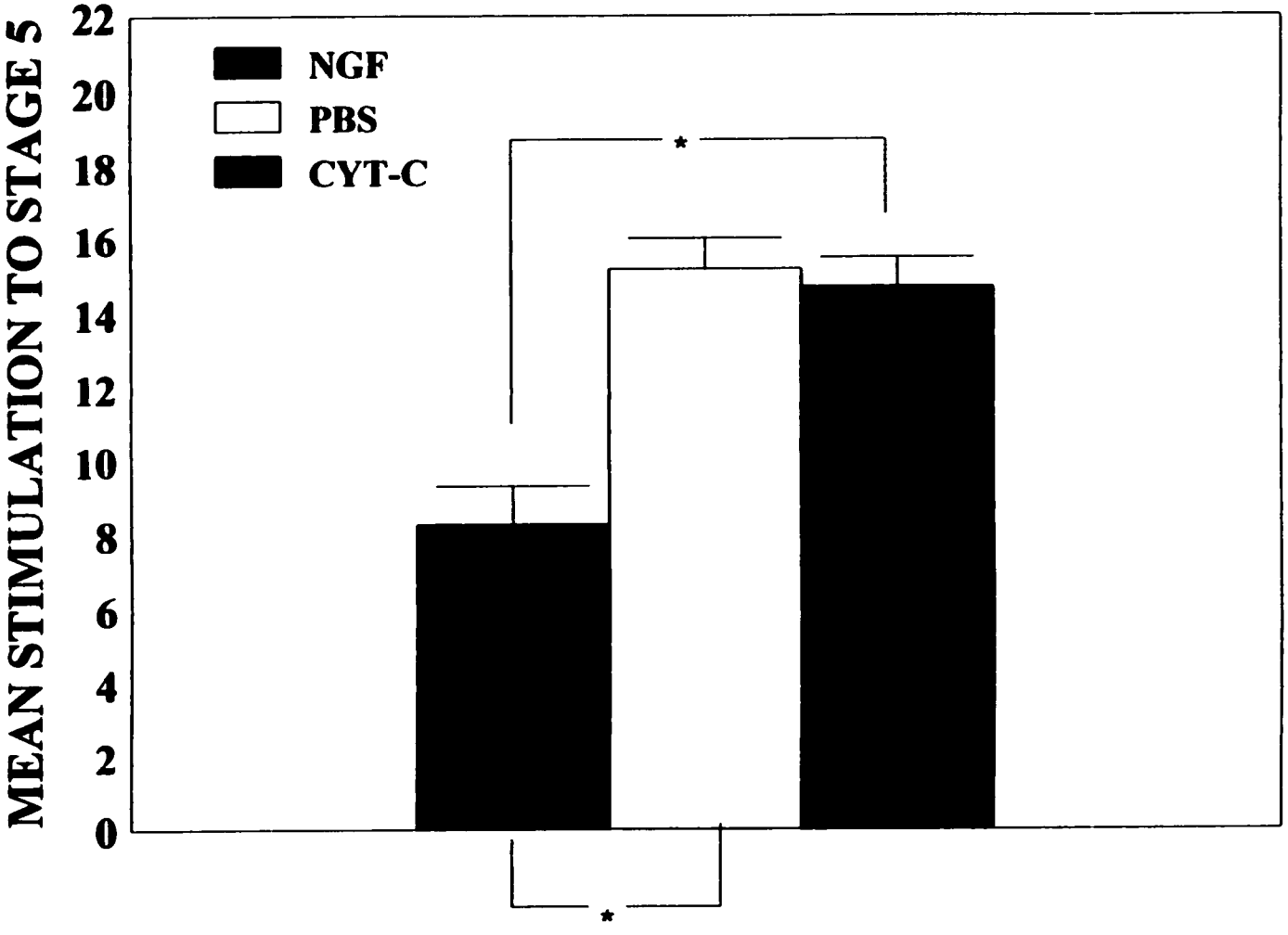

MEAN STIMULATION TO STAGE 5
22
20
18
16
14
12
10
8
6
4
2
0
NGF
PBS
CYT-C
*
*

Figure 2.5 Photomicrographs of Timm stained sections showing the kindling-induced synaptic reorganization in CA3 region induced by amygdaloid kindling. Representative examples of area CA3 of a non-kindled PBS-infused rat (a), a kindled PBS-infused rat (b), and a kindled NGF-infused rat (c). Arrows point to Timm granules in the stratum oriens of the CA3.

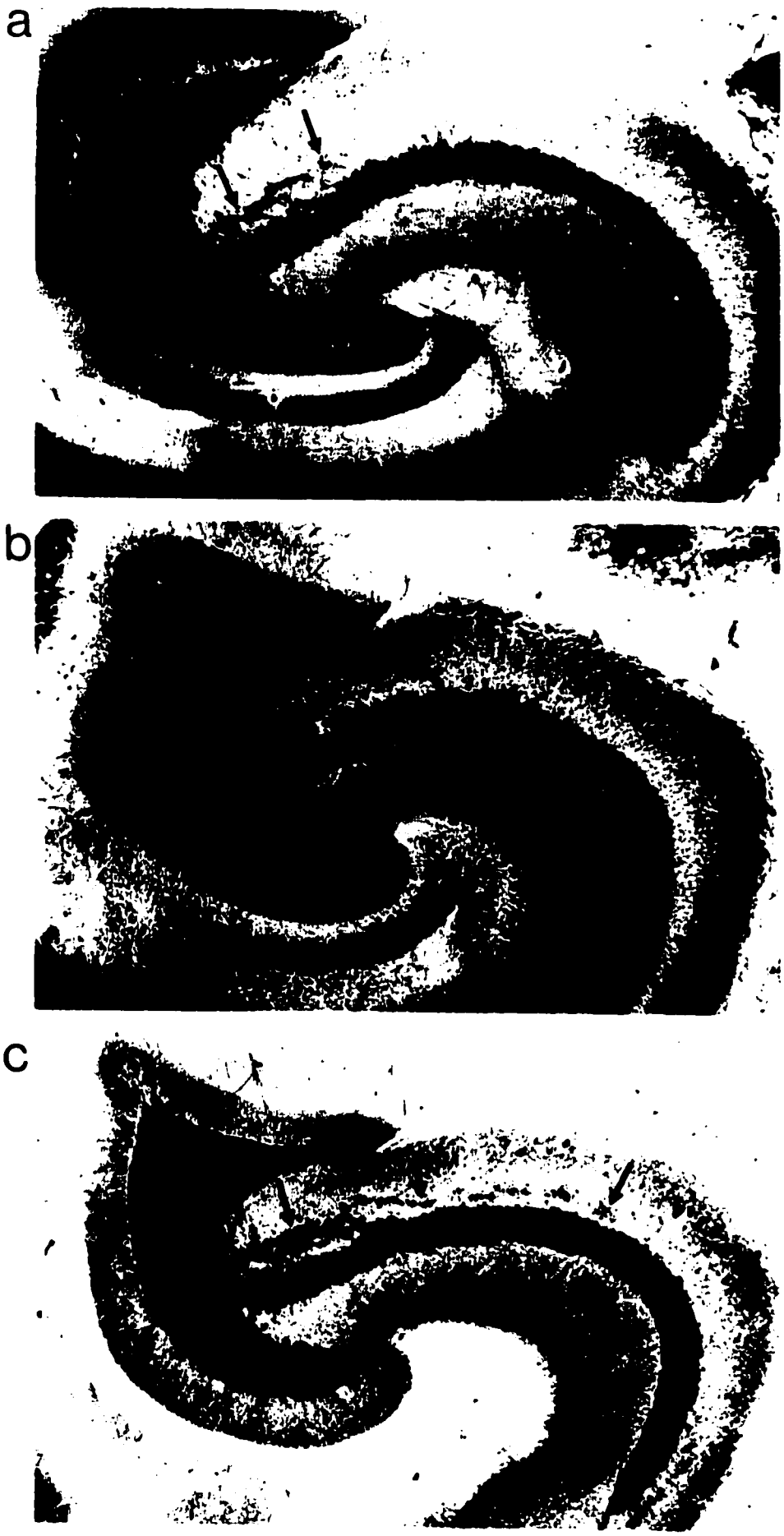

a
b
c

Figure 2.6 Photomicrographs of Timm staining in IML region. Representative examples of IML region of a non-kindled PBS-infused rat (a), a kindled PBS-infused rat (b), and a kindled NGF-infused rat (c). Arrows point to the band of Timm staining in the IML region. We do not see the dense band of Timm granules following amygdala kindling that has been reported following perforant path kindling (e.g. Sutula et al., 1988). We did, however, see an increase in the density of Timm staining in the IML following combined kindling and NGF infusion.

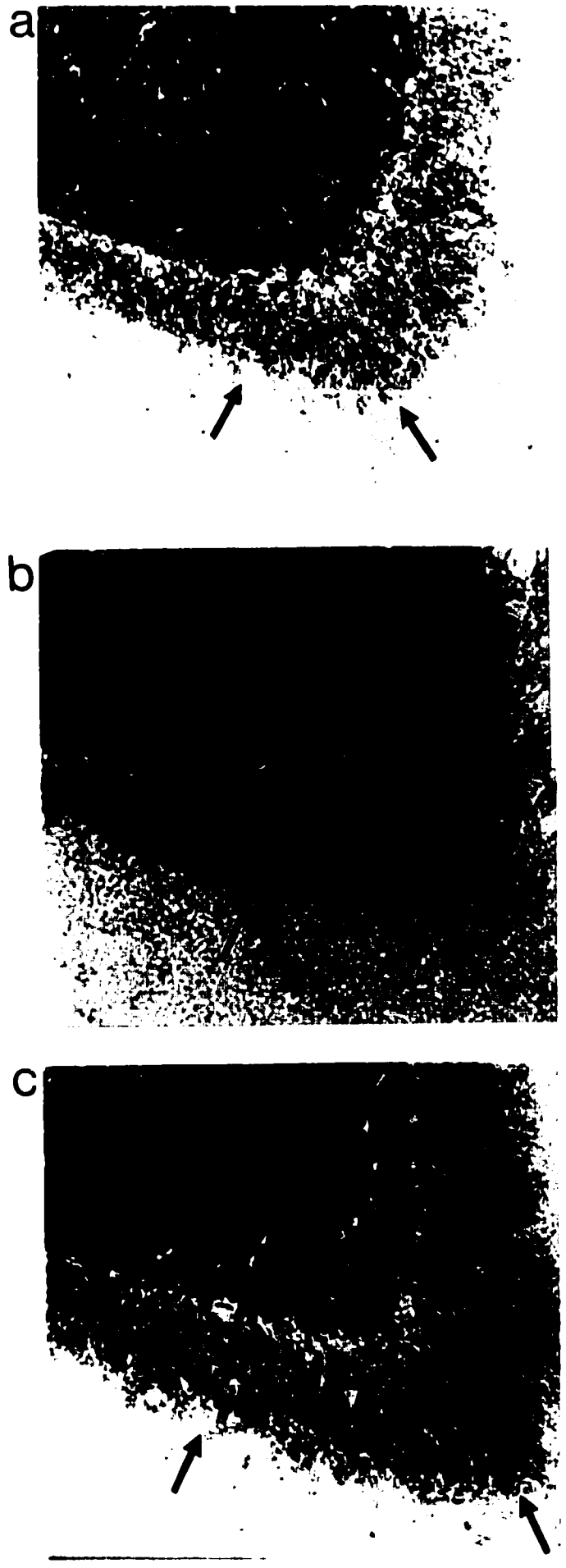

a
b
c

Figure 2.7 Timm granule density in CA3 region expressed as relative optical density (ROD) as a function of cursor position for all groups. Non-kindled and kindled control groups contain combined data for cytochrome C and PBS groups because no differences were found between these groups using a three-way ANOVA and subsequent post-hoc comparisons. Similarly, no differences in Timm granule density from ipsilateral and contralateral hippocampi were found ($p>0.05$), so data were combined for graphical presentation. Values represent mean ROD as a function of cursor position ± S.E.M. for the NGF-kindled group (n=7), kindled control group (PBS-infused: n=6; cytochrome C-infused: n=5), non-kindled control group (PBS-infused: n=7; cytochrome C-infused: n=6), and NGF-infused non-kindled group (n=5).

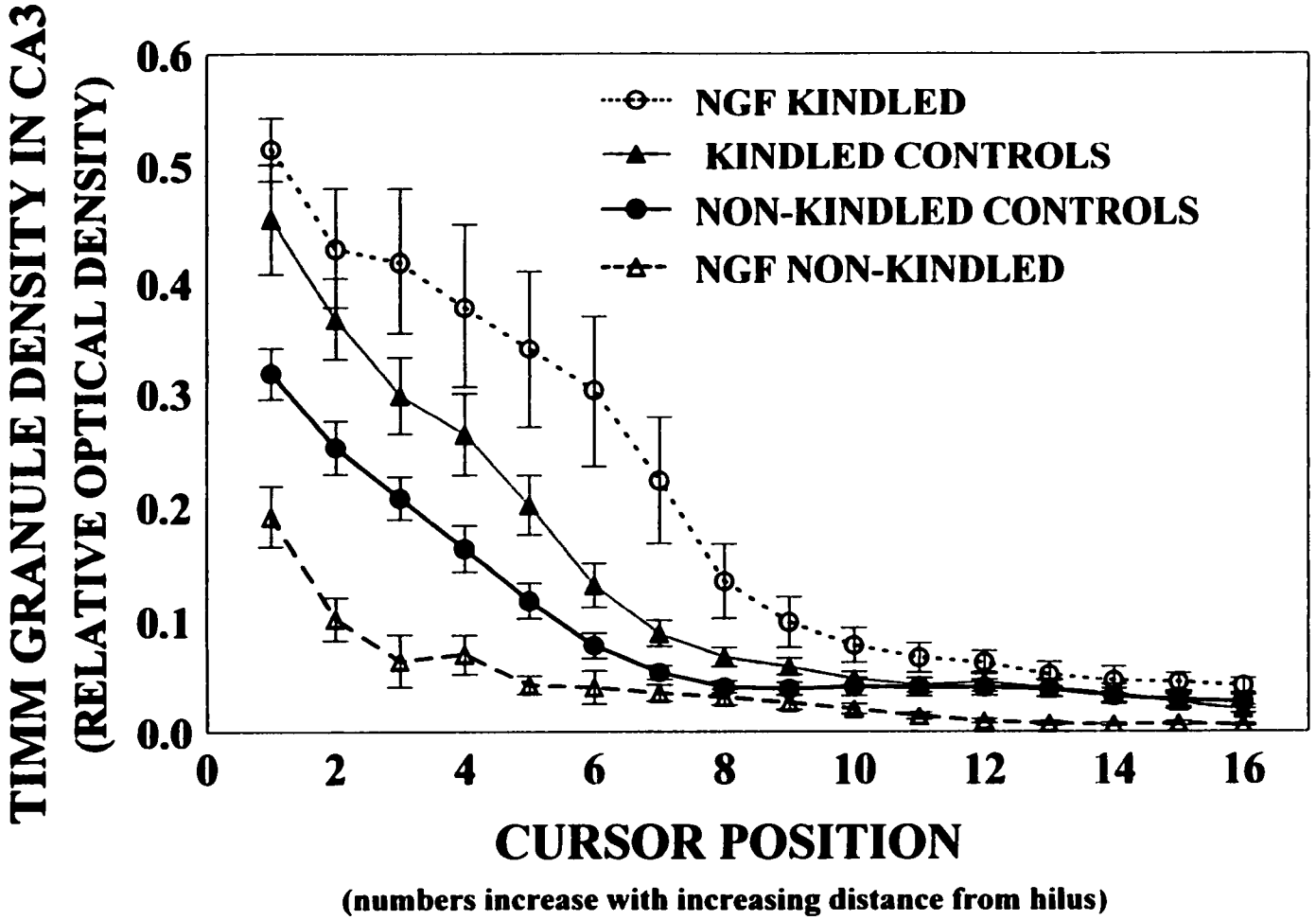

TIMM GRANULE DENSITY IN CA3
(RELATIVE OPTICAL DENSITY)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
2
4
6
8
10
12
14
16
NGF KINDLED
KINDLED CONTROLS
NON-KINDLED CONTROLS
NGF NON-KINDLED
CURSOR POSITION
(numbers increase with increasing distance from hilus)

