EFFECTS OF NIFEDIPINE ON KINDLING AND MOSSY FIBRE SPROUTING

THE EFFECT OF THE VOLTAGE-GATED CALCIUM CHANNEL BLOCKER, NIFEDIPINE, ON KINDLING AND KINDLING-INDUCED MOSSY FIBRE SPROUTING

By

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Abstract

Kindling epileptogenesis has been associated with a number of different forms of neuroplasticity in the hippocampus, including mossy fibre sprouting and an increase in both intracellular calcium and zinc. The purpose of this thesis was to determine whether interfering with the influx of calcium via the voltage gated calcium channels would interfere with kindling- induced plasticity. Both kindled and control rats were injected with either 5 or 25mg/kg of the L-type voltage gated calcium channel blocker, nifedipine, or a control vehicle, DMSO (dimethylsulfoxide). The kindled groups received a kindling stimulation twice a day for 11 days. It was revealed that both doses of nifedipine significantly increased afterdischarge duration ($p<0.001$) and furthermore, both doses of nifedipine were capable of significantly interfering with the rate of kindling ($p<0.001$). Three weeks following the last kindling stimulation, rats were perfused and brain tissue was processed according to the Timm method. The density of Timm granules, an indication of the level of intracellular zinc in the mossy fibre pathway, was quantified. The results of this analysis revealed that 25mg/kg of nifedipine is capable of significantly reducing the amount of intracellular zinc in both the IML ($p<0.04$) and the CA3 ($p<0.01$) region of the mossy fibre pathway, regardless of whether the rats had received kindling stimulations or not. These results provide support for the notion that nifedipine (5 or

25mg/kg) is an effective anticonvulsant agent. These results also suggest that, at a sufficient dose (25mg/kg), nifedipine can reduce the amount of intracellular zinc in the mossy fibre pathway in both kindled and non-kindled animals, suggesting that nifedipine may be a useful therapeutic agent for pathologies that have been associated with zinc-induced neurotoxicity.

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List of Abbreviations

AD	afterdischarge
ADD	afterdischarge duration
ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
Ca^{2+}	calcium
DMSO	dimethylsulfoxide
EEG	electroencephalogram
GAP	growth associated protein
IE	immediate early
IML	inner molecular layer
IP	intraperitoneal
MCID	micro computer imaging device
NMDA	N-methyl-D-aspartate
PP	perforant path
TLE	temporal lobe epilepsy
Zn^{2+}	zinc

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CHAPTER 1

General Introduction

1.0 Temporal Lobe Epilepsy

Epilepsy is the second most common neurological disorder after stroke, and is defined by brief, recurrent, spontaneous seizures (Tortora & Anagnostaka, 1990). Epileptic seizures are caused by the abnormal and highly synchronous discharge of millions of neurons in the brain. The resulting nerve impulses often lead to involuntary muscle contraction, sensations of stimuli not present, and possibly inhibition of the waking centres in the brain leading to a loss of consciousness (Tortora & Anagnostaka, 1990).

The epilepsies refer to a heterogeneous group of chronic neurological disorders with diverse etiologies, electrographical and behavioural seizure patterns, and pharmacological sensitivities (Simonato, 1993). In adults, the most common and devastating form is temporal lobe epilepsy (TLE), particularly partial complex temporal lobe epilepsy (Simonato, 1993), which accounts for approximately 40% of all cases in adults. TLE is characterized by repeated episodes of confused behaviour, stereotyped motor behaviour, and often a loss of consciousness. Individuals with TLE often do not respond favourably to the presently available anticonvulsant drugs, with only 25% gaining

complete seizure control (McNamara, Bonhaus, & Shin, 1993). Therefore, a significant number of people affected by the disorder are unable to lead normal lives. They are, for example, unable to drive a motor vehicle, which often leads to the inability to gain and/or maintain employment (McNamara, et al., 1993). For nearly 30 years investigators have used an animal model of epilepsy, termed kindling, to investigate the mechanisms underlying TLE.

1.1 Kindling Epileptogenesis

Kindling is a phenomenon that was discovered by Graham Goddard and his colleagues nearly 30 years ago, and is now a well established animal model of human temporal lobe epilepsy (TLE). The typical kindling procedure involves the repeated application of a convulsant agent, usually electrical stimulation of a particular brain region. The consequence of this treatment is a progressive intensification of seizure activity, leading to the development of a permanent seizure- prone state (Goddard, McIntyre, & Leech, 1969). Electrical kindling typically involves the application of brief (1 or 2 second), low intensity trains of sine wave or biphasic electrical pulses at a frequency of 60 Hz. These trains are usually applied once daily through a chronically implanted electrode (Goddard et al., 1969). The initial stimulus typically does not evoke any detectable change in behaviour, although it will elicit a focal afterdischarge (brief seizure-like event recorded on the EEG) (Racine, 1972b; McNamara, 1989). The stimulation trains must induce afterdischarges (AD) in order to produce kindling. The

AD 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. Subsequent stimulations lead to a progressive lengthening and spread of electrical seizure which is paralleled by the development of a behavioural seizure (Racine, 1972a, b). Behaviourial seizures typically evolve through the following stages as defined by Racine (1972b): Class 1-Facial clonus (mouth and facial twitches), Class 2- clonic head movements(nodding), Class 3-Contralateral forelimb clonus, Class 4- clonic rearing, and Class 5- rearing and falling (loss of postural control).

The temporal lobe structures are particularly prone to seizures. Thresholds are low and fewer stimulations are required to reach a fully kindled state (class 5 seizure) (Racine, 1972a, b). The amygdala is by far the most commonly kindled structure. The amygdala has a very low seizure threshold and requires only 10-15 stimulations to develop a class 5 seizure (Racine, 1972a, b). Once the enhanced sensitivity (3 class 5 seizures) has developed, the animal is said to be fully kindled. This enhanced sensitivity is long-lasting and there is some evidence that it may be permanent (Goddard et al., 1969; Wada et al., 1974). For example, it has been demonstrated that amygdala kindling persists with relatively little loss following intervals of up to twelve months (Goddard et al., 1969; Wada et al., 1974), and following these long delays, kindled animals rarely require more than one or two stimulations to show maximal behavioural and electrographic responses.

The kindling model allows us to differentiate between the epileptic state and the development of that state (McNamara et al., 1993). Furthermore, kindling is one of the few models that offers complete control over the epileptic agent as well as one of the few models of neural plasticity in the adult animal that involves a permanent neural reorganization (Racine, 1978).

1.2 Kindling as a model of epilepsy

There are a number of fundamental similarities between the kindling phenomenon and human temporal lobe epilepsy: the EEG activity recorded from intracerebral electrodes during kindled seizures and seizures experienced by humans with TLE are similar; the behavioural manifestations during kindled seizures TLE are similar; both types of seizures respond similarly to conventional anticonvulsant agents; and there are comparable structural alterations in hippocampal circuitry. Let us now consider those points in more detail.

Both kindled discharges and TLE discharges consist of rhythmic polyspikes evolving into polyspike and wave patterns (Sato, Racine & McIntyre, 1990). Furthermore, the presence of interictal spike transients in EEG recordings from the hippocampus and the amygdala is another feature common to both the kindling model and partial complex seizures in humans (Sato, et al., 1990). Finally, both kindled seizures and partial complex seizures in humans lead to a transient increase in the frequency of interictal spikes (Sato, et al., 1990).

Seizures are often accompanied by very characteristic motor behaviors. According to Sato et al. (1990) the behavioural patterns of class 1 and 2 kindled seizures are remarkably similar to those of complex partial seizures of presumed temporal origin in humans. The arrest of ongoing activity, oral automatisms and reduced responsiveness to external stimuli are common features of kindled and human seizures. Finally, the clonic motor components of secondary generalized limbic seizures are also analogous in rats and humans.

The kindling model is widely used for the evaluation of drugs believed to possess anticonvulsant properties (Albertson, Peterson, & Stark, 1980). The anticonvulsant pharmacology of kindled seizures in animals and partial complex seizures in humans is remarkably similar. The anticonvulsant agents phenobarbital, carbamazepine, valproate, and diazepam effectively suppress both kindled (amygdala) seizures and complex partial seizures (Wada & Osawa, 1976). Conversely, anticonvulsants effective in the treatment of petite mal but *not* complex partial seizures, such as ethosuximide and trimethadone, are ineffective against kindled seizures (Sato, et al., 1990). Finally, investigations of the kindling model have led to new compounds, such as WB-CPI, that have potential anticonvulsant activities (Albertson et al., 1980).

Kindling has been associated with a number of permanent structural changes in the brain, including synaptic reorganization of connections of the glutamatergic mossy fibre pathway that originates from the hippocampal dentate granule cells. These mossy fibres normally innervate the polymorph and the CA3 neurons and provide very little

innervation of structures within the granular or molecular layers of the dentate gyrus. However, as a result of kindling, collateral sprouting of the mossy fibres has been observed in the supergranular layer, the hilus, and in the CA3 region of the hippocampus (Sutula et al., 1988). The axonal sprouting and reorganization of the mossy fibre pathway also occurs in humans suffering with TLE (Sutula et al., 1988).

Hippocampus sclerosis tends to be the most commonly found lesion in human partial complex epilepsy (Sutula, Cascino, Cavazos, Parada, & Ramirez, 1989). Although there is still some controversy about the extent of hippocampus damage in kindling, there have been some reports of neuron losses similar to those reported in humans.