Figure 2.8 Timm granule density in IML region expressed as ROD for kindled-infused controls (n=11), non-kindled-infused controls (n=18) and the NGF-infused kindled group (n=6). Timm granule density was increased in the NGF-kindled group relative to the kindled control group ($p<0.05$) and the non-kindled group ($p<0.01$). Values represent mean Timm granule density (ROD) as a function of group ± S.E.M. * indicates $p<0.05$

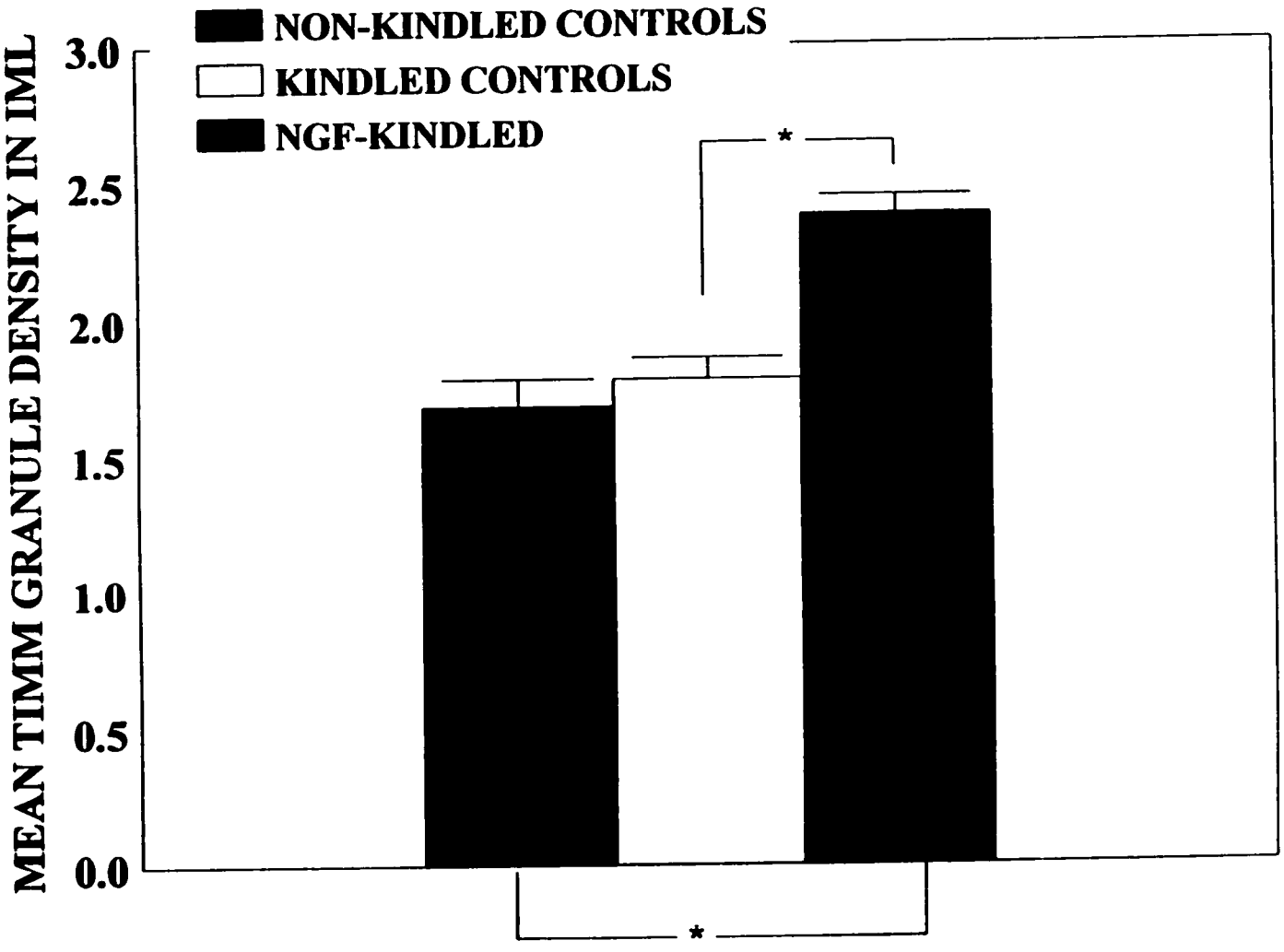

NON-KINDLED CONTROLS
KINDLED CONTROLS
NGF-KINDLED
MEAN TIMM GRANULE DENSITY IN IML
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*

Figure 2.9 Neuronal density counts in the hilar region. Mean hilar neuronal density as a function of group. A three-way ANOVA and subsequent post hoc comparisons revealed no differences between the non-kindled PBS (n=7), cytochrome C (n=6), and NGF (n=5) groups (non-kindled controls) ($p>0.05$) and no differences between the kindled PBS (n=6) and cytochrome C (n=5) groups (kindled controls) ($p>0.05$). Data for these non-kindled and kindled animals were combined, respectively. Values represent mean hilar cell density ± S.E.M. Mean hilar cell density was decreased by approximately 15% in the kindled control group relative to both the non kindled animals and the kindled NGF-infused group (n=7) ($p<0.05$). * indicates $p<0.05$

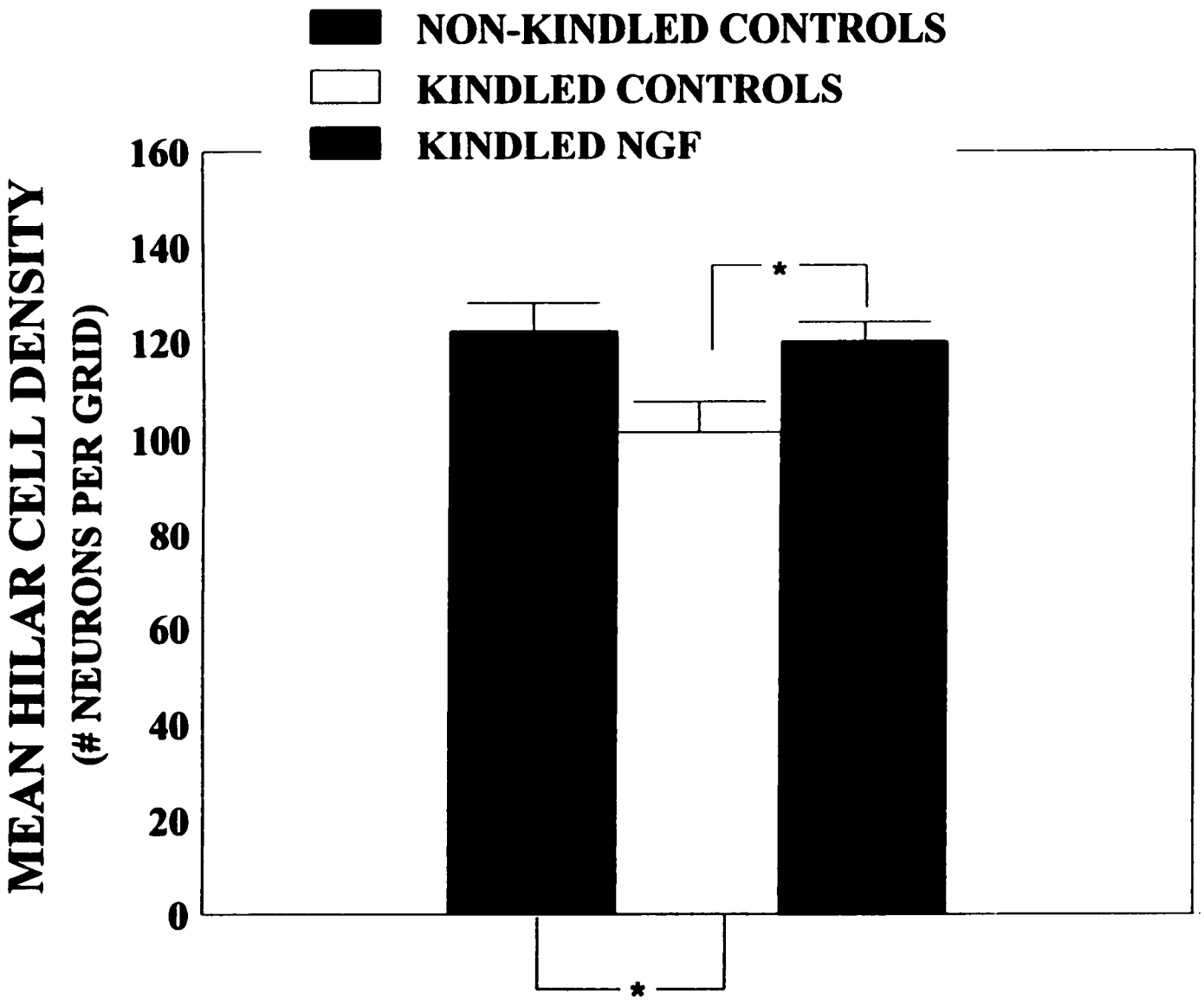
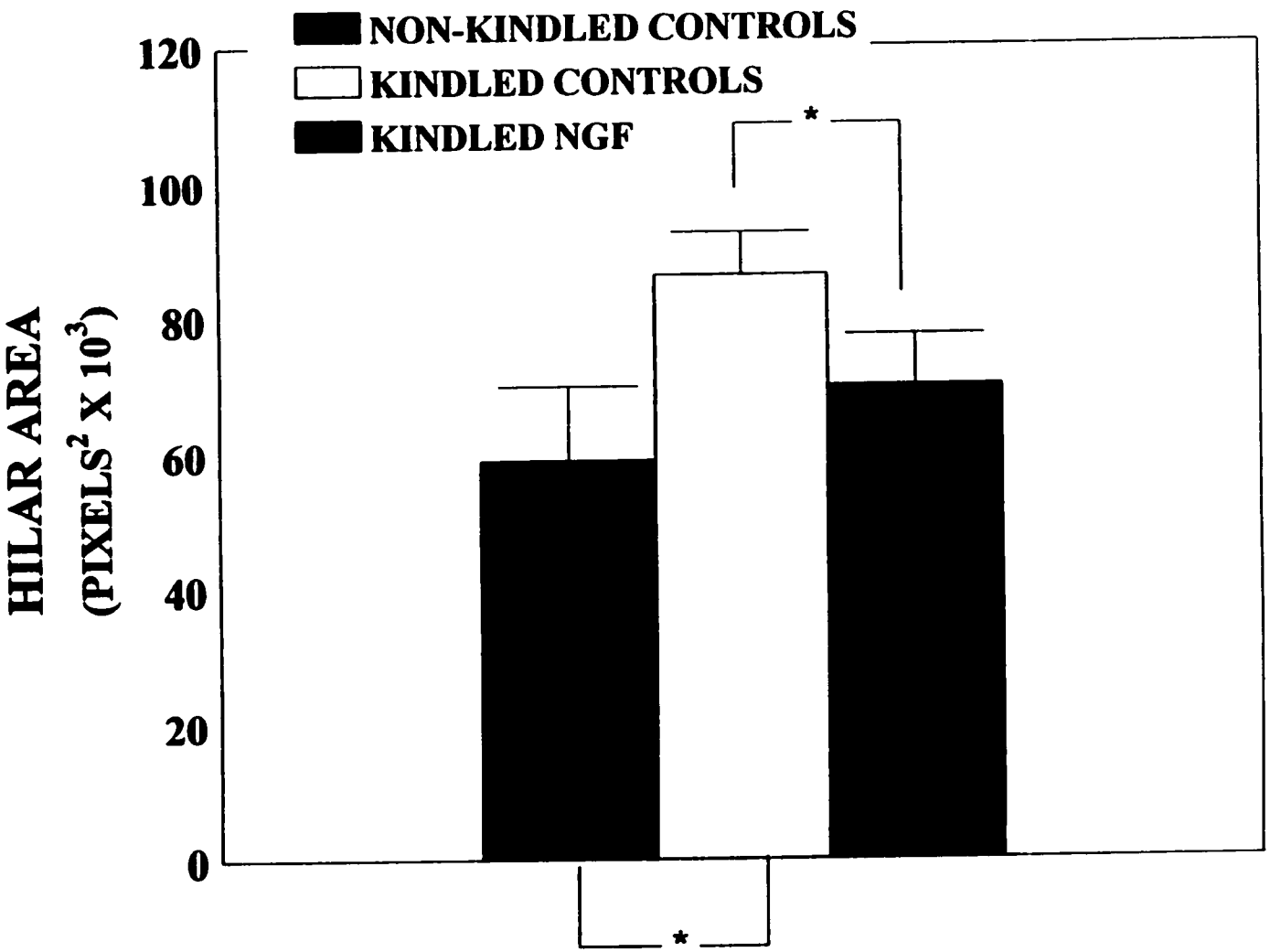

NON-KINDLED CONTROLS
KINDLED CONTROLS
KINDLED NGF
MEAN HILAR CELL DENSITY
(# NEURONS PER GRID)
160
140
120
100
80
60
40
20
0
*
*

Figure 2.10 Mean hilar area as a function of treatment condition. A three-way ANOVA and subsequent post hoc comparisons revealed no differences between the non-kindled PBS (n=7), cytochrome C (n=6) and NGF groups (n=5) (non-kindled controls) ($p>0.05$) and no differences between the kindled PBS (n=6) and cytochrome C (n=5) groups (kindled controls) ($p>0.05$). Data for these non-kindled and kindled animals were combined, respectively. Values represent mean hilar area expressed in microns^2 ± S.E.M. Mean hilar cell area was increased by approximately 15% in the kindled control group relative to the non kindled animals and the kindled NGF-group (n=7) ($p<0.05$). * indicates $p<0.05$


NON-KINDLED CONTROLS
KINDLED CONTROLS
KINDLED NGF
HILAR AREA
(PIXELS^2 X 10^3)
120
100
80
60
40
20
0
*
*

Chapter 3

Is the NGF-induced increase in kindling rates and mossy fiber sprouting mediated via the cholinergic system?

In Study 1, we demonstrated that intraventricular administration of NGF accelerates kindling rates and enhances mossy fiber sprouting. These findings with NGF are compatible with those of Van der Zee et al. (1995) and Rashid et al. (1995), who demonstrated that intraventricular infusion of NGF-inhibitors retards kindling rates and reduces mossy fiber sprouting. Taken together, these results indicate that NGF plays an important role in regulating the development of kindling and kindling-induced neural growth.