A common criticism of the kindling model is that it rarely results in spontaneous seizures (Majkowski, 1986). It is clear, however, that the development of spontaneous seizures in the kindling model is simply dependent upon the number of stimulations applied. Long-term periodic stimulation of the amygdala, hippocampus, or entorhinal cortex reliably leads to the appearance of spontaneous seizures (Pinel & Rovner, 1978)

The kindling phenomenon has been documented to occur in many species including frogs (Morrell & Tsuru, 1979), mice (Leech & McIntyre, 1976), rabbits (Whieldon &VanHarreveld, 1950), dogs (Watanabe, 1936), cats (Alsonso-De Florida & Delgado, 1958, Wada & Sato, 1974), monkeys (Wada, Mizoguchi & Osawa, 1978) and baboons (Wada & Osawa, 1976), therefore, it highly likely that it also occurs in humans. Furthermore, research also suggests that kindling can occur in humans (McNamara,

1989). Specifically, partial complex seizures are associated with: 1) A time-dependent spread of epileptogencity independent of tissue pathology; 2) A some kind of LTP persisting interictally with respect transmission of activity between limbic structures; 3) Lasting changes in behaviour following repeated limbic stimulation with and without associated changes in epileptogencity.

TLE patients have a much better outcome if surgery, to remove the temporal lobe focus, occurred closer to the date of first seizure than after longer intervals (Perrin & Hoffman, 1979). This finding supports the idea that TLE, like kindling, is an incrementally developing seizure disorder. Furthermore, Jackson (1931) described the seizure progression of untreated human epilepsy as one in which each seizure was progressively more intense. Based on these findings it seems reasonable that an understanding of kindling and its underlying mechanisms could help to further understand the pathology of human epilepsy.

1.3 Mossy Fibre Sprouting

Kindling has been associated with a number of permanent structural changes in the brain, including synaptic reorganization of connections of the glutamatergic mossy fibre pathway that originates from hippocampal dentate granule cells. The mossy fibres have an unusually high zinc content and therefore, the trajectory of mossy fibre axons and the location of their synaptic terminals can be readily identified at the light and

ultrastructural level with the Timm method, which allows us to identify neural tissue containing heavy metals (Sutula, Xiao-Xian, Cavazos, & Scott, 1988).

It is presently not clear whether the glutamatergic mossy fibre sprouting contributes to, or is simply an effect of, kindling or epilepsy. However, a number of investigators believe that this reorganization of the mossy fibre pathway may contribute to the synchronization and epileptiform activity of neurons in the hippocampus (Houser, 1992). There appears to be a positive correlation between mossy fiber sprouting and the behavioural progression of seizures (Sutula, et al., 1988). Furthermore, both the induced susceptibility to seizures and the alterations in synaptic connectivity of the mossy fibre pathway appear to be permanent (Sutula et al., 1988). It is therefore possible that an aberrant and enhanced mossy fibre synaptogenesis impairs hippocampal function and contributes to the development and maintenance of epilepsy.

1.4 NMDA Receptors

The excitatory amino acids, glutamate and aspartate, are believed to subserve excitatory synaptic transmission throughout the mammalian central nervous system. Three receptor subtypes have been defined pharmacologically, based on the agonists that selectively bind to them: N-methyl-D-aspartate (NMDA), kainate, and quisqualate (McLennan, 1984). NMDA receptors and their associated ion channels possess the unique property of being blocked by magnesium ions when the cell is at rest, it is only by depolarization that the magnesium ion is dislodged from the channel in a voltage

dependent manner (Gilbert, 1988). Therefore, NMDA receptors do not appear to participate in low frequency synaptic transmission, but are activated by glutamate and aspartate only following sufficient membrane depolarization (Gilbert, 1988). NMDA receptor responses have been well characterized. Once activated, the response is defined by slow rise times, prolonged, rhythmic depolarizations, and regular bursts of action potentials (Gilbert, 1988). It is believed that action at the NMDA receptors is critical for the development and possibly the maintenance of kindling epileptogenesis. Glutamate, aspartate and their antagonists have been shown to be capable of inducing epileptiform activity (Peterson, Collins, & Bradford, 1983). Furthermore, a number of NMDA-receptor antagonists have been shown to have strong anticonvulsant properties in both acute (Croucher, Collins, Meldrum, 1982) and chronic seizure models (Gilbert, 1988). Finally, it has further been revealed that glutamate release is significantly increased following generalized kindled seizures (Peterson, et al., 1983).

The initial kindling stimulations induce a long-lasting increase in excitatory synaptic transmission (Racine et. al., 1975; Sutula & Steward, 1986). In the dentate gyrus, this kindling-induced potentiation effect is at least partially mediated by the NMDA subfamily of glutamate-gated channels (Sutula, Koch, Golarai, Watanbe, & McNamara, 1996). In the same system, kindling results in a long-lasting change in cellular physiology in response to synaptic activation of the NMDA subtype of excitatory amino acid receptors (Mody , Reynolds, Salter, Carlen, & MacDonald, 1990). It is believed that this initial physiological alteration is dependent on substantial increases in

intracellular calcium followed by a complex, evolving sequence of gene expression. There are also slowly evolving changes in neurotrophins, neurotrophic factor receptors and axonal growth associated proteins such as GAP-43 (Sutula, 1996).

Sutula and colleagues used the non-competitive NMDA receptor antagonist (MK-801) to determine whether blocking the NMDA receptor could prevent the long-term, kindling-induced structural and functional alterations of the neurons in the dentate gyrus. They demonstrated that a subcutaneous injection of 5mg/kg of MK-801 thirty minutes prior to each kindling stimulation significantly prolonged the mean afterdischarge duration, impaired the progression of kindling to the stage of class five seizures and impaired the development of the kindling-induced mossy fibre sprouting (Sutula, et al., 1996). These observations suggest that the NMDA receptor plays a key role in the signaling cascade that translates the initial transmembrane alterations induced by kindling stimulation into long-term alterations of neuronal structure and function.

1.5 Voltage-gated Calcium Channels

Voltage-gated calcium (Ca^{2+}) channels have been typed according to their physiological properties (Catterall & Striessnig, 1992). To date at least 4 different types have been found: L, N, T and P, each of which is activated by different levels of depolarization, has different times of inactivation and has a specific pharmacology. Voltage-dependent calcium fluxes are ultimately dependent on membrane potential. For

instance, Low-voltage-activated, or T-type, Ca2+ channels are activated at relatively negative membrane potentials, have a small single channel conductance, and mediate a transient Ca2+ current that is important in determining the frequency of action potential generation in neurons and cardiac muscle cells. High-voltage-activated Ca2+ channels require a more positive membrane potential for activation and include L, N, and P channel types. N- and P-type Ca2+ channels are *inactivated* at membrane potentials more positive than -40mV and have an intermediate single channel conductance compared with T and L types. They are expressed primarily in neurons, where they play an important role in neurotransmitter release. L-type Ca2+ channels are not strongly *inactivated* by depolarization to -40mV, have the largest single-channel conductance among the voltage-gated Ca2+ channels, and have both slow voltage-dependent and more prominent Ca2+-dependent mechanisms of inactivation. They are the molecular target for the actions of the therapeutically useful Ca2+ channel antagonists, used in the treatment of angina and hypertension (Catterall, et al., 1992).

1.6 Voltage-gated Calcium Channel Blockers

The existence of highly specific binding sites for various classes of calcium channel blockers has been demonstrated in various tissues including the brain (Quirion, Lal, Olivier, Robitaille, Van Nair, Ford & Stratford, 1988). It now appears that different classes of calcium antagonists such as the dihydropyridines, the phenylalkylamines, and

diltiazem interact with various binding sites (Quiron et al., 1988). The dihydropyridines are the most commonly studied calcium channel blocker, and they have become a clinically valuable agent for a number of human disorders, including cardiovascular disease and epilepsy.

The dihydropyridines are calcium antagonists which primarily modulate the voltage-dependent gating of the L-type Ca2+ channels. Dihydropyridine binding sites are highly conserved among the L-type Ca^{2+} channels found in skeletal muscles, heart and neurons (Catteral, & Striessnig, 1992). Dihydropyridines can function either as channel activators or as inhibitors; enantiomeric pairs often have opposite influences on channel function (Catterall & Striessnig, 1992). Binding of the dihydropyridine Ca2+ channel antagonists is not strongly influenced by repetitive activation of the Ca2+ channel, which indicates that binding is not dependent on channel state. However, inhibition of the Ca2+ channel by dihydropyridines is enhanced by prolonged depolarization. Dihydropyridines modulate the function of the L-type Ca2+ channels by favouring distinct modes of channel gating. Modulation of calcium channel gating is believed to be the mechanism by which several neurotransmitters, hormones, and therapeutic agents mediate their effects on cell function (Pietrbon & Hess, 1990). For instance, strong depolarizations will drive the channel into a mode of gating characterized by long openings and high open-state probability (gating mode 2) (Pietrobon, et al., 1990). Activators favour gating mode 2 with long single channel openings, whereas inhibitors favour gating mode 0 in which the channel fails to open in response to depolarization (Catterall, et al., 1992).

Therefore, dihydropyridines such as nifedipine, nimodipine and flunarizine, which favour gating mode 0, prevent voltage-gated calcium channels from opening and thereby prevent the extracellular Ca2+ from entering the cell through L-type calcium channels.