It is not yet clear, however, how NGF mediates these effects. It has been established that the biological effects of NGF are mediated primarily via its high-affinity receptor, TrkA (Kaplan et al., 1991; Klein et al., 1991; Chao, 1992), but expression of these receptors is most pronounced in the cholinergic neurons in the basal forebrain. At present there is little evidence for TrkA receptors in the hippocampus (Holtzman et al., 1994;1995). NGF also binds to the low-affinity p75 receptor (Bothwell, 1991) and p75 receptor immunoreactivity has been reported in both the CA3 hippocampal pyramidal layer and the dentate gyrus of colchicine-treated animals (Pioro and Cuello, 1990). Although it is still controversial whether the p75 receptor is capable of mediating the biological effects of NGF, cooperative interactions with Trk receptors to increase affinity of neurotrophin binding and signalling efficiency (Chao & Hempstead, 1995). Thus, it remains unclear whether NGF can mediate its effects directly on hippocampal neurons.

Alternatively, it is possible that NGF acts indirectly via the high-affinity Trk A receptors

on cholinergic neurons in the basal forebrain. As outlined earlier, it has been well-established that these neurons are sensitive to NGF. Specifically, intraventricularly injected ^{125}I-NGF labels cholinergic neurons (Nishio et al., 1992), chronic intraventricular administration of NGF increases both choline acetyltransferase (ChAT) activity and high affinity choline transport in the rat brain (Hefti et al., 1984), and exogenous NGF promotes axonal outgrowth in the lesioned septohippocampal pathway (Hagg et al., 1990). By contrast, anti-NGF infusion blocks cholinergic sprouting following kainic acid administration (Holtzman and Lowenstein, 1995), blocks the collateral sprouting of hippocampal fibers following entorhinal cortex lesions (Van der Zee et al., 1992), and reduces ChAT immunostaining in basal forebrain cholinergic neurons (Van der Zee et al., 1995). These same cholinergic systems are involved in kindling (Arnold et al., 1973; Cain, 1989), and cholinergic antagonists have been shown to retard the development of kindling in a dose-dependent manner (Arnold et al., 1973; Westerberg and Corcoran, 1987). Thus, an enhanced synthesis of acetylcholine in basal forebrain neurons induced by NGF infusions could lead to increased kindling rates. Whether such increases in acetylcholine synthesis could affect mossy fiber sprouting remains to be determined. To address this issue, we investigated the effects of the cholinergic agonist pilocarpine hydrochloride and the antagonist scopolamine hydrochloride on kindling-induced mossy fiber sprouting.

MATERIAL AND METHODS

Animals and Surgical Procedures

Adult male Long-Evans hooded rats (n=36) weighing between 300 and 400 grams were used. Rats were maintained on an *ad libitum* feeding schedule, housed individually, and kept on a

12 hour on/12 hour off light cycle. Using stereotaxic procedures, rats were anesthetized with sodium pentobarbitol (65 mg/kg) and implanted with a bipolar electrode made from Teflon-coated wires (diameter= 190μm) into the right perforant path. Stereotaxic coordinates (Paxinos and Watson, 1985) were -7.6 mm posterior and 4.1 mm lateral to the bregma and 3.3 mm below the skull surface. The electrode was held in place by dental acrylic and four stainless steel screws inserted into the skull. Following a two week recovery period, rats were randomly assigned to either a control condition (n=18) or a kindled condition (n=18). These two conditions were each further subdivided into three groups (n=6 per group) to be treated intraperitoneally with either a cholinergic antagonist (scopolamine hydrochloride, 30 mg/kg; Sigma Chemicals), 0.1 M phosphate buffered saline (PBS; pH=7.4), or a cholinergic agonist (pilocarpine hydrochloride, 15 mg/kg; Sigma Chemicals). Kindled rats were injected twice a day for 7 days prior to kindling and were injected twice a day during the 11 day kindling period. The two daily injections were 6-7 hours apart. Controls were injected twice a day for a total of 19 days and remained in the colony for the duration of the experiment. During the experiment, all rats were weighed daily to ensure that they did not lose more than 10% of their original body weight due to the injections of the drugs.

Kindling

Kindled rats were injected with either scopolamine, PBS, or pilocarpine thirty minutes prior to each kindling stimulation. Rats were stimulated twice daily with interstimulus intervals of at least 6 hours, for a total of 11 days. Each stimulation comprised a one second train of one-millisecond pulses at a frequency of 60 Hz and a pulse intensity ranging from 500-700

microamperes. This was sufficient to trigger epileptiform afterdischarges of greater than five seconds following each stimulation. The durations of the afterdischarges were recorded in the electroencephalograph recordings from the perforant path electrode. The behavioural progression of kindling was evaluated by an experimenter blind to the experimental conditions. The behavioural seizure stage after each stimulation was rated according to Racine's classification (1972). Following kindling, rats remained in the colony for 14 days to allow mossy fiber sprouting to occur. According to Cavazos et al. (1991) mossy fiber sprouting reaches asymptotic levels by 14 days following the last kindling stimulation.

Histologic Analyses

At Day 39 post-surgery, kindled and non-kindled rats were anesthetized with sodium pentobarbitol (65 mg/kg) and were perfused transcardially with a 50 mL sodium sulfide solution (Adams et al., 1997) at room temperature. Following perfusion, brains were removed and frozen in methyl butane cooled to - 40°C. Horizontal serial 40 micron sections of the hippocampal area 4.1 at 7.1 ventral to bregma were sectioned using a cryostat at -18°C and mounted on chromium potassium sulfate-coated slides. Section depth was determined according to the stereotaxic atlas of Paxinos and Watson (1985). Sections were stained using a modified Timm method (Sutula et al., 1988; Van der Zee et al., 1995) for the analysis of mossy fiber sprouting. To ensure that brain sections were from comparable levels, four sections from the hippocampal area at 4.1- 7.1 mm ventral to bregma and 750 microns apart were selected from each brain across all rats. Slides were then coded and all subsequent analyses were conducted by an observer who was unaware of the experimental treatment.

Quantification of Timm Histochemistry

Timm granule density was evaluated in the CA3 and IML regions using computer-assisted semi-quantitative densitometry as described in Study 1 (Adams et al., 1997a).

RESULTS

Behavioural Progression of Kindling

A repeated measures ANOVA was conducted to evaluate the behavioural progression of kindling in the scopolamine-, PBS-, and pilocarpine-injected groups as a function of stimulation number. There was a significant Group x Stimulation Number interaction: $F(42, 294)=1.67$, $p<0.008$. Post-hoc Tukey tests showed that kindling rates were significantly increased in the pilocarpine group compared to the PBS ($p<0.05$) and the scopolamine ($p<0.01$) groups, and kindling rates were significantly faster in the PBS group relative to the scopolamine group ($p<0.01$) Figure 3.0 shows the behavioural progression of kindling for all three groups.. A 1-way ANOVA with subsequent post-hoc Tukey tests was used to calculate the mean number of stimulations required to reach a stage 5 seizure as a function of group (Fig. 3.1). The pilocarpine group required approximately 64 % fewer stimulations to reach a stage 5 seizure compared to the PBS group ($p<0.05$) and approximately 74 % fewer stimulations compared to the scopolamine group ($p<0.001$). The PBS group required approximately 27% fewer stimulations to reach a stage 5 seizure compared to the scopolamine group ($p<0.01$).

Afterdischarge Duration Analyses

A repeated measures ANOVA was conducted to evaluate afterdischarge duration as a

function of stimulation number. As expected, afterdischarge (AD) duration significantly increased as a function of stimulation number across all groups ($p < 0.001$) (data not shown). However, afterdischarge duration as a function of stimulation number did not differ between the groups ($p > 0.05$).

Mossy Fiber Sprouting Analyses

A 3-way ANOVA (6x(2x 16)) with one between variable (Group) and 2 within variables (Brain Hemisphere [left or right] and Cursor Position [1-16; starting at the hilus]) was conducted for the analysis of Timm densitometry in the CA3 region. Analyses were done on both raw densitometry measures and on measures corrected for background density. The results were nearly identical. Statistical analyses revealed no differences in density of background staining in the stratum radiatum across all groups, indicating that there was no influence of seizure activity on the staining in the stratum radiatum. As in Study 1, there was a main effect for Cursor Position ($p<0.001$), showing that the density of Timm granules was greatest in the hippocampal CA3 area near the hilus and decreased with increasing distance from the hilus in all animals. This main effect was further qualified by a significant Group x Cursor Position interaction ($p<0.001$) (Fig. 3.2). Post-hoc analyses revealed that Timm granule density was significantly enhanced in the PBS- and pilocarpine- kindled groups (uppermost curves; Fig. 3.2) relative to all non-kindled control groups and the scopolamine-kindled group (lower curves; Fig. 3.2) ($p<0.05$). This enhancement was further increased in the pilocarpine-kindled group (uppermost curve; Fig 3.2) compared to the kindled-PBS group ($p<0.05$). There were no differences in Timm granule density in the CA3 region between the three control conditions and the kindled-scopolamine group

($p>0.05$).

A 4-way ANOVA (6x(4x2x9)) with one between variable (Group) and 3 within variables (Section[1-4 ventral to dorsal], Brain Hemisphere [left or right], and Cursor Position[1-9]) was conducted for the analysis of Timm density in the IML region. There was a main effect for Group ($p<0.0001$) (Fig. 3.4). Post-hoc analyses revealed increased Timm granule density in the pilocarpine-kindled group compared to the PBS-kindled group ($p<0.05$). Timm granule density in the IML region of both these groups, however, was enhanced compared to the scopolamine-kindled group and all non-kindled groups ($p<0.05$). There were no differences in Timm granule density between the scopolamine-kindled groups and all non-kindled groups ($p>0.05$).

DISCUSSION

These findings provide evidence that the cholinergic system plays a role in *both* kindling and kindling-induced mossy fiber sprouting. These data support past research showing that cholinergic agonists accelerate the behavioural progression of kindling and that cholinergic antagonists retard the development of kindling (e.g., Cain, 1992). Moreover, the present study shows that the cholinergic agonist pilocarpine enhances kindling-induced mossy fiber sprouting in the CA3 and IML regions of the hippocampus, whereas the cholinergic antagonist, scopolamine, blocks kindling-induced mossy fiber sprouting in the CA3 and IML regions. These data suggest that the cholinergic system may contribute to the long-term structural and functional alterations that are characteristic of the kindled state. Furthermore, these findings provide support for the hypothesis that NGF infusions may mediate kindling-induced mossy fiber sprouting via the cholinergic system.

There is evidence that certain cholinergic inputs may not be *necessary* for mossy fiber sprouting to occur, even though it may provide a modulatory function. Mohapel et al. (1997) investigated the effects of fimbria fornix (FF) transections on perforant path kindling and kindling-induced mossy fiber sprouting. The FF contains one of the major cholinergic inputs to the hippocampus. Transection of the FF produces chronic epileptiform discharge in the hippocampal formation and induces mossy fiber sprouting in the IML of the dentate gyrus (Corcoran et al., 1998). There are receptors for NGF and other neurotrophins on cholinergic terminals in the hippocampus that project through the FF, and it is possible that mossy fiber sprouting depends on NGF acting on these cholinergic terminals (Corcoran et al., 1998). If this is the case, it might be predicted then, that transection of the FF should reduce or preclude sprouting induced by kindling (Corcoran et al., 1998). In contrast, however, Mohapel et al. (1997) found that sprouting in kindled rats with FF transections was greater than sprouting in kindled controls or transected controls. A more robust *damage*-induced activation of sprouting may have overwhelmed any signs of a lost modulatory effect from cholinergic systems. In any case, these findings indicate that mossy fiber sprouting may not require the presence of cholinergic or other fibers that project through the FF (Corcoran et al., 1998).

There is evidence, however, showing that the cholinergic system modulates *activity*-dependent plasticity in other brain systems. For example, acetylcholine has been demonstrated to enhance the induction of LTP in the hippocampal CA1 region (Hirotsu et al., 1989; Blitzer et al., 1990; Tanaka et al., 1989; Huerta & Lisman, 1994) and in the dentate gyrus (Burgard & Sarvey, 1989). It has been suggested that this may be related to the cholinergic enhancement of stimulation-initiated protein synthesis via an NMDA-dependent mechanism (Feig & Lipton, 1993)

and to cholinergic potentiation of NMDA receptor-mediated responses (Markram & Segal, 1990).

These data raise the possibility that a similar mechanism may mediate the cholinergic potentiation of kindling-induced mossy fiber sprouting in the present study. As described earlier, kindling produces a long-lasting increase in excitatory synaptic transmission (Sutula & Steward, 1986) which is mediated in part by the NMDA family of glutamate-gated ion channels in the granule cells of the dentate gyrus (Mody & Heinemann, 1987; Mody et al., 1988). This increase in excitatory synaptic transmission is accompanied by a complex sequence of gene expression, which includes transient increases in the expression of transcription factors, and more slowly evolving changes in neurotrophin levels, neurotrophic factor receptors and axonal growth associated proteins. According to Sutula et al. (1996), the initial seizure-induced changes in NMDA-dependent synaptic transmission and gene expression are followed by more slowly evolving cellular alterations such as mossy fiber sprouting in the dentate gyrus. Moreover, recent research has shown that the administration of the NMDA receptor antagonist MK-801 impairs the progression of kindling and reduces kindling-induced mossy fiber sprouting (Sutula et al., 1996). These data suggest that the NMDA receptor is an important component of a molecular pathway that mediates both kindling and seizure-induced mossy fiber sprouting. These findings are also consistent with the possibility that NMDA-dependent gene expression induced by repeated seizures contributes to the activity-dependent, long-term modification of neuronal structure and function during kindling (Sutula et al., 1996). These data parallel those observed in the present study showing that the cholinergic system plays a role in both kindling and kindling-induced mossy fiber sprouting. These data also raise the possibility that the NMDA receptor and the cholinergic system may work synergistically to mediate kindling and kindling-induced mossy

fiber sprouting.

FIGURES and CAPTIONS

Chapter 3

Figure 3.0 Behavioral progression of seizure activity. Treatment with pilocarpine accelerates the behavioral progression of kindling, whereas treatment with scopolamine retards the progression of kindling. Values represent mean seizure stage ± standard error of the mean (S.E.M.) for pilocarpine-kindled (n=5), PBS-kindled (n=6) and scopolamine-kindled (n=5) animals.

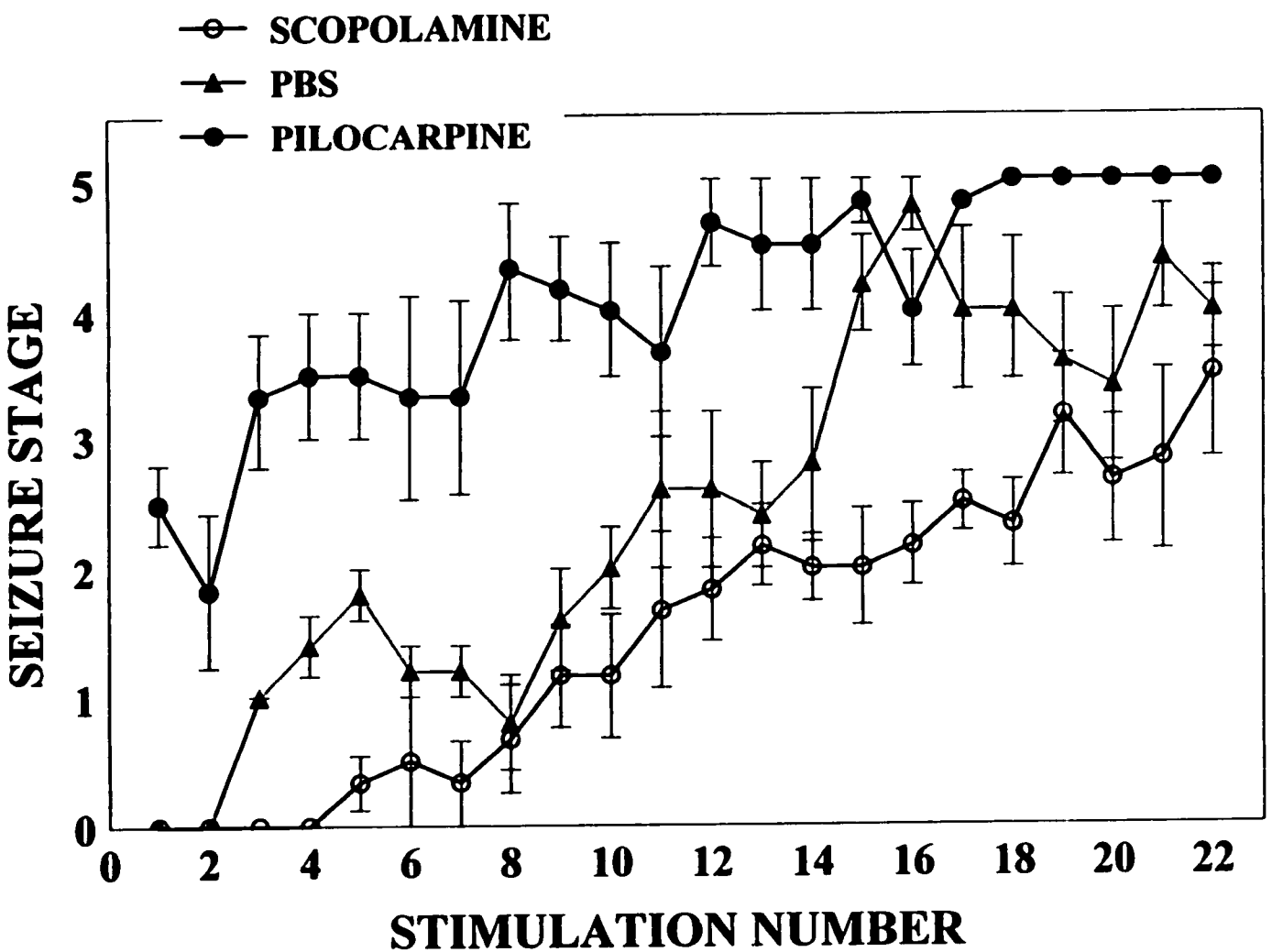

SCOPOLAMINE
PBS
PILOCARPINE
SEIZURE STAGE
0
1
2
3
4
5
0
2
4
6
8
10
12
14
16
18
20
22
STIMULATION NUMBER

Figure 3.1 Behavioural progression of kindling. The pilocarpine group (n=5) required approximately 64% fewer stimulations to reach a stage 5 seizure compared to the PBS group (n=6) ($p<0.05$) and approximately 74 % fewer stimulations compared to the scopolamine group (n=5) ($p<0.001$). The PBS group required approximately 27% fewer stimulations to reach a stage 5 seizure compared to the scopolamine group ($p<0.01$). Values represent mean number of stimulations ± S.E.M. required to reach 3 consecutive stage 5 seizures. * indicates $p<0.05$

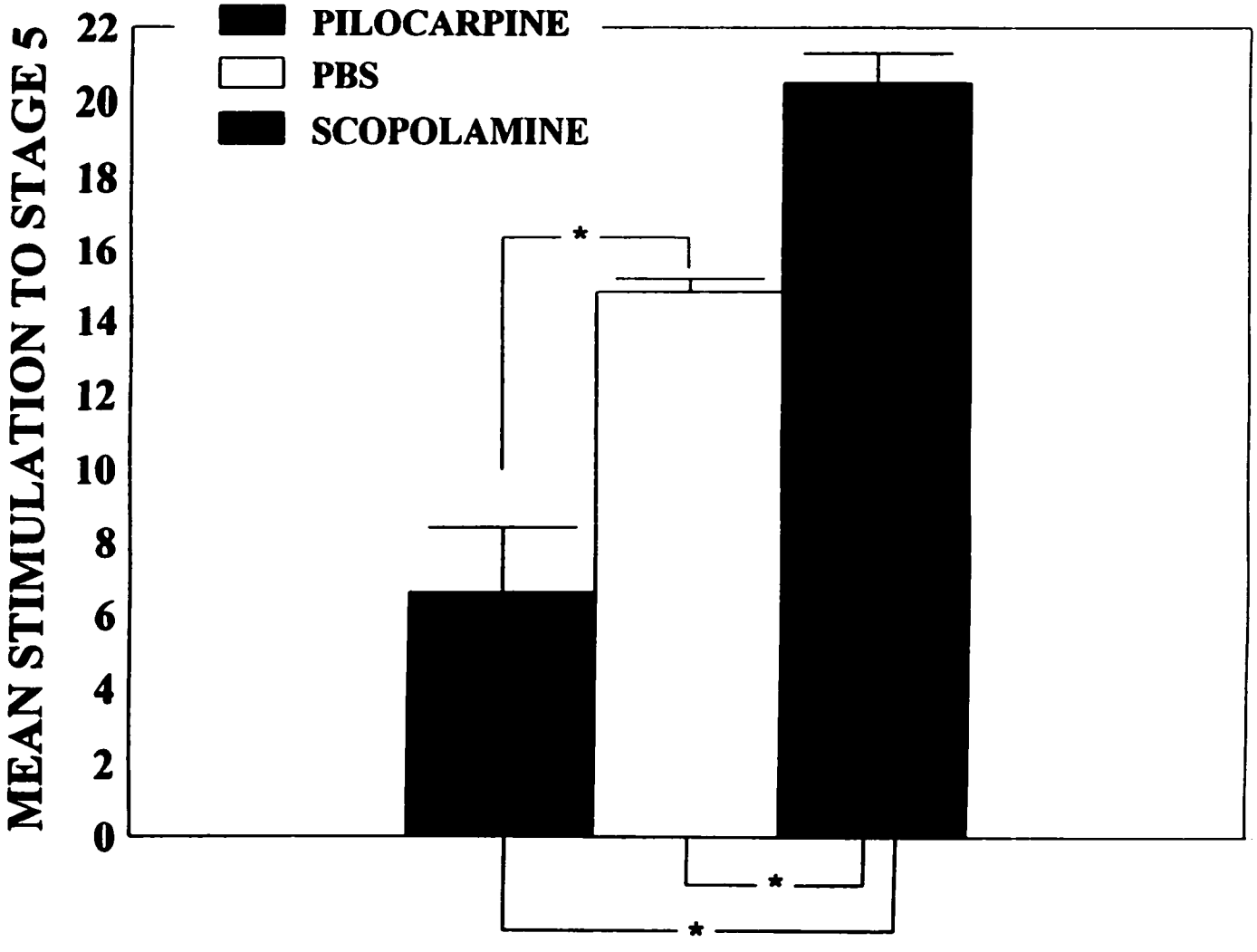

PILOCARPINE
PBS
SCOPOLAMINE
MEAN STIMULATION TO STAGE 5
22
20
18
16
14
12
10
8
6
4
2
0
*
*
*

Figure 3.2 Timm granule density in CA3 region expressed as relative optical density (ROD) as a function of cursor position for all groups. No differences in Timm granule density from ipsilateral and contralateral hippocampi were found ($p>0.05$), so data were combined for graphical presentation. Timm granule density was significantly enhanced in the PBS- and pilocarpine-kindled groups (uppermost curves) relative to all non-kindled control groups and the scopolamine-kindled group (lower curves) ($p<0.05$). This enhancement was further increased in the pilocarpine-kindled group (uppermost curve) compared to the kindled-PBS group ($p<0.05$). There were no differences in Timm granule density in the CA3 region between the three control conditions and the kindled-scopolamine group ($p>0.05$). Values represent mean ROD as a function of cursor position ± S.E.M. for all groups .

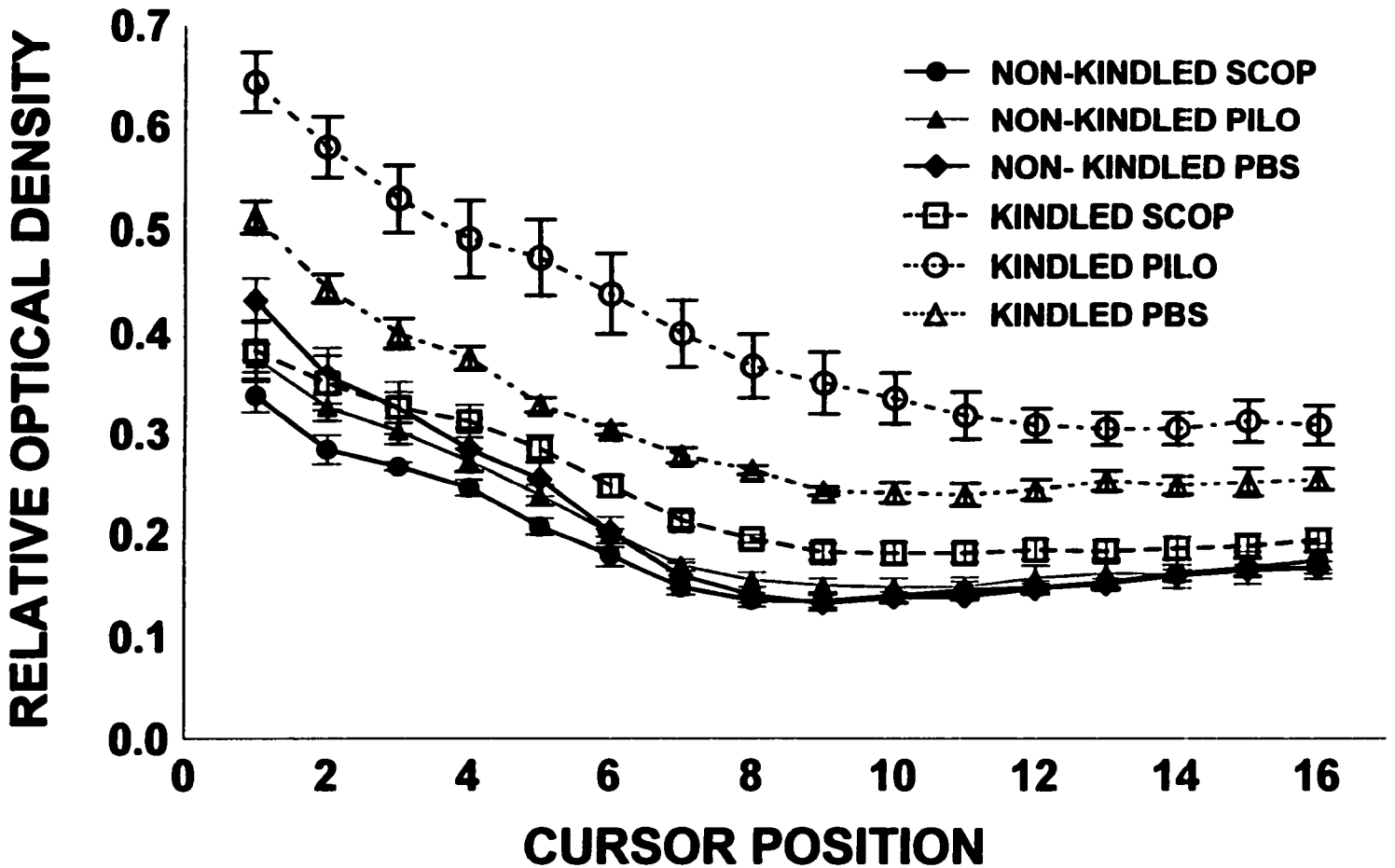
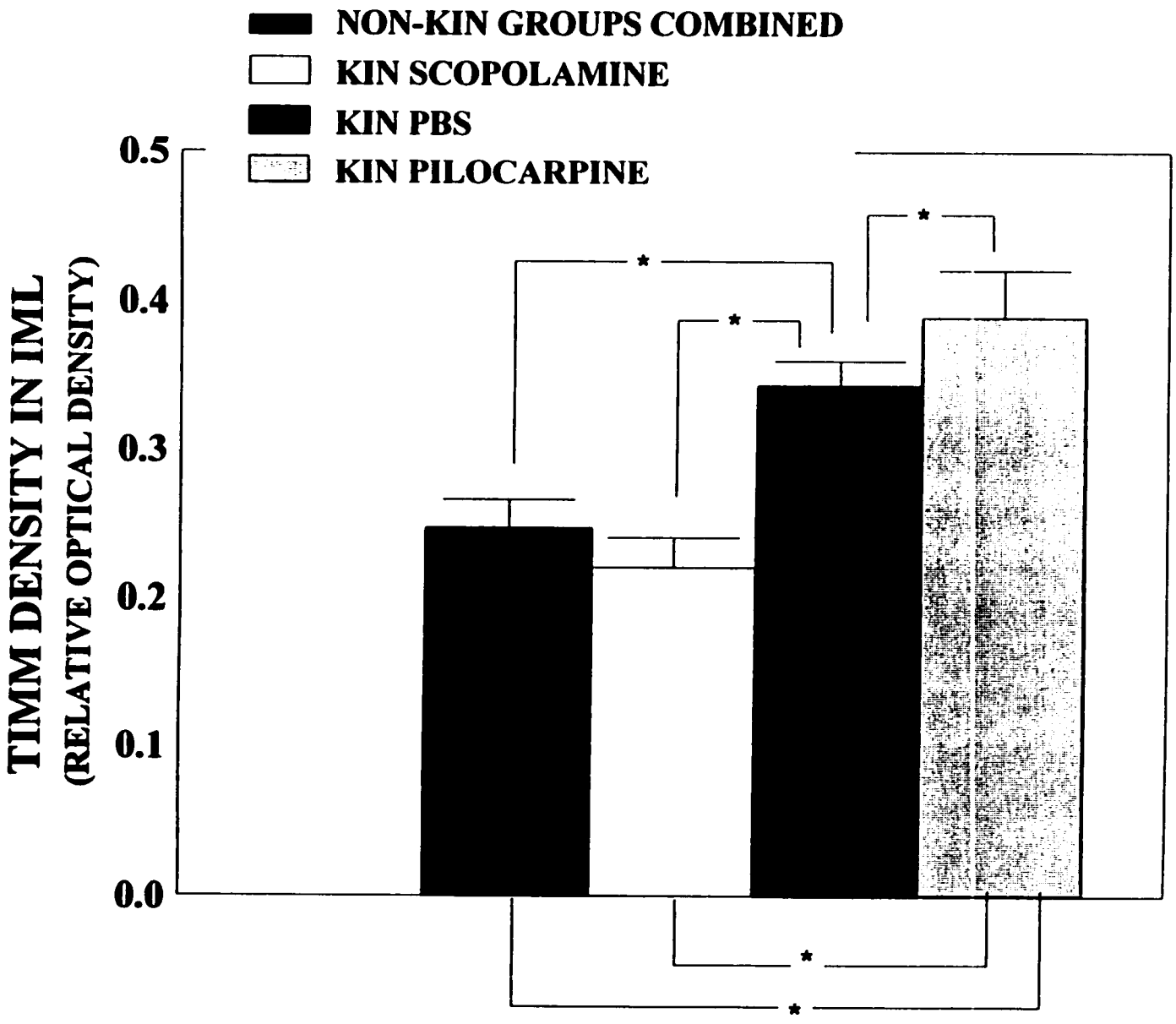

RELATIVE OPTICAL DENSITY
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
2
4
6
8
10
12
14
16
CURSOR POSITION
NON-KINDLED SCOP
NON-KINDLED PILO
NON- KINDLED PBS
KINDLED SCOP
KINDLED PILO
KINDLED PBS

Figure 3.3 Timm granule density in IML region expressed as ROD for non-kindled groups combined (n=16), scopolamine-kindled (n=5), PBS-kindled (n=6), and pilocarpine-kindled (n=6). Timm granule density was increased in the pilocarpine-kindled group relative to the kindled-PBS group ($p<0.05$), the kindled-scopolamine group and all non-kindled groups ($p<0.01$). Timm granule density was increased in the PBS-kindled group compared to the kindled scopolamine group and all non-kindled controls. There were no significant differences between the kindled-scopolamine group and the non-kindled groups. Values represent mean Timm granule density (ROD) as a function of group ± S.E.M. * indicates $p<0.05$


NON-KIN GROUPS COMBINED
KIN SCOPOLAMINE
KIN PBS
KIN PILOCARPINE
TIMM DENSITY IN IML
(RELATIVE OPTICAL DENSITY)
0.5
0.4
0.3
0.2
0.1
0.0
*
*
*
*
*

Chapter 4

If mossy fiber sprouting is activation-induced as opposed to damage-induced, can non-epileptogenic trains trigger mossy fiber sprouting?