1.7 Calcium Current Flow and Excitatory Amino Acid Receptors

Activation of excitatory amino acid receptors is accompanied by a decline in extracellular Ca2+ levels. Bading and colleagues (1995) revealed that glutamate leads to an increase in intracellular Ca2+ in hippocampal neurons and that this influx of Ca2+ is mediated by both L-type voltage sensitive calcium channels and N-methyl-D-aspartate (NMDA) receptor channels (Bading, Segal, Sucher, Dudek, Lipton, & Greenberg, 1995) A number of studies have demonstrated that NMDA receptor channels, which are permeable to Ca2+ ions, and L-type calcium channels represent the major sites of calcium entry into neurons (Bading, et al., 1995). Activation of excitatory amino acid receptors, particularly of the NMDA type, has been shown to induce calcium fluxes across the neuronal membrane and electrophysiological studies have indicated that entry of calcium into the neurons might represent a potential substrate for epileptogenesis (Vezzani, Wu, Stasi, Angelico, & Samanin, 1988). In fact, epileptiform bursts are often associated with influx of calcium ions into nerve cells, and a decrease in the extracellular concentration of calcium precedes the onset of seizures in many experimental models of epilepsy (Vezzani et. al., 1988). Furthermore, there is evidence for an increased synaptic activation of NMDA receptors on kindled granule cells and significantly larger decreases

in the extracellular calcium concentrations in the kindled dentate gyrus following repetitive activation of the perforant pathway (Mody, & Heinemann, 1987).

1.8 Calcium Transients and Neural Plasticity

Calcium is an important second messenger that plays a role in a variety of neuronal functions, including transmitter release and secretion, regulation of growth cone behaviour and gene activation (Kocsis, Rand, Lankford, Stephen, & Waxman, 1994).

Electrical activity is capable of strengthening, weakening, or breaking synaptic connections, or regulating growth cone motility to affect neurite outgrowth and synaptogenesis (Fields, 1994). Genetic sensitivity to environmental input is provided by a class of genes called immediate early (IE) genes. These IE genes are rapidly transcribed in response to a variety of external stimuli and they regulate the transcription of secondary genes, to produce structural and functional alterations in neurons (Fields, 1994). Nerve impulses are converted into transcellular signals by calcium ions entering through voltage-gated calcium channels. Both growth cone motility and the transcription of early genes can be influenced by calcium fluxes (Fields, 1994). Also, the glutamate released during synchronous neuronal firing (i.e. seizures) appears to be associated with an increase in intracellular calcium levels and the activation of the brain-derived neurotrophic factor (BDNF) gene (Kokaia, Gunilla, Ringstedt, Bengzon, Kokaia, Siesjo, Persson, & Lindvall, 1993).

1.9 Voltage-gated Calcium Channel Blockers & Kindling Epileptogenesis

As previously discussed, the dentate gyrus granule cells possess pharmacologically distinct voltage-dependent calcium channels (Blaxter, Carlen, & Niesen, 1989). Furthermore, some, or all of these channels appear to be altered by kindling epileptogenesis (Mody, et al., 1990). Kindling in the dentate gyrus appears to involve both an NMDA receptor-mediated change in calcium regulation (via both NMDA receptor channels and voltage gated calcium channels) and a loss of putative intraneuronal calcium buffering protein Calbindin-D_{28k} (Vezzani, et al., 1988). These changes result in an increased level of intracellular calcium in the neurons of the dentate gyrus.

A number of voltage-gated calcium channel blockers have shown effective anticonvulsant properties. Mody and colleagues revealed that the organic calcium blockers flunarizine and nifedipine protect hippocampal-kindled rats from fully developed convulsions, and that flunarizine protects rats against convulsions induced by intrahippocampal injections of quinolinic acid, an endogenous activator of NMDA receptors (Mody, Reynolds, Salter, Carlen & MacDonald, 1990). These results suggest that alterations in calcium-dependent processes or calcium currents underlie, at least partially, the neuronal hyperexcitability that leads to kindling-induced seizure activity.

A study completed by Yamada and Bilkey (1991) revealed that the dihydropyridine calcium antagonist, nifedipine, when administered to adult rats 30

minutes prior to each kindling stimulation, leads to the same pattern of results that are produced by an injection of MK-801 given at least 30 minutes before each kindling stimulation. MK-801 is a non-competitive NMDA receptor antagonist. Specifically, they both significantly retard the rate of kindling, as well as significantly increase the mean afterdischarge duration. These results suggest that voltage-gated calcium channel antagonists and NMDA receptor antagonists may influence the same kindling mechanisms. The common link is the attenuation of the seizure-induced increase in intracellular calcium.

1.10 Experimental Rationale

The purpose of the present experiment was to determine whether an L-type calcium channel blocker, nifedipine, could interfere with the development of kindling-induced mossy fibre sprouting. The organic dihydropyridine calcium channel antagonist, nifedipine, has been preferred for this research because of its specificity for L-type voltage-gated calcium channels and its reliability in clinical use (Yamada, & Bilkey, 1991). Assuming that voltage-gated calcium channel antagonists influence kindling epileptogenesis via the same mechanism as NMDA receptor antagonists, it is hypothesized that nifedipine, like the NMDA receptor antagonist, MK-801, will attenuate kindling-induced mossy fibre sprouting.

CHAPTER 2

THE EFFECT OF THE VOLTAGE-GATED CALCIUM CHANNEL BLOCKER, NIFEDIPINE, ON KINDLING-INDUCED MOSSY FIBRE SPROUTING

Part 1- THE EFFECTS OF DIMETHYLSULFOXIDE (DMSO) ON KINDLING AND KINDLING-INDUCED MOSSY FIBRE SPROUTING.

Rationale

Dimethylsulfoxide is an organic solvent that has been used as a vehicle for nifedipine in past research (Yamada, et al., 1991). Nifedipine is more readily absorbed by neurons when dissolved in DMSO (Yamada, et al. 1991). Since DMSO is capable of increasing the transport of nifedipine across the cell membrane, we wanted to ensure that this change in membrane permeability did not also effect the rate of kindling epileptogenesis, the associated sprouting of the mossy fibre pathway, or the levels of intracellular heavy metals.

Methods

Animals and Surgical Procedures

Adult male Long-Evans hooded rats (n=22) weighing between 300-500g were used. Rats were maintained on an ab lib feeding schedule, were individually housed, and were kept on a 12 hour on/12 hour off light cycle.

Employing stereotaxic procedures, rats were anaesthetized with sodium pentobarbital (65mg/kg) and implanted with a bipolar recording/stimulating electrode. Electrodes were made with teflon-coated, 125 μm, stainless steel wires targeting the right perforant path. The stereotaxic coordinates for the perforant path were -7.6mm posterior and +4.8mm lateral to bregma, and -3.3mm ventral from brain surface. The electrode was held in place by dental acrylic and three stainless steel screws inserted into the skull.

The rats were given three weeks to recover from surgery and then randomly assigned to either kindled (n=12) or a non-kindled group (n=10). These groups were further subdivided into two groups to be treated with either saline (100 ml) or DMSO (100 ml) 30 minutes prior to kindling stimulation.

Kindling

Rats were given an intraperitoneal (IP) injection of either saline or DMSO 30 minutes prior to each kindling stimulation. They were stimulated twice daily with interstimulus intervals of at least 6 hours, for a total of 11 days. Each stimulation comprised a one-second stimulus train of one-millisecond pulses at a frequency of 60 Hz and a pulse intensity of 500uA. This was sufficient to trigger epileptiform afterdischarges of greater than five seconds for each stimulation. The durations of the afterdischarges were measured from electroencephalograph (EEG) recordings of the signal from the perforant path electrode. The behaviourial progression of kindling was evaluated by recording the behaviourial seizure stage according to Racine's classification

(1972). Animals were regarded as fully kindled when they exhibited three consecutive stage 5 seizures. Non-kindled controls were give IP injections of either saline or DMSO twice daily with interinjection intervals of at least 6 hours, for 11 days.

Histological Analyses

Three weeks following the last stimulation, the rats were anaesthetized with sodium pentobarbital (65mg/kg) and perfused transcardially with 50 mL sodium sulfide solution (8.9g $Na_2S.9H_20$, 10.9g sucrose, 1.19 g $Na_2PO_4.H_2O$ per 100 ml dH_2O) at room temperature. Brains were frozen in isopentane cooled to -40^{0}C and stored at -70^{0}C until sectioning. Forty-micron horizontal sections were taken through the region of the brain containing the hippocampus using a cryostat which was set at -18^{0}C. The sections were mounted on chromium potassium sulphate-coated slides. To ensure that the brain sections included in the data analysis were comparable, 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. Forty serial sections were taken. From these, 8 sections were analyzed, corresponding to depths of 4.94-7.34 mm ventral to bregma. These sections were 400 microns apart and each was matched against the landmarks in the corresponding atlas sections. Sections were stained using a modified Timm method (Sutula et al.., 1988; Adams et al., 1998) for analysis of mossy

fibre sprouting. To ensure objectivity in the data analyses, slides were coded and analyses were completed by an observer unaware of the treatment of the animal.

Timm Densitometry

The Timm method stains neural elements containing heavy metals (e.g., the high Zn^{2+} content of the mossy fibre axons of the dentate granule cells). To reduce variability in Timm staining between groups, sections from animals in different groups were processed simultaneously.

Tissue sections were examined at 50x magnification by creating a digitized image using an MCID imaging system (Micro Computer Imaging Device, Brock University, St. Catherine's, Ontario, Canada) connected to a light microscope (Zeiss Axioskop) with a high resolution CCD camera (MTI CCD 72). 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, beginning at the base of the hilar region. The density of Timm granules in the inner molecular layer (IML) was measured at 12 adjacent cursor positions by placing the central two cursor positions above the genu of the hilus and 5 cursor positions to the right and the left of these. Background values were provided by cursors placed in the stratum radiatum of the CA3 as described by Adams et al. (1997). 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. Density

measurements were evaluated from 8 brain sections per rat at different section levels for both the right and left sides of the brain.