In Study 1, we demonstrated that intraventricular nerve growth factor infusions increase kindling-induced mossy fiber sprouting in the absence of neuronal loss. These findings appear to exclude the possibility that mossy fiber sprouting is triggered by any readily detectable neuron losses. These findings also raise the possibility that kindling-induced mossy fiber sprouting may be dependent on the co-involvement of neuronal activation and growth factors.

The purpose of this study was to investigate whether non-epileptogenic neuronal activation (i.e., stimulation that does not evoke an epileptiform afterdischarge and presumably does not produce degenerative effects) can induce mossy fiber sprouting. One example of non-epileptogenic stimulation is the brief high-frequency stimulus trains used to induce long-term potentiation (LTP). As described earlier, LTP refers to an increase in synaptic strength produced by high frequency stimulation of excitatory afferents and can be defined as a stable, long-lasting increase in the amplitude of post-synaptic responses evoked in a neuronal pathway following activation of that pathway with brief tetanic stimulation (Bliss & Lomo, 1972). In this study, long-term potentiation was induced in the dentate gyrus by the application of brief, high frequency trains to the perforant path. To date, there have been no reports of either LTP-induced mossy fiber sprouting or LTP-induced neuronal degeneration following perforant path stimulation.

MATERIALS AND METHODS

Animals and Surgery

Sixteen male Long-Evans rats weighing between 300-400 g were used. Rats were anaesthetized with sodium pentobarbitol (65 mg/kg) and implanted with a bipolar electrode made from teflon-coated stainless steel wires (diameter 120 μm) in the right perforant path. Stereotaxic coordinates were 7.6 mm posterior and 4.1 mm lateral to bregma and 3.3 mm below the skull surface. The electrode was held in place by dental acrylic and three stainless steel screws inserted into the skull. Following a 2-week recovery period, rats were randomly assigned to either an LTP condition (n=8) or a control condition (n=8). Implanted control rats remained in the colony for 32 days post-surgery.

A stimulating (in the perforant path) and a recording electrode (in the dentate gyrus) are typically implanted in the brain when monitoring the progression of perforant path to dentate LTP. In the present study, we did not implant electrodes in the dentate gyrus to monitor the progression of potentiation because it would have substantially interfered with subsequent histological analyses of mossy fiber sprouting in the hippocampus (i.e., it is possible that the implantation of an electrode in the dentate gyrus could produce damage, which could cause mossy fiber sprouting). To eliminate this possibility, we used only a stimulating electrode in the perforant path. Although we did not monitor the progression of LTP in the present study, the stimulation protocol that was used reliably triggers LTP in our laboratory.

LTP Stimulations

To ensure that epileptogenic activity was absent from rats prior to LTP stimulation, a

switching circuit was used to record field activity from the perforant path electrode during the first session of high frequency stimulation. Rats in the LTP group were given one stimulation sesssion a day for 11 consecutive days. During each session, thirty, 8-pulse trains were delivered to the perforant path at a rate of one train every 10 seconds. The pulse frequency within the trains was 400 Hz and the pulse intensity was 1000μA. During the last session of LTP stimulations, post-train electrical activity was recorded again to ensure the continued absence of epileptogenic activity. Control rats were match-handled. Rats remained in the colony for 7 days following the delivery of LTP trains to allow maximal levels of mossy fiber sprouting.

Perfusion and Histology

On Day 32 post-surgery, rats were anesthetized with sodium pentobarbitol and perfused with 50 mL of a sodium sulfide solution (Adams et al., 1997) at room temperature. Following perfusion, brains were frozen in isopentane cooled to - 40°C on dry ice and stored at -70°C. Horizontal 40μm sections of the hippocampal area at 4.1-8.1 mm ventral to bregma were sectioned using a cryostat at -18°C and sections were mounted on chromium potassium sulphate-coated slides.

Brain sections were processed using a modified Timm method (Van der Zee et al., 1995) for the analysis of mossy fiber sprouting. To minimize variability in Timm staining between groups, tissue sections from the experimental and control groups were processed simultaneously. To ensure objectivity in the data analysis, slides were coded and all subsequent analyses were done by an observer who was unaware of the treatment of the animal.

Quantification of Timm Histochemistry

Timm granule density was evaluated in the hippocampal CA3 and IML regions using computer-assisted semi-quantitative densitometry as described in Study 1 (Adams et al., 1997).

RESULTS

Timm Granule Density in the CA3 region

A 4-way ANOVA [2x(10x2x8)] [Group (LTP or control) x Section Depth (ventral to dorsal) x Brain Hemisphere (left and right) x Cursor Position] was conducted on the Timm granule density ratios obtained from the CA3 region. There was a significant Group x Section Depth x Cursor Position interaction: $F(63,819)=1.89$; $p<0.001$, showing that Timm granule density was increased in the LTP groups at cursor positions that are closer to the dentate gyrus and in more dorsal brain sections compared to the implanted controls (Fig. 4.0).

Timm Granule Density in the IML region

A [2x(10x2x9)] [Group x (Section Depth x Brain Hemisphere x Cursor Position)] ANOVA was conducted on the Timm granule densities obtained from the IML region. There was a significant Group x Section Depth x Cursor Position interaction: $F(72, 936)= 1.65$, $p<0.001$, showing that Timm granule density was greatest in the LTP group in the genu region of the dentate gyrus and in more ventral sections compared to the control group (Fig. 4.1).

DISCUSSION

Summary of Results and Implications

These findings provide evidence that LTP trains induce mossy fiber sprouting in the hippocampal CA3 and IML regions following perforant path stimulation. Moreover, these findings suggest that mossy fiber sprouting can occur in the absence of neuronal degeneration and provide support for the hypothesis that mossy fiber sprouting is dependent on neuronal activation. Additional support for this hypothesis is provided by Escobar et al. (1997), who demonstrated that the induction of LTP at the mossy fiber-CA3 synapse produces mossy fiber sprouting in the CA3 region. LTP at the mossy fiber-CA3 synapse is dependent on the activation of opioid receptors, which is believed to be due to opioid peptides contained in and released by the mossy fibers (Derrick et al., 1992). Escobar et al. (1997) directly stimulated the mossy fibers to determine whether synaptogenesis occurs in response to mossy fiber LTP induction. Seven days following LTP induction, Timm staining was reported to be more prominent in the CA3 region on the side contralateral to the stimulation compared to non-stimulated controls. Unlike the present study, however, Escobar et al. (1997) did not attempt to quantify LTP-induced mossy fiber sprouting. Taken together, these two studies suggest that mossy fiber sprouting can be induced by non-epileptogenic forms of stimulation.

Activity-Dependent Plasticity and Neurotrophic Factors

It has been reported that seizure-induced changes in hippocampal neurotrophic factor RNA and protein expression are correlated with synaptic reorganization (Gall, 1993). This raises the possibility that neurotrophic factors may play an important role in the activity dependent plasticity

in epileptogenesis. Similarly, recent research has shown enhanced neurotrophin and neurotrophin receptor mRNA expression specific to LTP in the hippocampus of freely moving rats (Bramham et al., 1996). These results provide additional evidence for a physiological role for neurotrophic factor and neurotrophic factor receptor regulation in activity-dependent synaptic plasticity in the adult hippocampus. Moreover, these findings suggest that an LTP-induced upregulation of growth factors may underlie the LTP-induced mossy fiber sprouting observed in the present study.

What is the Functional Significance of LTP-Induced Hippocampal Plasticity?

It has been suggested that the long-lasting increase in the efficacy of synaptic transmission observed in LTP may be supported by structural synaptic modifications (Lynch et al., 1988; Lynch & Baudry, 1991; Bliss & Collingridge, 1993). Examples of LTP-induced structural modifications include: 1) increases in synaptic numbers in the dentate gyrus of both young and aged rats (Geinisman et al., 1991; 1992), 2) increases in the size of dendritic spines (Van Herreveld & Fifkova, 1975; Fifkova & Van Harreveld, 1977), and 3) increases in the size of dendritic spine heads (Desmond & Levy, 1981). Furthermore, it has been demonstrated that induction of LTP in the dentate gyrus is followed by a significant and marked increase in the number of axospinous synapses with multiple, completely partitioned zones (Geinisman et al., 1993). Although no research to date has explicitly demonstrated that neurotrophins mediate these LTP-induced structural modifications, it is likely that neurotrophins play a role in this form of plasticity.

Although it is feasible that LTP-induced synaptic modifications, including LTP-induced mossy fiber sprouting, may represent a structural substrate of the persistent enhancement of synaptic responses that define LTP, additional studies demonstrating a causal relationship

between these structural modifications and the long-term increases in synaptic efficacy observed in LTP are required.

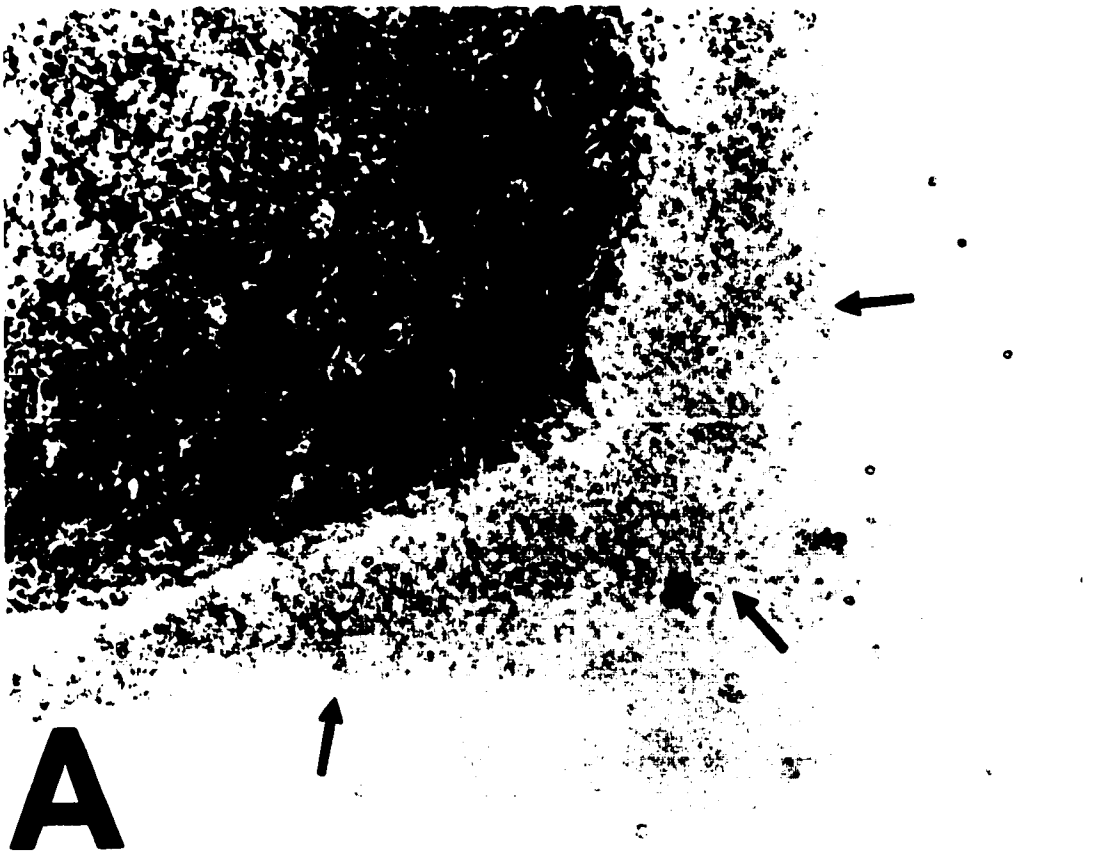
FIGURES and CAPTIONS

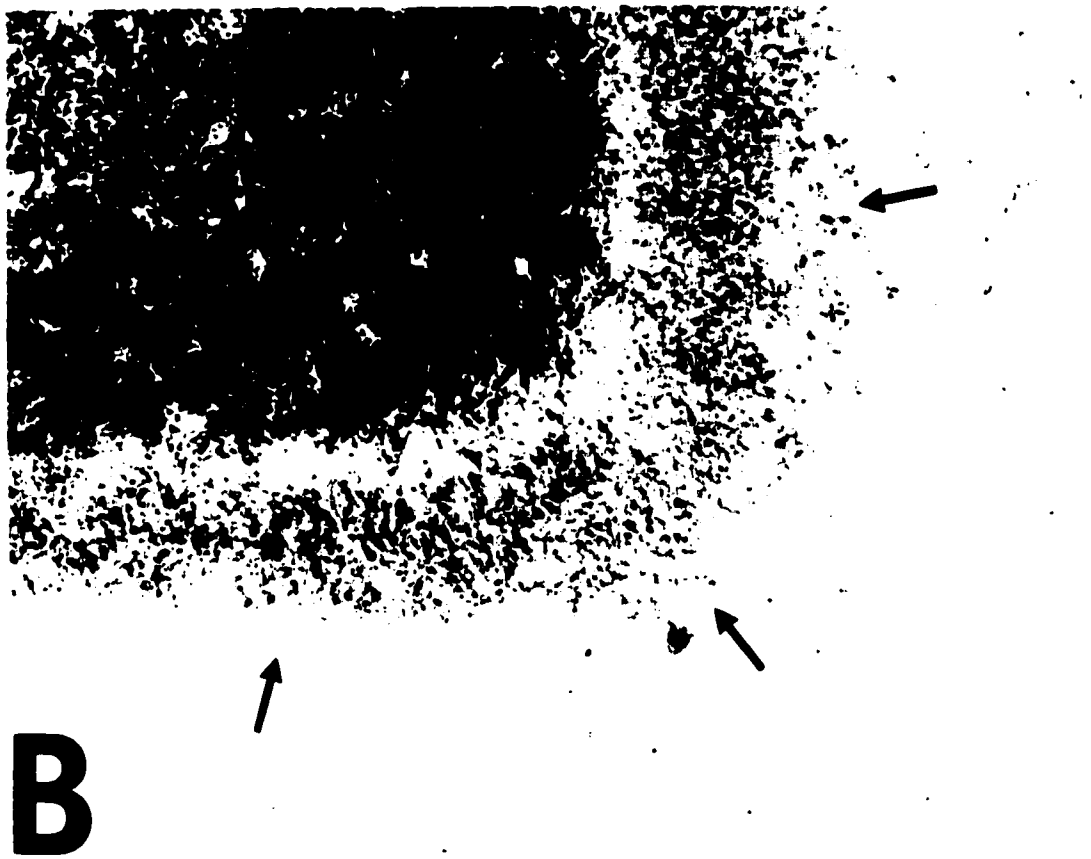
Chapter 4

Figure 4.0 Photomicrographs of CA3 region in implanted control (A) and LTP (B) rats. Timm granule density is increased in the LTP groups at cursor positions that are closer to the dentate gyrus and in more dorsal brain sections compared to the implanted controls. Arrows point to Timm granules.