Results

Kindling Results

The rats treated with DMSO 30 minutes prior to each kindling stimulation exhibited the same pattern of seizure development as rats treated with saline. An ANOVA revealed that the class of seizure increased significantly as a function of the number of stimulations in both groups $F(21,189)= 4.42$; ($p<0.001$). Afterdischarge duration (ADD) increased significantly in both groups as a function of the number of stimulations $F(21,189) = 9.66$; ($p<0.001$). On average, the number of stimulations that the rats treated with DMSO required to reach 3 consecutive stage 5 seizures (mean 14.8±2.6) was not significantly different from the number of stimulations required to induce 3 consecutive stage 5 seizures in the rats treated with saline (mean=16 ±3.1), $F(1,10)=.088$; ($p>0.05$) (See Figure 1). Afterdischarge duration, either as a function of stimulation or as a measure of cumulative duration, also did not differ between groups, DMSO (mean= 29.3±1.6) and saline (mean=28.2±1.7), $F(1,10)=.228$; ($p>0.05$) (See Figure 2).

Mossy fiber sprouting analyses

The amount of Timm labeling did not differ significantly between the groups $F(15,120) = 0.20$; ($p > 0.05$). The data revealed that injecting DMSO prior to each kindling stimulation did not interfere with (or accelerate) the kindling-induced mossy fibre sprouting observed in the IML and the CA3 (See Figure 3) regions of the hippocampus. Based on these findings, we surmised that it was reasonable to use DMSO as a vehicle for nifedipine for the purposes of the present study.

Part 2- THE EFFECTS OF 5mg/kg NIFEDIPINE ON KINDLING AND KINDLING-INDUCED MOSSY FIBRE SPROUTING.

Rationale

An experiment completed by Yamada and colleagues (1991) revealed that nifedipine (5mg/kg) is capable of significantly retarding the development of kindling and significantly increasing the afterdischarge duration in kindled animals. This pattern of results is consistent with the results revealed by Sutula and colleagues (1996) when MK-801 was used to suppress kindling. However, in addition to significantly retarding the progression of seizures and significantly increasing mean afterdischarge duration in kindled rats, MK-801 also significantly interfered with kindling-induced sprouting of the mossy fibre pathway (as revealed by Timm method). Since MK-801 and nifedipine had remarkably similar effects on the kindling process (both behaviourally and electrophysiologically), it seemed reasonable that nifedipine could also block the mossy fiber sprouting that is associated with kindling epileptogenesis. Furthermore, since Yamada and colleagues (1991) were able to successfully interfere with the kindling process with only 5mg/kg of nifedipine it was deemed reasonable that the same dose would also be sufficient to interfere with kindling-induced sprouting of the mossy fibre pathway.

Methodology

Adult male Long-Evans rats (n=22) weighing 300-500g were used. The exact protocol detailed above was maintained. Since it was revealed that DMSO did not significantly affect either kindling epileptogenesis or the associated mossy fibre sprouting, DMSO was used as the vehicle. Both the kindled (n=12) and non-kindled (n=10) rats were injected IP with either vehicle (DMSO) alone or 5mg/kg of nifedipine (Sigma, N7634) dissolved in DMSO in a dark room 30 minutes prior to each kindling stimulation. Rats in the kindling groups were given an IP injection of either nifedipine (n=6) or DMSO (n=6) 30 minutes prior to each kindling stimulation.

Results

Kindling Results

The stage of kindling increased significantly as a function of the number of stimulations in both groups $F(21, 189)=9.66$, ($p< 0.001$). There was also a main effect of kindling on afterdischarge duration (ADD) $F(21, 189)= 4.42$, ($p< 0.001$). The nifedipine-treated group exhibited significantly larger ADDs (mean= 56±4.5 seconds) than the group treated with DMSO alone (mean= 33±3.6 seconds): $F(1,9)= 5.68$, ($p=0.0411$) (See Figure 5). On the other hand, nifedipine significantly retarded the rate of kindling: $F(1,8)= 11.88$, ($p=0.009$). The group treated with nifedipine required significantly more stimulations to reach a fully kindled state (3 stage 5 seizures) (mean=

21.6 ±0.3) than the DMSO-treated control group (mean= 11.88 ±2.1) (See Figure 4).

Mossy Fibre Sprouting Analyses

A 1-way ANOVA revealed that there were no significant differences in Timm granule labeling between the groups in either the CA3 $F(21,119)= 0.49$ ($p>0.05$) or the IML $F(3,11)=0.63$($p>0.05$) region of the hippocampus. However, the trend in the data suggested that nifedipine (5mg/kg), coupled with activation, may have suppressed the Timm labeling. In both the CA3 (See Figure 6) and the IML region (See Figure 7) of the hippocampus, nifedipine (5mg/kg) appeared to interfere with either the kindling induced sprouting of the mossy fibre pathway or Timm granule density [labeling of zinc in the granule cells].

Part 3-THE EFFECTS OF NIFEDIPINE (25mg/kg) ON KINDLING AND KINDLING INDUCED MOSSY FIBRE SPROUTING.

Rationale

Five mg/kg of nifedipine injected prior to each kindling stimulation resulted in a trend suggesting that the calcium channel blocker might be capable of interfering with the kindling-induced increase in Timm granule density. If so, increasing the dose of nifedipine from 5mg/kg to 25 mg/kg might produce significant differences.

Methodology

Adult male Long-Evans rats (n=12) weighing 300-500g were used. The protocol detailed above was maintained, except that the kindled (n=6) and non-kindled (n=6), rats were injected IP with 25mg/kg of nifedipine.

Results

Kindling Results

The kindling results were consistent with those produced by 5mg/kg nifedipine (Part 2 of present study). Both the stage of kindling ($p < 0.001$) and afterdischarge duration (ADD) ($p < 0.001$) increased significantly as a function of the number of stimulations. The group treated with 25 mg/kg nifedipine exhibited significantly longer

cumulative ADDs (mean = 162± 35.9 secnds) than the group treated with either 5mg/kg nifedipine (mean =64.64± 10.4 seconds) or DMSO alone (mean= 33.5± 3.7 seconds) $F(2,13)=10.92$ ($p=0.002$) (See Figure 5). It was revealed through a 1-way ANOVA that 25mg/kg nifedipine significantly retards the rate of kindling. The group treated with nifedipine (25mg/kg) required significantly more stimulations to reach a fully kindled state (3 stage 5 seizures) (mean= 19.7 ±1.60) than the group treated with DMSO (mean= 11.88 ±2.1) $F(1,9)= 5.176$ ($p<0.05$) (See Figure 4). The number of stimulations required to reach a fully kindled state, however, was not significantly different between the groups treated with lower and higher doses of nifedipine: $F(1,10)=0.014$ ($p>0.05$) (See Figure 4). Either kindling was not suppressed in a dose-dependent manner by the dihydropyridine voltage gated calcium channel blocker, nifedipine, or both dose levels were already high enough to produce an asymptotic effect.

Mossy Fibre Sprouting Analysis

Nifedipine (25mg/kg) significantly interfered with Timm labeling in both the IML ($F(2,27)= 2.78$: $p<0.04$) (See Figure 7) and CA3 (See Figure 6) ($F(5,27)= 9.29$: $p=0.00$) region of the hippocampus. Both the kindled and the non-kindled groups treated with 25mg/kg nifedipine exhibited a lower density of Timm labeling than either the groups (kindled or non-kindled) treated with 5mg/kg nifedipine or the groups (kindled or non-kindled) treated with DMSO only. Since both kindled and non-kindled groups treated with nifedipine (25mg/kg) exhibited reduced levels of Timm granule density, we are

unable to say that nifedipine blocked kindling-induced sprouting. These results indicate that nifedipine (25mg/kg) must interfere directly with the labeling of zinc in the mossy fibre pathway, regardless of whether the animals were exposed to kindling stimulation or not.

FIGURES and CAPTIONS

Figure 1. The behavioural progression of seizure activity as a function of group. The number of stimulations that the DMSO-treated group required before reaching a fully kindled state (3 stage 5 seizures) was not significantly different from that of the saline-treated group $F(1,10)=0.088;(p>0.05)$.

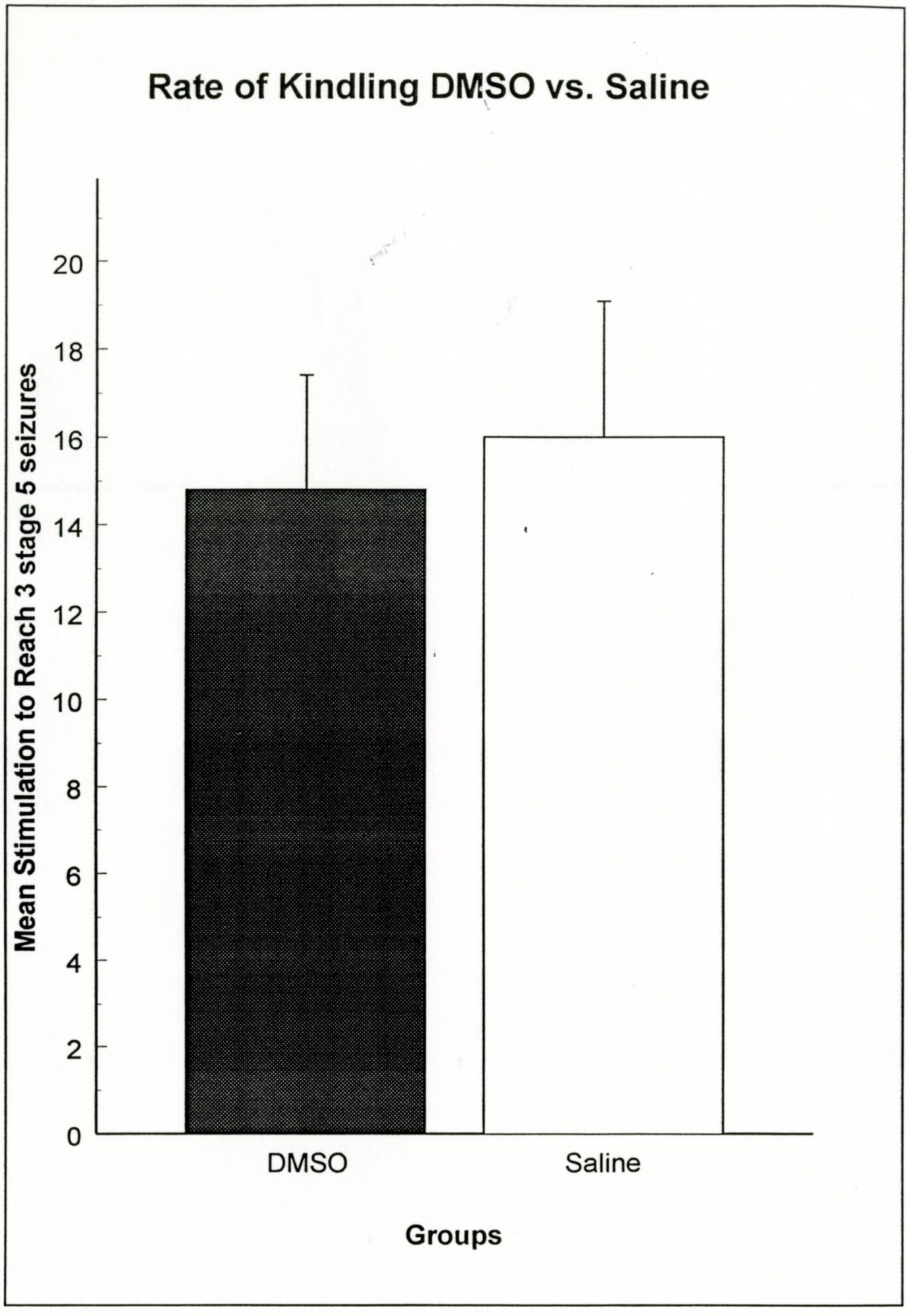

Rate of Kindling DMSO vs. Saline
Mean Stimulation to Reach 3 stage 5 seizures
0
2
4
6
8
10
12
14
16
18
20
DMSO
Saline
Groups

Figure 2. Afterdischarge duration as a function of group. The cumulative mean afterdischarge duration of the group treated with DMSO was not significantly different than that of the group treated with saline $F(1,10)=0.228$; ($p>0.05$).

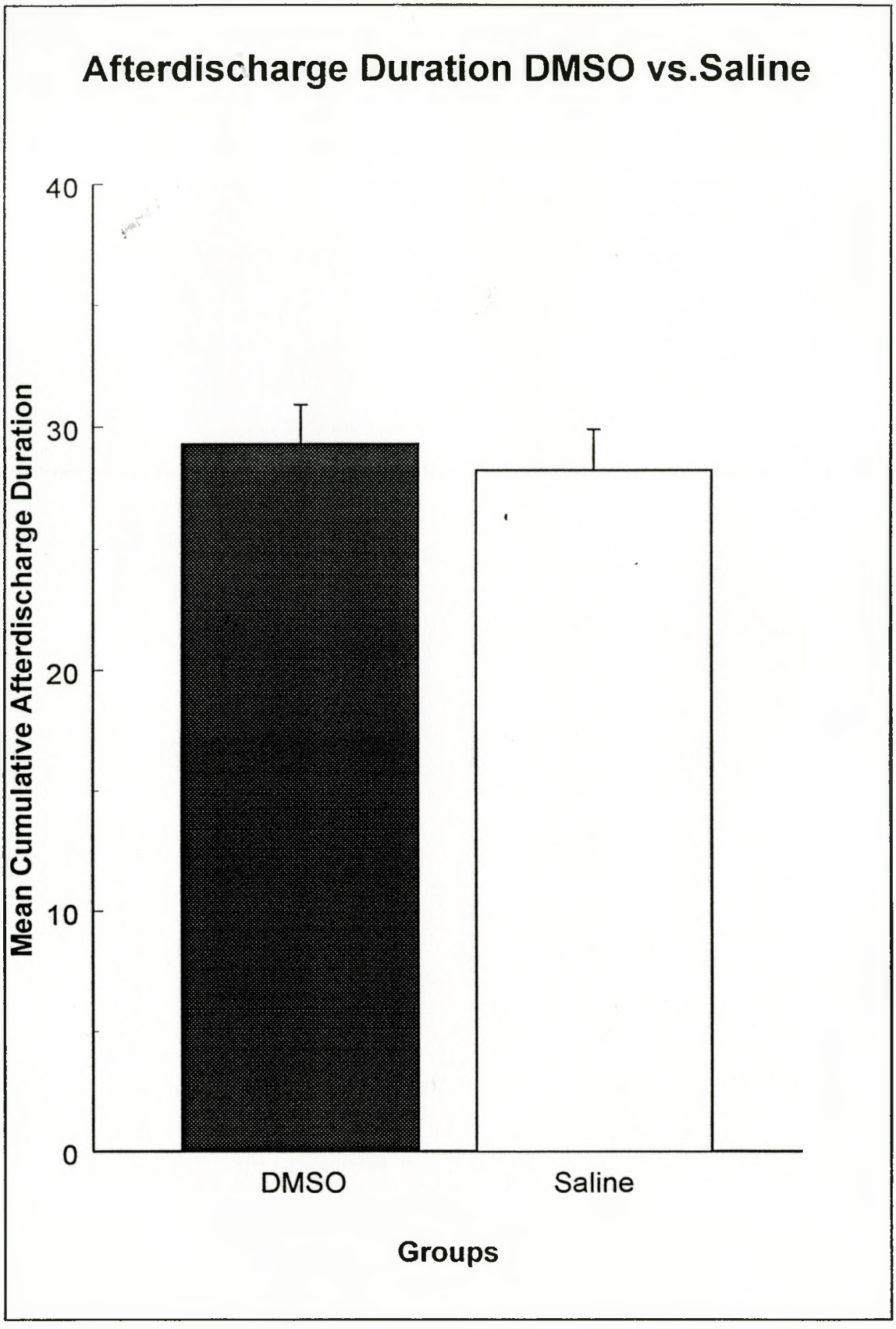

Afterdischarge Duration DMSO vs.Saline
Mean Cumulative Afterdischarge Duration
40
30
20
10
0
DMSO
Saline
Groups

Figure 3. Timm granule density as a function of cursor placement for both saline treated and DMSO treated animals. The amount of Timm labeling in the CA3 region of the hippocampus did not differ significantly between the group treated with DMSO and the group treated with saline $F(15,120) = .20$; ($p > 0.05$).