A
B

Figure 4.1 Photomicrographs of Timm granule density in IML region in control (A) and LTP (B) rats. Timm granule density was greatest in the LTP group in the genu region of the dentate gyrus and in more ventral sections compared to the control group. Arrows point to Timm granules.


A


B

Chapter 5

Can reactive gliosis account for kindling-induced changes in hilar area?

Recent research, including data obtained in Study 1, has shown that kindling causes an increase in the size of the hilar region. To date, potential mechanisms underlying this effect have not been investigated.

One possibility is that kindling-induced changes in hilar area may be related to reactive gliosis (Bertram & Lothman, 1993). Reactive gliosis is generally characterized by: 1) the proliferation and hypertrophy of glial cell bodies and processes, and 2) dramatic increases in the levels of glial fibrillary acidic protein (GFAP) and GFAP mRNA (Torre et al., 1993). Furthermore, increased GFAP immunostaining is considered to be a biochemical hallmark denoting the transformation of normal glial cells to reactive glial cells (Torre et al., 1993).

Moreover, recent research has demonstrated that kindling upregulates GFAP mRNA and protein levels in a time-dependent manner (Hansen et al., 1991; Torre et al., 1993), and that kindling causes glial cell hypertrophy and proliferation (Khurgel et al., 1992). It has also been reported that kindling-induced reactive gliosis can occur in the absence of neuronal loss or degeneration (Steward et al., 1991).

The objectives of this study were to determine whether kindling-induced reactive gliosis could account for kindling-induced changes in hilar area. To accomplish this, we evaluated whether kindling-induced changes in hilar area and kindling-induced changes in reactive gliosis follow a similar time course. Hilar area and reactive gliosis were evaluated at 7 days, 1 month, and 2 months post-kindling compared to non-kindled, implanted controls.

MATERIALS AND METHODS

Animals and Surgery

Adult male Long-Evans hooded rats (n=20) weighing between 300-350 g were used. Rats were maintained on an *ad lib* feeding schedule, housed individually, and kept on a 12 hour on/12 hour off light cycle. Using stereotaxic procedures, rats were anaethetized with sodium pentobarbitol (65 mg/kg) and were implanted with a bipolar electrode made from teflon-coated stainless steel wires in the right perforant path. Stereotaxic coordinates for the perforant path were 7.6 mm posterior and 4.1 mm lateral to bregma, and 3.3 mm ventral to the brain surface. The electrode was held in place by dental acrylic and three stainless steel screws inserted into the skull. Following a two-week recovery period, rats were randomly assigned to either a kindled (n=15) or a non-kindled (n=5) group. Non-kindled control rats remained in the colony for 32 days post-surgery.

Kindling

Kindled rats received a 1 second train of 1 msec pulses at a frequency of 60 Hz and pulse intensity of 500-700μA twice a day for 11 days. Progression of kindling was monitored behaviourally using Racine's classification scale (Racine, 1972) and electrophysiologically using an electroencephalogram of the evoked epileptiform afterdischarges (AD). Following kindling, rats were randomly assigned to one of three groups: 7 days (n=5), 1-month (n=5), and 2-months (n=5). Kindled rats remained in the colony for the assigned time period following the last kindling stimulation.

Perfusion and Histology

Following the assigned time period, rats were perfused with 200 mL of 0.1M phosphate buffered saline (PBS; pH=7.4) followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB; pH=7.4) at 4°C. Brains were post-fixed in 4% paraformaldehyde in 0.1M PB for 24 hours and then stored in a 20% sucrose solution (20g sucrose/100g 0.1M PBS) for 24 hours at 4°C for cryoprotection. Brains were subsequently frozen in isopentane, cooled to -40°C, and stored at -70°C

Horizontal serial 30μm sections were cut using a sliding microtome and section depth was determined according to the stereotaxic atlas of Paxinos and Watson (1986). To ensure that brain sections included in the data analysis were from comparable levels, we selected six pairs of adjacent sections (12 sections/brain) of the hippocampal area at 4.1-7.1 mm ventral to bregma and 600μm apart to be processed for immunocytochemistry.

Sections were incubated overnight with monoclonal anti-GFAP antibody (clone G-A-5, Boehringer Mannheim, Laval Quebec, Canada, 1:200) at 4°C. After washing in 0.1M phosphate buffer (PB), sections were incubated with biotinylated anti-mouse IgG (BA-2000); Dimension Laboratories, Mississauga, Ontario, Canada, 1:200) for 1 hour at room temperature. After washing in PB, sections were then incubated with Vectastain ABC reagent (Vector Laboratories) for 45 minutes at room temperature. Finally, after washing in PB, sections were incubated with 70 mg of diaminobenzidine (Sigma Chemicals) and 30μl of 30% H_2O_2 in 100 mL of 0.1M PB for 60-90 seconds until the desired staining intensity developed. To ensure comparable levels of immunostaining, tissue from all groups was always batch processed. Following immunostaining, tissue sections were mounted on chrom alum-coated slides. One section from each adjacent pair

of sections (6 sections/brain) was also counterstained with Cresyl violet for the determination of hilar area. Slides were then coded and all subsequent analyses were conducted by an observer who was unaware of the treatment of the animal to ensure objectivity in the data analysis.

Hilar Area Measurements

For the evaluation of hilar area, horizontal sections immunostained with GFAP and counterstained with Cresyl violet were examined at 50x magnification by creating a digitized image with the MCID system attached to a light microscope, as described in Study 1 (Adams et al., 1997).

Quantification of GFAP Immunostaining

Figure 5.0 shows representative examples of GFAP immunostained sections in a control rat (A), in rats at 7 days post-kindling (B), 1 month post-kindling (C), and 2 months post-kindling (D). These sections clearly show increases in glial cell size and possibly number in the 7-day and 1-month groups, suggesting that kindling-induced glial cell changes may involve both glial cell hypertrophy and proliferation. Given that increased GFAP immunostaining is considered to reflect reactive gliosis (Torre et al., 1993), hilar GFAP immunostaining was used to quantify reactive gliosis in the present study.

Horizontal sections immunostained with GFAP were examined at 400x magnification using MCID. Hilar GFAP immunostaining was evaluated in a hilar field (0.2mm x 0.48 mm) starting at the hilar end of the CA3/CA4 for each brain section, using MCID's target detection feature. This feature permits image components to be separated into valid targets and background

based on the optical density of the target. Glial cell bodies and processes were regarded as valid targets in the present study. A target acceptance criterion was established using a segmentation range between the upper and lower density thresholds of the target. That is, pixels lying within the segmentation range were regarded as valid targets whereas pixels lying outside of the range were ignored as background. The segmentation range was set manually by decreasing the thresholding value until a blue overlay display, designating the thresholded area completely occupied the glial cell bodies and processes (i.e., the target) but not the background. Figure 5.1 shows MCID images of GFAP immunostained hilar fields (A) before, and (B) after density thresholding. The mean proportional area (i.e., the proportion of the field that is occupied by the target) of GFAP immunostaining was calculated for each hilar field per section.

RESULTS

Kindling Rates and Afterdischarge Duration

As expected, repeated measures ANOVAs confirmed that behavioural seizure stage significantly increased as a function of stimulation number across all groups ($p<0.05$; data not shown), and there was no significant difference in the behavioural progression of kindling between the groups ($p>0.05$). Also, AD duration significantly increased as a function of stimulation number across all three kindled groups ($p<0.05$; data not shown), but did not differ among the three groups. These data suggest that there were no differences in either the behavioural or the electrographic progression of kindling between the three kindled groups. Therefore, any subsequent differences between the groups cannot be attributed to differences in response to the kindling procedure.

Hilar Area Analyses

A (4 x (2 x 6)) ANOVA (Group x (Brain Hemisphere x Section Level)) was conducted to evaluate hilar area. There was a main effect for Group: $F(3,16)= 22.22$; $p<0.001$ (Fig. 5.2). Post-hoc Tukey tests showed that mean hilar area was significantly increased by approximately 47% post-kindling ($p<0.001$) and remained elevated at 1-month post-kindling ($p<0.01$). There were no significant differences between the 1-week and 1-month kindled groups ($p>0.05$). Hilar area measures declined back to control levels by 2 months post kindling ($p<0.01$). These findings suggest that kindling-induced increases in hilar area are not permanent.

GFAP Immunostaining Analyses

A (4 x (2 x 6)) ANOVA (Group x (Brain Hemisphere x Section Level)) was conducted to evaluate the mean proportional area of hilar GFAP immunostaining. There was a main effect for Group: $F(3,16)=44.24$, $p<0.001$ (Fig. 5.3). Post-hoc Tukey tests showed that the mean proportional area of GFAP immunostaining was significantly increased by approximately 57% at 7 days post-kindling, remained elevated at 1-month post-kindling but declined back to control levels at 2 months post-kindling. There were no differences between the 7-day and 1-month post-kindling groups ($p>0.05$), and there were no differences between the control and the 2-month post-kindling groups ($p>0.05$). These findings are similar to those obtained by Hansen et al. (1991), who demonstrated that GFAP protein levels remained elevated at 1 week post-kindling but declined back to control levels at 2 months post-kindling. These data suggest that kindling-induced upregulation of GFAP is transient.

DISCUSSION

The findings in the present study provide the first evidence that kindling-induced changes in hilar area and kindling-induced changes in GFAP immunostaining levels follow a similar time course. In addition, these findings provide support for the hypothesis that kindling-induced changes in hilar area are mediated by kindling-induced glial cell changes.

If Kindling Does Not Produce Damage, What Triggers Kindling-Induced Reactive Gliosis?

The present study showed that reactive gliosis may be a potential mechanism underlying kindling-induced increases in hilar area. It has been widely established that various types of CNS injury, including those mediated by degenerative processes, pharmacological agents, and mechanical lesions can result in reactive gliosis (Torre et al., 1993). A number of different studies, however, have failed to report any form of neuronal degeneration following kindling. If kindling does not produce damage (i.e., cell loss), why does reactive gliosis occur in the kindled brain?

There is some experimental evidence to suggest that reactive gliosis may also be induced in circumstances that are not related to tissue damage. For example, following localized CNS injury, transient levels of GFAP can be detected in regions that are distant from the site of injury, in brain areas where cellular degeneration does not occur (Bonthius, 1994; Hajos et al., 1990; Hozumi et al., 1990). Moreover, following denervating lesions, glial responses can be detected before there is any evidence of axonal degeneration (Steward et al., 1990; 1992). These findings raise the possibility that signals unrelated to tissue damage (i.e., kindling) may be capable of inducing reactive gliosis (Bonthius et al., 1994).

Kindling has been reported to produce reactive gliosis in a range of different brain regions (Racine et al., 1989; Hansen et al., 1990; Steward et al., 1991; Hawrylak et al., 1993). More specifically, the primary effect appears to be hypertrophy of these cells and their processes, accompanied by an increase in GFAP mRNA, total GFAP and GFAP immunoreactivity (Racine et al., 1989; Hansen et al., 1990; Steward et al., 1991). There is also evidence to suggest that this kindling-induced reactive gliosis occurs in the absence of neuronal degeneration (Khurgel et al., 1995). For example, Khurgel et al. (1995) found no evidence of neuronal degeneration in kindled brains using a degeneration-sensitive cupric stain, but did observe kindling-induced reactive gliosis in the amygdala, pyriform cortex, entorhinal cortex and hippocampus. These data suggest that kindling-induced reactive gliosis may not be related to neuronal degeneration, and that kindling-induced reactive gliosis may be associated with abnormal neuronal activity (Khurgel et al., 1995).

What is the purpose of kindling-induced reactive gliosis?

Recent research indicates that reactive gliosis may actually provide a permissive substratum for neurite extension (Represa et al., 1994; Ridet et al., 1997). Under certain conditions, it is now believed that glial cells surrounding axons may play a key role in determining CNS regenerative capacity by promoting axonal outgrowth (Sivron & Schwartz, 1995).

These findings raise the possibility that kindling-induced reactive gliosis may be related to kindling-induced mossy fiber sprouting (Represa et al., 1995). Evidence to support this possibility has been provided by studies demonstrating that glial cells produce a variety of surface and extracellular matrix molecules, which may act as signals that affect process outgrowth, cell movement, protein synthesis, and synaptogenesis (Represa et al., 1995). For example, glial cells

synthesize and secrete cell adhesion molecules, such as neural cell adhesion molecules (NCAM) or tenascin, and extracellular matrix molecules, such as laminin or fibronectin (Sanes, 1989; Represa et al., 1995). Furthermore, it has been proposed that these components may act as either a permissive substrate that enhances axonal outgrowth and helps axons to reach their targets (Edelman, 1986; Rutihauser & Jessel, 1988; Sanes, 1989), or as a repulsive substrate that forms barriers to inhibit growth (Faissner & Kruse, 1990). Therefore, it is possible the reactive astrocytes that express adhesion and substrate molecules following seizure activity may determine the specificity of mossy fiber innervation (Represa et al., 1995).

Support for this notion is provided by the finding that NCAM immunoreactivity is increased on cytoplasmic membranes of axon shafts that are believed to be mossy fiber contacts and on the cytoplasmic membranes of reactive astrocytes at the axon-glial contacts following seizure activity in the hippocampus (Le Gal La Salle et al., 1992; Niquet et al., 1992). It has been readily established that NCAMs are involved in the establishment and maintenance of contact between nerve cells (Edelman, 1986). Thus, these findings suggest that NCAMs may play an important role in regulating the sprouting of mossy fibers following seizure activity (Niquet et al., 1992). Moreover, it has been demonstrated that tenascin-C immunoreactivity was observed in areas in which axonal degeneration did not occur, whereas areas in which reactive synaptogenesis occurred (i.e., the CA3 area and the molecular layer) were devoid of tenascin-C immunoreactivity (Niquet et al., 1995). It has been suggested that tenascin-C may constitute a repulsive substrate for neurons and may contribute to axonal pathfinding by creating a barrier to neuronal growth (Niquet et al. ,1995). These findings indicate that, by expressing adhesion molecules with trophic or repulsive properties, reactive glial cells may influence mossy fiber sprouting in the hippocampus

following seizure activity (Represa et al., 1995). Recent research has also shown that reactive astrocytes express increased levels of growth factors, growth factor receptors and cytokines (Ridet et al., 1997), which may also play a role in mediating kindling-induced synaptic reorganization.

Future research employing interventions to block reactive gliosis in kindling may be useful to gain a better understanding of the role of reactive gliosis in kindling. Several neuroscience groups are in the process of developing anti-gliotic molecules, including antibodies against reactive astrocyte markers, specific antisense oligonucleotides for the corresponding genes, or chemicals to interfere with the physiology of reactive astocytes (Ridet et al., 1997). The effectiveness and the specificity of these different anti-gliotic treatments remains to be determined. In summary, this study provided the first evidence that reactive gliosis may mediate kindling-induced changes in hilar area and it represents a step towards understanding the role of reactive gliosis in kindling.

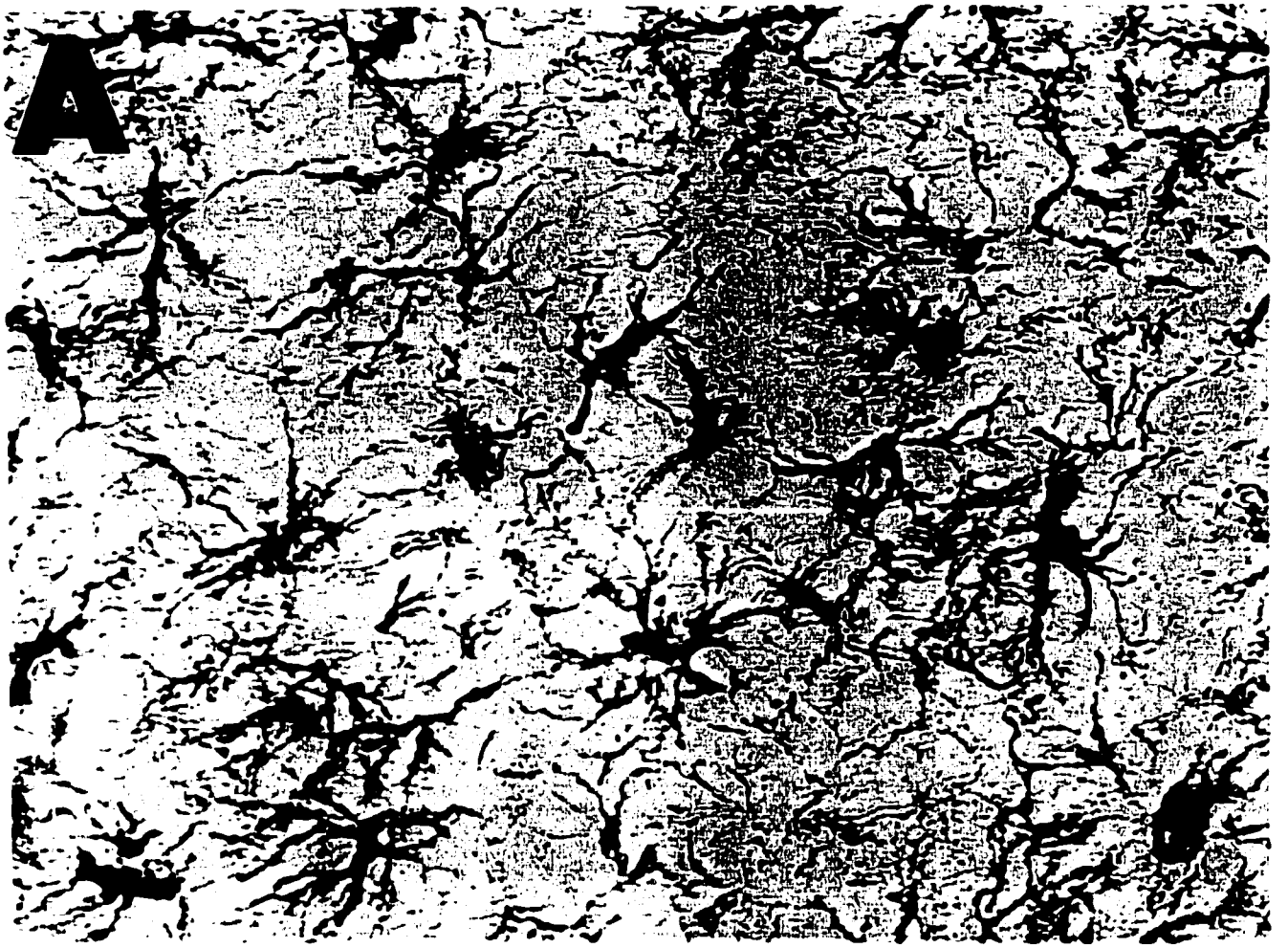
FIGURES and CAPTIONS

Chapter 5

Figure 5.0 Photomicrographs of GFAP immunostaining in the hilar region at 200x magnification. Representative examples of GFAP immunostained sections in a control rat A), in a rat at 7 days post-kindling B), in a rat at 1 month post-kindling C), and in a rat at 2 months post-kindling D). Note that reactive gliosis is evident in both the 1-week and 1-month groups, but is not apparent in either the control or the 2-month groups.


A
B
C
D

Figure 5.1 MCID images of GFAP immunostained hilar fields A) before, and B) after density thresholding, as indicated by the blue colour overlay. Note that after density thresholding, only blue target regions contribute to the proportional area measurement of GFAP immunostaining for each hilar field.


A

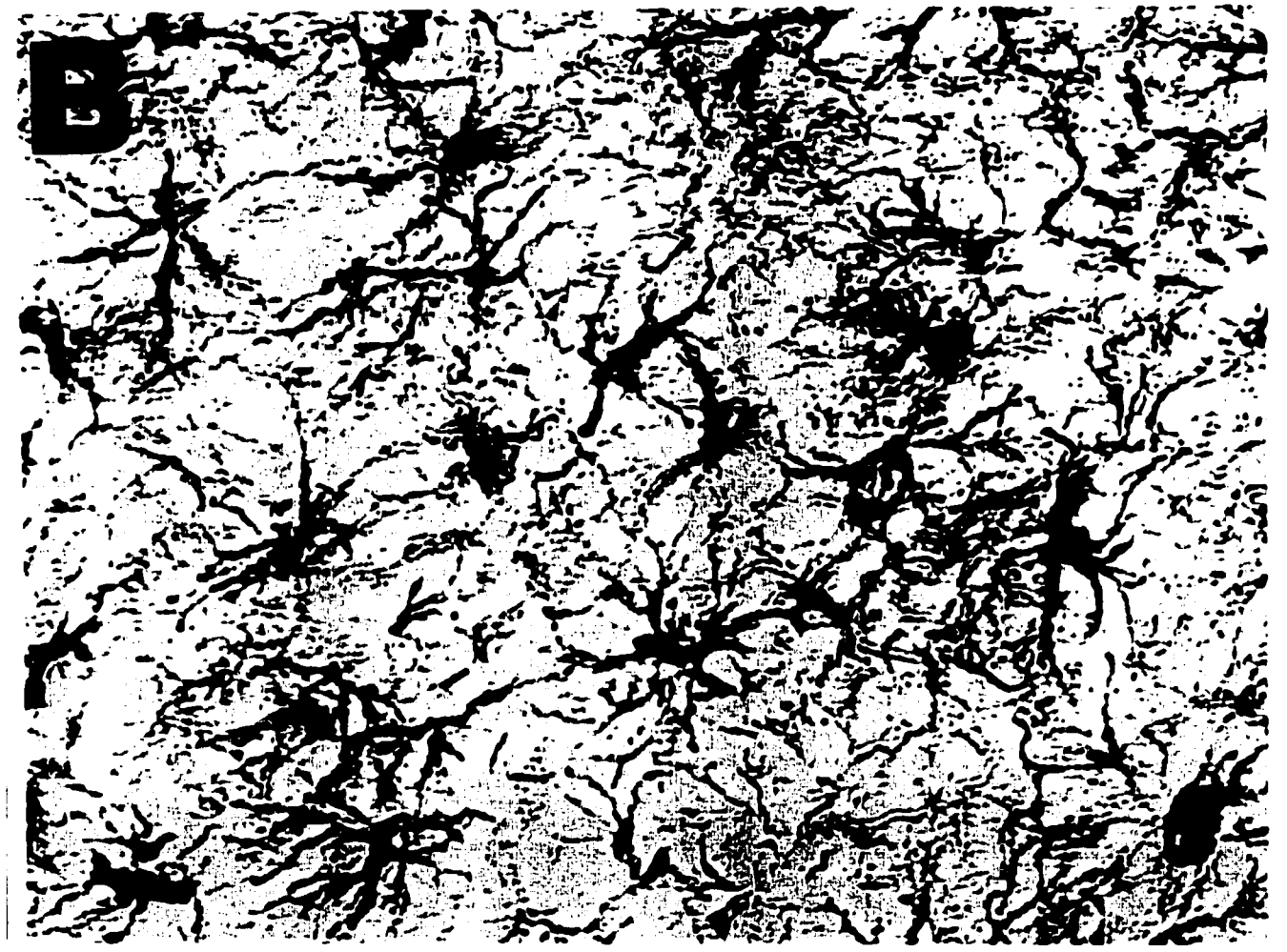

B

Figure 5.2 Mean proportional area of hilar GFAP immunostaining as a function of treatment condition. A 3-way ANOVA with subsequent post-hoc comparisons showed that mean area GFAP immunostaining as a proportion of the total field was significantly elevated at 1 week post-kindling (n=5) and remained elevated at 1 month post-kindling (n=5) ($p<0.05$), but decreased to control levels by 2 months post-kindling ($p<0.05$) (n=5). Mean proportional area of GFAP immunostaining was not significantly different between either the 1-week and the 1-month groups ($p>0.05$), or between the control (n=5) and the 2-month groups ($p>0.05$). * indicates $p<0.05$

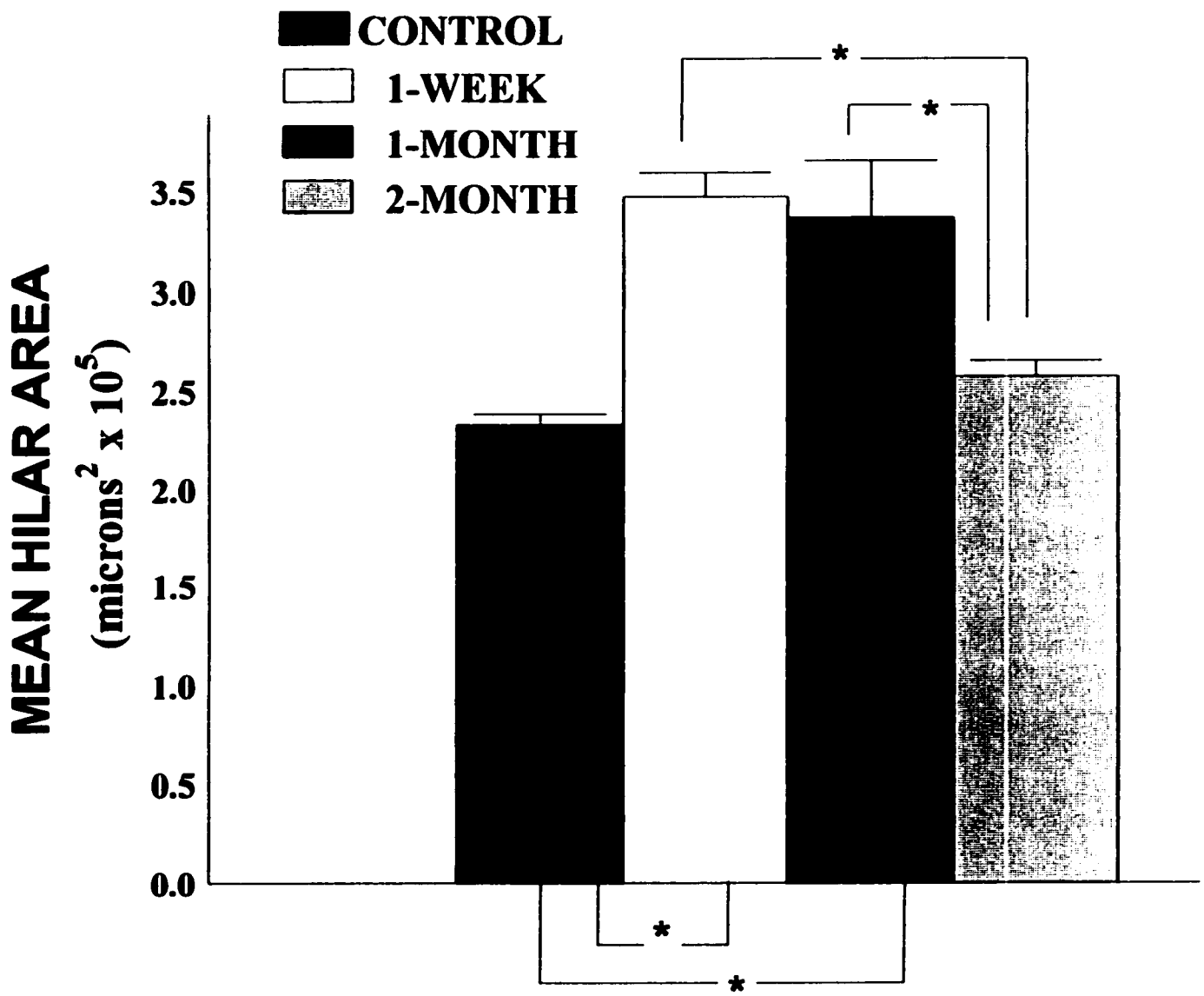
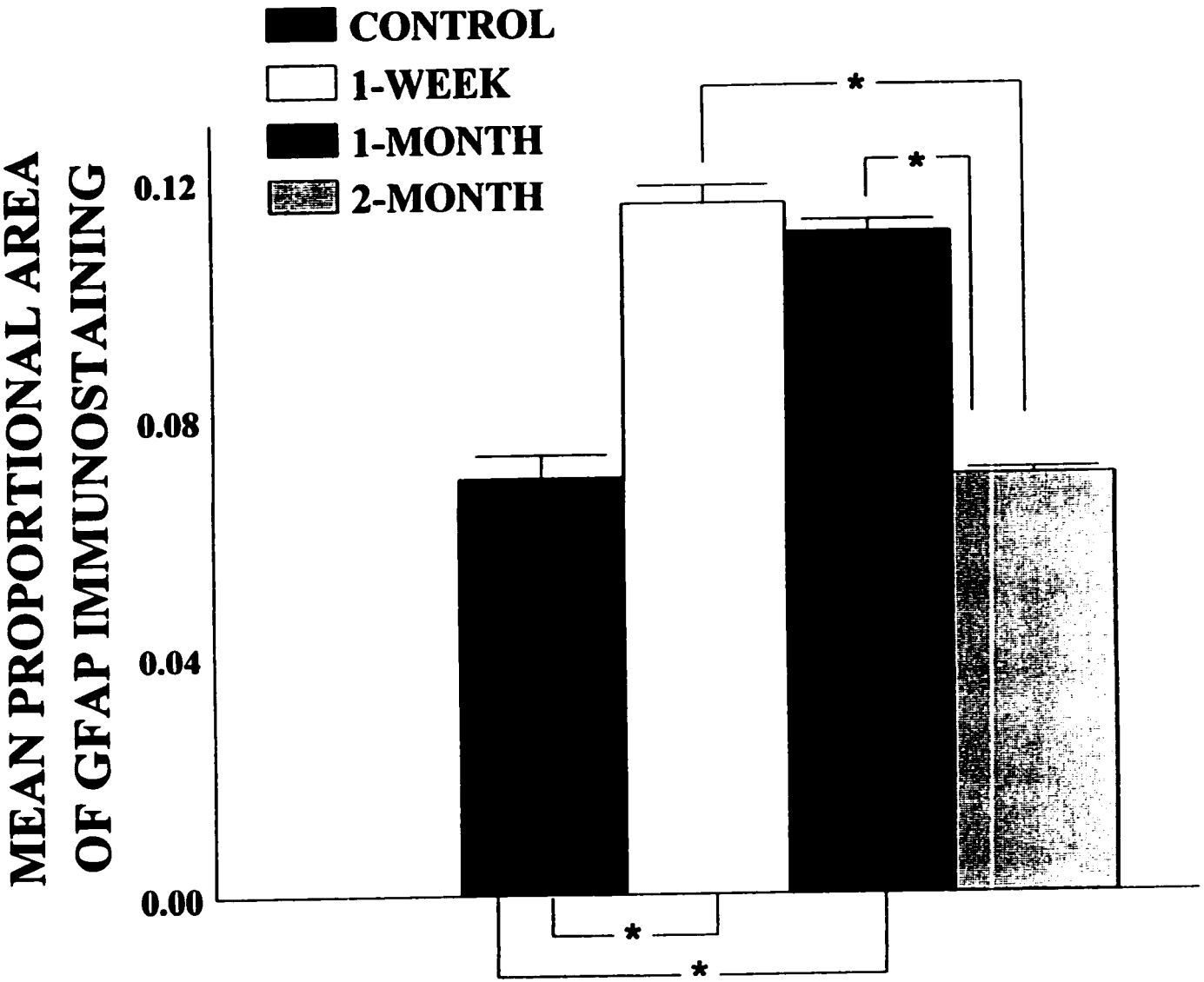