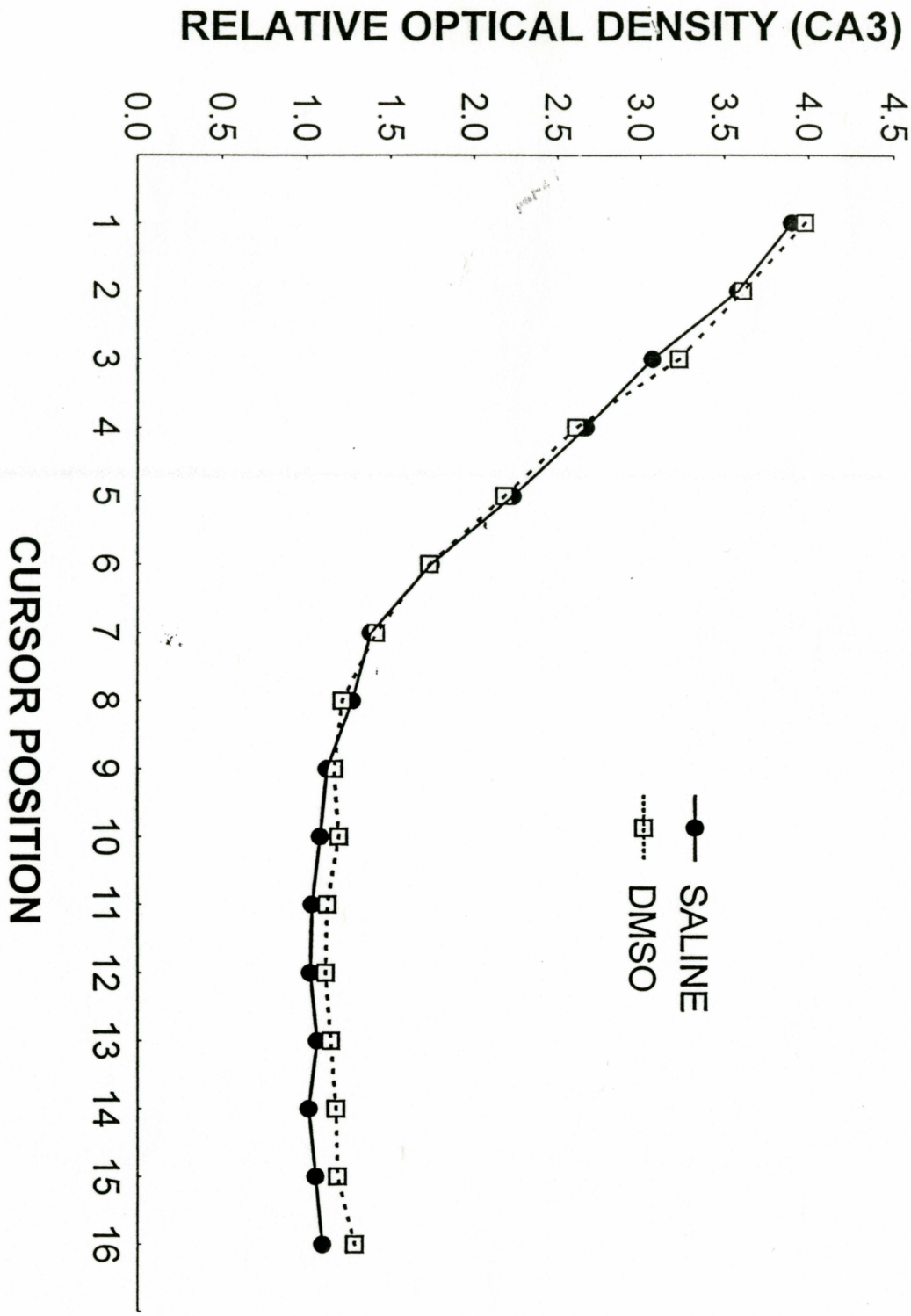

RELATIVE OPTICAL DENSITY (CA3)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
CURSOR POSITION
SALINE
DMSO

Figure 4. The behavioural progression of seizure activity as a function of group. The number of stimulations that the DMSO-treated group required before reaching a fully kindled state (3 class 5 seizures) was significantly lower than either of the nifedipine-treated groups (5 or 25 mg/kg) ($p<0.05$). However, the number of stimulations required to reach a fully kindled state was not significantly different between the two nifedipine groups.

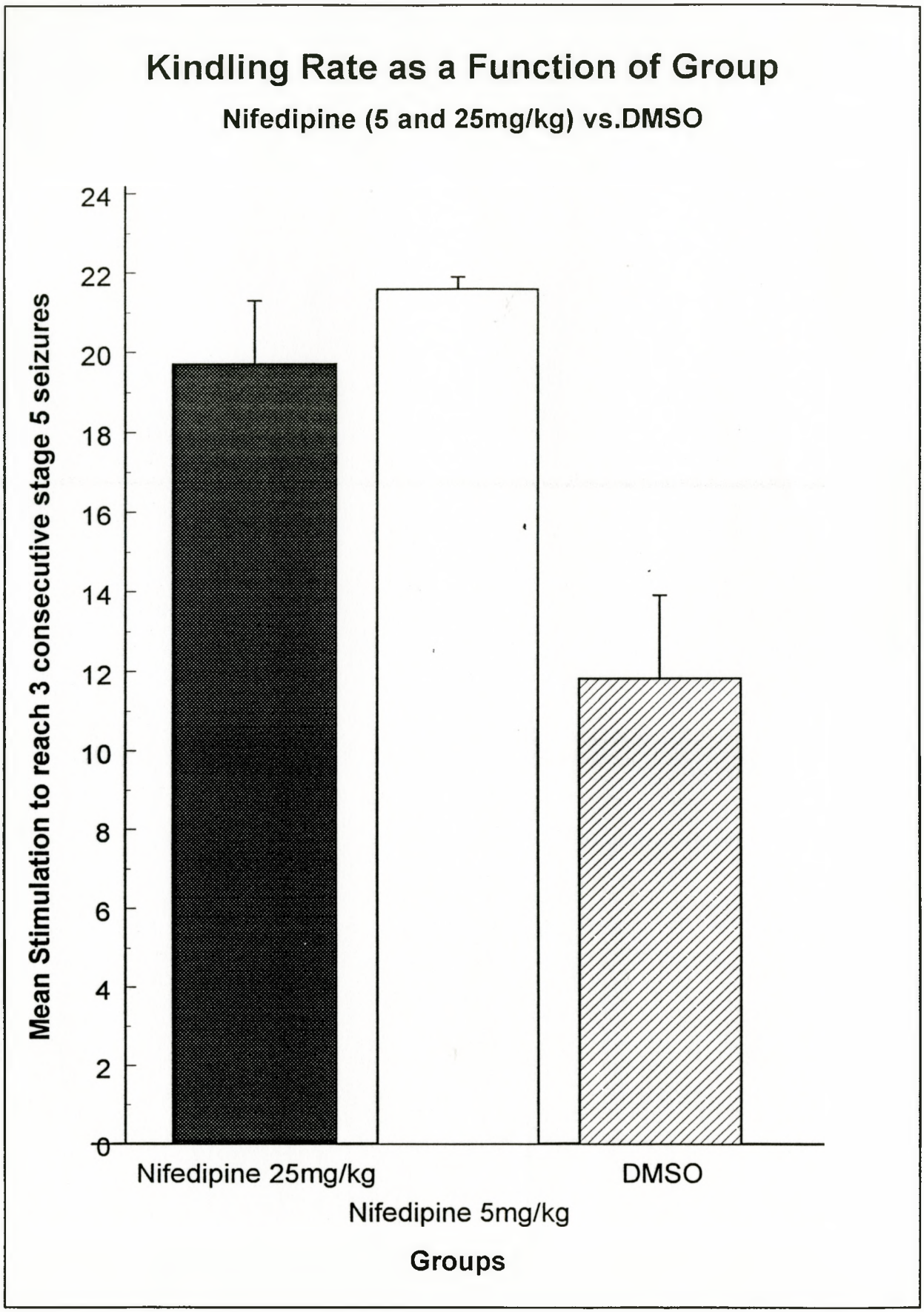

Kindling Rate as a Function of Group
Nifedipine (5 and 25mg/kg) vs.DMSO
Mean Stimulation to reach 3 consecutive stage 5 seizures
24
22
20
18
16
14
12
10
8
6
4
2
0
Nifedipine 25mg/kg
Nifedipine 5mg/kg
DMSO
Groups

Figure 5. Afterdischarge duration as a function of group. The cumulative mean afterdischarge duration was significantly longer in those animals treated with nifedipine than in those animals treated with DMSO ($p<0.05$). Furthermore, the animals treated with 25mg/kg of nifedipine exhibited significantly longer afterdischarges than those animals treated with 5mg/kg of nifedipine ($p<0.05$).