CONTROL
1-WEEK
1-MONTH
2-MONTH
MEAN HILAR AREA
(microns^2 x 10^5)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*

Figure 5.3 Mean hilar area as a function of treatment condition. A 3-way ANOVA with subsequent post-hoc comparisons showed that mean hilar area was significantly increased at 1 week post-kindling (n=5) and remained elevated at 1 month post-kindling (n=5) ($p<0.05$), but declined back to control levels at 2 months post-kindling (n=5) ($p<0.05$). Mean hilar area was not significantly different between either the 1-week and the 1-month groups ($p>0.05$), or between the control (n=5) and the 2-month groups ($p>0.05$). Values represent mean hilar area expressed in μm^2 ± SEM. * indicates $p<0.05$


CONTROL
1-WEEK
1-MONTH
2-MONTH
MEAN PROPORTIONAL AREA
OF GFAP IMMUNOSTAINING
0.12
0.08
0.04
0.00
*
*
*
*

Chapter 6

General Discussion

General Summary of Thesis Findings. The primary objective of this thesis was to gain a better understanding of hippocampal plasticity as expressed by kindling-induced structural changes in the dentate gyrus and mossy fiber system. In Study 1, we investigated whether kindling-induced mossy fiber sprouting in the hippocampal region is triggered by neuronal loss (i.e., damage) or by neuronal activation. To address this issue, we used the neurotrophin, NGF, as a tool to dissociate kindling-induced neuronal loss from kindling-induced mossy fiber sprouting. Data from Study 1 showed that kindling-induced mossy fiber sprouting can proceed in the absence of kindling-induced neuronal loss. These data suggest that kindling-induced mossy fiber sprouting is activation-induced. Moreover, data from Study 2 provided the first evidence that the cholinergic system plays a role in mediating kindling-induced mossy fiber sprouting.

To further investigate whether kindling-induced mossy fiber sprouting is activation-induced, we evaluated whether mossy fiber sprouting can be induced by non-epileptogenic stimulation, using stimulation parameters designed to induce LTP. Data from Study 3 provided some of the first evidence that mossy fiber sprouting can be induced by activation in the absence of neuronal damage.

Finally, in Study 4, we evaluated the time course for kindling-induced changes in hilar area. To date, there has been little research on this form of kindling-induced plasticity. Data from Study 4 provided the first evidence that kindling-induced changes in hilar area are not permanent and that reactive gliosis may be a potential mechanism underlying kindling-induced hilar area changes.

Damage-Induced Plasticity and Kindling

Is Damage Necessary for Kindling-Induced Mossy Fiber Sprouting? Findings from Study 1 and Study 3 showed that damage is not necessary for the development of kindling-induced mossy fiber sprouting. As outlined earlier, kindling has been reported to produce neuronal loss in the hilar region of the hippocampus and the mossy fibers may then sprout as a consequence of this partial denervation in their target region. Results from Study 1, however, show that the intraventricular administration of NGF increases kindling rates and enhances mossy fiber sprouting in the absence of any detectable hilar cell loss. These findings appear to dissociate kindling-induced mossy fiber sprouting and kindling-induced neuronal loss. In Study 1, kindling-induced mossy fiber sprouting appears to be due to the co-involvement of growth factors and activation. Results from Study 3 show that mossy fiber sprouting can be triggered by LTP trains, which provides additional evidence that mossy fiber sprouting is activation-induced as opposed to damage-induced. As outlined earlier, it is likely that LTP-induced mossy fiber sprouting is also due to the co-involvement of growth factors and activation.

Could there be undetected kindling-induced neuronal damage in Study 1? Although hilar neuronal loss was not detected in Study 1, the possibility that a more subtle form of kindling-induced denervation of synaptic targets cannot be excluded. To date, recent research investigating kindling-induced neuronal loss have used biased counting methods (i.e., profile counts and assumption-based methods), including the neuronal counts done in Study 1. Recent stereological research, however, suggests that these classical methods of counting neurons can produce inaccurrate counts (Coggeshall & Lekan, 1996). For example, in Study 1, we evaluated mean hilar neuronal density in kindled and non-kindled animals. This counting method is referred to as the

method of areal densities (Coggeshall & Lekan, 1996). It involves determining areal densities in the control and experimental condition and determining their ratios. For instance, using this method, if a 30% decrease in mean hilar neuronal density were observed in the kindled condition compared to the non-kindled condition, we would conclude that kindling causes a 30% decrease in neuronal density.

There are problems, however, associated with this counting procedure. According to Coggeshall & Lekan (1996), there is no simple relation between profile counts and neuron number. It should be noted that we are counting profiles of neurons (i.e., labelled structures in a histologic section) and not entire neurons themselves. Therefore, the necessary assumption is that changes in profile densities are reflecting changes in neuron numbers, even if the profile numbers are not the same as neuron numbers. Coggeshall & Lekan (1996) state that neurons can change in size and shape following a perturbation, resulting in a modification of profile densities and ratios unrelated to changes in neuron numbers. Moreover, if a population of cells is lost, it is generally a specific population which changes profile densities and ratios disproportionately to alterations in neuron numbers (i.e., if large neurons are lost, there will be a disproportionate drop in profile numbers because large neurons are sectioned into more profiles than small neurons) (Coggeshall & Lekan, 1996). Another problem is that the reference space may change, which could affect neuronal densities or ratios, even if neuron numbers remain constant (i.e., if kindling causes a 50% increase in hilar area, neuronal densities would decrease by 50% if no neurons were lost). This problem is referred to as the reference trap (Braedengaard & Gundersen, 1986; Pakkenberg et al., 1991). Therefore, according to Coggeshall and Lekan (1996), although commonly used, the method of areal densities may not provide an accurate estimate of total number or changes in numbers

because the number of profiles is dependent on many variables besides neuron numbers. Based on these arguments, it would be useful to compare neuronal counts in kindled and non-kindled brains using stereological techniques. It should be noted, however, that the experiments outlined in Study 1 had already been reported by others using similar counting procedures. It is quite clear, even with these procedures, that the large neuronal losses reported by Cavazos et al. (1991) are not found in the strain of rat used in the experiments of Study 1. Future experiments from the Racine laboratory will utilize stereological counting procedures.

Activation-Induced Plasticity and Kindling

Can neural activation trigger mossy fiber sprouting? Study 1 showed that kindling-induced mossy fiber sprouting was likely due to the combination of neural activation and growth factors as opposed to neural damage. To study this further, we investigated whether non-epileptogenic stimulation (i.e., LTP trains) could induce mossy fiber sprouting because there is no evidence showing that LTP is accompanied by neural damage. Findings from Study 3 provided further evidence that mossy fiber sprouting can be induced by neural activation. These data also provided support for the notion that neural damage may not be necessary to trigger kindling-induced mossy fiber sprouting. In the case of kindling, then, mossy fiber sprouting may be a consequence of the high-frequency ictal discharge that is the hallmark of kindling.

How does activation trigger mossy fiber sprouting? There is increasing evidence that high levels of neuronal activity like those occurring during seizure activity are associated with changes in gene expression (Mody, 1993). A number of different genes is regulated by excessive neuronal activity include those encoding neurotrophic factors and their tyrosine kinase receptors, glutamate

receptors, neuroactive peptides, potassium channels, transcription regulatory proteins, extracellular proteases and glutamic acid decarboxylase (GAD). In the case of NGF, it is clear that seizure activity increases NGF levels in the brain and Study 1 provides evidence that seizure-induced increases in NGF may play an important role in mediating kindling-induced mossy fiber sprouting.

Kindling induces a long-lasting increase in excitatory synaptic transmission in the dentate gyrus, which is mediated in part by the NMDA subfamily of glutamate-gated ion channels in granule cells (Mody & Heineman, 1987; Mody et al., 1988). This increase in excitatory transmission is accompanied by a complex sequence of gene expression which involves transient increases in transcription factors (e.g., Dragunow & Robertson, 1987; Morgan & Curran, 1991), and more slowly evolving changes in neurotrophins (Gall & Isackson, 1989; Enfors et al., 1991; Gall, 1993; Lindval et al., 1994), neurotrophic factor receptors (Bengzon et al., 1993; Burgra et al., 1994) and other growth-associated proteins (Bendotti et al., 1993; Meberg, 1993). It has been suggested that these seizure-induced changes in NMDA-dependent synaptic transmission and gene expression are followed by other cellular alterations that include mossy fiber sprouting (Sutula et al., 1996).

Sutula et al. (1996) showed that the NMDA antagonist MK-801 impedes the progression of kindling and impairs mossy fiber sprouting. These findings suggest that the NMDA receptor plays a role in modifying the organization of hippocampal circuits in the adult brain. Moreover, the results from Study 2 provided the first evidence that another neurotransmitter system, the cholinergic system, may also play a role in modifying patterns of connectivity in the adult hippocampus. In Study 2, we showed that the cholinergic agonist pilocarpine accelerates the development of kindling and enhances mossy fiber sprouting, whereas the cholinergic antagonist

scopolamine retards the development of kindling and impedes mossy fiber sprouting. Although it is not known how the cholinergic system mediates these effects, data from Study 2 clearly suggest that the cholinergic system is involved in kindling-induced mossy fiber sprouting. Moreover, the results from Study 2 also suggest that NGF may enhance mossy fiber sprouting by enhancing cholinergic activity in the brain.

What is the significance of seizure-induced mossy fiber sprouting? As outlined earlier, the evidence that mossy fiber sprouting accompanies kindling is compelling (e.g., Sutula et al., 1988; Cavazos et al., 1991; Represa & Ben-Ari, 1992; Represa et al., 1993). Based on these findings, it has been suggested that seizure-induced mossy fiber sprouting may actually contribute to the pathophysiology of epileptogenesis by creating abnormal, excitatory connections in the brain.

Alternatively, the possibility should not be excluded that mossy fiber sprouting may simply be an epiphenomenon of seizure activity, playing no role in epileptogenesis. There is some research to suggest that kindling can proceed in the absence of mossy fiber sprouting. For example, as described earlier, Longo and Mello (1997) showed that the protein synthesis inhibitor, cycloheximide, can block kainate- or pilocarpine-induced mossy fiber sprouting without interfering with subsequent epileptogenesis, which suggests that mossy fiber sprouting may not play a causal role in kindling.

There is also evidence showing that mossy fiber sprouting can be dissociated from kindling epileptogenesis. For example, Mohapel et al. (1997) demonstrated that, in kindled rats with transections of the fimbria-fornix, mossy fiber sprouting was increased, whereas kindling rates were retarded. In addition, Armitage et al. (1997) demonstrated that the time course of rapid (low

frequency) kindling and the time course for mossy fiber sprouting were different. More specifically, Armitage et al. (1997) found no evidence of increased Timm granule density in the IML region after rapid kindling with low-frequency amygdaloid stimulation when rats were killed 24 hours following the first stage 5 seizure (a mean of approximately 6 days after the first AD). Although this time interval was too short for significant levels of mossy fiber sprouting to occur (although it may be possible that very low levels of mossy fiber sprouting are present 6 days following the onset of rapid kindling), it is conceivable that mossy fiber sprouting would occur after rapid kindling if rats were sacrificed at a longer time interval following kindling. To examine this possibility, Armitage et al. (1997) allowed rapidly kindled rats to survive approximately two weeks after the first stage 5 seizure and found variable but higher levels of Timm granules in the IML region compared to control rats. These findings suggest that sprouting of the mossy fibers into the IML of the dentate gyrus is not a prerequisite for the development of rapid kindling. These findings provide additional support for the hypothesis that mossy fiber sprouting may not be related to the development or maintenance of kindling.

In summary, this thesis examined different forms of kindling-induced hippocampal plasticity and provided new insight into the mechanisms underlying activation-induced structural plasticity. To date, it is not clear whether kindling-induced hippocampal plasticity is adaptive or maladaptive. One possibility is that kindling-induced hippocampal changes may represent an adaptive response to seizure activity and may lead to a recovery of function following seizure activity. Alternatively, it is possible that kindling-induced hippocampal plasticity may be a pathological response to seizure activity and may actually contribute to the development and/or

maintenance of the kindled state. It is also possible that some forms of kindling-induced hippocampal plasticity may have no functional significance and may not contribute to the epileptogenic state. A better understanding of the mechanisms underlying the relationship between kindling and kindling-induced plasticity, however, could represent a potential site for therapeutic intervention in the treatment of epileptogenesis.

Summary of Conclusions:

1. Kindling-induced mossy fiber sprouting can proceed in the absence of neural damage, which suggests that kindling-induced mossy fiber sprouting is activation-induced, as opposed to damage-induced.

2. Nerve growth factor (NGF) plays a role in kindling and kindling-induced mossy fiber sprouting.

3. The cholinergic system is involved in kindling-induced mossy fiber sprouting.

4. Hippocampal mossy fiber sprouting can be induced by non-epileptogenic stimulation that does not cause neural degeneration (i.e., LTP trains.)

5. Kindling-induced reactive gliosis can account for kindling-induced changes in hilar area.

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