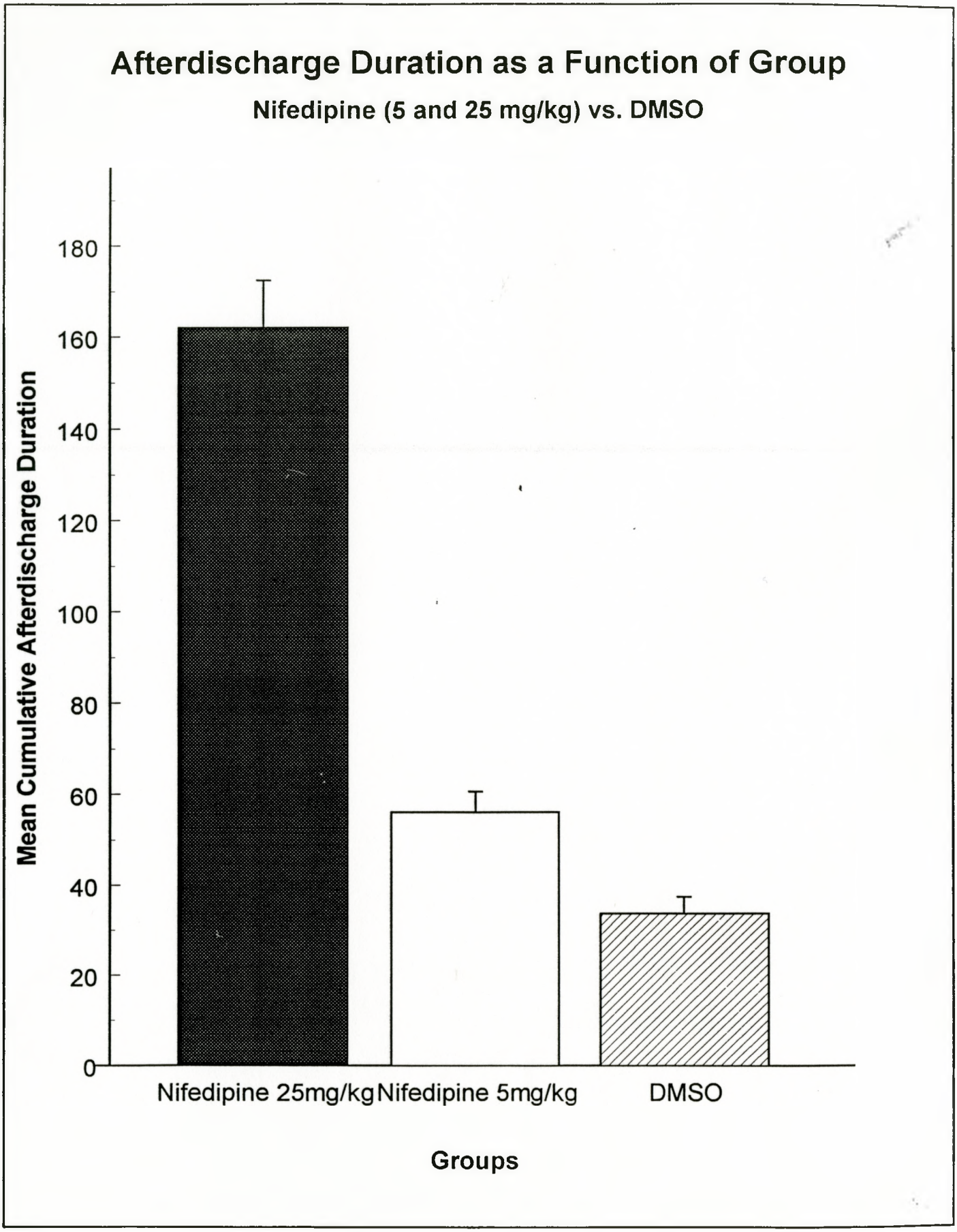

Afterdischarge Duration as a Function of Group
Nifedipine (5 and 25 mg/kg) vs. DMSO
Mean Cumulative Afterdischarge Duration
180
160
140
120
100
80
60
40
20
0
Nifedipine 25mg/kg
Nifedipine 5mg/kg
DMSO
Groups

Figure 6. Timm granule density as a function of group. The cumulative amount of Timm labeling revealed in the CA3 region of the hippocampus did not differ significantly between the groups, kindled and non-kindled, treated with 5mg/kg of nifedipine and groups, kindled and non-kindled, treated with DMSO only (21, 119)=0.49 ($p>0.05$). However, the cumulative amount of Timm labeling revealed in the CA3 region of the hippocampus was significantly reduced in those animals, kindled or non-kindled, that were treated with 25mg/kg of nifedipine when compared to animals, kindled or non-kindled, treated with either 5mg/kg of nifedipine or DMSO ($F(2,27)=9.29$:$p<0.001$).

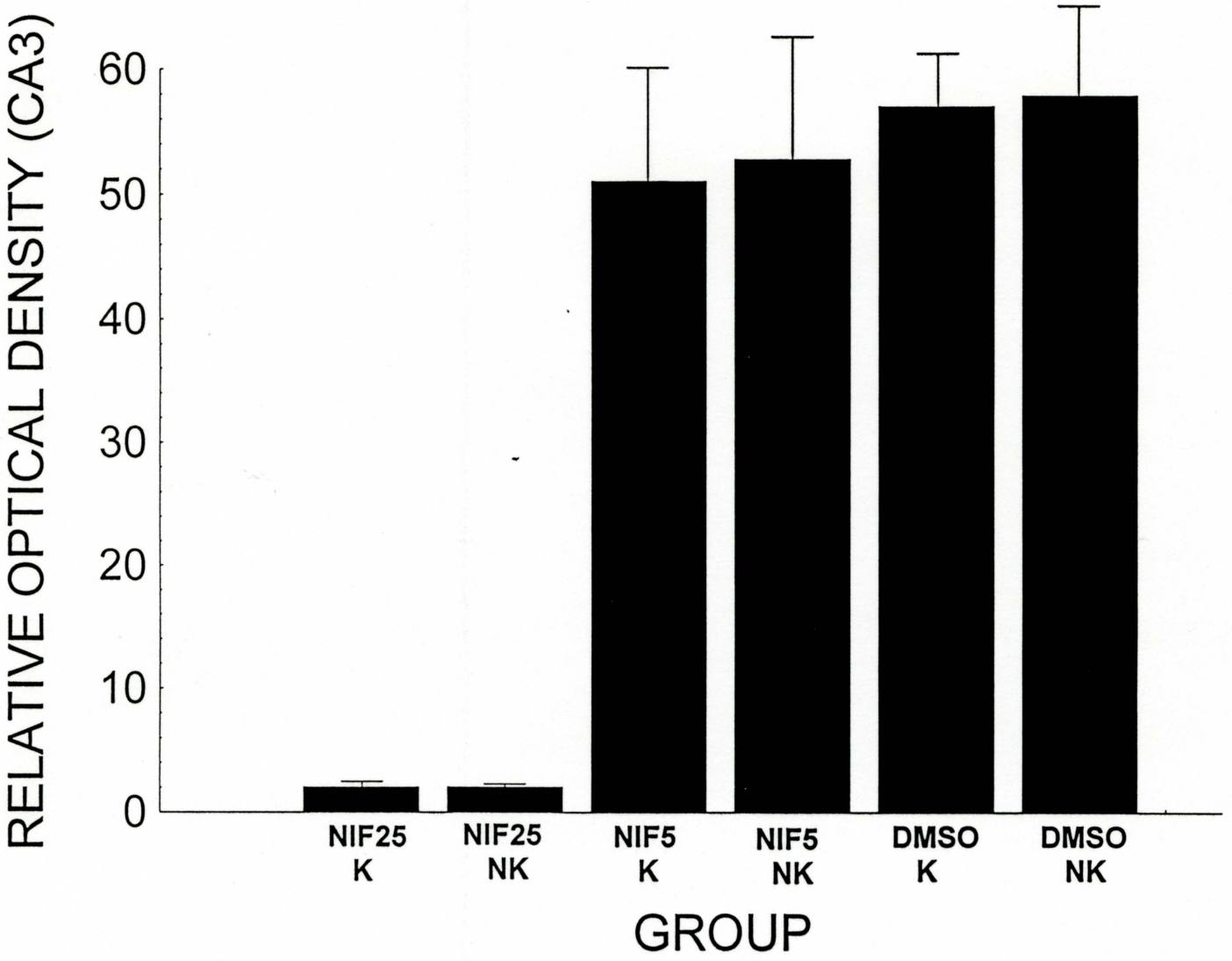
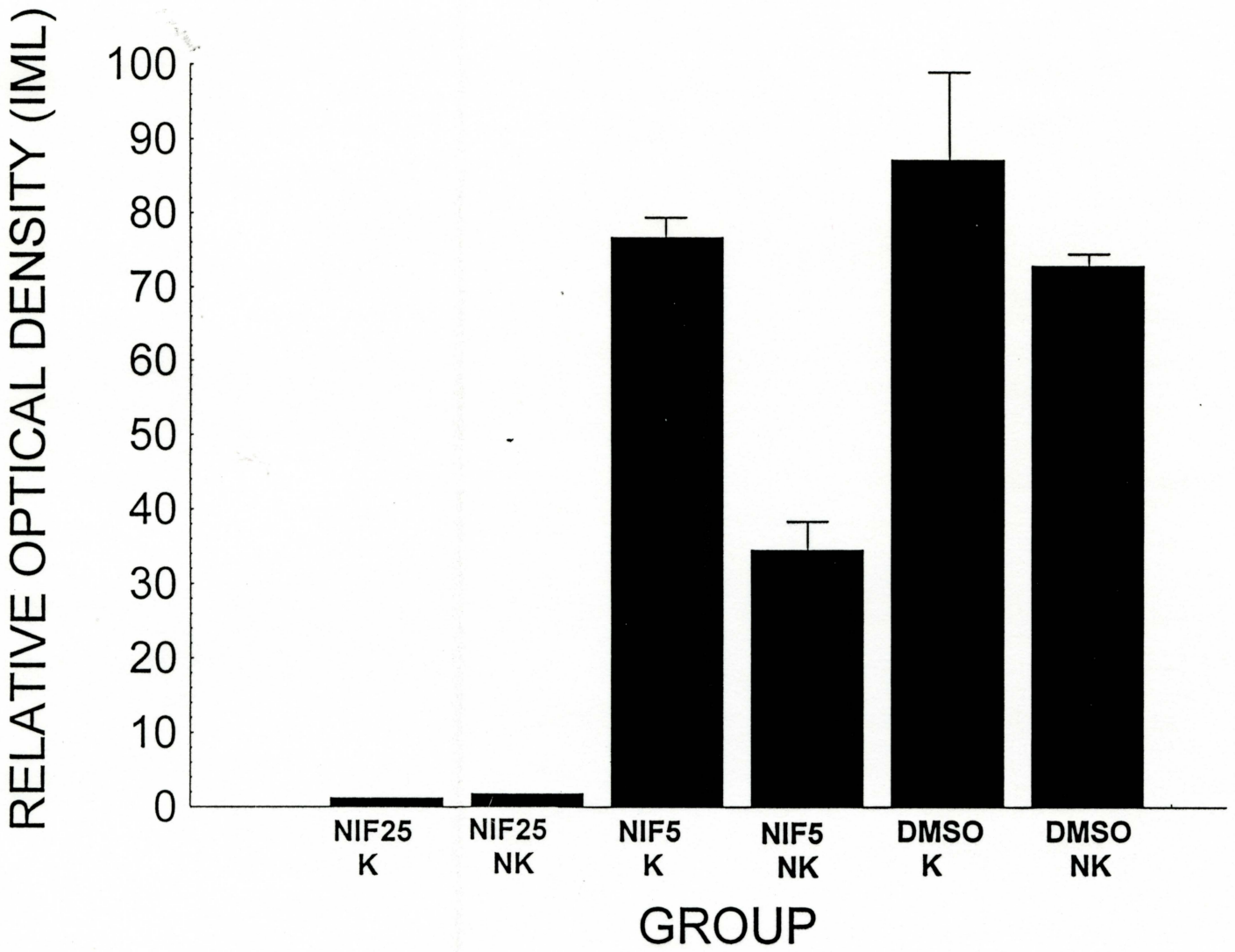

RELATIVE OPTICAL DENSITY (CA3)
60
50
40
30
20
10
0
NIF25 K
NIF25 NK
NIF5 K
NIF5 NK
DMSO K
DMSO NK
GROUP

Figure 7. Timm granule density as a function of group. The cumulative amount of Timm labeling revealed in the IML region of the hippocampus did not differ significantly between the groups, kindled and non-kindled, treated with 5mg/kg of nifedipine and groups, kindled and non-kindled, treated with DMSO only (3, 11)=0.63 ($p>0.05$). The cumulative amount of Timm labeling revealed in the IML region of the hippocampus was significantly reduced in those animals, kindled or non-kindled, that were treated with 25mg/kg of nifedipine when compared to animals, kindled or non-kindled, treated with either 5mg/kg of nifedipine or DMSO ($F(2,27)=2.78$: $p<0.04$).


RELATIVE OPTICAL DENSITY (IML)
100
90
80
70
60
50
40
30
20
10
0
NIF25 K
NIF25 NK
NIF5 K
NIF5 NK
DMSO K
DMSO NK
GROUP

CHAPTER 3

General Discussion

The purpose of this thesis was to determine whether blocking L-type calcium channels via the dihydropyridine calcium channel blocker, nifedipine, would reduce or inhibit kindling induced mossy fibre sprouting. Collateral sprouting in this pathway was evaluated by the density of Timm staining, a measure of intracellular zinc labeling, in either the CA3 or IML region of the hippocampus. Since the influx of calcium is thought to be involved in both the development of seizures and activation-induced neural growth, and since the voltage-gated calcium channels are considered the main route of entry for calcium ions, nifedipine was expected to retard both the rate of kindling and the level of kindling-induced mossy fibre sprouting.

The kindling results described in the present study are consistent with past research. The stage of kindling and afterdischarge duration increased as a function of the number of stimulations received (Racine, 1972). Both the 5mg and 25mg doses of nifedipine significantly retarded the rate of kindling and increased the mean afterdischarge duration (Yamada & Bilkey, 1991). These results confirm that calcium influx plays an important role in the development of perforant-path kindling, and might provide further support for the notion that voltage-gated calcium channel blockers might provide effective anticonvulsant agents (Mody et al., 1990). The longer cumulativ

afterdischarges experienced by the rats treated with nifedipine suggest that blocking the influx of calcium, which is typically enhanced during kindling, results in a weaker discharge. Weaker discharges may sustain themselves for longer periods of time before shutting themselves down by exhaustion, depolarization block, or the recruitment of inhibitory systems. Since MK-801 also significantly increases cumulative afterdischarges in addition to interfering with the progression of seizures, the pattern of results revealed in the present study provide further support for the hypothesis that nifedipine and MK-801 may be interfering with the kindling process via the same mechanism (by suppressing calcium influx). Voltage-dependent NMDA receptor channels and the voltage-gated calcium channels are the primary routes of entry for calcium. Therefore, blocking either channel will reduce the levels of intracellular calcium, attenuate cell discharge, and slow the propagation of seizures to secondary brain regions. The outward signs of this sequence of events during the kindling process is a reduction of the motoric manifestations of kindling.

The present study did not reveal a significant difference in kindling measures between groups treated with 5mg/kg or 25mg/kg nifedipine, suggesting that a peak effect may have already been reached at the lower dose level. Since Sutula and colleagues (1996) only tested a 5mg/kg dose of MK-801, it is not clear whether larger doses would produce larger effects.

Kindling has been associated with synaptic reorganization of connections of the glutamatergic mossy fibre pathway that originate from hippocampal dentate granule cells

(Ikegaya, Yoshida, Saito, & Nishiyama, 1997). Ten percent of the total hippocampal zinc is contained within the hippocampal mossy fibres and the remaining 90% is distributed throughout all grey matter and is referred to as "background zinc" (Howell, Welch, Frederick, 1984). Electrical stimulation applied to the dendrites of granule cells selectively facilitates the influx of zinc into mossy fibres. The largest increase occurs in the hilus followed by a smaller increase in the CA3 region (Howell, et al., 1984). The mossy fibres normally innervate the polymorph and the CA3 neurons and provide very little innervation of structures within the granule cell or molecular layers of the dentate gyrus. However, collateral sprouting of the mossy fibres has been observed to extend into the supergranular layer of the dentate gyrus, as well as into the hilus and the CA3 region, as a result of kindling epileptogenesis (Howell, et. al., 1984). Furthermore, the turnover rate of zinc in the mossy fibres is accelerated by electrical stimulation of the dentate gyrus (Howell, et al., 1984), and hippocampal zinc levels are significantly increased by kindling epileptogenesis (Mody & Miller, 1984).

It was reasoned that treatment with nifedipine would affect kindling induced mossy fibre sprouting in much of the same way as MK-801 (Sutula et al., 1991). That is, that nifedipine would block kindling induced sprouting in both the CA3 and the IML regions of hippocampus. However, the present results suggest that nifedipine alters these regions in a very unique way. All of the groups treated with nifedipine, *whether they had been kindled or not*, exhibited less Timm granule density than those groups treated with DMSO alone. Furthermore, when the dose of nifedipine was increased from 5mg/kg to

25mg/kg, the observed difference in Timm labeling (less Timm labeling in groups treated with nifedipine both kindled and non-kindled) became significant. Since Timm staining is a histological technique designed to identify neural tissue containing heavy metals (Sutula, et al., 1988), and since the mossy fibre pathway is rich in zinc, it can be reasoned that nifedipine reduces the amount of intracellular zinc that is normally present in hippocampal granule cells.

The mammalian brain contains high concentrations of divalent zinc ions, particularly in the hippocampus and the neocortex (Freund & Reddig, 1994). Ultrastructural analysis has revealed that zinc is localized in the synaptic vesicles of excitatory nerve terminals (Freund et al., 1994). It is believed that zinc ions' route of entry is by permeation through calcium channels (Freund et al., 1994). Freund and colleagues (1994) reported that nimodipine caused a complete and potent inhibition of the neurotoxicity induced by AMPA/zinc. Based on these results they reasoned that AMPA-induced depolarization leads to the opening of the L-type voltage gated calcium channels, resulting in an increase in both intracellular calcium and zinc. Furthermore, Busselberg and colleagues revealed that zinc is capable of blocking voltage-activated calcium currents in mammalian neurons (Busselberg, Michael, Evans, Carpenter, & Haas, 1992). They revealed that N, L, and T calcium currents are blocked (approx. 80%) at concentrations over 200 uM zinc. Zinc presumably competes with calcium for a binding site within the channel or close to its opening (Busselber et al., 1992). Based on these findings it is possible that blocking the L-type voltage-gated calcium channels with nifedipine restricted the influx of zinc into the

mossy fibres, and therefore reduced the level of zinc available for reaction with the sulphide which is the basis of the Timm staining method.

Animals treated with nifedipine showed lower than baseline levels of Timm staining. This decreased staining may be due to a loss of presynaptic zinc via a physiological release mechanism (Sloviter, 1984), and the inability of the cells to replenish intracellular zinc due to the nifedipine-induced block of calcium/zinc channels. Alternatively, the reduced levels of calcium may have led to a change in zinc disposition, rendering the zinc unable to react with the sulphide, which is the basis of the Timm stain. In either case, the results presented in this thesis cast some doubt on the idea that blocking the NMDA receptors via MK-801 results in a blocking of kindling-induced mossy fibre sprouting (Sutula et al., 1996). Since blocking the NMDA receptors reduces the levels of depolarization achieved, it would also be expected to interfere with the opening of the voltage-dependent calcium channels. NMDA antagonists, then, might also reduce the amount of intracellular zinc and consequently interfere with the Timm stain.

In view of the results presented in this thesis, conclusions should not be drawn about the occurrence of mossy fibre sprouting solely on the basis of intracellular zinc levels, as quantified by the Timm method. As illustrated in the present experiments, zinc levels are affected by many factors such as kindling activation, and the availability of voltage-gated calcium channels. We cannot conclude with any confidence that blocking voltage-gated calcium channels does or does not interfere with kindling induced sprouting of the mossy fibre pathway. However, we can conclude that blocking voltage-

gated calcium channels of the L type reduces the progression of seizures and reduces intracellular zinc levels in the hippocampal mossy fibres. In order to determine whether nifedipine alters kindling-induced sprouting, another method of quantifying mossy fibre sprouting should be used, one which does not rely solely on the density of intracellular zinc.

It has been revealed that intense exposure to zinc can be neurotoxic, killing cortical neurons after several minutes (Sensi, Canzoniero, Yu, Ying, Koh, Kerchner, & Choi, 1997). Since this neurotoxicity appears to be mediated by zinc influx, primarily through voltage-gated calcium channels and also through NMDA receptor-gated channels, (Koh, & Choi, 1994) the results in the present experiment provide us with a potential method for preventing zinc-induced neurotoxicity that can occur as a result of prolonged seizures (Sloviter, 1985) or transient global cerebral ischemia (Koh, Suh, Gwag, He, Hsu, & Choi, 1996). Therefore, it is possible that since nifedipine possesses the ability to interfere with the neuronal influx of zinc, it could also provide therapeutic benefits for other neuronal pathologies that have been associated with zinc-induced neurotoxicity.

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